RESEARCH ARTICLE

Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring

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Abstract

Geothermal environments are a suitable habitat for nitrifying microorganisms. Conventional and molecular techniques indicated that chemolithoautotrophic nitrite-oxidizing bacteria affiliated with the genus Nitrospira are widespread in environments with elevated temperatures up to 55 °C in Asia, Europe, and Australia. However, until now, no thermophilic pure cultures of Nitrospira were available, and the physiology of these bacteria was mostly uncharacterized. Here, we report on the isolation and characterization of a novel thermophilic Nitrospira strain from a microbial mat of the terrestrial geothermal spring Gorjachinsk (pH 8.6; temperature 48 °C) from the Baikal rift zone (Russia). Based on phenotypic properties, chemotaxonomic data, and 16S rRNA gene phylogeny, the isolate was assigned to the genus Nitrospira as a representative of a novel species, for which the name Nitrospira calida is proposed. A highly similar 16S rRNA gene sequence (99.6% similarity) was detected in a Garga spring enrichment grown at 46 °C, whereas three further thermophilic Nitrospira enrichments from the Garga spring and from a Kamchatka Peninsula (Russia) terrestrial hot spring could be clearly distinguished from N. calida (93.6–96.1% 16S rRNA gene sequence similarity). The findings confirmed that Nitrospira drive nitrite oxidation in moderate thermophilic habitats and also indicated an unexpected diversity of heat-adapted Nitrospira in geothermal hot springs.

Introduction

Chemolithoautotrophic nitrite-oxidizing bacteria (NOB) derive their energy by oxidizing nitrite to nitrate, thus performing the second step of aerobic nitrification. Nitrite has a central position in the nitrogen cycle, connecting aerobic nitrification and anaerobic denitrification pathways. In the past few years, our knowledge about organisms involved in biological nitrate transformation has been extended: first, it was discovered that nitrite is involved in anammox—the anaerobic oxidation of ammonia with nitrite as an electron acceptor (Strous et al., 1999). Second, it was found that bacteria belonging to the candidate division ‘NC10’ are able to couple the anaerobic oxidation of methane to denitrification (Raghoebarsing et al., 2006). In both cases, nitrite is converted to dinitrogen gas. Later on, nitrite was identified as an electron donor for anoxygenic photosynthesis (Griffin et al., 2007). Thus, in natural environments, NOB compete for nitrite with other organisms of the nitrogen cycle such as denitrifiers and anammox bacteria (Vlaeminck et al., 2010) and for oxygen with heterotrophic bacteria and ammonia-oxidizing bacteria (Liu et al., 2008). Although nitrification is an aerobic process, members of Nitrospira were found to persist under anoxic conditions (Altmann et al., 2004), to have typical metabolic pathways of microaerophilic bacteria (Lück et al., 2010), and to coexist with planctomycetes performing anammox along an oxygen gradient in a reactor-system (Third et al., 2001). Here, the nitrite-oxidizing activity increased under ammonium limitation, identifying the availability of nitrogen compounds as one selective factor for the competition between the different organisms.
Recent molecular surveys on nitrification in geothermal systems focused on microorganisms affiliated with different lineages of ammonia-oxidizing Archaea (AOA) (Spear et al., 2007; Weidler et al., 2007; Reigstad et al., 2008; Zhang et al., 2008), whereas NOB were mostly unattended. As shown below, all 16S RNA gene sequences of NOB reported so far for terrestrial geothermal systems up to 62°C grouped with the genus *Nitrospira*. Reports about the affiliation of sequences derived from thermal springs at 42°C to *Nitrospira* (Weidler et al., 2007) could not be confirmed after comparative sequence analysis. In addition to terrestrial hydrotherms, *Nitrospira*-related 16S rRNA gene sequences were detected in deep-sea vents at the Mid-Atlantic Ridge (Lopez-Garcia et al., 2003). However, these sequences belong to a phylogenetic cluster without any cultured representative, which falls outside the monophyletic group comprising all currently known sublineages of the genus *Nitrospira* (Daims et al., 2001), and the function of nitrite oxidation needs to be confirmed for these organisms. The family *Nitrospiraceae* (Garrity & Holt 2001) comprises two other genera: *Leptospirillum* and *Thermodesulfovibrio* as well as 'Candidatus Magnetobacterium bavaricum' without the capacity to oxidize nitrite. To study the microbial diversity of NOB in geothermal habitats, only those 16S rRNA gene sequences from previous studies that belonged to the genus *Nitrospira* were taken into account. Genus *Nitrospira*-like rRNA gene sequences were retrieved from geothermal systems with geographically different locations including a hot spring in Thailand with a water temperature up to 55°C (Kanokratana et al., 2004), subterranean thermal Alpine springs (Austria) with a temperature of 42°C (Weidler et al., 2007), and a gold mine hot water stream in Japan with temperatures above 50°C (Hirayama et al., 2005). Furthermore, Anitori et al. (2002) detected *Nitrospira*-like sequences in a South Australian radon-containing hot spring with temperatures up to 63°C.

In addition to the molecular surveys mentioned above, several attempts to cultivate thermophilic nitrifiers originating from hot springs were performed, which focused mainly on AOA (de la Torre et al., 2008; Hatzenpichler et al., 2008). Otherwise, thermophilic representatives of NOB enriched so far exclusively belong to the genus *Nitrospira* (Lebedeva et al., 2005). However, their biology has not been characterized in detail due to the lack of pure or highly enriched cultures. The objective of this work was to bridge this knowledge gap, and here, we describe the first moderately thermophilic species of *Nitrospira*, isolated from the Gorjachinsk hot spring (Russia). Additionally, three moderately thermophilic enrichments from the Garga hot spring, termed GaII, Ga3a, and Ga9-4, which were partly characterized in our previous report (Lebedeva et al., 2005), have been included in this study. For phylogenetic comparison, we analyzed another enrichment of these NOB derived from a hot spring at Kamchatka Peninsula (Russia) (Ns4).

**Materials and methods**

**Source of bacteria**

Deposits and microbial mat material were sampled from the terrestrial Gorjachinsk geothermal spring, located in the Baikal rift zone (Buryat Republic, Russia) in March 2006. The water temperature at the sampling site was 48°C, the pH was 8.6, the mineralization was 8.54 mM and the alkalinity was 2.79 mM. The concentrations of the nitrogen compounds NH₄⁺, NO₂⁻, and NO₃⁻ were 10, 0.26, and 9.35 μM, respectively. The moderately thermophilic nitrite-oxidizing enrichment culture was derived from a microbial mat sample, consisting of two layers. The upper layer was friable, dark green, and 0.1 cm thick, whereas the one beneath was light green with a gelatinous structure and 0.9 cm thick. The sampling site of the terrestrial hot spring in the Uzon Caldera (Kamchatka Peninsula, Far East Russia) was characterized as follows: the temperature was 45°C, the pH was 6.5, and the concentration of ammonium amounted to 34.4 μM. Nitrite or nitrate was not detectable. Environmental data and values of water analyses for the Garga spring in the Baikal rift zone are given by Lebedeva et al. (2005).

**Cultivation**

NOB in the original mat material from the Gorjachinsk spring were quantified using the MPN technique (Schmidt & Belser, 1994). Primary enrichments of moderately thermophilic NOB were grown at 46°C without agitation in mineral salts medium supplemented with 0.3 mM of nitrite as the only energy source. The pH of the basal salts medium (Ehrich et al., 1995) was adjusted by sodium carbonate buffer up to 9.2 after sterilization and declined to 8.4 within 2 days. Later on, if not stated otherwise, the final isolate was cultivated at 46°C in a medium with 1 mM of nitrite and a pH of about 7.6. Cultivation was always conducted in the dark and the oxygen concentration amounted to 4–5 mg L⁻¹. Growth became visible by nitrite consumption concomitant with nitrate production and nitrite was regularly replenished to increase the cell density. Stable development of nitrite-oxidizing enrichments of spiral-shaped cells was achieved after three transfers into a fresh mineral medium with 10% inoculum. Four other *Nitrospira* enrichments from the Garga spring (Lebedeva et al., 2005) and Kamchatkan spring were similarly cultivated using incubation temperatures of 42°C (Ga9-4 and Ga3a) and 46°C (GaII and Ns4).
Physiological properties

The influence of organic matter was checked in mineral salts medium with 1 mM of nitrite, supplemented with 10 mg L−1 of pyruvate or acetate, succinate, formate, or 20 mg L−1 of yeast extract at pH 7.6. The effect of vitamins prepared according to Balch et al. (1979) was also tested. The ability for organotrophic growth was investigated in a complex medium described by Lebedeva et al. (2008). The purity of the isolate was checked by inoculating complex organic medium as mentioned above. After 10 days of incubation at 46 °C, cultures were checked for the absence of turbidity. In addition, the purity of the culture was controlled by phase-contrast microscopy and by denaturing gradient gel electrophoresis (DGGE) analyses using a primer set that targets most Bacteria (341F/907R) (Lane, 1991).

Electron microscopy

Electron microscopy of whole cells and of ultrathin sections was carried out as described by Spieck & Lipski (2011). The samples were observed using a transmission electron microscope (model JEM 100C or LEO-906E, Zeiss).

Analytical procedures

Nitrite and nitrate concentrations were determined quantitatively by HPLC according to Spieck & Lipski (2011).

DNA isolation, PCR, and DGGE analyses

DNA from NOB enrichments was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Partial 16S rRNA gene fragments were amplified by PCR with a semi-specific primer set (341F/662R) targeting the genus Nitrospira and some other organisms (Lane, 1991; Alawi et al., 2007) or with a primer set that targets most Bacteria (341F/907R) (Lane, 1991). For DGGE analysis, a 5′-GC clamp was added to the forward primer. DGGE (Muyzer et al., 1993) was performed with a gradient from 40% to 70% denaturants, and was run at 59 °C and 110 V for 17 h. Bands were extracted from the gel, reamplified, and the partial 16S rRNA gene sequences obtained were compared with public databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Cloning of the 16S rRNA gene

To obtain near full-length sequences for phylogenetic analysis, the 16S rRNA genes were amplified using the primers 27F/1492R (Lane, 1991) or the Nitrospira-targeting primer pair 616V/1158R (Maixner et al., 2006). Purified PCR products were cloned and sequenced as described by Off et al. (2010). 16S rRNA gene sequences derived in this study have been submitted to the GenBank database under accession numbers HM485587–HM485591.

Phylogenetic analysis

All 16S rRNA gene sequences were imported into the ARB software package (Ludwig et al., 2004). Chimeric sequences were checked for using PINTAIL (Ashelford et al., 2005) and omitted from the dataset. After automatic sequence alignment with SINA (http://www.arb-silva.de/aligner/) and manual curation, phylogenetic analyses were performed as described elsewhere (Daims et al., 2001). Only sequences longer than 1400 nucleotides were retained in the alignment. Partial sequences were added to the final tree using the ARB_PARSIMONY function without changing the overall tree topology.

Fatty acid analyses

Biomass for fatty acid extraction was harvested from enrichments grown in 1.5 L medium in 3 L flasks. These large-scale cultures were incubated for several months and were regularly fed with a sterile nitrite solution. Cells of Nitrospira were harvested by centrifugation at 15 000 g for 30 min when dense brownish flocs had developed. The cells from the cultures were washed with 0.9% NaCl (w/v) and stored at −20 °C. Saponification of fatty acids with 15% NaOH in 50% methanol, acid methylation with 6N HCL in 50% methanol, and extraction of fatty acid methyl esters (FAMEs) were performed as described by Sasser (1990). The extracts of fatty acid methyl esters were analyzed by GC-MS using a Hewlett-Packard model 5890 series II gas chromatograph equipped with a 5% phenyl methyl silicone capillary column and a model 5972 mass-selective detector. Chromatography and fatty acid analyses were carried out as described by Lipski & Altenendorf (1997) and by Lipski et al. (2001).

Results

Enrichment and isolation

Moderately thermophilic NOB were enriched using microbial mat material from the Gorjachinsk hot spring inoculated in mineral salts medium with 0.3 mM of nitrite at 46 °C and pH 8.4. Nitrite consumption was obtained within 3–4 weeks. Afterwards, the culture was transferred monthly.
The cell amount of NOB in the mat material was determined to be $7.0 \times 10^5$ cells mL$^{-1}$. One year after starting cultivation, DGGE analyses of 16S rRNA gene fragments obtained with the Bacteria-targeting primer set revealed one dominant band with a position characteristic for Nitrospira-associated PCR products and the presence of two weak bands belonging to concomitant bacteria (not shown). After three dilution series with mineral salts medium at pH 7.6, heterotrophic bacteria were no longer detected when inoculated into a complex organic medium. The isolate was named ‘Ns10’ and used for subsequent morphological, phylogenetic, and physiological characterization. The other moderately thermophilic enrichments of Nitrospira derived from the hot springs Garga and in Kamchatka Peninsula were obtained using a comparable approach, but not investigated in detail. In the DGGE mentioned above, DNA from the enrichments Ns10, GaII, and ‘Candidatus Nitrospira bockiana’ revealed very similar melting behavior in the lower range of the gradient, whereas Nitrospira moscoviensis and ‘Candidatus Nitrospira defluvi’ produced a DNA band in the upper part of the gel (not shown).

**Morphology**

In the liquid medium, the cells occurred mainly as free-living planktonic cells. The cell shape ranged from loosely wound spirals with a variable number of coils (Fig. 1a and c) to slightly curved and even straight rods (Fig. 1b). The cell dimensions ranged from 0.3 to 0.5 μm in width and from 1.0 to 2.2 μm in length. In whole-cell preparations, flagellation of Nitrospira cells was observed (Fig. 1a). As seen in thin sections, cells reproduce by binary fission (Fig. 1d). Isolate Ns10, like other Nitrospira, possessed an enlarged periplasmic space and contained glycogen-like deposits as carbon storage (Fig. 1d).

**Differentiation by DGGE**

Partial 16S rRNA gene amplicons of various mesophilic and moderately thermophilic Nitrospira cultures were screened by DGGE analysis using the genus Nitrospira-targeting primer set. As shown in Fig. 2, only one band was obtained for each sample, excluding the presence of several Nitrospira in the same culture in amounts above the detection limit of DGGE (about 1% of the total bacterial community) (Muyzer et al., 1993). While an identical band was observed for the closely related enrichment GaII, the migration behavior of the band derived from isolate Ns10 was clearly different from amplicons from the other cultures (N. moscoviensis, ‘Candidatus N. bockiana,’ and Ns4 from Kamchatka).

**Phylogenetic affiliation**

Comparative analysis of the near full-length 16S rRNA gene sequence of isolate Ns10 with that from other Nitrospira strains and isolates revealed that it is not closely affiliated with any previously described Nitrospira sublineages (Daims et al., 2001) (Fig. 3). Isolate Ns10 clusters together with the highly similar enrichment GaII from the Garga spring (99.6% 16S rRNA gene sequence similarity), forming a new sublineage (referred to as sublineage VI) within the genus. A partial 16S rRNA gene sequence obtained from a subsurface geothermal water stream (AB113588, Hirayama et al., 2005) falls on the same branch of the phylogenetic tree (Fig. 3), suggesting that lineage VI comprises Nitrospira adapted to elevated temperatures. A second, apparently distinct branch of thermophilic Nitrospira contains the Garga spring enrichment Ga3a and another new Nitrospira enrichment (Ns4), which was obtained from a hot spring in Kamchatka Peninsula (95.3% and 96.1% 16S rRNA gene sequence similarity, respectively). In contrast, enrichment Ga9-4 from the Garga spring (93.6% 16S rRNA gene sequence similarity to Ns10) does not belong to any of these lineages, but

**Fig. 1.** Morphology of Nitrospira calida Ns10, stained with phosphowolframic acid (a) or uranyl acetate (b,c). (a) Spiral-shaped cell with flagella, scale bar = 500 nm; (b) straight rod; (c) spiral cell. (d) Thin section preparation, showing the binary fission of a cell. Scale bar (b)–(d) = 200 nm.
Nitrospira cultures. Amplification was performed with a semi-specific primer set (341F/662R) targeting the genus Nitrospira. From left to right: line 1, enrichment Gall growing at 46°C; line 2, enrichment Ns4 derived from Caldera vulkano Uzon hot spring (Kamchatka) grown at 46°C; line 3, isolate Ns10 incubated at 46°C; line 4, ‘Candidatus Nitrospira bockiana’ (Ns42) grown at 37°C; line 5, Nitrospira moscoviensis grown at 37°C.

is related more closely to sublineage II of the genus Nitrospira (Fig. 3). Additional 16S rRNA gene sequences, which were retrieved from warm environments in other studies, are distributed among various lineages of the genus Nitrospira. Hence, thermophilic Nitrospira are phylogenetically diverse and, as shown for the Garga spring, members of different thermophilic Nitrospira lineages can occur in the same habitat.

Physiological properties

Growth experiments with the enrichment revealed that Ns10 has a maximum nitrite oxidation rate at 46–52°C (Supporting Information, Fig. S1). Keeping in mind that the temperature range of the nitrite-oxidizing activity of Nitrospira can depend on the amount of nitrite (Lebedeva et al., 2008), two different initial concentrations of nitrite (0.3 and 2.5 mM) were checked. In both cases, the culture Ns10 was able to oxidize nitrite within a temperature range of 37–58°C. A substrate concentration of 0.9 mM nitrite was oxidized stoichiometrically to nitrate within 5 days using an incubation temperature of 46°C. Growth of the enrichment Ns10 was inhibited by nitrite concentrations above 6 mM.

The enrichment culture was growing in a broad pH range between pH 7.0 and 8.8 with an optimum at pH 7.8 (Fig. S2). None of the organic compounds or vitamins tested stimulated nitrite oxidation of isolate Ns10. Chemoorganotrophic growth was not observed.

Characterization of the fatty acid composition

Lipids of the thermophilic Nitrospira enrichment cultures from the Gorjachinsk (Ns10) and Garga (GaII) geothermal springs were analyzed after growth at 37°C (only Ns10) and 45–46°C. The major fatty acids were hexadecanoic acid, the cis-7 isomer of hexadecenoic acid, and 11-methyl-hexadecanoic acid in different concentrations (Table 1). Enrichment Ns10 showed a high abundance of 11-methyl-hexadecanoic acid (19% and 38%, respectively) in combination with the cis-7 isomer of hexadecenoic acid (14% and 9%, respectively). In contrast, the enrichment GaII from the Garga hot spring showed a high abundance of the fatty acid 16:1 cis-7 (46%), but 11-methyl-hexadecanoic acid was detectable only in trace amounts (0.8%) here.

Discussion

The diversity of nitrifiers in geothermal environments has received significant research attention in the last few years, and most of these ecological studies have focused on AOA (de la Torre et al., 2008; Hatzenpichler et al., 2008). Compared with the relatively high phylogenetic diversity of AOA in hot environments (Zhang et al., 2008), the diversity of thermophilic NOB seems to be low and restricted to Nitrospira. However, it is not clear whether the detection of nitrate in situ at temperatures above 80°C and at a low pH (Reigstad et al., 2008) results from the activity of hyperthermophilic nitrite oxidizers or may be due to spontaneous, chemical oxidation of nitrite in acid solution (Cai et al., 2001). Furthermore, nitrite is unstable at high temperatures upwards 70°C and is partly auto-oxidized to nitrate even at neutral pH values (E. Spieck, unpublished data).

In contrast to thermophilic AOA, studies on the cultivation and identification of thermophilic NOB are still rare. Nevertheless, our previous results on nitrifying bacteria enriched from the geothermal spring Garga based on molecular and cultivation approaches provided evidence for a high diversity of Nitrospira and their ability to grow at temperatures up to 60°C (Lebedeva et al., 2005). However, so far, no thermophilic isolates or near full-length 16S rRNA gene sequences of these Nitrospira were available. Here, we report on the isolation of a moderately thermophilic chemolithoautotrophic Nitrospira with optimal activity at 46–52°C. A significant feature that distinguished isolate
Ns10 from the Nitrospira species described is the broad temperature range at which nitrite oxidation was observed, ranging from 37 to 58 °C. Similar growth temperatures had been observed previously for the closely related enrichment GaII (Lebedeva et al., 2005). Although the enrichment procedure was carried out at pH 8.4 near the ambient pH of the spring, the optimum pH of the final isolate was 7.8, with a range of growth between pH 7.0 and 8.8. This pH range corresponds to the preferences of alkali-tolerant microorganisms, which are active between pHs 4–7.0 and 8.5 and have a optimum pH of 8.5 (Kevbrin et al., 2004). It is supposed that alkali-protected microsites are important to ensure nitrifying activity in the alkaline habitat. Furthermore, Nitrospira might be protected in situ from pH shifts by very stable microcolonies (Larsen et al., 2008).

The fatty acid profiles of the enrichments Ns10 and GaII resembled those of other members of the genus Nitrospira (Lipski et al., 2001; Spieck et al., 2006; Lebedeva et al., 2008). They are clearly different from known profiles of other nitrite-oxidizing genera such as Nitrobacter, Nitrococcus, Nitrospina, and the new candidate genus Nitrotoga (Alawi et al., 2007). The characteristic lipid combination of the genus Nitrospira is the dominating hexadecanoic acid accompanied by the cis-7 and/or the cis-11 isomere of hexadecenoic acid. The cis-9 isomere of this fatty acid is
usually present in low amounts or lacking. Some representatives of the genus produced 11-methyl-hexadecanoic acid, which up to now has been detected exclusively in *Nitrospira*. Enrichment Ns10 showed high amounts of this characteristic branched lipid, resulting in a fatty acid profile similar to that of the species ‘Candidatus *N. bockiana*’ (Lebedeva *et al*., 2008). This similarity reflects the close phylogenetic proximity between Ns10 and ‘Candidatus *N. bockiana*’ (Fig. 3). The enrichment GaII, although having a 16S rRNA gene almost identical to Ns10, showed a remarkably high shift of 11-methyl-hexadecanoic to cis-7-hexadecenoic acid. This irregularity points to the need for further membrane lipid composition analyses within the different sublineages of the genus *Nitrospira*. This heterogeneity and flexibility of the fatty acid profiles may be an important avenue for the understanding of the membrane adaptation potential to extreme environments such as hot geothermal springs. In particular, the impact of the genus-specific fatty acid 11-methyl-hexadecanoic acid on the membrane viscosity needs to be analyzed in more detail. The fact that this compound was found for almost all cultures growing at an elevated temperature, but absent in mesophilic ones, suggests an influence on membrane fluidity (Table 1). Three different fatty acids (16:1 cis-7, 16:1 cis-11, and 16:0 11-methyl) are typical for *Nitrospira* and can be used as biomarker molecules for the *in situ* detection of these NOB in natural environments. Furthermore, the development of clusterspecific 16S rRNA gene-directed FISH probes will be helpful for identification on the species level.

Interestingly, the phenotypic properties of the genus *Nitrospira* (like the composition of the fatty acids) within a single 16S rRNA gene sequence cluster are not identical for the enrichment cultures GaII and Ns10, as mentioned above. This observation is in agreement with the concept of ‘microevolution,’ meaning the differentiation of individuals in populations (Sikorski, 2008), and corresponds with the finding that the enrichment Ns10 was able to grow at 37°C, whereas this was not the case for the enrichment GaII. So far, only one pure culture from each of the *Nitrospira* sublineages I, II, IV, and V is available. Therefore, further isolates are required to compare the physiological features of strains belonging to the same sequence cluster.

DGGE is a valuable and fast technique for molecular monitoring of enrichment cultures (Ward *et al*., 1997). A complex DGGE migration pattern of PCR amplicons from various moderately thermophilic *Nitrospira* cultures indicated the presence of different species. Consistently, the phylogenetic analyses of near full-length 16S rRNA gene sequences derived from these cultures showed that they belong to three different phylogenetic sublineages of the genus (Fig. 3). Previous analyses (Daims *et al*., 2001) showed a remarkably different environmental distribution of the *Nitrospira* sublineages I–IV in sewage treatment plants, soil, freshwater, and marine habitats. A shared feature like adaptation to elevated temperatures in members from different sublineages indicates that a strict habitat partitioning does not apply to all *Nitrospira* and that members of different sublineages can have overlapping ecological niches. Similarly, mesophilic representatives of sublineages I and II were detected in the same wastewater treatment plants where they had different nitrite concentration optima (Maixner *et al*., 2006). It is thus tempting to speculate that the phylogenetically different organisms GaII, Ga3a, and Ga9-4, which occur in the same geothermal spring, differ in yet unidentified physiological traits that enable niche separation as a basis for their coexistence instead of the competitive exclusion of any one of them. One unique feature common to all moderately thermophilic cultures of *Nitrospira* described here is the high nitrite sensitivity, contrasting the more robust mesophilic cultures, with the exception of some marine cultures (Off *et al*., 2010). This

### Table 1. Fatty acid profiles of *Nitrospira* cultures of Gorjachinsk (Ns10) and Garga (GaII) geothermal springs

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Ns10 (37°C)</th>
<th>Ns10 (45°C)</th>
<th>GaII (46°C)</th>
<th><em>N. moscoviensis</em> M1 (37°C)</th>
<th><em>N. marina</em> 295 (28°C)</th>
<th>Candidatus Nitrospira bocckiana Ns47 (47°C)</th>
<th>Candidatus Nitrospira defluvii A17 (28°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.1</td>
<td>0.4</td>
<td>1.1</td>
<td>0.5</td>
<td>1.4</td>
<td>2.1</td>
<td>7.4</td>
</tr>
<tr>
<td>15:0 iso</td>
<td>–</td>
<td>–</td>
<td>1.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.1</td>
</tr>
<tr>
<td>16:1 cis-7</td>
<td>13.7</td>
<td>9.2</td>
<td>45.8</td>
<td>5.4</td>
<td>30.4</td>
<td>18.3</td>
<td>3.0</td>
</tr>
<tr>
<td>16:1 cis-11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>36.2</td>
<td>15.5</td>
<td>–</td>
<td>42.2</td>
</tr>
<tr>
<td>16:0</td>
<td>57.3</td>
<td>45.0</td>
<td>42.1</td>
<td>19.8</td>
<td>36.5</td>
<td>48.8</td>
<td>15.8</td>
</tr>
<tr>
<td>16:0 11-methyl</td>
<td>19.0</td>
<td>38.3</td>
<td>0.8</td>
<td>33.7</td>
<td>0.8</td>
<td>20.2</td>
<td>–</td>
</tr>
<tr>
<td>16:0 3OH</td>
<td>1.0</td>
<td>0.4</td>
<td>–</td>
<td>0.3</td>
<td>–</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>18:1 cis-11</td>
<td>0.5</td>
<td>0.6</td>
<td>–</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>5.1</td>
</tr>
<tr>
<td>18:0</td>
<td>4.0</td>
<td>3.3</td>
<td>2.2</td>
<td>0.8</td>
<td>8.7</td>
<td>2.1</td>
<td>3.0</td>
</tr>
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</table>

Data for *Nitrospira moscoviensis* M1, *Nitrospira marina* 295 and Candidatus Nitrospira bocckiana Ns47 from Lipski *et al.* (2001); data for Candidatus Nitrospira defluvii A17 from Spieck *et al.* (2006). Compounds below 2% in all cultures were omitted from the table.

Incubation temperatures are given in parentheses.

-- not detected.
finding correlates with the low nitrogen loads measured in the geothermal springs investigated in this study.

The discoveries of thermophilic AOA (de la Torre et al., 2008; Hatzenpichler et al., 2008) and moderately thermophilic nitrite-oxidizing Nitrospira (Lebedeva et al., 2005 and this study) show that complete nitrification is possible in thermal environments and raise the question of whether this N-cycle process evolved under hot conditions (see also Hatzenpichler et al., 2008). The genus Nitrospira contains a considerable number of moderately thermophilic organisms in different phylogenetic sublineages (Fig. 3), but none of these thermophiles forms a phylogenetically deep-branching group. In contrast, the deepest branching known Nitrospira group, sublineage IV, consists of mesophilic marine NOB without any known thermophilic member (Fig. 3). Thus, phylogeny does currently not support a thermophilic origin of Nitrospira, but rather indicates that the thermophilic clusters could represent independent, secondary adaptations to high temperatures. Clearly, more research will be required to gain a better insight into very deep-branching organisms affiliated to Nitrospira. For example, if the yet uncharacterized bacteria detected in hydrothermal sediments (Lopez-Garcia et al., 2003) are able to oxidize nitrite, they could indeed be modern representatives of an ancient thermophilic Nitrospira lineage.

**Taxonomic considerations**

Based on the results of this study, we propose the classification of the moderately thermophilic nitrite-oxidizing Nitrospira isolate Ns10 into a new species, Nitrospira calida, with Ns10 as the type strain.

The short description of *Nitrospira calida* sp. nov. is as follows.

Named with reference to growth at a high temperature (latin calida = hot). The short description of *Nitrospira calida* sp. nov. is as follows.

The organism is phylogenetically allocated to the genus *Nitrospira*, Gram-negative cell wall, motile. Multiplication by binary fission. Obligately chemolithoautotroph. Oxidizes nitrite to nitrate. Carbon dioxide is used as the sole carbon source. Aerobic. The highest rate of nitrite consumption occurs at 46–52 °C within a temperature range of 37–58 °C. Cells range from loosely wound spirals to slightly curved or straight rods. Cell dimensions differ from 0.3 to 0.5 μm in width and from 1.0 to 2.2 μm in length. Did not show ability for mixotrophic or chemoorganotrophic growth. Major fatty acids are 16:0, 16:1 cis-7 and 16:0 11-methyl.

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**References**


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### Supporting Information

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

**Fig. S1.** Temperature dependence of nitrite consumption by *Nitrospira calida* Ns10.

**Fig. S2.** Influence of pH on the nitrite consumption of *Nitrospira calida* Ns10.

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