

Physiological and phylogenetic characterization of a novel lithoautotrophic nitrite-oxidizing bacterium, '*Candidatus Nitrospira bockiana*'

E. V. Lebedeva,¹ M. Alawi,² F. Maixner,³ P.-G. Jozsa,² H. Daims³ and E. Spieck²

Correspondence

E. Spieck

spieck@mikrobiologie.
uni-hamburg.de

¹Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospect 60-let Oktyabrya 7/2, Moscow 117312, Russia

²Universität Hamburg, Biozentrum Klein Flottbek, Mikrobiologie, Ohnhorststr. 18, D-22609 Hamburg, Germany

³Department für Mikrobielle Ökologie, Universität Wien, Althanstr. 14, A-1090 Wien, Austria

A new isolate of a lithoautotrophic nitrite-oxidizing bacterium was obtained from internal corrosion deposits from a steel pipeline of the Moscow heating system. The organism oxidized nitrite as the sole energy source and fixed carbon dioxide as the only carbon source. The cells were extremely pleomorphic: loosely wound spirals, slightly curved and even straight rods were detected, as well as coccoid cells. The highest rate of nitrite consumption (1.5 mM nitrite as substrate) was measured at 42 °C, with a temperature range of 28–44 °C. In enrichment cultures with *Nocardioides* sp. as an accompanying organism, optimal oxidation of 5.8 mM nitrite occurred at 45 °C, with a range of 28–48 °C. Neither pyruvate nor yeast extract stimulated nitrification. Organotrophic growth was not observed. Phylogenetic analysis of 16S rRNA gene sequences revealed that the novel isolate represents a new sublineage of the genus *Nitrospira*. On the basis of physiological, chemotaxonomic and molecular characteristics, the name '*Candidatus Nitrospira bockiana*' is proposed.

INTRODUCTION

Lithoautotrophic nitrifying bacteria (NB) mediate the process of sequential oxidation of ammonia to nitrite and nitrate, known as nitrification. Physiologically, they are separated into ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively). Initially, NB were grouped in the family *Nitrobacteraceae*. Differentiation of the genera was accomplished on the basis of physiological and morphological characteristics and the presence and arrangement of intracytoplasmic membranes (ICMs) (Watson *et al.*, 1989). Molecular techniques such as 16S rRNA gene sequencing have provided evidence for the phylogenetic heterogeneity of the NB (Teske *et al.*, 1994; Ehrich *et al.*, 1995; Purkhold *et al.*, 2000).

Abbreviations: AOB, ammonia-oxidizing bacteria; DGGE, denaturing gradient gel electrophoresis; EPS, extracellular polymeric substances; FISH, fluorescence *in situ* hybridization; ICMs, intracytoplasmic membranes; NB, nitrifying bacteria; NOB, nitrite-oxidizing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of '*Candidatus Nitrospira bockiana*' and the enrichment clone are EU084879 and EU084880, respectively.

A supplementary figure of the DGGE profile showing the increasing purity of the enrichments is available with the online version of this paper.

Until recently, all of the recognized genera of bacterial ammonia oxidizers have been limited to two phylogenetically distinct groups affiliated to the classes *Betaproteobacteria* and *Gammaproteobacteria* within the phylum *Proteobacteria*. The first ammonia oxidizers of the domain *Archaea*, within the phylum *Crenarchaeota*, were isolated by Könneke *et al.* (2005). Existing genera of NOB have been classified taxonomically under two phyla in the domain *Bacteria*. The genera *Nitrobacter*, *Nitrococcus* and *Nitrospira* belong to the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* within the phylum *Proteobacteria*. The genus *Nitrospira* represents a monophyletic lineage within the deep-branching bacterial phylum *Nitrospira* (Ehrich *et al.*, 1995; Spieck & Bock, 2001), where they occur together with '*Candidatus Magnetobacterium bavaricum*' and members of the genera *Leptospirillum* and *Thermodesulfovibrio* (Garrrity & Holt, 2001). So far, the genus *Nitrospira* is represented by two recognized species: *Nitrospira marina* (Watson *et al.*, 1986) and *Nitrospira moscoviensis* (Ehrich *et al.*, 1995), isolated from marine and freshwater habitats, as well as by '*Candidatus Nitrospira defluvi*' (Spieck *et al.*, 2006), highly enriched from activated sludge. Species of the genus *Nitrospira* are regarded to be obligate lithotrophs with the ability to perform mixotrophic growth (Watson *et al.*, 1986).

Investigations performed by cultivation-independent molecular methods (Burrell *et al.*, 1998; Hovanec *et al.*, 1998; Daims *et al.*, 2001) and immunological techniques (Bartosch *et al.*, 1999) clearly indicated that *Nitrospira*-like bacteria are widely distributed in different natural and engineered ecosystems. For example, it was shown that *Nitrospira*-types are the most common nitrite-oxidizers in wastewater treatment plants (Juretschko *et al.*, 1998) and not *Nitrobacter*-types as was previously thought according to culture-based studies. *Nitrospira*-like bacteria were further detected in various soils (Bartosch *et al.*, 2002; Noll *et al.*, 2005) and freshwater sediments (Stein *et al.*, 2001; Altmann *et al.*, 2003). Recently, such organisms were also found to be present in thermophilic communities when studied by molecular and classical techniques (Kanokratana *et al.*, 2004; Lebedeva *et al.*, 2005).

In this study, we describe the physiological and phylogenetic characterization of a novel *Nitrospira*-like bacterium. The organism originated from internal corrosion deposits from a steel pipeline of the Moscow heating system, Russia. The first evidence for the existence of this novel *Nitrospira*-like bacterium was based on data from lipid profiles; in particular, the absence of the lipid component 16:1*cis*11, a feature characteristic of the recognized species of the genus *Nitrospira* (Lipski *et al.*, 2001; Spieck *et al.*, 2006). The novel nitrite-oxidizing bacterium is provisionally named 'Candidatus Nitrospira bockiana' (following the naming convention of Murray & Stackebrandt, 1995).

METHODS

Source of bacteria. The enrichment was carried out using material from internal corrosion deposits from the steel return pipeline of the Moscow heating system, Russia. Samples were collected during repair work in summer 1995. The temperature of water in the pipeline ranged from 40 to 70 °C, depending on the heating season. A detailed description of the habitat from which the bacteria were obtained has been reported previously (Rozanova *et al.*, 2003).

Cultivation. Primary enrichment of NOB was obtained at 42 °C in basal salt medium (Ehrich *et al.*, 1995) supplemented with 1 mM of nitrite as the only energy source. Later, if not stated otherwise, cultivation of enrichment cultures was carried out at 45 °C in 250 ml flasks with 100 ml mineral medium supplemented with 3–7 mM of nitrite. The final isolate was cultivated at 42 °C with 0.3–3 mM of nitrite. Growth was detected by measuring nitrite consumption; nitrite was regularly replaced to increase the cell density. Percoll density-gradient centrifugation and subsequent serial dilutions in a medium supplemented with 0.3 mM of nitrite were performed as described by Ehrich *et al.* (1995).

The influence of organic matter was checked in a mineral medium with 3 mM of nitrite supplemented with pyruvate (55 mg l⁻¹) or yeast extract (20 mg l⁻¹). The effect of vitamins, prepared according to Balch *et al.* (1979), was also tested. A complex medium was used to check whether the enriched organisms were capable of organotrophic growth. For this purpose, the basal mineral medium without nitrite was supplemented with pyruvate (55 mg l⁻¹) as carbon source and yeast extract (150 mg l⁻¹) and peptone (150 mg l⁻¹) as nitrogen sources. The pH of the medium was adjusted to 7.6. Growth was monitored by light microscopic observations.

Isolation and investigation of *Nocardioides* sp. Samples of enrichment 5 were streaked onto plates of solid complex medium as mentioned above and incubated at 42 °C. Single colonies were selected and spread onto plates again to obtain a pure culture. The ability of the isolate, identified as *Nocardioides* sp., to perform denitrification and nitrate reduction was tested in 20 ml tubes filled with complex medium supplemented with 1 g l⁻¹ of sodium nitrate. The tubes also contained Durham tubes for collecting any gas formed by denitrification.

Analytical procedures. Nitrite and nitrate concentrations were determined quantitatively by HPLC in an automated system (MT2; Kontron Instruments). Separation was achieved by ion pair chromatography with a Hypersil ODS C18 column (125 × 4.6 nm) (Meincke *et al.*, 1992). Detection was performed by UV absorption at 225 nm. Consumption of nitrite was detected qualitatively by the Griess-Ilosvay spot test (Schmidt & Belser, 1982). Cell density was determined under a light microscope by direct cell counting.

Electron microscopy. Electron microscopy of whole cells and of ultrathin sections was carried out as described by Spieck *et al.* (2006) and samples were viewed in a transmission electron microscope (model 420, Philips or LEO-906E, Zeiss).

Denaturing gradient gel electrophoresis (DGGE) analyses. The partial 16S rRNA genes in the enrichment cultures were amplified by PCR with the eubacterial primer set 346F/907R (Muyzer *et al.*, 1998). A GC-clamp was added to the forward primer. DGGE was performed at a temperature of 59 °C with a gradient from 50–80 % denaturants. Bands were extracted from the gel and reamplified and the partial 16S rRNA gene sequences were compared with those available on publicly accessible databases by using the Basic Local Alignment Search Tool program (BLAST, NCBI).

Fluorescence *in situ* hybridization. Aliquots of the enrichments were prepared for fluorescence *in situ* hybridization (FISH) by fixation in paraformaldehyde according to Daims *et al.* (2005). Following fixation, the biomass was spotted onto microscope slides and FISH was performed according to Manz *et al.* (1992) and Daims *et al.* (2005). The applied oligonucleotide probes were S-G-Ntspa-0662-a-A-18 (target group: genus *Nitrospira*) (Daims *et al.*, 2001), S*-Ntspa-0712-a-A-21 (target group: phylum *Nitrospira*) (Daims *et al.*, 2001), S*-Ntspa-1151-a-A-20 (target group: sublineage II of the genus *Nitrospira*) (Maixner *et al.*, 2006), S-G-Nbac-1035-a-A-18 (=NIT3; Wagner *et al.*, 1996) (target group: genus *Nitrobacter*) and the EUB probe mix that detects almost all known *Bacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999). The probes were 5'-labelled with either the FLUOS dye [5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester] or the sulfoindocyanine dye Cy3. The *Nitrospira*- and *Nitrobacter*-specific probes were used together with competitor oligonucleotides according to Daims *et al.* (2001) and Wagner *et al.* (1996). All probes and competitors were obtained from Thermo Scientific (Germany). Fluorescence signals were recorded with a confocal laser scanning microscope (LSM 510 Meta; Zeiss) by using an Ar ion laser to detect FLUOS (488 nm excitation wavelength) and a HeNe laser (543 nm) to detect Cy3, respectively.

Cloning and phylogenetic analysis of 16S rRNA genes. Bacterial 16S rRNA genes were amplified by PCR, cloned and sequenced as described by Maixner *et al.* (2006) with the only modification that instead of extracted genomic DNA, 1.5 µl of *Nitrospira* enrichment was added directly to the PCR reaction mix for 16S rRNA gene amplification. Sequence alignments and phylogenetic analyses were carried out using ARB software (Ludwig *et al.*, 2004) according to Daims *et al.* (2001).

RESULTS

Enrichment and isolation of '*Candidatus Nitrospira bockiana*'

The scheme of enrichment and isolation procedure for the novel *Nitrospira* isolate is presented in Table 1. The process was monitored by microscopic observations, FISH, DGGE and 16S rRNA gene sequencing followed by phylogenetic analysis. The enrichment of NOB from material from corrosion deposits (as described above) resulted in the primary enrichment culture of spiral-shaped cells,

cultivated at 42 °C (enrichment 1). In subculture, the consumed nitrite was regularly replaced to obtain a high cell density for the following working steps (enrichment 2). After application of the Percoll technique and subsequent dilution, enrichment 3 was obtained. Electron microscopic observations of ultrathin sections revealed that the most abundant cells resembled those of members of the genus *Nitrospira* (Fig. 1). Rarely encountered cells exhibited structural features of cells of members of the genus *Nitrobacter*, with a polar cap of intracytoplasmic membranes (data not shown). FISH with probes specific for the genus and phylum *Nitrospira* revealed that *Nitrospira* cells

Table 1. Scheme of enrichment and isolation of '*Candidatus Nitrospira bockiana*'

Treatment	Culture	Incubation temperature	Methods used to characterize culture	Composition of the culture
Mineral medium inoculated with environmental sample	Enrichment 1	42 °C	Phase-contrast microscopy	Spiral shaped cells
Cultivation with regular replacement of consumed nitrite	Enrichment 2	42 °C	Phase-contrast microscopy	Spiral shaped cells
Percoll density-gradient centrifugation with subsequent dilution series resulted in harvesting the most diluted (10^{-7}) nitrite-oxidizing culture	Enrichment 3	42 °C	Electron microscopy, ultrathin sections	<i>Nitrospira</i> -like cells + <i>Nitrobacter</i> -like cells + different contaminants
			16S rRNA partial gene sequence FISH	' <i>Candidatus Nitrospira bockiana</i> ' + <i>Nitrospira moscoviensis</i> -like ' <i>Candidatus Nitrospira bockiana</i> ' + <i>N. moscoviensis</i> -like
			Purity test in complex medium	Several types of contaminants
Incubation at elevated temperature	Enrichment 4	45 °C	Electron microscopy, ultrathin sections FISH	<i>Nitrospira</i> -like cells (no <i>Nitrobacter</i>) ' <i>Candidatus Nitrospira bockiana</i> ' (no <i>N. moscoviensis</i> -like cells). Morphologically highly variable nitrite-oxidizing culture
Percoll density-gradient centrifugation with subsequent dilution series resulted in harvesting the most diluted (10^{-7}) nitrite-oxidizing culture	Enrichment 5	45 °C	Electron microscopy, ultrathin sections	2 types of cells: <i>Nitrospira</i> -like + Gram-positive contaminant
			DGGE with subsequent 16S rRNA partial gene sequence	2 bands corresponding to ' <i>Candidatus Nitrospira bockiana</i> ' and <i>Nocardioides</i> sp. (contaminant)
			Purity test in complex medium	One type of contaminant
Dilution series from a highly enriched culture resulted in harvesting the most diluted (10^{-8}) nitrite-oxidizing culture.	Final isolate	42 °C	Electron microscopy, negative staining	Morphologically highly variable <i>Nitrospira</i> cells
			DGGE with subsequent 16S rRNA partial gene sequence FISH	Only one band, corresponding to ' <i>Candidatus Nitrospira bockiana</i> ' ' <i>Candidatus Nitrospira bockiana</i> ' - no <i>N. moscoviensis</i> -like
			Purity test in complex medium	No contaminant

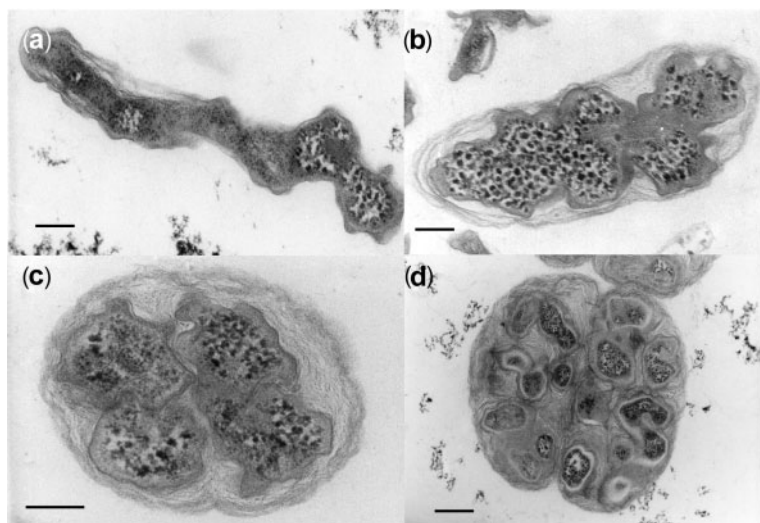


Fig. 1. Electron micrographs of ultrathin sections of cells of '*Candidatus Nitrospira bockiana*' derived from the Moscow heating system (enrichment 3) showing the different stages in the formation of microcolonies. (a) Single planktonic cell. (b) Secretion of EPS accompanied by a decrease in cell length. (c) Small microcolony, where two cells are embedded in a common matrix of EPS. (d) Microcolony of *Nitrospira* containing multiple cells of variable shapes. Bars, 0.25 µm (a–c); 0.5 µm (d).

were abundant in the enrichment, but that it also contained at least two different *Nitrospira* populations (Table 1). Whereas most *Nitrospira* cells were detected only by the genus- and phylum-specific probes, a minor fraction also hybridized to probe S⁻-Ntspa-1151-a-A-20, which is specific for the sublineage II of the genus *Nitrospira* (the *Nitrospira moscoviensis* lineage). At this point, phylogenetic analyses already indicated that the culture contained novel *Nitrospira*-like bacteria (see below). The temperature shift up to 45 °C provided a selective means for discrimination of the novel *Nitrospira* isolates from members of the genus *Nitrobacter* and members of the sublineage II *Nitrospira* (enrichment 4). A second Percoll gradient centrifugation, followed by a dilution series, resulted in enrichment 5. Here, DGGE results indicated that only one type of contaminant was present. To increase the abundance of *Nitrospira* cells, the consumed nitrite was regularly replenished in order to obtain a highly enriched culture with visible turbidity. This culture was the starting material used for several dilution series. The enrichment process was monitored by DGGE analysis, where the intensity of the band belonging to the accompanying organism decreased (see Supplementary Fig. S1 in IJSEM Online). The so-called final isolate, containing '*Candidatus Nitrospira bockiana*' was obtained at the dilution step of 10⁻⁸.

Since this *Nitrospira*-like bacterium was extremely pleomorphic, several tests were done to verify the absence of contaminants and other nitrite oxidizing species: (i) no heterotrophic growth in complex medium was observed, and (ii) DGGE analyses showed that after the purification procedures, the contaminant had disappeared and only one *Nitrospira*-like nitrite-oxidizing bacterium was present (see Supplementary Fig. S1). No members of the genus *Nitrospira* affiliated to sublineage II, members of the genus *Nitrobacter* or any other bacteria except '*Candidatus Nitrospira bockiana*' were detected by FISH (Fig. 2). Thus, based on the results obtained by these monitoring

strategies, the final enrichment was probably a pure culture of '*Candidatus Nitrospira bockiana*'.

Morphology of '*Candidatus Nitrospira bockiana*'

In enrichment 3 and in the culture of the final isolate, the morphology of the *Nitrospira*-like cells differed significantly. In enrichment 3, most *Nitrospira*-like cells were long, spiral rods. At this stage, a transition in cell shape suggesting a life cycle was observed. Initially, very long spiral rods occurred as planktonic cells (Fig. 1a). Then the cell length decreased, a process that was accompanied by intense formation of

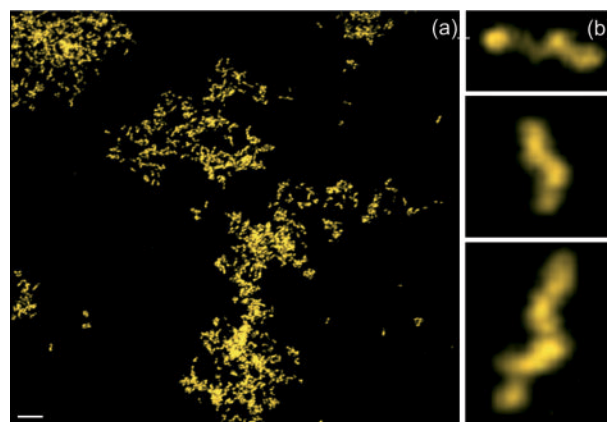


Fig. 2. (a) Detection of '*Candidatus Nitrospira bockiana*' in the final isolate by FISH with probes S-G-Ntspa-0662-a-A-18 (red) and the EUB338 probe mix (green). *Nitrospira* cells appear yellow because of hybridization to all probes. Note the absence of any other bacteria. The contrast of this image was adjusted by image processing software. Bar, 5 µm. (b) Single cells of '*Candidatus Nitrospira bockiana*' detected by FISH. The spiral morphology is clearly visible. The images of these cells were extracted from image (a) and then digitally magnified.

extracellular polymeric substances (EPS) (Fig. 1b). Cells continued to divide inside small microcolonies (Fig. 1c) and finally, numerous short cells were organized in a dense layer of a biofilm matrix, where the spiral cell shape could hardly be detected anymore (Fig. 1d). In contrast, the cells of the final isolate occurred mainly as free-living planktonic cells and demonstrated extremely high morphological variability. Their cell shape ranged from loosely wound spirals (Fig. 3a) with a variable number of coils to slightly curved and even straight rods (Fig. 3b) as well as coccoid cells with a diameter of 0.9 μm (Fig. 3d). The size of the spiral- and rod-shaped cells ranged from 0.3 to 0.6 μm in width and from 1.0 to 2.5 μm in length. The absence of motility was characteristic for all enrichment stages. Cells reproduced by binary or by unequal fission (Fig. 3c). As has already been shown for *Nitrospira moscoviensis* (Spieck *et al.*, 1998), coccoid cells were found to contain membrane-bound particles (Fig. 3e),

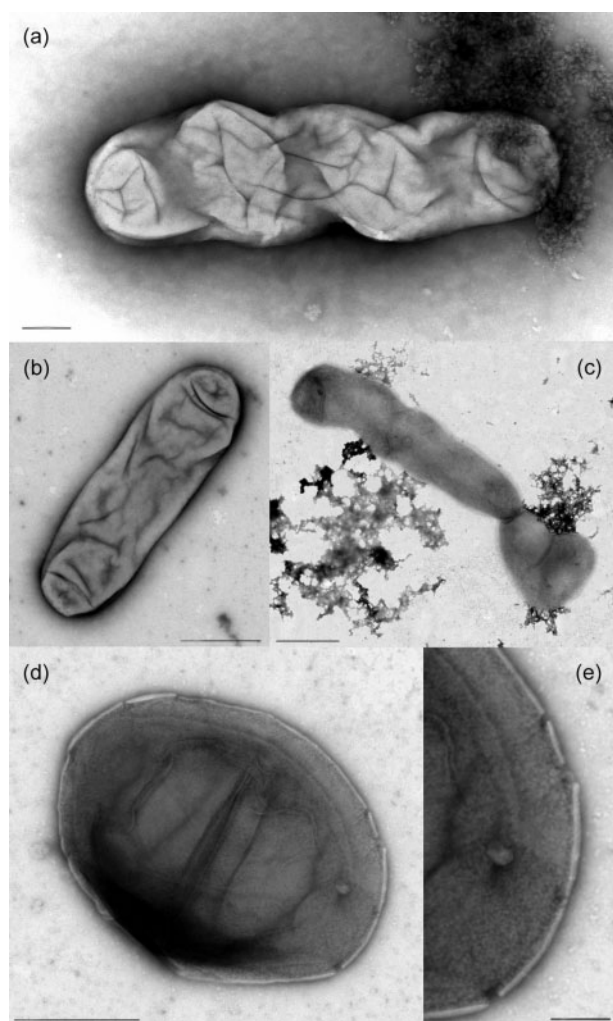


Fig. 3. Overall shape of ‘*Candidatus Nitrospira bockiana*’, stained with uranyl acetate. (a) Spiral rod; (b) long straight rod; (c) unequal fission; (d) coccoid cell; (e) section of (d) showing membrane-bound particles in detail. Bars, 0.2 μm (a); 0.5 μm (b–d); 0.1 μm (e).

as revealed by negative staining. The particulate nitrite oxidizing system was localized on the periplasmic side of the cytoplasmic membrane, shining through the outer membrane in partially lysed cells.

During all enrichment stages, the ultrastructure of the cells was typical of that of members of the genus *Nitrospira* (Fig. 1a and b). In the observed ultrathin sections, the *Nitrospira*-like bacteria possessed an enlarged periplasmic space that is characteristic for this genus (Watson *et al.*, 1986). No intracytoplasmic membranes or carboxysomes were found. As shown by electron microscopy, cells often contained glycogen and polyphosphate-like deposits as storage compounds.

Physiological properties

Physiological investigations started with enrichment 5, which was characterized as a co-culture of ‘*Candidatus Nitrospira bockiana*’ and *Nocardioides* sp. (see Supplementary Fig. S1). Enrichment 5 had the highest rate of nitrite consumption at around 45 °C (Fig. 4) at a pH of 7.6–8.0. The oxidation of nitrite correlated with an increase in the cell concentration (data not shown). The doubling time of the total cell number was between 8–9 h in a mineral medium with 6.8 mM nitrite, which was oxidized within 4 days. Enrichment 5 was characterized by a wide tolerance of high nitrite concentrations, a characteristic previously noted for ‘*Candidatus Nitrospira defluvii*’ (Spieck *et al.*, 2006). The substrate was oxidized at concentrations up to 26–30 mM (Table 2).

In contrast to enrichment 5, the temperature optimum of the final isolate dropped to 42 °C (Fig. 4). The highest tolerated nitrite concentration was also reduced to 18 mM (Table 2). The rate of nitrite consumption of the final isolate was significantly lower when compared with enrichment 5, e.g. 5 mM of nitrite was consumed within 10 days. Optimal growth occurred in a mineral salt medium with a relatively low nitrite concentration (0.3–1.5 mM). The addition of organic substances or vitamins did not enhance nitrite oxidation. Chemo-organotrophic growth was also not observed.

It was found that the temperature range of the nitrite-oxidizing activity largely depended on the amount of nitrite. Starting with a low substrate concentration of 0.3 mM, enrichment 5 and the final isolate were able to oxidize nitrite between 17 and 48 °C and 17 to 44 °C, respectively, while 1.5 mM nitrite was oxidized within a temperature range of 28–48 °C and 28–44 °C, respectively. Using a higher concentration of 6 mM nitrite, growth of enrichment 5 was restricted to an even narrower temperature range of 37 to 48 °C.

Characterization of the contaminant

Based on phylogeny using partial 16S rRNA gene sequences, the accompanying heterotrophic organism was

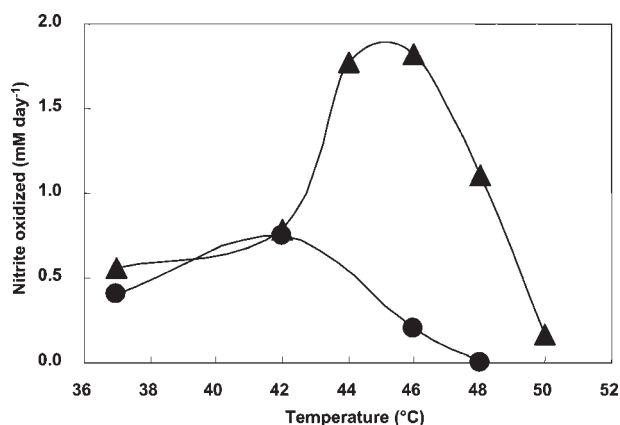


Fig. 4. Temperature optima for nitrite consumption by enrichment 5 and 'Candidatus Nitrospira bockiana'. ▲, enrichment 5; inoculum was taken from a pre-culture grown at 45 °C. Nitrite consumption was measured between day 3 and 4 with 5 mM as substrate concentration. ●, final isolate; inoculum was taken from a pre-culture grown at 42 °C. Nitrite consumption was measured between day 4 and 5 with 1.5 mM as substrate concentration.

identified as *Nocardioides* sp. in the family *Nocardiaceae*, a member of the phylum *Actinomycetales* (Yoon *et al.*, 2005). The *Nocardioides* sp. was Gram-positive, aerobic, rod-shaped ($0.4\text{--}0.7 \times 0.5\text{--}1.3 \mu\text{m}$) or coccoid ($0.7 \mu\text{m}$ in diameter) cells. No motile forms were observed. The organism was able to perform reduction of nitrate, but not complete denitrification.

Phylogenetic analysis

Phylogenetic analyses of 16S rRNA genes revealed that the final isolate, 'Candidatus Nitrospira bockiana', was a novel *Nitrospira*-like bacterium which did not group with any previously known sublineages (Daims *et al.*, 2001) of the genus *Nitrospira* (Fig. 5). Three partial 16S rRNA gene sequences of 'Candidatus Nitrospira bockiana' (1158 nucleotides) from the final isolate were analysed and found to be highly similar to each other (99.8–99.9% gene sequence similarity). The few base differences were probably due to PCR or sequencing errors. From earlier enrichments (but not from the final isolate), 16S rRNA gene sequences were retrieved for a second *Nitrospira*-like bacterium that fell into sublineage II of the genus *Nitrospira*, a lineage that also contains the cultured species *Nitrospira moscoviensis* (Fig. 5). This finding is consistent with the results of FISH with different *Nitrospira*-specific probes shown above.

DISCUSSION

In this paper, we describe the isolation and physiological characterization of a novel lithoautotrophic nitrite-oxidizing bacterium. This is the second investigation of a novel species of a nitrite-oxidizing bacterium belonging to the genus *Nitrospira* derived from the Moscow heating system. It is interesting that in the first enrichments obtained in this study at least two different strains of *Nitrospira* were found. The coexistence of various *Nitrospira* strains in the same environment has been earlier reported in studies on activated sludge and biofilms (Maixner *et al.*, 2006) and in grassland soils (Freitag *et al.*, 2005). Similarly, the coexistence of different strains of *Nitrospira* in a hot spring of the

Table 2. Comparison of key characteristics of the different species belonging to the genus *Nitrospira*

Taxa: 1, 'Candidatus Nitrospira bockiana'; 2, 'Candidatus Nitrospira defluvii'; 3, *Nitrospira moscoviensis*; 4, *Nitrospira marina*. Data were taken from Spieck *et al.* (2006), Spieck & Bock (2001) and Lipski *et al.* (2001).

Characteristic	1	2	3	4
Cell morphology	Spirals, curved and straight rods or coccoid cells	Short, slightly curved cells or spiral-shaped rods	Irregularly shaped cells or spiral-shaped rods	'Comma-shaped' cells or spiral-shaped rods
Size (μm)	$0.3\text{--}0.6 \times 1.0\text{--}2.5$ or 0.9×0.9	$0.2\text{--}0.4 \times 0.7\text{--}1.7$	$0.2\text{--}0.4 \times 0.9\text{--}2.2$	$0.3\text{--}0.4 \times 0.8\text{--}1.0$
Turns	1–4	1–4	1–3	1–12
Tendency to aggregate	Present	Strong	Present	Weak
Optimum growth temperature (°C)	44–46*, 42†	28–32	39	28
Medium	Non-marine	Non-marine	Non-marine	Marine
Phylogenetic affiliation in genus <i>Nitrospira</i>	Novel sublineage	Sublineage I	Sublineage II	Sublineage IV
Dominant lipids	16:1 <i>cis</i> 7, 16:0, 16:0 11 methyl	16:1 <i>cis</i> 11, 16:0	16:1 <i>cis</i> 11, 16:0, 16:0 11 methyl	16:1 <i>cis</i> 7, 16:1 <i>cis</i> 11, 16:0
Tolerance against nitrite (mM)	26–30*, 18†	20–25	15	6

*Enrichment 5.

†Final isolate.

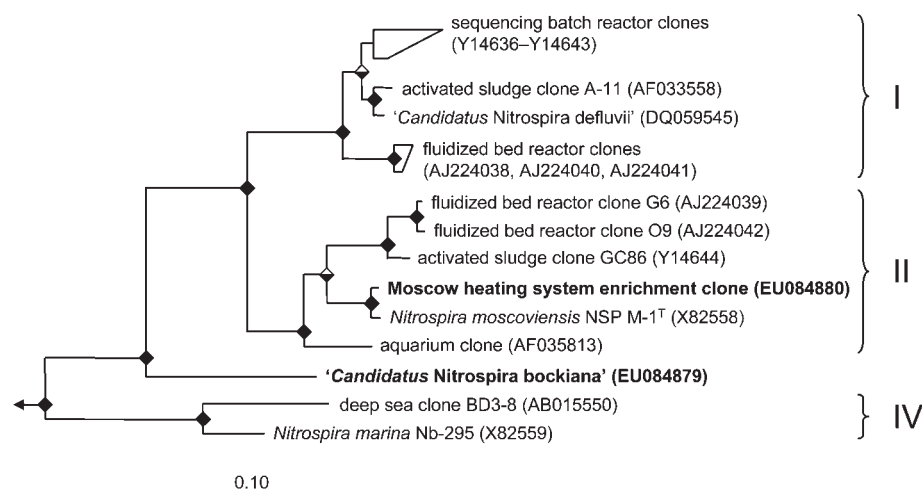


Fig. 5. Phylogenetic affiliation of '*Candidatus Nitrospira bockiana*'. The phylogenetic tree is based on 16S rRNA genes of selected *Nitrospira*-like bacteria. Sequences of '*Candidatus Nitrospira bockiana*' and of another *Nitrospira*-like bacterium, which was found only in the early enrichment, are highlighted in bold. The tree calculation was performed using the maximum-likelihood algorithm of the ARB software package (Ludwig *et al.*, 2004). A 50% conservation filter for the genus *Nitrospira* was applied to 1158 informative positions of the 16S rRNA genes. Partially filled diamonds represent quartet puzzling reliability values $\geq 70\%$. Filled diamonds symbolize additional high parsimony bootstrap support ($\geq 90\%$) based on 100 iterations. Previously defined (Daims *et al.*, 2001) sublineages of the genus *Nitrospira* (numbers I, II and IV) are shown with brackets. Bar, 10% estimate sequence divergence.

Baikal rift zone was suggested by partial 16S rRNA gene sequence analysis of DNA fragments (Lebedeva *et al.*, 2005).

Adaptation to higher temperatures is a physiological feature which distinguishes the novel *Nitrospira*-like isolate from *Nitrospira moscoviensis* and *Nitrospira marina* as well as from '*Candidatus Nitrospira defluvii*' (Table 2). During the enrichment and isolation procedures, this characteristic was used successfully in order to discriminate '*Candidatus Nitrospira bockiana*' from the genus *Nitrobacter* and members of the sublineage II of the genus *Nitrospira*.

The painstaking isolation of '*Candidatus Nitrospira bockiana*' described in this study is the result of 12 years of work. The main reasons for the difficulties in purification were the abilities of the organism to form extracellular polymeric substances and to form microcolonies that also enclosed contaminants. Once purification procedures for free-living planktonic cells were employed, an enrichment that could be further purified by dilution techniques was finally obtained. The results of classical (no growth observed in complex medium) and molecular (DGGE, FISH) tests suggest that the finally-obtained culture was free from contaminants and other NOB. However, the presence of low numbers of other organisms cannot be excluded as the detection limit of FISH is 10^3 – 10^4 cells ml^{-1} (Amann, 1995) and populations below 1% of the total bacterial community may not be detectable by DGGE (Muyzer *et al.*, 1993).

It is interesting to note that *Nitrospira* strains growing in co-culture with a *Nocardioide* sp. (enrichment 5) could resist higher nitrite concentrations and were shown to

possess a higher temperature tolerance (Table 2) when compared with the final isolate. Keeping in mind the loss of organic matter by autotrophically growing NOB (Rittmann *et al.*, 1994; Kindaichi *et al.*, 2004), *Nitrospira* strains might supply the *Nocardioide* sp. with organic carbon for cell growth. The reason for the stimulation of *Nitrospira* strains by the contaminant is still unknown and remains to be clarified in further studies.

The data from lipid profiles were in accordance with the results of the 16S rRNA sequence analyses and suggest that '*Candidatus Nitrospira bockiana*' is related, but not identical, to the recognized species of the genus *Nitrospira*. As shown by Lipski *et al.* (2001), the culture contains the dominant lipid components 16:0; 16:0 11 methyl and 16:1*cis*7 and lacks 16:1*cis*11, a lipid profile that is typical of the other recognized *Nitrospira* species. Based on physiological differences, chemotaxonomic data and on 16S rRNA gene sequence analysis, we provisionally designate this organism as '*Candidatus Nitrospira bockiana*' (as per the naming convention proposed by Murray & Stackebrandt, 1995).

Description of '*Candidatus Nitrospira bockiana*'

Nitrospira bockiana (bock.i.a.na. N.L. fem. adj. *bockiana* named after Professor Eberhard Bock, a microbiologist who devoted his research to the investigation of NB).

The organism is phylogenetically related to the genus *Nitrospira*. Gram-negative. Multiplication takes place by binary as well as by unequal fission. A chemolithoautotroph that oxidizes nitrite to nitrate and is able to use carbon

dioxide as a sole carbon source. The highest rate of nitrite consumption occurs at 42 °C, with a temperature range between 28 and 44 °C. Pleomorphic cells range from loosely wound spirals with a variable number of coils to slightly curved and even straight rods, as well as coccoid cells (0.9 µm diameter). The width of the spiral and rod-shaped cells ranges from 0.3 to 0.6 µm and the length ranges from 1.0 to 2.5 µm. Major fatty acids are 16:0; 16:0 11 methyl and 16:1 cis7. Neither pyruvate nor yeast extract stimulates nitrite oxidation. Organotrophic growth is not observed.

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