RESEARCH ARTICLE

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 $\leq$ 57.9 °C, all attempts to enrich NOB from > 10 hot springs at $\geq$ 61 °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 ($\geq$ 97%) 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 $\pm$ 1.20 to 23.0 $\pm$ 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 NO_2^- 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*, *Nitrococcus*, *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; Altmann *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 *Nitrospira*, 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; Dodsworth *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} $CaCl_2 \bullet 2H_2O$, 5.8 mg L^{-1} $NaHCO_3$, 0.5 g L^{-1} NaCl, 150 mg L^{-1} KH_2PO_4, and 1 mL L^{-1} of the following stock solutions: $MnCl_2 \bullet 4H_2O$ (52 mg L^{-1}), H_3BO_3 (40 mg L^{-1}), $ZnSO_4 \bullet 7H_2O$ (34 mg L^{-1}), $Na_2MoO_4 \bullet H_2O$ (34 mg L^{-1}), $CuSO_4 \bullet 5H_2O$ (25 mg L^{-1}), $FeSO_4 \bullet 7H_2O$ (0.97 mg L^{-1}), and $MgCl_2 \bullet 6H_2O$ (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 $NaNO_2$ and had a headspace composition of either full air or N_2 : air (3 : 1, v : v). Enrichments from China and Armenia contained 0.1 mM $NaNO_2$ and had a headspace composition of N_2/air (3 : 1, v : v). Maintenance cultures for all springs were incubated at 50 °C. NO_2^- 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.

Table 1. NO_2^- oxidation activity in inoculated enrichment cultures

Location	Spring	GPS location at source	pH	Temperature (°C)	NO_2^- oxidation activity
Great Basin, US	GBS	N40.84139°	6.80	80.0	–
		W119.61889°	7.03	65.0	–
			7.26	50.0	+
Tengchong, China	Zimeiquan, Rehai	N24.95102	8.98	84.7	–
		E98.43613			
	Gumingquan, Rehai	N24.57060°	9.40	83.5	–
		E98.43615°			
	Direchi, Rehai	N24.95009°	8.29	83.0	–
		E98.43807°	8.33	74.3	–
			8.39	69.4	–
			8.56	61.2	–
	Jinze, Ruidian	N23.44138°	6.71	80.6	–
		E98.46004°			
	Gongxiaoshe, Ruidian	N25.44012°	7.29	73.8	–
		E98.44081°			
	Shuirebaozha, Rehai	N24.95002°	8.27	72.1	–
		E98.43728°			
	Qiaobianrequan, Rehai	N24.95044°	*c.* 7	57.9	+
		E98.43650°	*c.* 7	48.5	+
	Sinter Apron, Rehai	n.d.	9.00	33.6	+
Armenia	Karvachar	N40.17417°	7.30	56.0	+
		E46.27500°			
	Jermuk	N39.96639°	7.05	53.0	+
		E45.68528°	7.05	50.0	+
	Jermuk G	N39.87944°	6.90	53.0	+
		E45.77417°			
	Arzakan	N40.68389°	7.20	44.0	+
		E44.74111			
	Hankavan	N40.63265°	7.00	44.0	–
		E44.48463°			

n.d., not determined.

Measurement of NOB activity and inhibition

For all experiments, NO_3^- and NO_2^- 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_2^- to NO_3^- was tested. Three enrichments and two uninoculated controls were spiked with 0.1 mM $NaNO_2$, and the conversion of NO_2^- to NO_3^- was monitored. When all NO_2^- was depleted, NO_3^- was measured and enrichments were spiked with an additional 0.1 mM NO_2^-. This process was repeated until 0.9 mM NO_3^- had accumulated.

Second, inhibition of NO_2^- oxidation activity by the inhibitor ClO_3^- was tested. Ten enrichments and six controls were incubated at 50 °C for 6 weeks at which time the NO_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 $NaClO_3$ was added to five of the enrichments. Controls included two uninoculated bottles without $NaClO_3$, two uninoculated bottles with 5 mM $NaClO_3$, and two inoculated bottles that were spiked with 5 mM $NaClO_3$ immediately after inoculation.

Finally, to test the loss of NO_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_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 (GRGTTTGATCCTGGCTCAG) and 1512uR (ACGGHTACCTTGTTACGACTT) (Burggraf

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 (CGGCAGTCCCCTCCGACCTT) and NSP8-R (ATGGGACGGGAAACCGTTCGGA), 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 Miniprep 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 he 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 $\leq$ 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 $\geq$ 61 °C were unsuccessful (n = 13), enrichments from $\leq$ 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_3^- with the depletion of NO_2^-, according to the reaction $NO_2^- + 1/2O_2 \rightarrow NO_3^-$, was used to verify NO_2^- 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

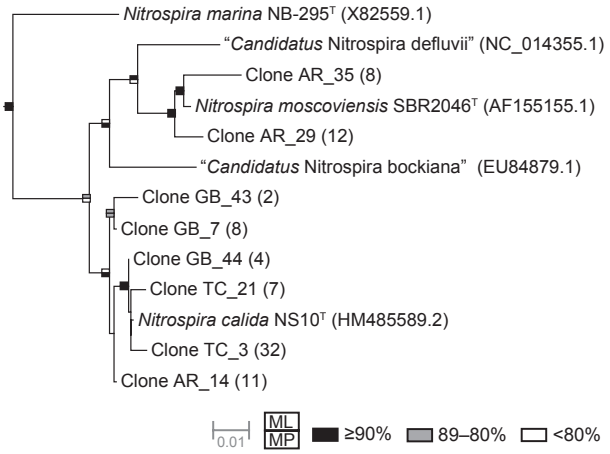

Fig. 1. Phylogenetic tree showing the relationship between NOB in enrichment cultures from GBS (US;GB), Rehai (China; TC), and Jermuk (Armenia; AR) and all cultivated *Nitrospira,*. species. 16S rRNA gene sequences were clustered using mothur at the 98% level. Representative OTUs are shown along with the number of clones represented in parenthesis ($n = 44$ per enrichment). Accession numbers and strain designations are included for cultivated *Nitrospira* species. Bootstrap values > 80% from maximum likelihood (ML) and maximum parsimony (MP) are shown.

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), *Derxia 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 NO_2^- 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 2. OTUs from GBS (US), Rehai (China), and Jermuk (Armenia) enrichments

		Cultured microorganism whose 16S rRNA gene has the highest BLASTN hit to the OTU representative sequence		
Representative sequence	No. in OTU*	Organism name and strain	Accession no.	Id. (%)†
GBS (US)				
Clone_GB_8	10	*Petrobacter succinatimandens* BON4	AY219713.1	89
Clone_GB_7	8	*Nitrospira calida* NS10	HM485589.1	98
Clone_GB_9	8	*Meiothermus timidus* RQ10	AJ871169.1	95
Clone_GB_3	8	*Azospira restricta* SUA2	NR044023.1	91
Clone_GB_44	4	*Nitrospira calida* NS10	HM485589.1	99
Clone_GB_2	4	*Geobacter hephaestius* VeEG4	AY737507.1	84
Clone_GB_43	2	*Nitrospira calida* NS10	HM485589.1	96
Clone_GB_43	2	*Nitrospira calida* NS10	HM485589.1	96
Rehai (China)				
Clone_TC_3	32	*Nitrospira calida* NS10	HM485589.1	99
Clone_TC_21	7	*Nitrospira calida* NS10	HM485589.1	99
Clone_TC_19	5	*Anoxybacillus amylolyticus* MR3C	NR_042225.1	93
Jermuk (Armenia)				
Clone_AR_29	12	*Nitrospira moscoviensis* NSP M-1	NR_029287.1	97
Clone_AR_14	11	*Nitrospira calida* NS10	HM485589.1	98
Clone_AR_35	7	*Nitrospira moscoviensis* NSP M-1	NR_029287.1	96
Clone_AR_19	4	*Anoxybacillus contaminans* R-1222	NR_029006.1	99
Clone_AR_10	3	*Meiothermus timidus* RQ10	AJ871169.1	95
Clone_AR_14	2	*Derxia gummosa* IAM14990	AB089481.1	92
Clone_AR_25	2	'*Candidatus* Chloracidobacterium thermophilum' B	CP002514.1	83
Clone_AR_34	2	*Ignavibacterium album* JCM16511	CP003418.1	98

*Number of sequences in the 16S rRNA gene library represented by the OTU.
†Percent identity to the OTU representative sequence.

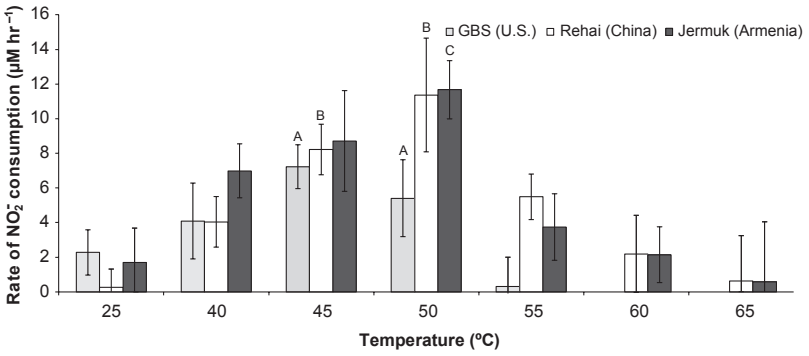

Fig. 2. Optimization of NO_2^- oxidation activity with respect to temperature in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia). NO_2^- and NO_3^- were monitored colorimetrically. The mean rate for each temperature is graphed. Error bars, standard deviation of mean ($n = 3$). Letters indicate rates within an enrichment that are not significantly different ($P > 0.05$; see Materials and methods).

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 × 10^5 *Nitrospira* 16S rRNA gene copies mL^{-1} and the Jermuk enrichment had 1.4–1.6 × 10^6 *Nitrospira* 16S rRNA gene copies mL^{-1}. Using these data, and assuming one 16S rRNA gene per genome (Lücker *et al.*, 2010) and one genome per cell (Åkerlund *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, NO_2^- 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 inhabitants of 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 NO_2^- concentration (Lebedeva *et al.*, 2008), we did not test this dependency in our enrichments. However, we did use low (0.3 mM) NO_2^- concentrations, and it has been reported that low NO_2^- 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 NO_2^- was used (Lebedeva *et al.*, 2011). While the effect of NO_2^- concentrations on the temperature ranges was not explored, we did determine the effect of initial NO_2^- concentration on NOB activity. The experiments revealed that initial NO_2^- 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 NO_2^- (0.1–0.3 mM) resulted in the shortest lag time (Fig. S5). These

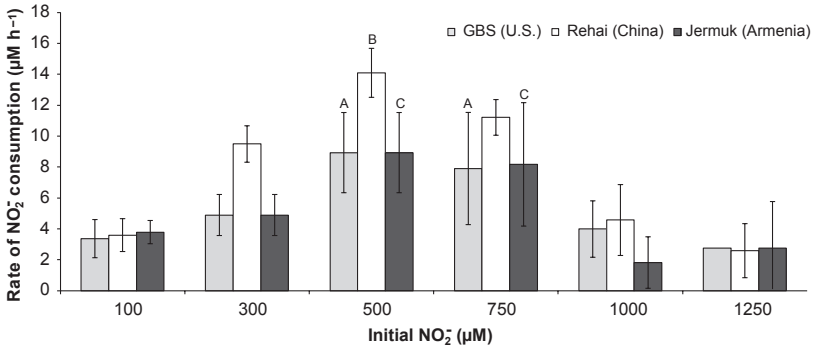

Fig. 3. Optimization of NO_2^- oxidation activity with respect to initial NO_2^- concentration in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia). NO_2^- and NO_3^- were monitored colorimetrically. The mean rate for each temperature is graphed. Error bars, standard deviation of mean ($n = 3$). Letters indicate rates within an enrichment that are not significantly different ($P > 0.05$; see Materials and methods).

results emphasize the importance of using low concentrations of NO_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_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, *Nitrosolancetus* 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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Supporting Information

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

Fig. S1. Stoichiometric conversion of NO_2^- to NO_3^- in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

Fig. S2. Inhibition of NO_2^- oxidation activity by ClO_3^- in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

Fig. S3. Lag of NO_2^- oxidation activity with respect to temperature in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).

Fig. S4. NO_2^- oxidation activities at 60 °C for GBS (US) and Rehai (China) enrichments.

Fig. S5. Lag of NO_2^- oxidation activity with respect to initial NO_2^- concentration in enrichment cultures from GBS (US), Rehai (China), and Jermuk (Armenia).