Short Communication

Archaea Dominate Ammonia Oxidizers in the Permian Water Ecosystem of Midland Basin

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We investigated the existence and characteristics of ammonia oxidizers in Permian water from Midland Basin. Molecular surveys targeting the amoA gene showed that only ammonia-oxidizing archaea (AOA) exist and have potential activity in this special environment. In contrast, no ammonia-oxidizing bacteria (AOB) were detected in the water. Phylogenetic analysis indicated that 72–89% of the total screened AOA clones were affiliated with those found in underground water, and 10–24% of the AOA clones were related to those found in marine water or sediments. Our results indicate AOA might be the most abundant ammonia-oxidizing microbes in this ecological niche.

Key words: Ammonia oxidizer, Permian water, Underground basin

The conversion of ammonia to nitrite is an important microbiological process for nitrification, which is performed by the key ammonia monooxygenase (AMO) enzyme encoded by the amo gene (23). It has traditionally been assumed that this step is carried out mainly by autotrophic ammonia-oxidizing bacteria (AOB) of the β- and γ-subgroups of proteobacteria (16, 21, 22). However, this view has recently been changed by the discovery of the amoA gene in archaea populations, thus raising the prospect of the presence of ammonia-oxidizing archaea (AOA) in different ecosystems (10, 17, 21, 29, 32). In fact, AOA were found to be more abundant than AOB in a range of terrestrial and marine ecosystems, suggesting that AOA can play a significant role in nitrogen biogeochemical cycling (2, 4, 10, 21, 32). There is increasing evidence that environmental conditions, such as pH (20), salinity (19, 24), and especially ammonium availability (7, 15, 18), can affect the distribution, abundance and activity of AOB and AOA. Although AOA and AOB have been investigated in diverse environments, their distribution and diversity in Permian groundwater remain unexplored.

The Permian Basin is a unique ecosystem which contains the remnant of an ancient ocean that existed during the Permian time (~250 million years ago) (33). The Permian Basin is a sedimentary basin largely contained in the western part of the U.S. It reaches from just south of Lubbock, Texas, to south of Midland and Odessa, extending westward into the southeastern part of the adjacent state of New Mexico. It is so named because it has one of the world’s thickest deposits of rocks from the Permian geologic period. In the long historical period, the Permian basin received outside water gradually through the penetration of surface water or input of deepwater (1). A significant feature of the sample is high nitrate concentration and relatively low ammonia concentration (Table S1), which draw attention to the importance of nitrification. It could be presumed that ammonia oxidizers play important roles in the transformation from ammonia to nitrate. The goal of this study was therefore to investigate the existence, abundance and activity of ammonia oxidizers in Permian water based on the amoA gene and to evaluate their potential function in nitrogen transformation in this specific underground water.

For the above purposes, Permian water samples were collected from a location in the Pecos Cenozoic Trough in Imperial, Texas (latitude 31, 16’, 16.93” N; longitude 102, 40’, 48.35”W) (Fig. S1) in December 2010 and July 2011, respectively. After sampling, the water was fixed with HgCl2 immediately for hydrochemical analysis. For preparing samples for DNA analysis, 1L Permian water was filtered through a 0.22 μm pore-size membrane filter and the folded filtered membrane was placed into a 2 ml tube. The same procedure as above was repeated to prepare samples for RNA analysis. A difference was that the folded filtered membrane was placed into a 2 ml tube containing RNA later solution (Life Technologies, Carlsbad, CA, USA). All membrane samples were kept at –80°C.

As displayed in Table S1, the salinities of the two Permian water samples were 17.5‰ and 15.5‰, respectively, equivalent to approximately half of the average salinity of the ocean. The water contained a high concentration of bicarbonate, which can be supplied as a carbon source for autotrophic microbes. The average concentration of ammonia in Permian water is 0.19 μM, lower than that in the marine euphotic zone (0.3 μM), but higher than that in the marine aphotic zone (0.01 μM) greatly. The nitrite concentration in Permian water (average 0.77 μM) exceeds that in seawater (5, 14). The aerobic environment may contribute to the
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accumulation of nitrate, because aerobic conditions do not favor the processes of denitrification and anammox. The mean ratio of N/P is 20.95, with no major deviation from general terrestrial or marine ecological environments. These hydrological parameters offer an ambient background for analyzing the interactional relationship between ammonia oxidizers and environmental conditions.

For performing the molecular investigation, DNA and RNA were extracted using a PowerWater DNA Isolation Kit and PowerWater RNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) with the standard protocol. Three specific primer sets were used for amoA gene amplification and qualification: Arch-amoAF and Arch-amoAR (10) for AOA, amoA-1F and amoA-2R (23) for β-AOB and A189- for/ A682-rev (22) for γ-AOB. In addition, the bacteria and archaea were detected with general primer sets 27f/1492r (9) and 21f/852r (8) respectively. All PCR products of the amoA gene were purified by cutting gel bands with the Qiagen II Gel Extraction Kit (Qiagen, Hilden, Germany) and then cloned into pCR 2.1 TOPO T-vector (Invitrogen/Life Technologies, Carlsbad, CA, USA) to construct the gene libraries. The positive clones TOPO-1 and TOPO-2 with an insert for a gene fragment were selected as standards for real-time quantitative PCR (q-PCR) to detect the amoA gene abundance of AOA and AOB, respectively. Q-PCR was run on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using power SYBR Green PCR Master Mix run on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using power SYBR Green PCR Master Mix run on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) to construct the gene libraries. The positive clones TOPO-1 and TOPO-2 with an inserted amoA gene fragment were selected as standards for real-time quantitative PCR (q-PCR) to detect the amoA gene abundance of AOA and AOB, respectively. Q-PCR was run on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Standard curves were prepared from the serial dilution (10^8–10^2 amoA copies by decimal dilution series) of plasmids containing environmental archaeal and beta-proteobacterial amoA gene sequences. The PCR efficiencies were 87–94% (average 90%) for archaeal amoA and 92–96% (average 93%) for beta-proteobacterial amoA. Correlation coefficients (R^2) for both assays averaged 0.98 (standard deviation of 0.01).

Fig. S2 shows that the amplification of AOA was positive, but the amplifications of β- and γ-AOB were both negative, even under low stringency PCR conditions. Furthermore, q-PCR detection showed that the abundance of AOA was approximately 3.4×10^4 copies mL^-1 water, but the abundance of AOB was not detectable (the detection limit of q-PCR was approximately 10 copies mL^-1 in positive control experiments for both AOA and AOB) (Table 1). The finding that only AOA, not AOB, were detected in Permian water with both general PCR amplification and real-time q-PCR detection suggests that AOA are the dominant and even exclusive contributors to ammonia oxidation in Permian underground water.

To gain insight into the diversity of AOA in Permian water, we constructed two clone libraries based on amoA amplification and real-time q-PCR detection. The cloned sequences were submitted to GenBank under accession numbers DQ738931-DQ738981, DQ738983-DQ739031, DQ739033-DQ739081 and DQ739083-DQ739131. GenBank accession numbers have also been deposited in the database of the Center for the Study of Ammonia Oxidation (CSAO, www.ammonia-oxidation.org).

We performed sequence analysis of the cloned inserts to construct the gene libraries. The positive clones TOPO-1 and TOPO-2 with an inserted amoA gene fragment were selected as standards for real-time quantitative PCR (q-PCR) to detect the amoA gene abundance of AOA and AOB, respectively. Q-PCR was run on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Standard curves were prepared from the serial dilution (10^8–10^2 amoA copies by decimal dilution series) of plasmids containing environmental archaeal and beta-proteobacterial amoA gene sequences. The PCR efficiencies were 87–94% (average 90%) for archaeal amoA and 92–96% (average 93%) for beta-proteobacterial amoA. Correlation coefficients (R^2) for both assays averaged 0.98 (standard deviation of 0.01).

Fig. S2 shows that the amplification of AOA was positive, but the amplifications of β- and γ-AOB were both negative, even under low stringency PCR conditions. Furthermore, q-PCR detection showed that the abundance of AOA was approximately 3.4×10^4 copies mL^-1 water, but the abundance of AOB was not detectable (the detection limit of q-PCR was approximately 10 copies mL^-1 in positive control experiments for both AOA and AOB) (Table 1). The finding that only AOA, not AOB, were detected in Permian water with both general PCR amplification and real-time q-PCR detection suggests that AOA are the dominant and even exclusive contributors to ammonia oxidation in Permian underground water.

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Table 1. A rchaeal amoA gene and β-proteobacterial amoA gene copy numbers in the samples from Permian water

| Sample No. | Archaeal amoA gene copies mL^-1 (s.d.) | β-proteobacterial amoA gene copies mL^-1 (s.d.) | Archaeal amoA mRNA copies mL^-1 (s.d.) | β-proteobacterial amoA mRNA copies mL^-1 (s.d.) |
|------------|----------------------------------------|-----------------------------------------------|------------------------------------------|-----------------------------------------------|
| Sample 1   | 1.96×10^4 (7.48×10^3)                  | U                                             | 1.93×10^3 (0.82×10^2)                     | U                                             |
| Sample 2   | 4.83×10^4 (1.39×10^3)                  | U                                             | 2.68×10^3 (3.99×10^2)                     | U                                             |

U: Undetectable.
estuarine sediments (EU025152), and the deep sea hydro-
thermal vent (EU427963). Using a 3% cutoff at the DNA
sequence variation to define an OTU, the non-asymptotic
rarefaction curves showed that the OTU number would be
higher if more clones were sequenced (Fig. S3).

To determine whether amoA genes from AOA and AOB
communities are actively transcribed in Permian water and
whether transcriptions are correlated with gene abundance,
we extracted total RNA from two samples and constructed
two amoA cDNA libraries. Then, 20 amoA cDNA clones
were randomly selected and sequenced. The phylogenetic
relationships of the deduced amino acid sequences were close
and were all clustered within AOA freshwater cluster (Fig.
S4), suggesting that the freshwater cluster AOA possibly play
an active role in ammonia oxidation in the Permian water.
Using a 3% cutoff at the DNA sequence variation to define
an OTU, the number of OTUs of cDNA clones has reached
the maximum. With the reverse transcription q-PCR method,
we detected that the transcript abundance of AOA was
approximately 2.3×10^3 copies mL^-1 (Table 1), while the
transcripts of AOB were under the detection limit. Corre-
sponding to the transcriptional activity of AOA communities,
the ammonia oxidation rate was detectable in Permian water.
A set of experiments with NaClO_3 as an inhibitor were
performed to determine the ammonia oxidation rate. Because
NaClO_3 can inhibit the oxidation reaction from nitrite to
nitrate, nitrite could be accumulated when NaClO_3 was
supplied to the reaction system (13). Thus, the increase of
nitrite can be regarded as the rate of ammonia oxidation. Fig.
2 shows that the ammonia oxidation rate was approximately
0.337 μM d^-1. However, ammonia oxidation activity was not
completely inhibited by ammonia oxidation inhibitor allyl-
thiourea (ATU) (Fig. S5). A previous study also reported that
the nitrification rate in the California Current dominated by
AOA was inhibited only partially by ATU (25) at the level
known to completely inhibit cultivated AOB (11, 28, 24).

The different response of AOB and AOA to ATU is possible
for their different mechanism of ammonia oxidation (28,
31). The difference in the relative abundance and expression
of bacterial and archael ammonia oxidizers may be due to
the different ability to adapt to the niche. Previous studies
(12, 30) have demonstrated that ammonia concentration
influences their abundance and activity and AOA prefer an
environment with a low ammonia concentration. Martens-
Habbena et al. (18) showed that Nitrospumilus maritimus
SCM1 have a high affinity for ammonium, as much as 200-
fold higher than that of AOB, and a low substrate threshold
of 10 nM NH_4, or less. The available ammonia in Permian
water was very low (mean 0.2 μM). The source of the
ammonia may be leakage of surface water, or organic
minerals contained in the surface water (1). Based on the
finding of AOA’s markedly high specific affinity for ammonia (16),
the low level of available ammonia in Permian water is maybe an important factor contributing to the survival
of AOA rather than AOB. In addition, experimental results
of PCR amplifications showed that bacteria and Achaea are
both present in Permian seawater (Fig. 1), suggesting that
the environment in Permian water was not favorable for the
survival of Achaea or bacteria. So the difference in the
abundance of AOA and AOB in Permian water possibly resulted from their own physiological and metabolic charac-
teristics adapting to the low ammonia environment.

Interestingly, the amoA gene phylogenies from Permian
water reveal that these sequences represent two distinct
clusters (Fig. 1). Approximately 80% AOA clones in Permian
water belong to the freshwater cluster, and they are closely
related to the AOA sequences retrieved from underground
water. Less than 20% clones are affiliated with the marine
cluster. The co-existence of freshwater and marine AOA is
consistent with the environments from which they were drawn
and strengthen the idea that environmental niches within
the AOA are reflected in the amoA phylogeny. It is possible
that that salinity is an important factor for AOA adapting to
ecological niches. Recent reports (2, 19, 24) support the idea
that salinity affects the community composition of AOA.

In this study, the measured salinity in the Permian water
from Midland basin is approximately half of average salinity
in the ocean. The salinity of Permian water is much lower
than that of the originally enwrapped sea water because
evidence suggests that ancient marine salinity was similar to
or higher than that of the modern sea (3). The decreased
salinity is the result of mixing with fresh water penetrating
from the surface continuously (1). Along with the penetrated
water, microorganisms including AOA possibly entered the
Permian basin. If marine cluster AOA are regarded as
indigenous to the original Permian water, freshwater cluster
AOA should be exogenous. Previous investigations suggested
that AOA have pronounced ecological niche separation based
on amoA gene phylogenies and the community composition
of AOA in the marine environment is distinct with that in
freshwater (2, 10, 25). With the change of the environment
in Permian sea water, the composition of ammonia oxidizers
may have changed to adapt to the new environment.
Gradually, freshwater cluster AOA have taken the dominant
position. However, the relationship between the molecular
information and the geological features needs further study.

In conclusion, the molecular information provided strong
evidence that AOA rather than AOB are responsible for the

![Fig. 2. Ammonia oxidation rates by the Permian water measured by adding NH_4Cl (final concentration 10 μM). Inhibited bottles were spiked with the nitrite-oxidation inhibitor NaClO_3 to achieve a final concentration of 10 mg L^-1. Water was filtered through a 0.22 μm pore-size membrane as a control.](image-url)
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ammonia oxidation process in Permian water. Environmental conditions, such as the available ammonia concentration and salinity, possibly shaped the population structure and function of ammonia oxidizers. To our knowledge, this is the first study indicating the potential role of AOA in the environment of Permian water. Future work will add the activity of the community and examine the factors controlling ammonia oxidizers’ diversity and abundance and how to adapt to a confined environment.

The GenBank accession numbers for the amoA gene sequences reported here are JX311735–JX311856.

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