RNA-Based Investigation of Ammonia-Oxidizing Archaea in Hot Springs of Yunnan Province, China

Running title: Ammonia-Oxidizing Archaea in Yunnan Hot Springs

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Revised for Applied and Environmental Microbiology
Mar. 18, 2010
Using RNA-based techniques and hot spring samples collected from Yunnan Province, China, we show that the *amoA* gene of aerobic ammonia-oxidizing archaea can definitely be transcribed at temperatures higher than 74°C and up to 94°C, suggesting that archael nitrification can potentially occur at near boiling temperatures.
Aerobic ammonia-oxidizing archaea (AOA) are one major group of microorganisms mediating the autotrophic ammonia oxidation (2), which is central to the global nitrogen cycle (9). AOA possess an ammonia monooxygenase (AMO), which is the enzyme responsible for catalyzing aerobic ammonia oxidation and its alpha-subunit is encoded by the amoA gene (15). Multiple amoA gene-based molecular studies have demonstrated that AOA can be adapted to a large gradient of environmental variables with respect to temperature (0.2-97°C) and pH (2.5-9.0) [See review by (2) and refs therein]. However, so far only moderately thermophilic “Candidatus Nitrososphaera gargensis” and thermophilic “Candidatus Nitrosocaldus yellowstonii” have been obtained in culture and show the capability of oxidizing ammonia at high temperatures: they can produce nitrite at 46°C and 60-74 °C at pH 7-8, respectively (1, 4). In addition, Reigstad et al. (12) demonstrated biological ex situ nitrification at 85°C and pH 3.0 using terrestrial hot spring samples. This indicated that the AMO enzyme is active at temperatures up to 85 °C. In the meanwhile, with the use of DNA-based molecular techniques, Reigstad et al. (12) and Zhang et al. (16) retrieved AOA amoA gene clone sequences from global terrestrial hot springs with a large gradient of pH (2.5-9.0) and temperature (38-97°C). However, the AOA amoA gene has never been transcribed from environments with temperatures higher than 74 °C. In the present study, we performed RNA-based studies investigating the abundance and diversity in hot springs (Temperature: 44.5-94.0°C; pH 2.4-9.0) of Yunnan Province in southwestern China.

A total of 11 hot spring samples were selected for field measurements and sample
collection (Table 1). Hach kits-based field measurements showed that temperatures of
the sampled hot springs ranged from 44.5°C to 94.0°C, and pH from 2.4 to 9.0 (Table
1). Mats or mat-containing sinter/sediments were collected and subjected to RNA
extraction with the use of FastRNA® Pro Soil-Direct Kit (Qbiogene, Inc. CA)
according to the manufacturer’s protocols. The resulting crude RNA was digested
with RNase-free DNase I (Takara, Japan). The DNase-digested RNA samples were
verified to be free of genomic DNA contamination by PCR amplification with primer
sets specific for total archaea, bacteria, and AOA according to conditions described
elsewhere (See Table S1 in the supplemental material and cited references for details).
The DNA-free RNA samples were reverse-transcribed into cDNA using the Promega
AMV reverse transcription system (Promega Corporation, Madison, WI) as
previously described (7). The archaeal *amoA* gene and total bacterial and archaean 16S
rRNA genes in the synthesized cDNA were quantified by qPCR (See Table S1 in the
supplemental material) according our previous studies (6, 7). Bacterial and archaean
16S rRNA gene abundances were on the order of 10^8-10^{10} copies per gram of solids,
and the AOA *amoA* gene abundance ranged from 4.5×10^4 to 3.52×10^6 copies per gram
of solids in the investigated hot springs (Table 2). The abundance of transcribed AOA
*amoA* gene in high-temperature hot springs is comparable to those in low-temperature
biotopes (7, 8, 11).
The cDNA samples were PCR amplified using AOA-specific primer sets (See
Table S1 in the supplemental material) as described previously (7). The resulting PCR
products were used for constructing the *amoA* gene clone libraries according to
established procedures (7). A total of 337 AOA amoA gene clones were randomly selected for sequencing and the obtained sequences (Table 1) were subjected to operational taxonomic units (OTUs) analysis using DOTUR 1.53 (13), with the cutoff of 2% and 5%, respectively (3). The diversity indices of Shannon (H') and Chao1 were also calculated using DOTUR. One sequence from each OTU was then selected as representative for phylogenetic analysis. The representative sequences were deposited in the GenBank database under accession numbers GQ226055- GQ226135.

The number of clones in each sample represented 54-100% coverage (at 2% cutoff) for the clone libraries (Table 2). The representative sequences at 2% cutoff, reference sequences from Zhang et al. (16), and amoA gene sequences of “Candidatus Nitrosopumilus maritimus”, “Candidatus Nitrososphaera gargensis”, and “Candidatus Nitroscaldus yellowstonii” were combined for phylegenetic analysis using the MEGA 4.1 (14). The amoA phylogenetic nomenclature in Zhang et al. (16) was employed in this study (Fig. 1). The phylogenetic analysis showed that only two amoA gene clones retrieved in this study were affiliated with the Cluster A named in Zhang et al. (16). In contrast, 99% of clones retrieved in this study were classified into the Cluster B and distributed into three groups: B.1, B.2 and B.5 (Fig. 1). The retrieved sequences in the B.1 and B.2 groups were related (90-99%) to those from Tengchong hot springs determined in Zhang et al. (16) (Fig. 1). The B.2 clones were related (identity 90-99%) to moderately thermophilic “Candidatus Nitrososphaera gargensis” (4). In addition, all clone sequences in the Cluster B were distantly (<80% identity) related to thermophilic “Candidatus Nitroscaldus yellowstonii” (1) (Fig. 1).
Previous studies indicated that environmental factors (e.g. ammonium concentrations, organic carbon, temperature, salinity, DO levels, pH, sulfide levels, and phosphate) may affect AOA distributions (2, 10). In order to evaluate the correlation of the measured geochemical variables with amoA gene abundance and diversity in this study, the simple Mantel tests were performed using the zt software (http://www.psb.ugent.be/~erbon/mantel/) according to established procedures (5). Significant positive correlation ($r > 0.5; P < 0.05$) was present between the AOA amoA gene abundance (either absolute or relative) and a number of environmental variables, but absent ($r < 0.5$) between the AOA amoA gene abundance and the measured environmental variables (See Table S2 in the supplemental material). Without further investigation, however, it is uncertain whether the observed positive correlations are real or just coincidental.

In summary, our data show that AOA amoA gene can definitely be transcribed in hot spring samples with temperatures higher than 74°C and up to 94°C. However, ex situ experiments are required to verify the potential activity of AOA at such high temperatures and to find the reasons for the observed correlations between the AOA amoA gene and measured environmental variables. This will be a major focus of our future research.

This research was supported by SRF for ROCS, SEM, China Postdoctoral Science Foundation (20090450457) and the China University of Geosciences (Beijing) Teaching Laboratory funds to HJ, National Basic Research Program of China to WJ Li (2010CB833800), National Science Foundation of China to CZ (40972211),
COMRA fund grant (DYXM-115-02–2-17) to PW, and Research Fund of the State Key Laboratory of Geological Processes and Mineral Resources (GPMR2008K08B) of China University of Geosciences-Beijing, and the 111 Project (No. B07011) of China to HD. The authors are grateful to Shicai Deng at China University of Geosciences, Beijing, Yan Li and Xiaoyang Zhi at Yunnan University and Libo Yu at Third Institute of Oceanography, SOA for helping with sample collection and qPCR work. The authors are grateful to three anonymous reviewers whose constructive criticisms significantly improved the quality of the manuscript.

REFERENCES


Table 1 Water chemistry and temperature of eleven hot springs in Yunnan Province, China, and description of samples collected from these hot springs. TDS = total dissolved solids. Samples from Tengchong, Yunnan Province are abbreviated as: DGG = Dagunguo; DGGTYQ = Dagunguo-Tiyinqu; YJQ = Yanjingquan; ZZQ = Zhenzhuquan; WMXX = Wuming-Xiaoxi. Samples from Longling, Yunnan Province are abbreviated as: DHB = Dahebian; BLZ1 = Balazhang1; BLZ2 = Balazhang 2. Samples from Eryuan, Yunnan Province are abbreviated as: NJYPZT = Niujie-Yongping-Zaotang; LZT1 = Laizitang1; LZT2 = Laizitang2.

<table>
<thead>
<tr>
<th>Sample (GPS location)</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>SO₄²⁻ (mM)</th>
<th>NO₃⁻ (μM)</th>
<th>NO₂⁻ (μM)</th>
<th>NH₄⁺ (μM)</th>
<th>Fe²⁺ (μM)</th>
<th>Sulfide (μM)</th>
<th>TDS (μM/L)</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGG (24°57'12.7&quot;/98°26'17.4&quot;)</td>
<td>84.0</td>
<td>7.6</td>
<td>3.13</td>
<td>1.02</td>
<td>8.23</td>
<td>1.18</td>
<td>&lt;0.04</td>
<td>9.69</td>
<td>5.95</td>
<td>Grey sinter</td>
</tr>
<tr>
<td>DGGTYQ (24°57'12.7&quot;/98°26'17.4&quot;)</td>
<td>87.0</td>
<td>2.4</td>
<td>5.21</td>
<td>0.34</td>
<td>0.97</td>
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<td>303.57</td>
<td>6.56</td>
<td>4.53</td>
<td>Black sediment</td>
</tr>
<tr>
<td>YJQ (24°57'03.7&quot;/98°26'09.5&quot;)</td>
<td>94.0</td>
<td>9.0</td>
<td>0.07</td>
<td>0.96</td>
<td>9.03</td>
<td>0.59</td>
<td>&lt;0.04</td>
<td>150.00</td>
<td>5.80</td>
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</tr>
<tr>
<td>ZZQ (24°57'03.7&quot;/98°26'09.5&quot;)</td>
<td>89.5</td>
<td>3.5</td>
<td>0.73</td>
<td>0.72</td>
<td>3.06</td>
<td>20.00</td>
<td>17.86</td>
<td>1.25</td>
<td>0.70</td>
<td>Brown sediment</td>
</tr>
<tr>
<td>WMXX (24°56'59.6&quot;/98°26'15.7&quot;)</td>
<td>64.8</td>
<td>6.7</td>
<td>0.34</td>
<td>3.52</td>
<td>7.58</td>
<td>&lt;0.5</td>
<td>0.71</td>
<td>&lt;0.3</td>
<td>2.33</td>
<td>Black microbial mat</td>
</tr>
<tr>
<td>DHB (24°39'37.2&quot;/98°43'11.9&quot;)</td>
<td>44.5</td>
<td>7.1</td>
<td>0.30</td>
<td>0.07</td>
<td>0.48</td>
<td>&lt;0.5</td>
<td>0.36</td>
<td>&lt;0.3</td>
<td>0.82</td>
<td>Black microbial mat</td>
</tr>
<tr>
<td>BLZ1 (24°39'23.3&quot;/98°40'03.4&quot;)</td>
<td>60.0</td>
<td>6.3</td>
<td>0.42</td>
<td>0.28</td>
<td>&lt;0.7</td>
<td>&lt;0.5</td>
<td>&lt;0.04</td>
<td>&lt;0.3</td>
<td>0.96</td>
<td>Black sediment</td>
</tr>
<tr>
<td>BLZ2 (24°39'23.3&quot;/98°40'03.4&quot;)</td>
<td>55.0</td>
<td>6.7</td>
<td>0.49</td>
<td>0.22</td>
<td>&lt;0.7</td>
<td>1.18</td>
<td>&lt;0.04</td>
<td>&lt;0.3</td>
<td>1.80</td>
<td>Black sediment</td>
</tr>
<tr>
<td>NJYPZT (26°15'01.2&quot;/99°59'22.2&quot;)</td>
<td>70.0</td>
<td>7.9</td>
<td>0.31</td>
<td>1.28</td>
<td>4.68</td>
<td>8.24</td>
<td>0.54</td>
<td>&lt;0.3</td>
<td>1.94</td>
<td>Rufous sediment</td>
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<td>LZT1 (26°14'57.1&quot;/99°59'31.0&quot;)</td>
<td>62.0</td>
<td>6.9</td>
<td>1.46</td>
<td>2.41</td>
<td>5.81</td>
<td>2.94</td>
<td>0.54</td>
<td>&lt;0.3</td>
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<td>Black sediment</td>
</tr>
<tr>
<td>LZT2 (26°14'58.3&quot;/99°59'32.6&quot;)</td>
<td>80.0</td>
<td>7.25</td>
<td>1.25</td>
<td>1.04</td>
<td>1.77</td>
<td>12.35</td>
<td>0.18</td>
<td>&lt;0.3</td>
<td>2.20</td>
<td>Black sediment</td>
</tr>
</tbody>
</table>
Table 2 Abundance of 16S rRNA gene and archaeal amoA gene and sequencing information for eleven hot spring samples collected from Yunnan Province, China.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA gene (copies g(^{-1}))</th>
<th>Archaeal amoA gene (copies g(^{-1}))</th>
<th>Coverage (%)</th>
<th>OTUs</th>
<th>H'</th>
<th>Chao1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria StdDev</td>
<td>Archaea StdDev</td>
<td>amoA StdDev</td>
<td>n†</td>
<td>2%</td>
<td>5%</td>
</tr>
<tr>
<td>DGG</td>
<td>1.58 × 10^9</td>
<td>2.94 × 10^9</td>
<td>7.94 × 10^9</td>
<td>1.76 × 10^10</td>
<td>4.50 × 10^10</td>
<td>2.17 × 10^11</td>
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<td>DGGTYQ</td>
<td>2.69 × 10^9</td>
<td>1.55 × 10^9</td>
<td>4.63 × 10^9</td>
<td>3.95 × 10^10</td>
<td>3.52 × 10^10</td>
<td>6.53 × 10^10</td>
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<tr>
<td>YQ</td>
<td>2.93 × 10^9</td>
<td>2.62 × 10^9</td>
<td>3.82 × 10^9</td>
<td>4.11 × 10^10</td>
<td>2.60 × 10^10</td>
<td>1.98 × 10^10</td>
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<td>ZZQ</td>
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<td>1.25 × 10^10</td>
<td>1.64 × 10^9</td>
<td>3.68 × 10^10</td>
<td>4.25 × 10^10</td>
<td>5.22 × 10^10</td>
</tr>
<tr>
<td>WMXX</td>
<td>8.95 × 10^9</td>
<td>2.94 × 10^9</td>
<td>2.28 × 10^9</td>
<td>1.99 × 10^10</td>
<td>1.25 × 10^10</td>
<td>1.91 × 10^10</td>
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<tr>
<td>DHB</td>
<td>1.22 × 10^10</td>
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<td>1.09 × 10^9</td>
<td>1.60 × 10^10</td>
<td>2.30 × 10^9</td>
<td>2.10 × 10^10</td>
</tr>
<tr>
<td>BLZ1</td>
<td>1.04 × 10^11</td>
<td>3.09 × 10^10</td>
<td>2.22 × 10^9</td>
<td>1.70 × 10^10</td>
<td>1.28 × 10^9</td>
<td>1.21 × 10^9</td>
</tr>
<tr>
<td>BLZ2</td>
<td>3.33 × 10^10</td>
<td>8.16 × 10^9</td>
<td>2.16 × 10^9</td>
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<td>NJYXZ</td>
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<tr>
<td>LZT1</td>
<td>1.60 × 10^9</td>
<td>4.32 × 10^10</td>
<td>1.99 × 10^10</td>
<td>2.49 × 10^10</td>
<td>5.62 × 10^10</td>
<td>4.22 × 10^10</td>
</tr>
<tr>
<td>LZT2</td>
<td>4.20 × 10^9</td>
<td>1.71 × 10^10</td>
<td>1.80 × 10^10</td>
<td>3.88 × 10^10</td>
<td>9.91 × 10^10</td>
<td>1.53 × 10^10</td>
</tr>
</tbody>
</table>

† n = number of clones. The diversity indices were derived from clone libraries.
Figure captions

Fig. 1. Neighbor-joining tree (partial sequences, ~635 bp) showing the phylogenetic relationships of archaeal amoA gene sequences cloned from this study and Zhang et al [2008 AEM 74 (20): 6417-6426] and amoA gene sequences of three AOA isolates or cultures. Clone sequences from this study are bolded. One representative clone within each OTU is shown and the number of clones within each OTU is shown in the parentheses. The classification system for Cluster A and B in Zhang et al. (2008) was employed in this study. Scale bars indicate the Jukes-Cantor distances. Bootstrap values of >50% (for 1000 iterations) are shown.
Fig. 1

Cluster A

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