Cultivation and characterization of thermophilic Nitrospira species from geothermal springs in the US Great Basin, China, and Armenia

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Abstract
Despite its importance in the nitrogen cycle, little is known about nitrite oxidation at high temperatures. To bridge this gap, enrichment cultures were inoculated with sediment slurries from a variety of geothermal springs. While nitrite-oxidizing bacteria (NOB) were successfully enriched from seven hot springs located in US Great Basin, south-western China, and Armenia at ³57.9 °C, all attempts to enrich NOB from > 10 hot springs at ³61 °C failed. The stoichiometric conversion of nitrite to nitrate, chlorate sensitivity, and sensitivity to autoclaving all confirmed biological nitrite oxidation. Regardless of origin, all successful enrichments contained organisms with high 16S rRNA gene sequence identity (³97%) with Nitrospira calida. In addition, Armenian enrichments also contained close relatives of Nitrospira moscoviensis. Physiological properties of all enrichments were similar, with a temperature optimum of 45–50 °C, yielding nitrite oxidation rates of 7.53 ± 1.20 to 23.0 ± 2.73 fmoles cell⁻¹ h⁻¹, and an upper temperature limit between 60 and 65 °C. The highest rates of NOB activity occurred with initial NO₂⁻ concentrations of 0.5–0.75 mM; however, lower initial nitrite concentrations resulted in shorter lag times. The results presented here suggest a possible upper temperature limit of 60–65 °C for Nitrospira and demonstrate the wide geographic range of Nitrospira species in geothermal environments.

Introduction
Nitrification, a two-step process that results in the production of nitrate from ammonia, is an important component of nitrogen cycle. In the first step of nitrification, ammonia is oxidized to nitrite and in the second step nitrite is oxidized to nitrate. As no known organism is capable of carrying out both steps, it can be important to consider each separately. The majority of research on nitrification has focused on ammonia oxidation, possibly because of the excitement surrounding the recent discovery of ammonia-oxidizing archaea (Könneke et al., 2005), the widely held assumption that ammonia oxidation is rate limiting in nature (Kowalchuk & Stephen, 2001), and the difficulty of cultivating nitrite-oxidizing bacteria (NOB) in the laboratory.

Known chemolithotrophic NOB belong to the genera Nitrobacter, Nitrooccus, Nitrotoga, Nitrospina, Nitrospira, and Nitrolancetus (Winslow et al., 1917; Watson & Waterbury, 1971; Watson et al., 1986; Alawi et al., 2007; Sorokin et al., 2012). While Nitrobacter was traditionally considered to be the most important NOB, several recent studies using cultivation-independent methods have indicated that Nitrospira may be more abundant than Nitrobacter in many environments (Hovanec et al., 1998; Schramm et al., 1999; Altmann et al., 2003, 2004). Members of the genus Nitrospira represent a monophyletic group of NOB within the bacterial phylum Nitrospirae
(Ehrich et al., 1995; Spieck & Bock, 2001). They are found in a wide variety of natural habitats such as freshwater sediments (Stein et al., 2001; Altman et al., 2003, 2004), soils (Bartosch et al., 2002; Noll et al., 2005; Attard et al., 2010), marine water (Watson et al., 1986), and geothermal springs (Kanokratana et al., 2004; Lebedeva et al., 2005, 2011). Despite the tremendous ecological importance of Nitrospiraa, knowledge about this group of NOB is limited.

All Nitrospira are hypothesized to be K-strategists with high substrate affinity and low maximum growth rate (Schramm et al., 1999; Kim & Kim, 2006; Huang et al., 2010). As such, Nitrospira tend to be adapted to low oxygen (Schramm et al., 1999) and nitrite availability (Schramm et al., 1999; Nogueira & Melo, 2006) and their growth can be inhibited by nitrite concentrations as low as 1.5 mM (Off et al., 2010). This property might be a contributing factor to the difficulty in cultivating Nitrospira in the laboratory.

To date, there are only five formally identified members of the genus Nitrospira, with several lineages detected only by cultivation-independent censuses (Lücker et al., 2010). Nitrospira marina is a marine mesophile originally isolated from the Atlantic Ocean off the Gulf of Maine (Watson et al., 1986). 'Candidatus Nitrospira defluvii' is a freshwater mesophile highly enriched from activated sludge (Spieck et al., 2006). Nitrospira moscoviensis and 'Candidatus Nitrospira bockiana', both isolated from Moscow heating systems, were reported to be moderately thermophilic with growth temperature optima of 39 and 42 °C and growth temperature ranges of 33–40 and 28–44 °C, respectively (Ehrich et al., 1995; Lebedeva et al., 2008). Most recently, the most thermophilic NOB isolate known, Nitrospira calida, was isolated from Gorjachinsk Hot Spring in the Lake Baikal area of Russia and shown to have a growth temperature optimum of 46–52 °C and an upper temperature for growth of 58 °C (Lebedeva et al., 2011).

Despite this progress, and more substantial progress on chemolithotrophic ammonia oxidation (Pearson et al., 2004; Hatzenpichler et al., 2008; Reigstad et al., 2008; de la Torre et al., 2008; Zhang et al., 2008a; b; Jiang et al., 2010; Dodsorth et al., 2011), very little is known about the oxidative nitrogen cycle at elevated temperatures. This study focuses on the enrichment and characterization of thermophilic NOB from geothermal springs in the US Great Basin, south-western China, and Armenia.

Materials and methods

Sample collection

Sediment samples along with spring water were collected into sterile 15-mL polypropylene tubes from the source and outflow of springs located in US Great Basin, Tengchong (south-western China), and Armenia (Table 1). Samples from the US Great Basin were collected from the circumneutral Great Boiling Spring (GBS) located in northern Nevada. Samples from Tengchong County, Yunnan Province, China, were collected from various locations within the Rehai (‘Hot Sea’) geothermal field, a high temperature, granite-hosted system with a large diversity of geothermal features (reviewed in Hedlund et al., 2012) and the carbonate-hosted Ruidian system (Meixiang & Wei, 1987; Zhang et al., 2008b). Samples from Armenia were collected from a variety of circumneutral, carbonate-buffered springs (Mkrtchyan, 1969).

Sediment slurries from Great Basin springs were transported to the laboratory without temperature control before being inoculated into a sterile medium (described below). Sediment slurries from Armenia were kept at 4 °C and were transported to the laboratory without temperature control, at which time they were inoculated into sterile media. Sediment slurries from Tengchong were inoculated into duplicate serum bottles containing the enrichment medium on site with one replicate being incubated in the spring at the collection site for 3–5 days before being transported to the laboratory and the remaining replicate being maintained without temperature control until reaching the laboratory. In the laboratory, all enrichments were incubated at the temperature of their collection site.

Media and cultivation

All enrichments were made using a mineral medium (modified from Ehrich et al., 1995) with the following composition: 10 mg L⁻¹ CaCl₂ • 2H₂O, 5.8 mg L⁻¹ NaHCO₃, 0.5 g L⁻¹ NaCl, 150 mg L⁻¹ KH₂PO₄, and 1 mL L⁻¹ of the following stock solutions: MnCl₂ • 4H₂O (52 mg L⁻¹), H₂BO₃ (40 mg L⁻¹), ZnSO₄ • 7H₂O (34 mg L⁻¹), Na₂MoO₄ • H₂O (34 mg L⁻¹), CuSO₄ • 5H₂O (25 mg L⁻¹), FeSO₄ • 7H₂O (0.97 mg L⁻¹), and MgCl₂ • 6H₂O (47 mg L⁻¹). The medium was sterilized by autoclaving at 121 °C for 60 min. The pH was 7.0 at 25 °C after autoclaving.

Enrichment cultures were incubated in either 160- or 25-mL stoppered serum bottles with the following total and liquid medium volumes (mL): 160/40 (v/v), 160/60 (v/v), and 25/10 (v/v). Primary enrichments from GBS (US Great Basin) at 80, 65, or 50 °C contained 1 mM NaNO₂ and had a headspace composition of either full air or N₂ : air (3 : 1, v : v). Enrichments from China and Armenia contained 0.1 mM NaNO₂ and had a headspace composition of N₂ : air (3 : 1, v : v). Maintenance cultures for all springs were incubated at 50 °C. NO₃⁻ was monitored weekly and replenished when depleted, and the gas phase was exchanged weekly. These maintenance cultures were used to inoculate enrichments for all further studies.
Measurement of NOB activity and inhibition

For all experiments, NO\textsubscript{2} and NO\textsubscript{3} concentrations were measured colorimetrically by diazotization with and without cadmium reduction, respectively, using commercial kits (LaMotte, Chestertown, MD) and a Spectronic 20D spectrophotometer (Milton Roy).

Biological nitrite oxidation was verified using three methods. For all three methods, cultures were inoculated with 1% inoculum from a culture maintained at 50 °C.

First, the stoichiometric conversion of NO\textsubscript{2} to NO\textsubscript{3} was tested. Three enrichments and two uninoculated controls were spiked with 0.1 mM NaNO\textsubscript{2}, and the conversion of NO\textsubscript{2} to NO\textsubscript{3} was monitored. When all NO\textsubscript{2} was depleted, NO\textsubscript{3} was measured and enrichments were spiked with an additional 0.1 mM NO\textsubscript{2}. This process was repeated until 0.9 mM NO\textsubscript{3} had accumulated.

Second, inhibition of NO\textsubscript{2} oxidation activity by the inhibitor ClO\textsubscript{3} was tested. Ten enrichments and six controls were incubated at 50 °C for 6 weeks at which time the NO\textsubscript{2} concentrations were increased to 0.5 mM. Samples (500 μL) were collected from each enrichment and control every 3 h for 30 h; after the first 15 h, 5 mM NaClO\textsubscript{3} was added to five of the enrichments. Controls included two uninoculated bottles without NaClO\textsubscript{3}, two uninoculated bottles with 5 mM NaClO\textsubscript{3}, and two inoculated bottles that were spiked with 5 mM NaClO\textsubscript{3} immediately after inoculation.

Finally, to test the loss of NO\textsubscript{2} oxidation activity after autoclaving, three active enrichments were incubated at 50 °C for 3 weeks at which time two of the enrichments were autoclaved. NO\textsubscript{2} concentrations were monitored for an additional 4 weeks to ensure inactivity.

Identification of NOB in enrichment cultures

Cells were lysed and DNA was isolated using a FastDNA SPIN kit for Soil (MP-Biomedicals, Solon, OH) according to the manufacturer’s protocol. Isolated DNA was stored at −20 °C until analysis. DNA was amplified by PCR using primers 9bF (GRTGTTTATCCCTGCTGCAAG) and 1512uR (ACGGTACCTTGTGTCGATCCCTG) (Burggraf

Table 1. NO\textsubscript{2} oxidation activity in inoculated enrichment cultures

<table>
<thead>
<tr>
<th>Location</th>
<th>Spring</th>
<th>GPS location at source</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>NO\textsubscript{2} oxidation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Basin, US</td>
<td>GBS</td>
<td>N40.84139° W119.61889°</td>
<td>6.80</td>
<td>80.0</td>
<td>–</td>
</tr>
<tr>
<td>Tengchong, China</td>
<td>Zimeiquan, Rehai</td>
<td>N24.95102</td>
<td>8.98</td>
<td>84.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Gumingquan, Rehai</td>
<td>E98.46613</td>
<td>9.40</td>
<td>83.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Direhi, Rehai</td>
<td>N24.95009° E89.43807°</td>
<td>8.29</td>
<td>83.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.33</td>
<td>74.3</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>8.39</td>
<td>69.4</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>8.56</td>
<td>61.2</td>
<td>–</td>
</tr>
<tr>
<td>Jinze, Ruidian</td>
<td>N23.44138°</td>
<td>6.71</td>
<td>80.6</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E98.46004°</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Gongxiaoshe, Ruidian</td>
<td>N25.44012° E98.44081°</td>
<td>7.29</td>
<td>73.8</td>
<td>–</td>
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<td></td>
<td>Shuirebaozha, Rehai</td>
<td>N24.95002° E98.43728°</td>
<td>8.27</td>
<td>72.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Qiaobianrequan, Rehai</td>
<td>N24.95044° E98.43650°</td>
<td>c. 7</td>
<td>57.9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c. 7</td>
<td>48.5</td>
<td>+</td>
</tr>
<tr>
<td>Sinter Apron, Rehai</td>
<td>n.d.</td>
<td>9.00</td>
<td>33.6</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Armenia</td>
<td>Karvachar</td>
<td>N40.17417° E46.27500°</td>
<td>7.30</td>
<td>56.0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Jermuk</td>
<td>N39.96639° E45.68528°</td>
<td>7.05</td>
<td>53.0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Jermuk G</td>
<td>N39.87944° E45.77417°</td>
<td>6.90</td>
<td>53.0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Arzakan</td>
<td>N40.68389° E44.74111</td>
<td>7.20</td>
<td>44.0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hankavan</td>
<td>N40.63265° E44.48463°</td>
<td>7.00</td>
<td>44.0</td>
<td>–</td>
</tr>
</tbody>
</table>

n.d., not determined.
et al., 1992; Eder et al., 1999). Each 25 µL reaction contained 5 µL of 5X Go Taq buffer (Promega, Madison, WI), 400 nM dNTP (Promega), 1 µL template DNA, 400 nM each of forward and reverse primer, and 0.125 U Go Taq DNA polymerase (Promega). Cycling conditions were as follows: an initial melting step of 95 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and a final elongation step of 7 min at 72 °C. Clone libraries were made using a TOPO TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer’s protocol. Forty-four clones for each spring were sequenced by Functional Biosciences (Madison, WI) using the Sanger method. Sequences were clustered with mothur (Schloss et al., 2009), using the average neighbor algorithm at the 98% level and representative sequences for each cluster were identified using BLASTn (Altschul et al., 1990). 16S rRNA gene sequences were aligned with Nitrospira reference sequences obtained from the NCBI database using Clustal W within BioEdit (Hall, 2005), and phylip was used to construct maximum-likelihood and maximum-parsimony trees with 100 bootstraps per tree (Felsenstein, 2005). Modified parameters in phylip were as follows: outgroup was set to Thermodesulfovibrio aggregans, input order of species was randomized, and the speedier but rougher analysis was turned off. Representative sequences were deposited into the NCBI database (accession numbers KC161229–KC161246). No PCR product was obtained in any enrichment using primers specific for archaea: 8aF and 1512uR (Burggraf et al., 1992; Eder et al., 1999).

Quantification of NOB in enrichment cultures
Quantitative PCR (qPCR) was performed on template DNA extracted from enrichment cultures using primer sets NSP8-F (CGGAGTCCCTCCGACCTT) and NSP8-R (A TGGGACGGGAAACCGTTCGGA), which were designed in this study to be specific for the Nitrospira 16S rRNA gene. Standard curves were produced using a dilution series of purified plasmids (pCR2.1-TOPO; QiAprep Spin Mini-Prep Kit; Qiagen) containing the near full-length N. calida 16S rRNA gene obtained from the GBS enrichment as part of the clone library study described above. All standard curve reactions were prepared in duplicate. Sample reactions were prepared in triplicate and coupled with negative controls (no template). Reactions (25 µL) were prepared in individual wells of an iQ 96-well PCR plate (Bio-Rad, Hercules, CA) and contained 12.5 µL of 2X PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD), 400 nM each primer, and 5 µL template DNA. The following cycling conditions were used: an initial melt cycle (95 °C for 3 min) followed by 45 cycles of melting (94 °C for 15 s), annealing (57 °C for 25 s), and extension (72 °C for 45 s), with data collection using a SYBR-490 filter enabled during the 72 °C step, followed by a melt curve 55–95 °C by 0.5 °C increments with 10 s at each step. Gene copy numbers were quantified, and threshold cycles and data analysis were carried out using an iCycler iQ Multicolor Real-time PCR Detection System with iCycler iQ OPTICAL SYSTEM Software v3.1 (Bio-Rad). Standard curves were log-linear and correlation coefficients ($r^2$) for regressions, and amplification efficiencies were 101.4% and 0.982, respectively. The melt curves for controls and enrichment amplifications were identical and sequencing of clone libraries with PCR products obtained directly from GBS microbial mat material confirmed their specificity for Nitrospira.

Measurement of physiological properties
Rate studies measuring the consumption of NO$_2^-$ quantitatively were used to determine the optimal conditions for metabolic activity of nitrite oxidizers in enrichment cultures. All enrichments for the rate experiments started with 0.1 mM NaNO$_2$ to minimize the lag time and were inoculated with 1% inoculum from an enrichment maintained at 50 °C with a NO$_2^-$ concentration 0.3 mM. NO$_2^-$ concentrations were determined immediately after inoculation and weekly thereafter, and NO$_2^-$ was replenished as needed with NaNO$_2$. After 6 (GBS and Jermuk enrichments) or 8 (Rehai enrichments) weeks, NO$_2^-$ concentrations were increased to 0.3 mM to initiate rate experiments. Cultures were assumed to be in a nongrowing condition (stationary phase), or growth occurring during rate experiments was assumed to be negligible. Samples (500 µL) were taken from each enrichment and control immediately and every 3 h thereafter for 24 h for NO$_2^-$ concentration measurements. Two uninoculated negative controls and three inoculated enrichments were used for each condition in each study. To determine the optimal incubation temperature, enrichments were incubated at the following temperatures concomitantly: room temperature (24–26 °C), 40, 45, 50, 55, 60, and 65 °C. Temperatures in all incubators were monitored with a traceable thermometer (VWR 46610-024) throughout the experiment and were within 1 °C of the target temperature.

The optimal initial NO$_2^-$ concentration was determined in a similar manner to the optimal temperature with the following exceptions: enrichments were prepared with 0.1, 0.3, 0.5, 0.75, 1, and 1.25 mM NO$_2^-$ and incubated in the same 50 °C incubator, and NO$_2^-$ concentrations were checked immediately, after 2 weeks of incubation, and weekly thereafter for 6 weeks at which time the rate study was performed as described for the temperature optimization.
Statistical analysis

Levels of significance for the optimal temperature and initial NO$_2^-$ concentration data were analyzed using the nonparametric Kruskal–Wallis H-test followed by a Mann–Whitney U-test with Bonferroni correction. A Student’s t-test was used to determine whether the rates of NO$_2^-$ oxidation activities at 60 °C for GBS and Rehai enrichments were significantly different than abiotic controls.

Results and discussion

Results of thermophilic NOB enrichments

Initially, thermophilic NOB enrichments were inoculated with 50, 65, and 80 °C sediments from GBS (US Great Basin) and incubated at in situ temperature with 1 mM NO$_2^-$ and a fully aerobic or suboxic atmosphere (N$_2$ : air, 3 : 1, v : v). The 65 and 80 °C enrichments remained inactive throughout the > 1 year of incubation, but 50 °C enrichments began to show activity, as evidenced by NO$_2^-$ consumption, after 4 months of incubation. Similar lag times were observed for enrichments regardless of headspace gas composition, but in subsequent enrichments, growth was more reproducible under microaerophilic conditions. In addition, when NO$_2^-$ concentrations were reduced to 0.1 mM, lag times were reduced to ≤ 2 weeks. Based on the results from GBS enrichments, all additional enrichments were microaerophilic, incubated at 50 °C, and with 0.1 mM initial NO$_2^-$ concentration.

Using the improved NOB enrichment procedure, samples used for inocula were obtained from a variety of geothermal features in south-western China and Armenia. While all enrichments inoculated from sites ≥ 61 °C were unsuccessful (n = 13), enrichments from ≤ 53 °C samples were all successful (n = 10), with the exception of one spring in Armenia (Table 1). For successful enrichments, NOB activity became apparent within 2 weeks of inoculation.

Enrichments from the outflows of GBS (50 °C), Qiaobianrequan (48.5 °C), and Jermuk Spring (53 °C) were chosen as representative cultures from the US Great Basin, south-west China (Rehai), and Armenia (Jermuk region), respectively, for further study. Once enrichments were stable, two different approaches were employed to try to obtain axenic NOB cultures. First, optical tweezers were used to inoculate single cells from GBS and Rehai enrichments (c. 90 cells total) into the same medium, autoclaved spent enrichment medium, or filtered spring water amended with 0.1 mM NO$_2^-$. Second, serial dilutions of the same cultures were used to inoculate fresh medium. To date, neither method has successfully produced pure cultures. However, NOB are known to be extremely difficult to isolate and, to date, only three species of Nitrospira have been formally described as axenic species (Watson et al., 1986; Ehrich et al., 1995; Lebedeva et al., 2011). Additionally, ‘C. Nitrospira bockiana’ took up to 12 years to isolate and has not yet been described formally as an axenic culture (Lebedeva et al., 2008). Given the difficulty to obtain thermophilic NOB pure cultures and recognizing both the opportunities and limitations of studying mixed microbial cultures, enrichment cultures were used to determine the optimal conditions for nitrite oxidation activity.

Evidence of biological nitrite oxidation

As pure cultures were not obtained, we developed a rigorous framework to determine whether nitrite oxidation activity in each representative culture was biological. First, the predicted accumulation of NO$_2^-$ with the depletion of NO$_3^-$, according to the reaction NO$_3^- + 1/2O_2 \rightarrow NO_2^-$, was used to verify NO$_3^-$ oxidation in enrichment cultures. The approximate 1 : 1 stoichiometric conversion of NO$_2^-$ to NO$_3^-$ confirmed NO$_2^-$ oxidation activity in all enrichments tested (Supporting Information, Fig. S1). Second, because ClO$_3^-$ is a specific inhibitor of biological NO$_2^-$ oxidation (Belser & Mays, 1980), it was used to further confirm NOB activity. In all cases, 5 mM ClO$_3^-$ completely inhibited NOB activity in freshly inoculated subcultures and the addition of 5 mM ClO$_3^-$ to active cultures stopped NOB activity within five hours (Fig. S2). Finally, autoclaving long-term enrichments led to cessation of nitrite oxidation activity. These experiments conclusively demonstrated biological nitrite oxidation activity in the cultures.

Composition of enrichment cultures

16S rRNA gene PCR, clone library construction, sequencing, and phylogenetic analysis were used to identify the dominant microorganisms in each representative culture (Fig. 1 and Table 2). This revealed the presence of organisms with high (96–99%) 16S rRNA gene identity to N. calida in enrichment cultures from all three springs. 16S rRNA gene sequences for other known NOB were not found in the GBS or Rehai enrichments; however, the Jermuk enrichments also contained close relatives of N. moscoviensis. The GBS enrichment contained four additional species-level groups including close relatives of Meiothermus timidus (95% identity), one unidentified delta proteobacterium with 84% identity to Geobacter hephaestius, and two different Betaproteobacteria, one with 91% identity to Azospira restricta and another with 89% identity to Petrobacter succinatimandens. The Rehai
enrichment was much less diverse with 39 of the 44 sequences identified as close relatives of *N. calida* and the remaining five sharing 93% identity with *Anoxybacillus amylolyticus*. The Jermuk enrichment contained five additional species-level groups including relatives of *Anoxybacillus contaminans* (99% identity), *Ignavibacterium album* (98% identity), *M. timidus* (95% identity), *Dexia gummosa* (92% identity), and an organism with 83% identity to 'Candidatus Chloracidobacterium thermophilum'.

**Physiological properties**

The three representative enrichment cultures were used to test whether they have similar physiological properties and to gain insight into the kinetics of thermophilic nitrite oxidation. Incubation temperatures of 45–50 °C resulted in the fastest rate of NO₂⁻/C₀₂ consumption per unit volume for all enrichments (Fig. 2). Similarly, temperatures of 40–45 °C for GBS enrichments and 50 °C for both Rehai and Jermuk enrichments resulted in significantly shorter lags than other temperatures tested (Fig. S3). The enrichment from Jermuk had a wider temperature range of nitrite oxidation, possibly due to the

<table>
<thead>
<tr>
<th>Table 2. OTUs from GBS (US), Rehai (China), and Jermuk (Armenia) enrichments</th>
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<tbody>
<tr>
<td>Representative sequence</td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>GBS (US)</td>
</tr>
<tr>
<td>Clone_GB_8</td>
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<tr>
<td>Clone_GB_7</td>
</tr>
<tr>
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<td>Clone_AR_34</td>
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*Number of sequences in the 16S rRNA gene library represented by the OTU.
†Percent identity to the OTU representative sequence.
co-existence of phylogenetically and possibly functionally distinct NOB related to N. calida and N. moscoviensis.

Because these cultures were not defined microbiologically, and changes in growth conditions during optimization could lead to significant changes in the structure of enriched communities, no effort was made to optimize nitrite oxidation activity with respect to NOB cell number. As a result, these data are useful to frame possible relationships between temperature and nitrite oxidation activity, in general, but not to describe optimal activity for particular NOB taxa. However, generalized Nitrospira per cell calculations can be made if nitrite oxidation activity is presumed to be carried out exclusively by Nitrospira. To optimize this assumption, NOB were quantified in enrichment cultures used for the 16S rRNA gene censuses from Jermuk and Rehai (50 °C, 0.3 mM nitrite; Fig. 1 and Table 2) using Nitrospira-specific 16S rRNA gene primers. The Rehai enrichment had 3.9–6.0 × 10⁹ Nitrospira 16S rRNA gene copies mL⁻¹ and the Jermuk enrichment had 1.4–1.6 × 10⁸ Nitrospira 16S rRNA gene copies mL⁻¹. Using these data, and assuming one 16S rRNA gene per genome (Lückner et al., 2010) and one genome per cell (Akerlund et al., 1995), rates of 7.53 ± 1.20 fmoles cell⁻¹ h⁻¹ for the Jermuk enrichment and 23.0 ± 2.73 fmoles cell⁻¹ h⁻¹ for the Rehai enrichment were calculated. These rates are comparable with those measured for other NOB, such as those in nitrifying fluidized bed reactors colonized by Nitrospira (0.02–21.6 fmol cell⁻¹ h⁻¹; Schramm et al., 1999; Fujita et al., 2010) and those in cultures of Nitrobacter (5.1–13.6 fmol cell⁻¹ h⁻¹) and Nitrococcus (6.7–11.4 fmol cell⁻¹ h⁻¹; reviewed in Prosser, 1990).

Although the initial rate experiments demonstrated slow activity at 60 °C for only the Jermuk enrichments, activity near the upper temperature limits was difficult to reproduce, despite careful temperature monitoring and all attempts to maintain reproducibility. Therefore, additional experiments were carried out with GBS and Rehai enrichments to rigorously determine whether activity could be demonstrated at 60 °C, which ultimately confirmed slow but significant activity for both cultures at 60 °C (Fig. S4). In contrast, despite several attempts to grow enrichments from all three locations at temperatures ≥ 65 °C, NO₂⁻ oxidation activity was never observed at these temperatures (Fig. 2). Although not conclusive, these results are in agreement with other studies (Lebedeva et al., 2005, 2011) and suggest an upper temperature limit of 60–65 °C for Nitrospira.

The temperature range, optimum, and maximum rate of NOB activity appeared to be lower for the GBS enrichments compared with the other two enrichments during most experiments, although per cell rates were not determined because DNA from the appropriate enrichment was consumed prior to development of the qPCR procedure. Thus, apparent physiological differences may be due to differences in Nitrospira abundance, use of the improved NOB enrichment procedures for the primary Rehai and Jermuk enrichments, or to the activities of other inhabitantsof the co-cultures. Thus, although these differences may reflect physiological differences between the NOB in the Great Basin enrichments and the Asian enrichments, we urge caution in interpreting the data this way.

Despite the fact that the temperature range for Nitrospira species can be dependent on NO₂⁻ concentration (Lebedeva et al., 2008), we did not test this dependency in our enrichments. However, we did use low (0.3 mM) NO₂⁻ concentrations, and it has been reported that low NO₂⁻ concentrations (0.3 vs. 1.5 mM) resulted in a wider temperature range for ‘C. Nitrospira bockiana’ (Lebedeva et al., 2008). In addition, the temperature range did not change for N. calida whether 0.3 or 2.5 mM NO₂⁻ was used (Lebedeva et al., 2011). While the effect of NO₂⁻ concentrations on the temperature ranges was not explored, we did determine the effect of initial NO₂⁻ concentration on NOB activity. The experiments revealed that initial NO₂⁻ concentrations of 0.5–0.75 mM resulted in the fastest rate of nitrite oxidation (Fig. 3); on the other hand, the lowest concentrations of NO₂⁻ (0.1–0.3 mM) resulted in the shortest lag time (Fig. S5). These
results emphasize the importance of using low concentrations of NO\textsubscript{2} when cultivating thermophilic *Nitrospira* and provide a plausible explanation for the lengthy initial lag time for GBS enrichments.

**Conclusions**

As evidence of their wide geographic range, *Nitrospira* species were found in enrichment cultures from geothermal features located in the US Great Basin, Tengchong, south-west China, and Armenia. The sensitivity of these enrichments to both oxygen and nitrite concentrations supports the hypothesis that *Nitrospira* species are K-strategists and emphasizes the need for low NO\textsubscript{2} concentration (< 1 mM) when cultivating thermophilic *Nitrospira*. Similar to previously published results (Lebedeva *et al.*, 2005, 2011; Sorokin *et al.*, 2012), the upper temperature limit for growth for the nitrite-oxidizing enrichments, regardless of origin, was between 60 and 65 °C. Together, the data presented here suggest there may be an upper temperature limit for chemolithotrophic nitrite oxidation of 60–65 °C. However, it is possible that NOB exist in these habitats or in other geothermal springs that defy the enrichment conditions applied here. For example, *Nitrosolancetetus* grows optimally with much higher nitrite concentrations (5–20 mM; Sorokin *et al.*, 2012). Therefore, *in situ* geochemistry studies and cultivation-independent work should both be applied to probe the possible existence of NOB at higher temperatures. If they confirm that 60–65 °C is the global upper temperature limit for NOB, then this limitation could potentially drive a shunt in the high-temperature nitrogen cycle whereby nitrite produced by AOA might be the dominant substrate fueling denitrification at temperatures > 65 °C.

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**References**


Wide geographic range of thermophilic *Nitrospira* species


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Stoichiometric conversion of **NO** _x_ /C0 to **NO** /C0 in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

**Fig. S2.** Inhibition of **NO** /C0 oxidation activity by **ClO** /C0 in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

**Fig. S3.** Lag of **NO** /C0 oxidation activity with respect to temperature in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

**Fig. S4.** **NO** oxidation activities at 60 °C for GBS (US) and Rehai (China) enrichments.

**Fig. S5.** Lag of **NO** oxidation activity with respect to initial **NO** concentration in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).