universität wien

# DISSERTATION

Titel der Dissertation

## The ecophysiology of nitrite-oxidizing bacteria in the genus *Nitrospira*: Novel aspects and unique features

angestrebter akademischer Grad

**Doktor der Naturwissenschaften (Dr. rer. nat.)**


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| Verfasser: | Frank Michael Maixner |
| Matrikel-Nummer: | 0409617 |
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| Betreuer: | Univ.-Prof. Dr. Michael Wagner |


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*meiner Familie*

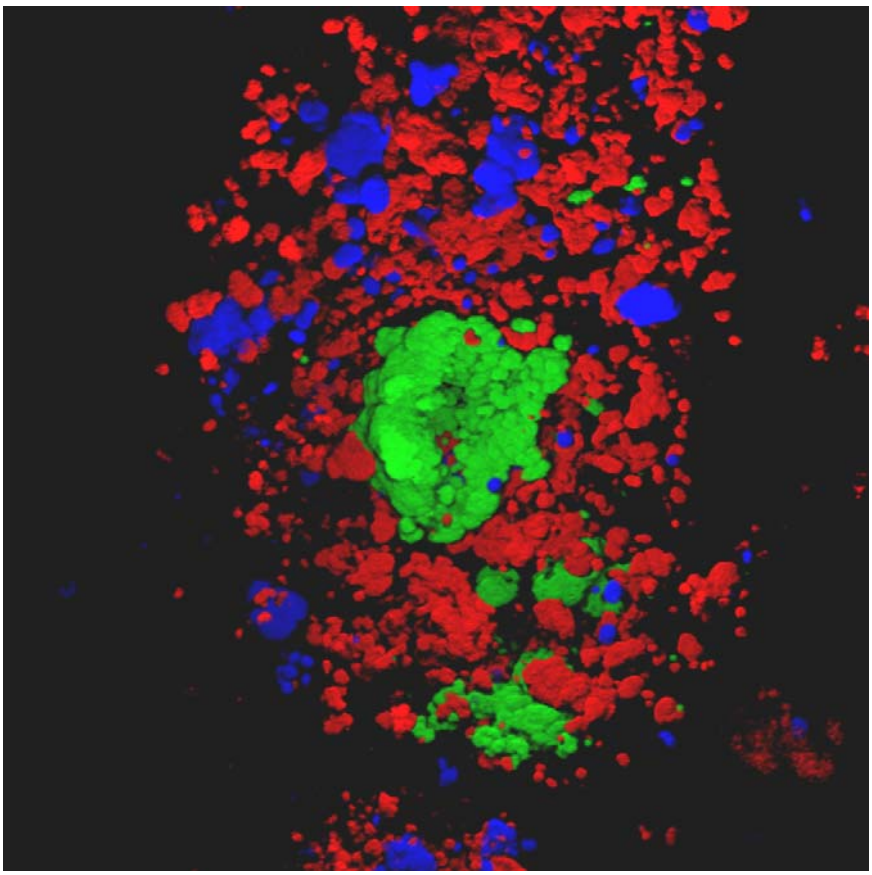
## Contents

## Abbreviations

| | |
|---|---|
| A | adenine |
| AmoA | ammonia monooxygenase subunit A |
| ARB | *Arbor* (software package comprising various tools for sequence and phylogenetic analysis) |
| AOA | ammonia-oxidizing archaea |
| AOB | ammonia-oxidizing bacteria |
| C | cytosin |
| CLD | chlorite dismutase |
| CLSM | confocal laser scanning microscope;<br>confocal laser scanning miocroscopy |
| DNA | deoxyribonucleic acid |
| *et al.* | *et alii* |
| Fig. | figure |
| FISH | fluorescence *in situ* hybridization |
| G | guanine |
| Hao | hydroxylamine oxidoreductase |
| N | nitrogen |
| *N. defluvii* | "*Candidatus* Nitrospira defluvii" |
| NOB | nitrite-oxidizing bacteria |
| Nxr | nitrite oxdioreductase |
| PCR | polymerase chain reaction |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| T | thymine |
| t | ton |
| U | uracile |
| wwtp | wastewater treatment plant |

# Chapter I

## General Introduction and Outline



**Front**: 3D visualization of the nitrifying community in a biofilm sample stained by fluorescence *in situ* hybridization with the probes S-*-Ntspa-1431-a-A-18 (sublineage I *Nitrospira*, labelled with Cy3, red), S-*-Ntspa-1151-a-A-20 (sublineage II *Nitrospira*, labelled with FLOUS, green) and NSO1225 (ammonia-oxidizing bacteria, AOB, labelled with Cy5, blue). AOB and nitrite-oxidizing bacteria (NOB) of the genus *Nitrospira* are in close vicinity to each other. Note the different spatial arrangement of the two *Nitrospira* sublineages relative to AOB in the biofilm (see also Chapter II).

# General Introduction

## 1. The Global Nitrogen Cycle

100 years after Fritz Haber patented the process to "synthesize ammonia from its elements", we nowadays live in a world which is highly dependent upon, and transformed by man-made fixed nitrogen (Erisman *et al.*, 2008). At first the "Haber-Bosch" nitrogen allowed large scale production of explosives resulting in millions of war victims. Second the ammonia is a starting product in several sectors in chemical industries producing plastics and synthetics. Most of the fixed nitrogen, however, is used to produce fertilizer in the forms of ammonium nitrate, calcium nitrate, ammonium bicarbonate, and several mixtures of nitrogen and phosphorous containing compounds. Since 1960, accompanied by an increase of the world population by 78% (since 1970), large scale production of synthetic nitrogen fertilizer supports at least half of the human population by increased food supply (Centre for Ecology and Hydrology, 2008, Galloway *et al.*, 2008) (Fig. 1).

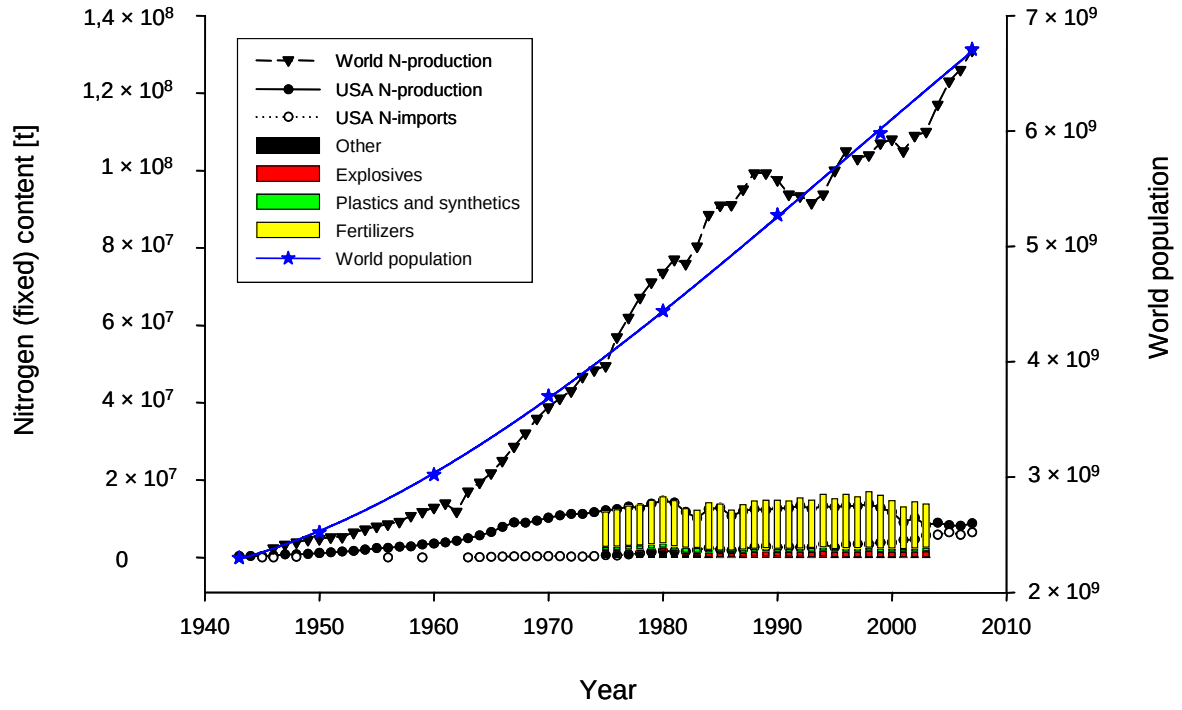



Fig. 1: Trends in worldwide industrial ammonia production (via the Haber-Bosch process) and human population throughout the last 70 years. Partitioning of synthetic fixed N to different branches of production in the United States of America between the years 1975 and 2003 indicated in bars, color-coded according to the legend. [Data sources: http://minerals.usgs.gov/ (industrial N-production); Chamie, 1999 (world population)].

Beside the nitrogen emitted to the atmosphere during fossil fuel burning the two major sources of anthropogenic nitrogen released into the environment are fertilizers and wastewater. Less than 30% of the synthetic nitrogen in fertilizers reaches the end- products (Smil, 2001). The remaining nitrogen is either lost via emission of elemental nitrogen, nitric oxides and ammonia or through leaching of nitrate. To counteract this loss often more fertilizer is used in agriculture than needed. A recent study addressed the problem of excessive nitrogen fertilization in agricultural areas in China (Ju *et al.*, 2009). Ju and coworkers tracked the fate of fertilizer nitrogen and could show that with a higher load of fertilizer, plants are less efficient at taking up synthetic nitrogen. As a result most of the nitrogen leached into the ground and surface water. A reduction of nitrogen fertilizer by two thirds and better fertilization timing would reduce environmental nitrogen contamination without compromising crop yields (Qiu, 2009).

The extraordinary population growth in the twentieth century resulted in a dramatically increased nitrogen deposition into the environment. On the one hand there is the aforementioned excess of fertilizer load due to the increased demand for food. Furthermore, the increasing population causes a higher wastewater deposition and mainly in less developed regions this wastewater ends up untreated in the environment. Therefore, beside better nitrogen fertilizer management the efficient elimination of nitrogen in modern wastewater treatment plants is a crucial process to reduce the import of nitrogen compounds into the environment.

The increased anthropogenic nitrogen deposition destabilized ecosystems in most parts of the world and resulted in major human health effects and unintended environmental consequences (Vitousek *et al.*, 1997, Galloway *et al.*, 2008). On the one hand, atmospheric reactions of emitted ammonia, nitric oxides, and sulfur oxides result in fine particle formation (Sharma, 2007), which increase the risk of cardiovascular and pulmonary diseases (Dominici *et al.*, 2006, Neuberger *et al.*, 2007). Additionally, nitrogen-compounds like ammonia and nitrite are highly toxic to aquatic life (Arthur *et al.*, 1987) and elevated nitrite and nitrate levels in drinking water can have severe health consequences to humans (Schneider & Selenka, 1974, Ward *et al.*, 2005). Beside these direct toxic effects of several nitrogen-compounds on living organisms, raised deposition of anthropogenic nitrogen compounds into natural habitats causes fatal ecological damages. Some environmental effects particularly noteworthy are coastal eutrophication due to import of significant amounts of nitrogen through rivers (Howarth & Marino, 2006) resulting in anoxia and hypoxia of the sea bottom (Rabalais, 2002, Rabalais *et al.*, 2002) or elevated nitrogen deposition linked to eutrophication of different

natural areas resulting in a considerable loss of floral biodiversity (Phoenix *et al.*, 2006). Beside the eutrophication of terrestrial and aquatic systems the interaction of the nitrogen and carbon cycle and the resulting global acidification has already and will further have severe consequences on earth climate (Gruber & Galloway, 2008). Recently, for example, aquatic invertebrates in nitrate-rich environments have been shown to emit the potent greenhouse gas nitrous oxide in quantitatively important amounts (Stief *et al.*, 2009).

In summary, this manmade nitrogen deposition caused an ecological imbalance into many ecosystems and the effects would have been even more dramatic without the high buffering capacity of nitrogen cycling microorganisms. However, to effectively monitor ecological changes due to anthropogenic pressure a better understanding of the mechanisms behind the global nitrogen cycle is urgently needed. It is important to understand the nitrogen cycle at a range of scales of biological organizations, beginning with the vast microbial diversity involved in nitrogen conversion and their interactions (Horner-Devine & Martiny, 2008). Especially during the last decade, groundbreaking and surprising findings in microbial ecology such as bacteria capable of anaerobic ammonia oxidation (ANAMMOX) (Mulder *et al.*, 1995) or the existence of ammonia oxidizing archaea (Treusch *et al.*, 2005, Könneke *et al.*, 2005) have shown that our knowledge of the nitrogen cycle and the involved microbial key players is still scarce.

The following sections will focus on nitrite-oxidizing bacteria (NOB) of the genus *Nitrospira*, one of the aforementioned key players in the nitrogen cycle but yet less intensively studied groups of NOB.

## 2. The Biological Nitrogen Cycle: Microorganisms keep the wheel turning

All forms of life require nitrogen as integral part of proteins and nucleic acids, and thus every living organism participates in the nitrogen-cycle. Microorganisms, however, catalyse essential steps in the global biochemical nitrogen-cycle that other organisms are not able to accomplish (Fig. 2). In microbial nitrogen fixation, gaseous dinitrogen is reduced by the enzyme nitrogenase to ammonia and subsequently assimilated into cell material (Zehr *et al.*, 2003). Ammonia is released during microbial decomposition of organic substances, a process termed ammonification or mineralization (McLain & Martens, 2005).

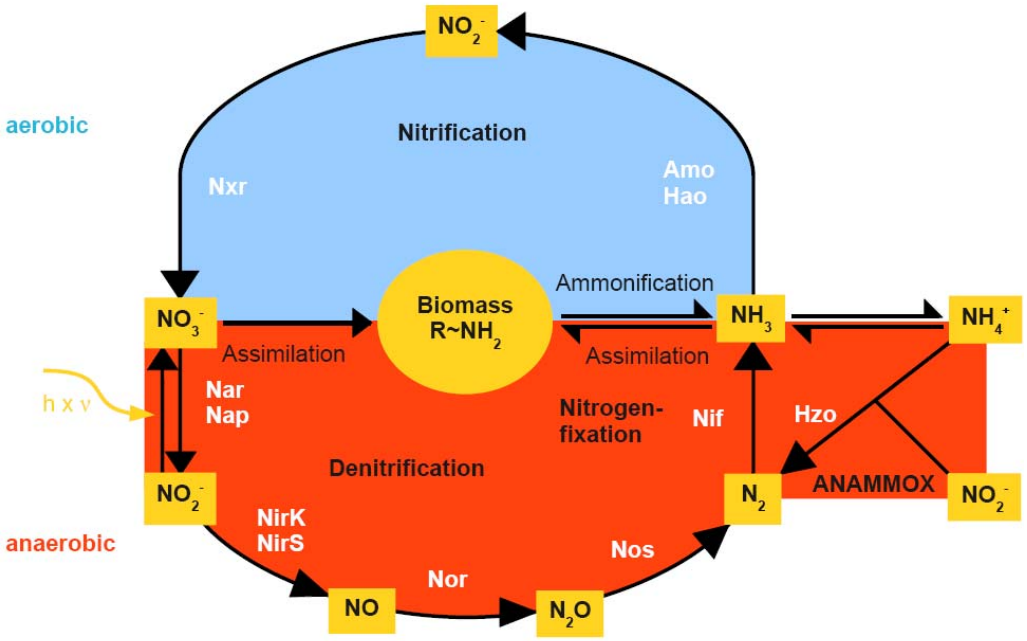



Fig. 2: Schematic representation of the N-cycle. Functional key enzymes involved in important redox-reactions of oxidized and reduced N-compounds (yellow boxes) are displayed in white with the following abbreviations: Amo, ammonia monooxygenase; Hao, hydroxylamine oxidoreductase; Nxr, nitrite oxidoreductase; Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; NirK/NirS, nitrite reductase; Nor, nitric oxide reductase; Nos; nitrous oxide reductase; Nif, nitrogenase; Hzo, hydrazine oxidoreductase.

At neutral pH most of the ammonia is available in form of ammonium. One part of the released ammonium is rapidly recycled and converted into amino acids in microorganisms and plants. However, especially in alkaline soils ammonia is lost via evaporation. The remaining ammonia/ammonium can be catabolized either aerobically or anaerobically in two distinct microbial key processes in the nitrogen-cycle. Under oxic conditions, microorganisms gain energy from oxidizing ammonia to nitrite and subsequently nitrite to nitrate, a process

termed nitrification catalysed by two distinct functional groups of chemolithotrophic prokaryotes: the ammonia-oxidizing bacteria (AOB) and archaea (AOA), and the nitrite-oxidizing bacteria (NOB) (Prosser, 1989, Bock & Wagner, 2006, Könneke *et al.*, 2005). In anoxic habitats, however, ammonium can be catabolized by physiologically specialized planctomycetes in a process called ANAMMOX (Kuenen, 2008). This reaction couples ammonium oxidation to nitrite reduction with dinitrogen as final product.

Whereas the gaseous product of the ANAMMOX process is released into the atmosphere, the nitrate produced via nitrification is either readily assimilated by plants and microorganisms or it is used by facultatively anaerobic organisms as the respiratory electron acceptor under anaerobic conditions (denitrification) (Zumft, 1997, Philippot & Hallin, 2005). Denitrification is widespread among different bacterial genera. Furthermore, the distribution of denitrification extends beyond the bacteria to the archaea and fungi, and surprisingly, complete denitrification was also discovered in a benthic foraminifer (Risgaard-Petersen *et al.*, 2006). In the first step of denitrification nitrate is reduced to nitrite, followed by the subsequent reduction of nitrite to nitric oxide. Furthermore, the gaseous nitric oxide serves in lieu of dioxygen as terminal electron acceptor and is reduced to nitrous oxide, which is subsequently reduced to dinitrogen. Hence, the denitrification and ANAMMOX processes return dinitrogen to the atmosphere from terrestrial and aquatic habitats, thereby completing the nitrogen-cycle. In addition to these anaerobic nitrogen-cycle processes, a recent study describes a previously unknown reaction in which anoxygenic phototrophic bacteria closely related to *Thiocapsa roseopersicina* use nitrite as an electron donor for photosynthesis (Griffin *et al.*, 2007). However, the ecological importance of these nitrite-oxidizing phototrophs and their influence on the global biochemical nitrogen-cycle remain to be determined.

The next sections deal with the aerobic key process of the nitrogen-cycle, nitrification. The main focus is on microorganisms involved in nitrite-oxidation, their phylogenetic assignment and their major biochemical properties.

## 3. Nitrification – the oxidation of ammonia to nitrate

Different inorganic substances can serve for a highly specialized group of microorganisms, the chemolihotrophs, as source of energy and reductants used for cell biosynthesis and maintenance (Peck, 1968, Kelly & Wood, 2006). Chemolithotrophs can be classified according to their substrate preference in functional groups such as iron oxidizers or hydrogen-oxidizing bacteria.

This chapter focuses on nitrification, the aerobic, sequential oxidation of ammonia to nitrite and nitrite to nitrate (Fig. 3). Nitrification is carried out by two spezialized groups of chemolithotrophic microorganisms: ammonia-oxidizing bacteria (AOB) and archaea (AOA), and nitrite-oxidizing bacteria (NOB). Since their discovery more than 100 years ago (Winogradsky, 1890) lithotrophic bacterial nitrifiers conventionally have been classified as one family, the *Nitrobacteriaceae* (Buchanan, 1917, Watson, 1971).

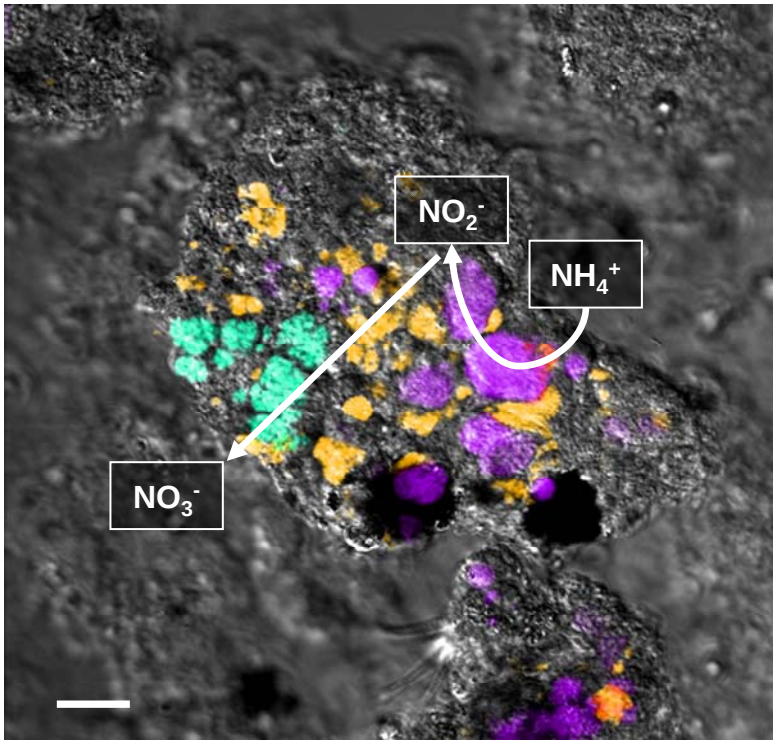



Fig. 3: Ammonia-oxidizing bacteria (AOB, purple) and nitrite-oxidizing bacteria (NOB, *Nitrospira* sublineage I, yellow, *Nitrospira* sublineage II, turquoise) detected by FISH in a nitrifying biofilm sampled from a sequencing batch biofilm reactor. Indicated in the image is the sequential oxidation of ammonia to nitrate during nitrification. Note the close co-aggregation of AOB and NOB in the biofilm. Bar = 20 µm.

However, by comparative analysis of small subunit rRNA gene sequences, as initially elaborated by Carl Woese and coworkers (Fox *et al.*, 1977, Woese, 1987), the nitrifier classification was totally revised. Comparative 16S rRNA sequence analysis revealed that most known AOB and NOB belong to different subclasses of *Proteobacteria* (Fig. 4) (Teske *et al.*, 1994, Purkhold *et al.*, 2000, Koops *et al.*, 2003, Alawi *et al.*, 2007). The only exception, however, are NOB of the genus *Nitrospira* that form a distinct phylum within the domain *Bacteria* (Ehrich *et al.*, 1995). Furthermore, it is tempting to speculate, that *Nitrospina*-like NOB, provisionally assigned to the delta-subclass of *Proteobacteria* (Teske *et al.*, 1994), represent in addition to *Nitrospira* an independent line of descent within the *Bacteria* (Fig 4).
In comparison to the 16S rRNA data, sequence analysis of the *amoA* gene, which encodes the alpha subunit of ammonia monooxygenase, suggested a similar evolutionary relationship of AOB (Purkhold *et al.*, 2000). Based on the phylogeny of both marker genes so far all isolated aerobic lithoautotrophic ammonia-oxidizer can be assigned to two monophyletic groups in the beta-subclass and gamma-subclass of the *Proteobacteria*. Recent studies, however, suggested *amo* gene homologues on metagenomic sequences derived from mesophilic crenarchaea (Venter *et al.*, 2004, Treusch *et al.*, 2005). These groundbraking findings could be confirmed by the successful enrichment and isolation of crenarcheal ammonia oxidizers from a marine aquarium and terrestrial hot springs (Könneke *et al.*, 2005, Hatzenpichler *et al.*, 2008, de la Torre *et al.*, 2008). The detection of AOA revised the long-lasting assumption that ammonia-oxidation would be restricted to few lineages of the *Proteobacteria* (Fig. 4).

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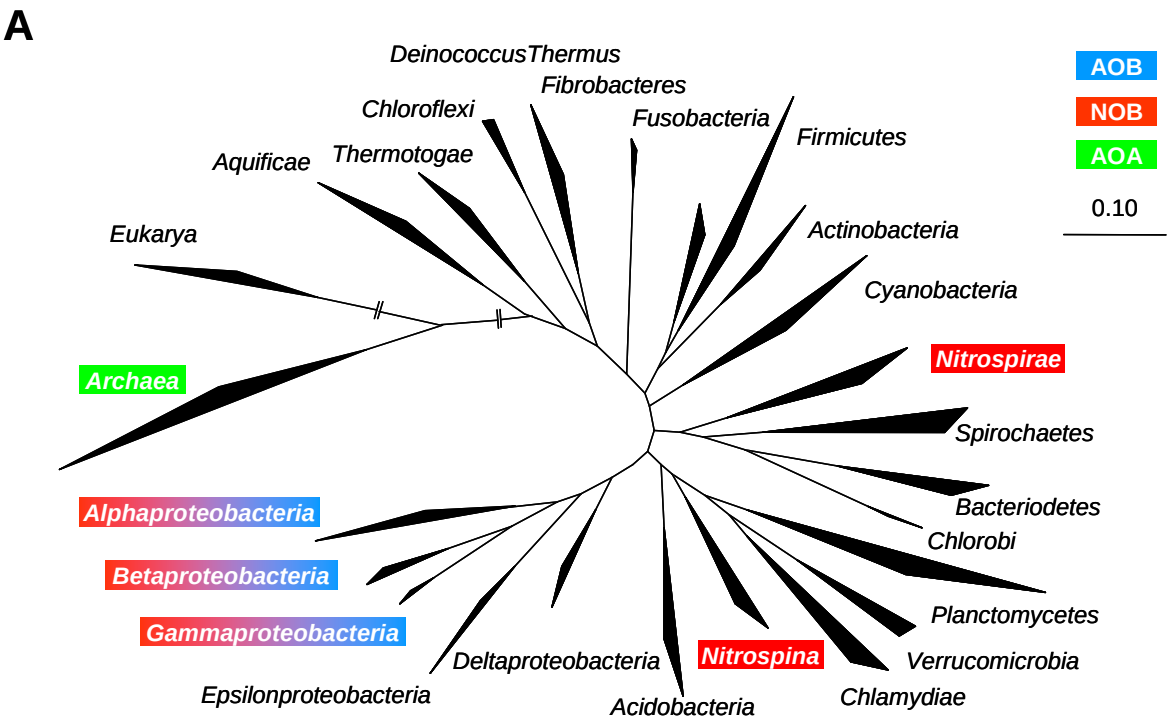
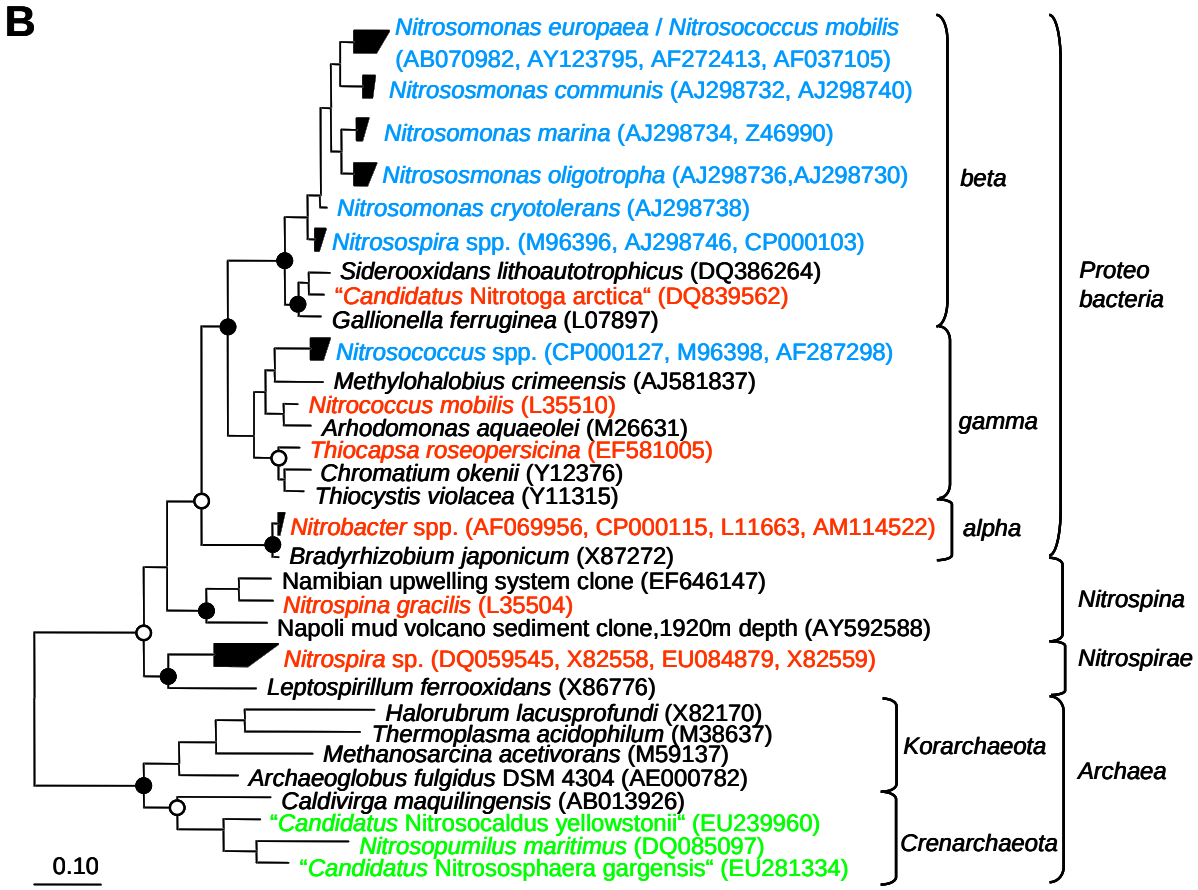
Fig. 4: 16S rRNA-based Maximum Likelihood trees displaying the phylogenetic affiliation of nitrifying microorganisms. Nitrite-oxidizing bacteria (NOB) are highlighted in red, ammonia-oxidizing bacteria (AOB) and archaea (AOA) are highlighted in blue and green, respectively. The tree in Fig. 4/A gives a general overview of the distribution of nitrifiers in the "tree of life", whereas the tree in Fig. 4/B illustrates a close-up view of Fig. 4/A representing all currently known nitrifying bacterial and archaeal genera and species. Both trees were calculated using the Maximum Likelihood algorithm implemented in the ARB software tool (Ludwig *et al.*, 2004). The partially filled circles at the tree nodes in Fig. 4/B represent quartet puzzling reliability values ≥ 70 % and filled circles symbolize additional high parsimony bootstrap support (≥ 90 %) based on 100 iterations. The bar indicates 10 % estimated sequence divergence.


A
DeinococcusThermus
Fibrobacteres
Chloroflexi
Fusobacteria
Firmicutes
Aquificae
Thermotogae
Actinobacteria
Eukarya
Cyanobacteria
Nitrospirae
Archaea
Spirochaetes
Alphaproteobacteria
Bacteriodetes
Chlorobi
Betaproteobacteria
Planctomycetes
Gammaproteobacteria
Deltaproteobacteria
Nitrospina
Verrucomicrobia
Epsilonproteobacteria
Acidobacteria
Chlamydiae
AOB
NOB
AOA
0.10


B
Nitrosomonas europaea / Nitrosococcus mobilis
(AB070982, AY123795, AF272413, AF037105)
Nitrososmonas communis (AJ298732, AJ298740)
Nitrosomonas marina (AJ298734, Z46990)
Nitrososmonas oligotropha (AJ298736,AJ298730)
Nitrosomonas cryotolerans (AJ298738)
Nitrosospira spp. (M96396, AJ298746, CP000103)
Siderooxidans lithoautotrophicus (DQ386264)
"Candidatus Nitrotoga arctica" (DQ839562)
Gallionella ferruginea (L07897)
Nitrosococcus spp. (CP000127, M96398, AF287298)
Methylohalobius crimeensis (AJ581837)
Nitrococcus mobilis (L35510)
Arhodomonas aquaeolei (M26631)
Thiocapsa roseopersicina (EF581005)
Chromatium okenii (Y12376)
Thiocystis violacea (Y11315)
Nitrobacter spp. (AF069956, CP000115, L11663, AM114522)
Bradyrhizobium japonicum (X87272)
Namibian upwelling system clone (EF646147)
Nitrospina gracilis (L35504)
Napoli mud volcano sediment clone,1920m depth (AY592588)
Nitrospira sp. (DQ059545, X82558, EU084879, X82559)
Leptospirillum ferrooxidans (X86776)
Halorubrum lacusprofundi (X82170)
Thermoplasma acidophilum (M38637)
Methanosarcina acetivorans (M59137)
Archaeoglobus fulgidus DSM 4304 (AE000782)
Caldivirga maquilingensis (AB013926)
"Candidatus Nitrosocaldus yellowstonii" (EU239960)
Nitrosopumilus maritimus (DQ085097)
"Candidatus Nitrososphaera gargensis" (EU281334)
beta
gamma
alpha
Proteo bacteria
Nitrospina
Nitrospirae
Korarchaeota
Crenarchaeota
Archaea
0.10

## 4. Nitrite-oxidizing bacteria

Nitrite oxidizers, catalyze the second step in nitrification, the oxidation of nitrite to nitrate. Based on early classifications of nitrite-oxidizing bacteria (NOB) considering the cell shape, the presence of characteristic cytoplasmic membrane structures (Watson *et al.*, 1989) and distinct fatty acid profiles (Lipski *et al.*, 2001) NOB were divided into four genera. This classification was confirmed, and recently extended to six genera, based on comparative 16S rRNA sequence analysis (Fig. 4/B):

i) The genus *Nitrobacter* belongs to the alpha-subclass of *Proteobacteria* and represents a phylogenetically young group (Seewaldt *et al.*, 1982, Orso *et al.*, 1994). Four described species currently represent this genus, *Nitrobacter winogradskyi* (Winslow *et al.*, 1917), *N. hamburgensis* (Bock *et al.*, 1983), *N. vulgaris* (Bock *et al.*, 1990) and *N. alkalicus* (Sorokin *et al.*, 1998). Interestingly, *Nitrobacter* is closely related to phototrophic bacteria, such as *Rhodopseudomonas palustris*, with which *Nitrobacter* shares common membrane structural arrangement (Teske *et al.*, 1994).

ii) Quite recently a novel cold adapted NOB has been enriched from a Siberian Arctic soil. The organism has been provisionally classified as "*Candidatus* Nitrotoga arctica" and is the first known NOB affiliated to the beta-subclass of *Proteobacteria* (Alawi *et al.*, 2007).

iii) The gamma-subclass of *Proteobacteria* harbours the marine NOB *Nitrococcus mobilis* (Watson & Waterbury, 1971). Together with the AOB *Nitrosococcus oceani*, *Nitroccoccus mobilis* is a member of the ectothiorhodospira branch of the gamma-subclass of *Proteobacteria*, suggesting that these nitrifying bacteria just like *Nitrobacter* are derived from photosynthetic ancestry (Teske *et al.*, 1994).

iv) In agreement with this hypothesis a recent study described an anoxygenic phototrophic bacterium (*Thiocapsa roseopersicina*), which is affiliated to the gamma-subclass of *Proteobacteria* and uses nitrite as electron donor for photosynthesis (Griffin *et al.*, 2007).

v) The genus *Nitrospina* encompasses two marine strains of *Nitrospina gracilis*, Nb-211 (from the Atlantic) and Nb-3 (from the Pacific). Both strains were provisionally classified to the delta-subclass of *Proteobacteria* (Teske *et al.*, 1994). However, phylogenetic analysis with a more encompassing 16S rRNA dataset suggests that *Nitrospina* forms an independent line of descent within the bacterial domain (Fig 4).

vi) Nitrite-oxidizers of the genus *Nitrospira* belong to a distinct phylum in the domain *Bacteria* (Ehrich *et al.*, 1995) (Fig 4). The detailed *Nitrospira* phylogeny and the description of the few isolated or enriched *Nitrospira*-like bacteria are addressed below (section 5.1).

Most work on the physiology and biochemistry of NOB was done with *Nitrobacter* species and our knowledge about the biochemical properties of all other known nitrite-oxidizing genera is only scarce. Therefore, it is important to keep in mind that most of the presented data cannot be generalized for all NOB.

Two electrons are abstracted when nitrite is oxidized to nitrate. The formation of nitrate is catalysed by the key enzyme of NOB, nitrite oxidoreductase (Nxr), according to the following equation:

$$\text{Nxr:} \qquad NO_2^- + H_2O \quad \leftrightarrow \quad NO_3^- + 2H^+ + 2e^-$$

The additional oxygen atom is derived from water (Aleem *et al.*, 1965). Most Nxr-related studies have been performed with different *Nitrobacter* strains (Tanaka *et al.*, 1983, Sundermeyer-Klinger *et al.*, 1984, Meincke *et al.*, 1992). In the absence of oxygen, the Nxr of *Nitrobacter* can reduce nitrate to nitrite. Therefore, the oxidation of nitrite is a reversible reaction (Sundermeyer-Klinger *et al.*, 1984). The Nxr of *Nitrobacter* is a integral membrane bound enzyme complex, which consists of at least two subunits, the large subunit NxrA and the small subunit NxrB (Sundermeyer-Klinger *et al.*, 1984, Meincke *et al.*, 1992). The mature holoenzyme contains molybdopterin as cofactor and several iron-sulfur centers (Ingledew & Halling, 1976, Sundermeyer-Klinger *et al.*, 1984, Meincke *et al.*, 1992). Immunocytochemical studies revealed that the Nxr of *Nitrobacter* is localized at the cytoplasmic face of the cell membrane and at the intracytoplasmic membranes (Spieck *et al.*, 1996).

Only a few studies about the Nxr of *Nitrospira* exist (Spieck *et al.*, 1998, Bartosch *et al.*, 1999, Chapter IV, this thesis). The results, however, indicated that the Nxr of *Nitrospira* differs remarkably from the Nxr of *Nitrobacter*. In contrast to the Nxr of *Nitrobacter*, the Nxr of *Nitrospira* was found to be membrane-associated in the periplasmic space (Spieck *et al.*, 1998). Furthermore, immunoblotting experiments and sequence analysis of Nxr subunits of *Nitrobacter* and *Nitrospira* revealed major differences in their apparent molecular masses and their phylogenetic affiliation (Bartosch *et al.*, 1999, Chapter IV, this thesis). Taken together, these results suggest a convergent evolution of the Nxr enzymatic function in two different nitrite-oxidizing systems.

Lithoautotrophic NOB fix carbon dioxide ($CO_2$) and about 80% of the energy generated by nitrite oxidation is used for $CO_2$ fixation (Spieck & Bock, 2005). Furthermore, the redox

potential of the $NO_2^-$/ $NO_3^-$ couple is extraordinarily high (+420 mV). Thus, similar to AOB/AOA, nitrite-oxidizing bacteria are extremely slow-growing organism with minimal generation times ranging from 10 h for *Nitrobacter vulgaris* (Bock *et al*., 1990) up to 90 h for *Nitrospira marina* (Watson *et al.*, 1986) under lithoautotrophic growth conditions.

Apart from aerobic nitrite oxidation several other metabolic properties are known for NOB. *Nitrobacter*, for example, thrives by denitrification in anoxic habitats (Freitag *et al.*, 1987, Bock *et al.*, 1988). Also *Nitrospira moscoviensis* can use nitrate as electron acceptor under anoxic conditions with hydrogen as electron donor (Ehrich *et al*., 1995). Furthermore, heterotrophic growth using a variety of different carbon compounds has been described for *Nitrobacter* for many years. This organotrophic growth, however, is inefficient and slow (Smith & Hoare, 1968, Bock, 1976). In contrast, *Nitrospira marina* grew best mixotrophically in a medium containing nitrite, pyruvate, yeast extract, and peptone (Ehrich *et al.*, 1995).

In summary, most available biochemical data of NOB comes from the easily culturable *Nitrobacter* species. Our knowledge about other NOB is still limited, mainly due to the lack of media to culture these fastidious and slow-growing organisms.

## 5. Nitrite-oxidizing bacteria of the genus *Nitrospira*

The next section includes a more detailed phylogenetic description of NOB of the phylum/genus *Nitrospira*, one of the aforementioned less intensively studied groups of NOB. Furthermore, the recent state of knowledge about their diversity and ecophysiology will be summarized. Finally, from this current status major ecological questions arose, which have been the basic framework for this thesis.

### 5.1 Phylogeny and Diversity of *Nitrospira*

Nitrite oxidizers of the genus *Nitrospira* form a distinct phylum in the domain *Bacteria*. The phylum *Nitrospira* comprises the genera *Nitrospira*, *Leptospirillum*, *Thermodesulfovibrio* and *Magnetobacterium* (Ehrich *et al.*, 1995), and several environmental clone groups, which are not yet assigned to any of these genera (Fig. 5/A).

The genus *Nitrospira* represents a large and highly diverse group, with only five isolated/ enriched representatives so far (Fig 5/B). The first two described species are *Nitrospira marina*, which was obtained from Atlantic Ocean samples (Watson *et al.*, 1986), and *Nitrospira moscoviensis* isolated from an urban heating system in Moscow (Ehrich *et al.*, 1995). However, several other attempts to selectively enrich *Nitrospira*-like bacteria from different environmental samples failed, mainly because *Nitrospira* was outcompeted by *Nitrobacter* when standard isolation procedures were used (Bartosch *et al.*, 1999). One determining factor for the outcome of the enrichment of NOB seems to be the initial nitrite concentration in the medium. Whereas *Nitrobacter* overgrew *Nitrospira*-like bacteria in media

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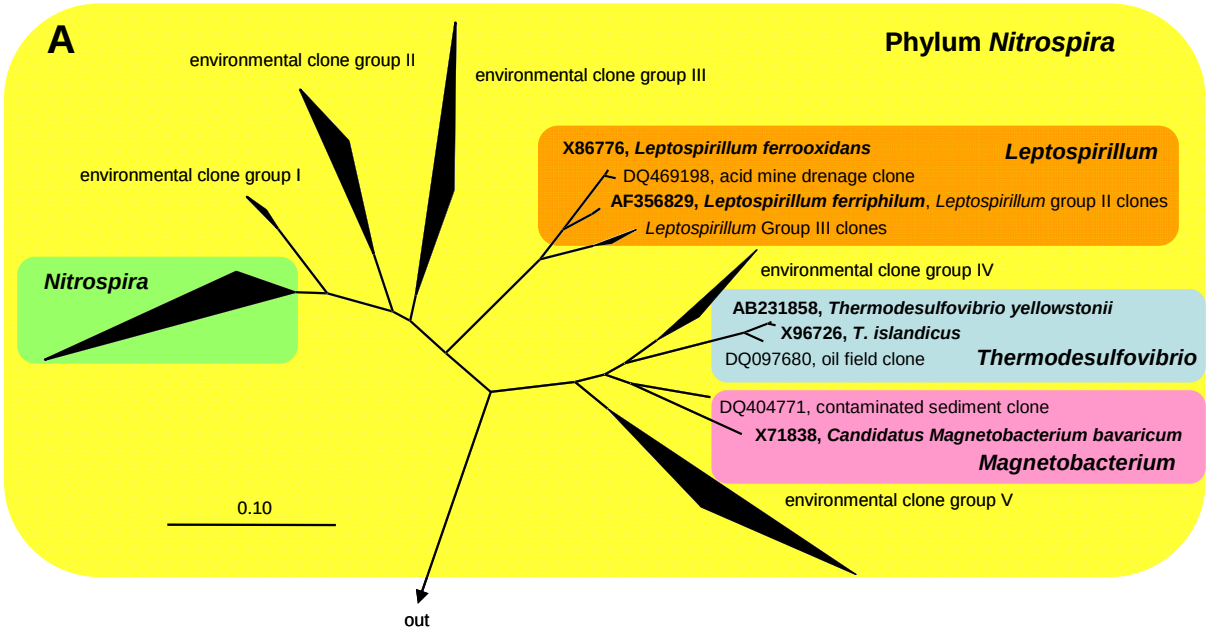
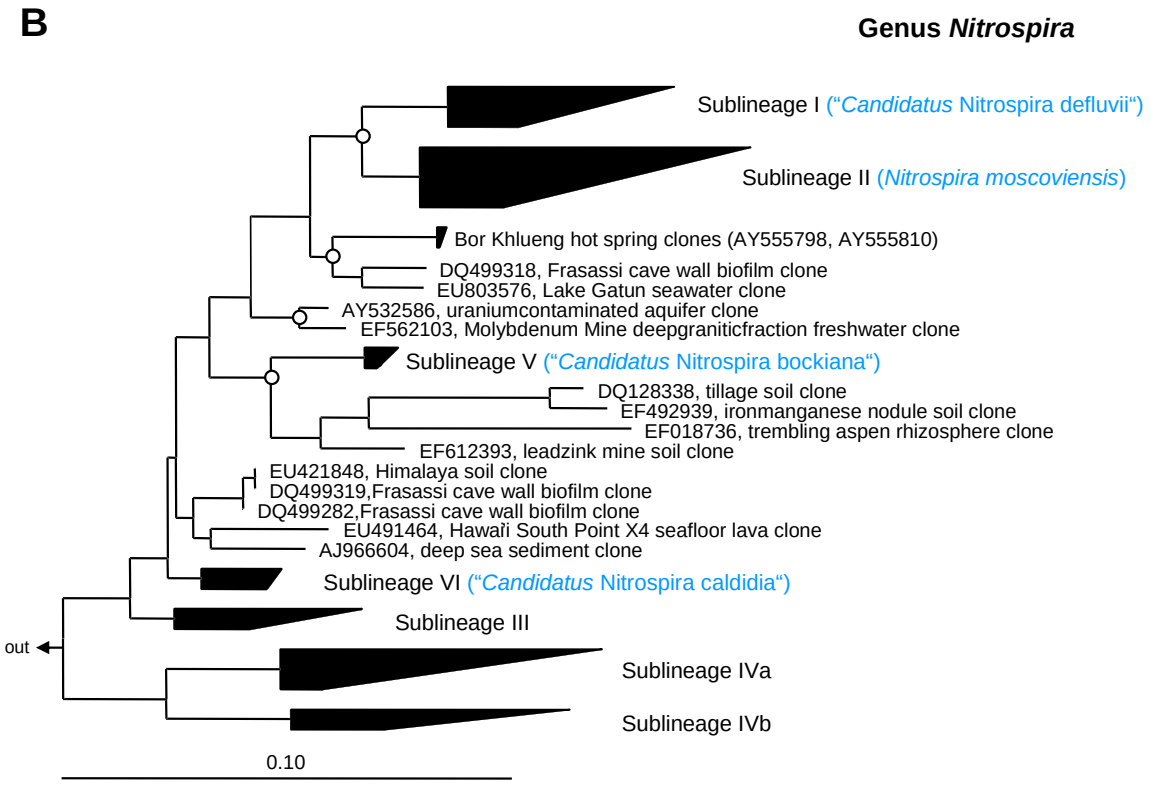
Fig. 5: 16S rRNA-based trees displaying the phylogeny of different genera (highlighted with different colours) within the phylum *Nitrospira* (A, highlighted yellow) and the phylogenetic affiliation of different sublineages within the genus *Nitrospira* (B). Sequences belonging to one sublineage have been clustered according to the sublineage definition of Daims and colleagues (Daims *et al.*, 2001) (Fig. 5/B). The so far isolated or enriched *Nitrospira*-like bacteria are displayed in brackets in blue behind the respective sublineage. For the un-clustered trees displaying the phylogeny within *Nitrospira* sublineages please refer to figure 1 in the Appendix. All trees were calculated using the Maximum Likelihood algorithm implemented in the ARB software tool (Ludwig *et al.*, 2004). The partially filled circles at the tree nodes in Fig. 5/B represent quartet puzzling reliability values ≥ 70 % and filled circles symbolize additional high parsimony bootstrap support (≥ 90 %) based on 100 iterations. The bar indicates 10 % estimated sequence divergence.


A
Phylum Nitrospira
environmental clone group II
environmental clone group III
environmental clone group I
X86776, Leptospirillum ferrooxidans
Leptospirillum
DQ469198, acid mine drenage clone
AF356829, Leptospirillum ferriphilum, Leptospirillum group II clones
Leptospirillum Group III clones
Nitrospira
environmental clone group IV
AB231858, Thermodesulfovibrio yellowstonii
X96726, T. islandicus
DQ097680, oil field clone
Thermodesulfovibrio
DQ404771, contaminated sediment clone
X71838, Candidatus Magnetobacterium bavaricum
Magnetobacterium
environmental clone group V
0.10
out


B
Genus Nitrospira
Sublineage I ("Candidatus Nitrospira defluvii")
Sublineage II (Nitrospira moscoviensis)
Bor Khlueng hot spring clones (AY555798, AY555810)
DQ499318, Frasassi cave wall biofilm clone
EU803576, Lake Gatun seawater clone
AY532586, uraniumcontaminated aquifer clone
EF562103, Molybdenum Mine deepgraniticfraction freshwater clone
Sublineage V ("Candidatus Nitrospira bockiana")
DQ128338, tillage soil clone
EF492939, ironmanganese nodule soil clone
EF018736, trembling aspen rhizosphere clone
EF612393, leadzink mine soil clone
EU421848, Himalaya soil clone
DQ499319,Frasassi cave wall biofilm clone
DQ499282,Frasassi cave wall biofilm clone
EU491464, Hawai'i South Point X4 seafloor lava clone
AJ966604, deep sea sediment clone
Sublineage VI ("Candidatus Nitrospira caldidia")
Sublineage III
out
Sublineage IVa
Sublineage IVb
0.10

with higher nitrite concentration (2 g $NaNO_2$ per liter), *Nitrospira*-like bacteria could be selectively cultivated in mixotrophic media containing 0.2 g $NaNO_2$ per liter (Bartosch *et al.*, 1999, Bartosch *et al.*, 2002). Subsequently, a combined approach using low nitrite concentration, percoll density gradient centrifugation for further purification, and serial dilutions led to a high enrichment (86%) of previously uncultured *Nitrospira*-like bacteria from activated sludge. The NOB has been classified as "*Candidatus* Nitrospira defluvii" (Spieck *et al.*, 2006) (Appendix: paper 1). Additionally, this elaborate strategy resulted in the cultivation of "*Candidatus* Nitrospira bockiana" from the internal corrosion deposits of a steel pipeline of the Moscow heating system (Lebedeva *et al.*, 2008) (Appendix: paper 2) and to the enrichment of the first thermophilic *Nitrospira*-like bacterium ("*Candidatus* Nitrospira caldida") from a microbial mat of the Gorjachinsk hot spring in the Baikal rift zone (Elena Lebedeva, manuscript in preparation). The isolated and enriched *Nitrospira*-like bacteria differ quite remarkably in their cell size and morphology. Furthermore, their optimal growth temperature ranges from 28°C up to 50°C and the organisms display a different tolerance against nitrite (Appendix: table 1). Taken together, these data reflect the various lifestyles and isolation sources of the cultured *Nitrospira*-like bacteria, which are members of different sublineages within the genus *Nitrospira* (Fig. 5/B). The small ribosomal subunit sequences of the five isolated or enriched *Nitrospira*-like bacteria, however, represent only a minor proportion of the 16S rRNA gene sequences that belong to the genus *Nitrospira*.

Extended phylogenetic analyses of the genus *Nitrospira* were mainly based on environmental clone sequences obtained from various habitats, including bioreactors (Juretschko *et al.*, 1998, Schramm *et al.*, 1998, Kindaichi *et al.*, 2004), different freshwater habitats (Hovanec *et al.*, 1998, Altmann *et al.*, 2003, Martiny *et al.*, 2003), soil, rhizosphere (Kim *et al.*, 2008, Lesaulnier *et al.*, 2008), hot springs (Lebedeva *et al.*, 2005), marine habitats (Foesel *et al.*, 2008, Santelli *et al.*, 2009) and sponges (Taylor *et al.*, 2007) (Appendix: figure 1). These comprehensive phylogenetic analyses extended the number of sublineages in the genus *Nitrospira* from four, as previously proposed by Daims and colleagues (Daims *et al.*, 2001), to six monophyletic sublineages (Fig. 5/B). Interestingly, some of these sublineages seem to represent a habitat-specific clustering of sequences, such as sublineage one, which almost exclusively consists of bioreactor clones, or sublineage four where all clones and cultures were derived from marine habitats or sponges (Appendix: figure 1).

In summary, cultivation and, moreover, 16S rRNA-based screenings have shown that NOB of the genus *Nitrospira* are a highly diverse group of organisms, and that these bacteria are

ubiquitously distributed in a wide range of aquatic and terrestrial ecosystems. However, it remains to be determined, whether this enormous phylogenetic diversity reflects differing physiological properties within the genus *Nitrospira*.

### 5.2 Ecopysiology of *Nitrospira*

Laboratory-based studies on the few cultivated *Nitrospira*-like bacteria revealed first insights into the physiology of *Nitrospira*. The abundance and *in situ* ecophysiology of *Nitrospira*-like bacteria in different habitats, however, remained for a long time largely unknown. The understanding of these NOB greatly increased with the introduction of different cultivation-independent techniques into microbial ecology (Wagner *et al.*, 2003). Mainly the application of FISH in combination with microautoradiography (MAR-FISH) or with microelectrodes uncovered novel aspects of their structural, functional and physiological properties.
First FISH analyses, aimed towards quantifying natural populations of nitrifiers, revealed that uncultured bacteria related to the genus *Nitrospira* and not *Nitrobacter* are the dominant NOB in aquaria, nitrifying laboratory-scale reactors, and full-scale wastewater treatment plants (Hovanec *et al.*, 1998, Juretschko *et al.*, 1998, Schramm *et al.*, 1998). Additionally, the *in situ* visualization could show that AOB and *Nitrospira*-like bacteria form within activated sludge flocs and biofilms compact microcolonies (consisting of hundreds to thousands of cells), which are often in close vicinity to each other (Fig. 3). Interestingly, larger *Nitrospira* cell aggregates in bioreactor samples contain multiple water-permeable channels, most likely facilitating nutrient- and gas-supply to the cells inside a colony (Daims *et al.*, 2001). Moreover, by combining FISH and microautoradiograhpy, Daims and colleagues could show that these *Nitrospira*-like bacteria took up under oxic conditions radiolabeled inorganic carbon (in forms of $HCO_3^-$ or $CO_2$) and pyruvate, suggesting a mixotrophic growth in the presence of pyruvate. In contrast, the *Nitrospira*-like bacteria incorporated the tested radioactive carbon sources neither under anoxic nor anaerobic conditions (Daims *et al.*, 2001). Another MAR-FISH study additionally revealed tight ecophysiological interactions between nitrifiers and heterotrophic bacteria in a carbon-limited autotrophic nitrifying biofilm with ammonium as sole energy source. The results indicated that heterotrophic bacteria thrive on the released soluble microbial products of nitrifiers, such as *Nitrospira* spp., and that there is an efficient carbon food web in the biofilm community (Kindaichi *et al.*, 2004). Nevertheless, these two studies represent so far the only ecophysiological characterizations of *Nitrospira*-like bacteria using the MAR-FISH approach. A further major advancement in microbial

ecology, beside MAR-FISH, was the combined application of FISH with microelectrodes. This approach revealed not only the spatial localisation of nitrifiers within a biofilm along different gas- and nutrient-gradients. Moreover, it was for the first time possible to record the *in situ* activity of AOB and NOB and to subsequently estimate, for example, the cell-specific activity of uncultured *Nitrospira*-like bacteria (Schramm *et al.*, 1998, Schramm *et al.*, 1999, Schramm *et al.*, 2000). The results obtained in these studies suggested that NOB of the genus *Nitrospira* are K strategists adapted to low oxygen and nitrite concentration, whereas *Nitrobacter*-like organism are r strategists, which overgrow *Nitrospira* spp. under conditions of elevated nitrite and oxygen concentrations.

Taken together, *Nitrospira* spp. exhibit in comparison to *Nitrobacter* spp. a higher affinity for nitrite and oxygen (Downing & Nerenberg, 2008). Considering the huge diversity within the genus *Nitrospira*, these low Km values might be advantageous in certain environments, enabling *Nitrospira*-like bacteria to thrive in ecological niches with constantly low substrate concentrations. One remarkable finding beside these ecophysiologal differences between *Nitrospira* and *Nitrobacter* was the co-existence of two *Nitrospira* populations in biofilm samples of nitrifying bioreactors (Schramm *et al.*, 1998, Schramm *et al.*, 1999). The two populations displayed a distinct spatial distribution pattern within the biofilm, suggesting a niche differentiation within the genus *Nitrospira* due to different physiological adaptations.

Nevertheless, beside these few depicted fundamental studies describing the *in situ* ecophysiology of *Nitrospira*-like bacteria, so far only a few studies indirectly referred to physiological properties of these NOB. In one of these studies, for example, Freitag and colleagues demonstrated the presence of different sublineage II *Nitrospira*-ecotypes in long-term fertilized and unfertilized agricultural grassland soils, also indicating a physiological diversification within the genus *Nitrospira* (Freitag *et al.*, 2005). Interestingly, a recent study showed that *Nitrospira*-like bacteria are more abundant in the rhizosphere compared to the bulk soil (DeAngelis *et al.*, 2009). However, it remains to be determined, whether these sublineage II *Nitrospira* metabolize the freshly introduced root exudates in the rhizosphere and therefore strongly respond to the root shooting. Finally, a study investigating temperature thresholds for bacterial symbiosis revealed a quick response of *Nitrospira*-like bacteria in sponges to elevated temperatures. A temperature shift from 27 °C to 33°C resulted in the loss of microbial sponge symbionts, such as sublineage IVb *Nitrospira* (Webster *et al.*, 2008).

Even though these few studies only touched on the question of the ecophysiology of *Nitrospira* in different habitats, the results already indicate various physiological adaptations within the genus *Nitrospira*. Whereas the theory behind the ecological differentiation of

*Nitrobacter* spp. and *Nitrospira* spp. is already well accepted (Schramm *et al.*, 2000), our knowledge about the physiological differentiation within the genus *Nitrospira* is still limited.

For example, a yet unresolved ecological question in this context is:

> Does the coexistence of different *Nitrospira* populations in the same habitat reflect the physiological adaptations to different environmental factors, such as nutrient concentration, oxygen availability, or temperature?

Furthermore, first *Nitrospira* genome sequences, which became available during this thesis, extended the question to:

i) Does the *Nitrospira* genome encode any resistance genes or genes indicating metabolic versatility, which could explain physiological adaptations to certain environments?
ii) Does the comparison of metabolic key enzymes (e.g. Nxr) of *Nitrospira* and *Nitrobacter* reveal any indications for different enzymatic properties supporting the distinct ecophysiological adaptations of these NOB?

These major ecological questions provided the theoretical framework for this thesis.

## Aims of this thesis

One major goal of this thesis was to gain more insight into the ecophysiology of *Nitrospira*-like bacteria by applying novel molecular *in situ* techniques and via in depth analysis of metagenomic data. Furthermore, in the course of this thesis another yet unresolved question was addressed, whether the Nxr subunits might be suitable as functional and also as phylogenetic markers for the detection of *Nitrospira*-like bacteria in environmental samples.

The niche differentiation between two coexisting uncultured populations of *Nitrospira*-like bacteria in nitrifying biofilm and activated sludge samples is described in **Chapter II**. Distinct spatial distribution patterns of the two *Nitrospira* sublineages relative to AOB suggested a niche differentiation with respect to their preferred concentrations of nitrite. A long term experiment revealed that representatives of the two sublineages indeed showed different response to nitrite concentration shifts. Population dynamics of *Nitrospira*-like bacteria demonstrated significant differences in the ecophysiological properties of closely related nitrifiers, a major requirement for niche differentiation in diverse habitats.

**Chapter III** offers insight into the first metagenome fragment of a *Nitrospira*-like bacterium ("*Candidatus* Nitrospira defluvii"). Surprisingly, a gene similar to genes encoding chlorite dismutases (CLD) was found on the *Nitrospira* contig. This enzyme, which has only been known from (per)chlorate reducing *Proteobacteria*, transforms toxic chlorite to chloride and oxygen. The biochemical activity of the non-proteobacterial CLD of *Nitrospira* was confirmed by heterologous expression in *E. coli* followed by enzymatic tests. The catalytic parameters of the recombinant CLD indicated that the gene encodes a highly active CLD. Furthermore, Western blot analysis revealed that the gene is expressed *in situ* by *Nitrospira*. The evolutionary origin of this enzyme family and the possible role of a CLD in a distinct physiological adaptation of this *Nitrospira*-like organism have been discussed in detail.

The study described in **Chapter IV** emerged as a spin-off from a larger environmental genomics project, which aimed at sequencing the whole genome of "*Candidatus* Nitrospira defluvii". Here, the goal was to identify and characterize in detail the genes comprising the key enzyme nitrite oxidoreductase in the genome of *N. defluvii*. The Nxr of *Nitrospira* consists of three subunits, which differ quite remarkably in sequence from the Nxr subunits of

*Nitrobacter* and *Nitrococcus*. Additionally, the Nxr beta subunit turned out to be a suitable functional and phylogenetic marker for the genus *Nitrospira*.

A summary of the presented studies is given in **Chapter V**.

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# Chapter II

## Nitrite concentration influences the population structure of *Nitrospira*-like bacteria

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Brief report

# Nitrite concentration influences the population structure of *Nitrospira*-like bacteria

**Frank Maixner,[1] Daniel R. Noguera,[2] Bettina Anneser,[1] Kilian Stoecker,[1] Gertrude Wegl,[1] Michael Wagner[1] and Holger Daims[1]***

[1]*Department für Mikrobielle Ökologie, Universität Wien, Althanstrasse 14, A-1090 Vienna, Austria.*

[2]*Department of Civil and Environmental Engineering, University of Wisconsin-Madison, 3216 Engineering Hall, 1415 Engineering Drive, Madison, WI 53706, USA.*

## Summary

**Chemolithoautotrophic nitrite oxidizers of the genus *Nitrospira* are a monophyletic but diverse group of organisms, are widely distributed in many natural habitats, and play a key role in nitrogen elimination during biological wastewater treatment. Phylogenetic analyses of cloned 16S rRNA genes and fluorescence *in situ* hybridization with newly developed rRNA-targeted oligonucleotide probes revealed coexistence of uncultured members of sublineages I and II of the genus *Nitrospira* in biofilm and activated sludge samples taken from nitrifying wastewater treatment plants. Quantitative microscopic analyses of their spatial arrangement relative to ammonia oxidizers in the biofilm and activated sludge flocs showed that members of the *Nitrospira* sublineage I occurred significantly more often in immediate vicinity to ammonia oxidizers than would be expected from random community assembly while such a relationship was not observed for *Nitrospira* sublineage II. This spatial distribution suggested a niche differentiation of these coexisting *Nitrospira* populations with respect to their preferred concentrations of nitrite. This hypothesis was tested by mathematical modelling of nitrite consumption and resulting nitrite gradients in nitrifying biofilms and by quantifying the abundance of sublineage I and II *Nitrospira* in activated sludge during incubations with nitrite in different concentrations. Consistent with the observed localization patterns, a higher nitrite concentration selected for sublineage I but suppressed sublineage II *Nitrospira*.**

Received 30 November, 2005; accepted 9 March, 2006. *For correspondence. E-mail daims@microbial-ecology.net; Tel. (+43) 1 4277 54392; Fax (+43) 1 4277 54389.

## Introduction

The development of cultivation-independent molecular detection methods for microorganisms has led to the discovery of an immense phylogenetic diversity of prokaryotes on all taxonomic levels and in virtually all analysed habitats (Hugenholtz *et al*., 1998; Rappé and Giovannoni, 2003). A recurring question in microbial ecology is how much functional differentiation is linked to this high phylogenetic diversity: Do all different phylotypes found in the same environmental sample represent organisms with different physiological properties and/or survival strategies? A related question is how much functional redundancy among different organisms can a complex microbial community harbour, i.e. to what extent can functionally similar microorganisms coexist without competitive exclusion? It has been suggested that organisms cannot live together unless their ecological niches are sufficiently different (e.g. May, 1981). Despite difficulties in establishing the validity of this competitive exclusion principle in natural ecosystems with spatial heterogeneity, temporally fluctuating conditions and complex predator–prey relationships (Begon *et al*., 1996), it seems reasonable to consider competition and niche differentiation as factors that influence the composition of microbial communities. Prominent examples of niche differentiation in the microbial world are the adaptations of coexisting marine planktonic bacteria, especially in regard to their photosynthetic characteristics (Man *et al*., 2003; Stomp *et al*., 2004). However, much less is known about niche differentiation of (mainly uncultured) bacteria in many other functional groups. These include organisms that play key roles in all ecosystems by catalysing essential chemical conversions such as the reactions of the biogeochemical nitrogen cycle.

Here we investigated niche differentiation between two coexisting uncultured populations of *Nitrospira*-like bacteria representing two different recognized phylogenetic sublineages within the genus *Nitrospira*. All cultivated members of this genus are chemolithoautotrophic nitrite

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**Table 1.** Oligonucleotides used as primers for the PCR-amplification of partial 16S rRNA genes of *Nitrospira*-like bacteria.

| Name | Specificity | Sequence (5′-3′) | Target site[a] | Reference |
|---|---|---|---|---|
| 616F | Domain *Bacteria* | AGA GTT TGA TYM TGG CTC[b] | 8–25 | Juretschko *et al.* (1998) |
| Ntspa1158R | Genus *Nitrospira* | CCC GTT MTC CTG GGC AGT | 1158–1176 | This study |

**a.** *Escherichia coli* numbering (Brosius *et al.*, 1981).
**b.** A shorter version of this primer than originally published was used.
The applied thermal cycling programme was: initial denaturation for 4 min at 95°C; followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C, and elongation for 60 s at 72°C; and final elongation for 7 min at 72°C.

oxidizers (Watson *et al.*, 1986; Ehrich *et al.*, 1995; Spieck *et al.*, 2006). *Nitrospira*-like bacteria occur in a large number of different natural habitats (Daims *et al.*, 2001) and are also the key nitrite oxidizers in biological wastewater treatment (Burrell *et al.*, 1998; Juretschko *et al.*, 1998; Daims *et al.*, 2001). Supporting evidence for niche partitioning was obtained by analysing, in biofilm and activated sludge, the spatial arrangement of the two *Nitrospira* populations relative to ammonia oxidizer microcolonies and by monitoring the population dynamics of these *Nitrospira*-like bacteria during a long-term competition experiment.

## Results and discussion

### *Detection and spatial arrangement of* Nitrospira

Nitrifying biofilm was taken from a continuously operated pilot-scale biofilm reactor (‘Biofor 2’) (Arnold *et al.*, 2000) receiving municipal wastewater, and nitrifying activated sludge was sampled on a full-scale municipal wastewater treatment plant (WWTP) near Gleisdorf, Austria (nitrifying-denitrifying stage with intermittent aeration; average influent concentrations: 90 mg $l^{-1}$ $NH_4^+$-N, 0.5 mg $l^{-1}$ $NO_3^-$-N; average effluent concentrations: 0.4 mg $l^{-1}$ $NH_4^+$-N, 15 mg $l^{-1}$ $NO_3^-$-N). The 16S rRNA genes of *Nitrospira* in these samples were amplified by specific polymerase chain reaction (PCR) with a universal forward and a newly developed reverse primer (Table 1). The amplicons were cloned and sequenced as described by Juretschko and colleagues (1998). In total, 36 clones were analysed. In either system, this approach detected two different *Nitrospira* populations, which belonged to sublineage I or II of the genus *Nitrospira* (Daims *et al.*, 2001) respectively (Fig. 1). The similarity of the retrieved *Nitrospira* sequences in sublineage I to those in sublineage II was between 92.8% and 93.6%, indicating that these sublineages represent different species (Stackebrandt and Goebel, 1994; Keswani and Whitman, 2001). In order to detect the different *Nitrospira*-like bacteria *in situ* in the samples, we designed, based on a large 16S rRNA sequence database (Ludwig *et al.*, 2004) with many sequences related to *Nitrospira* (Daims *et al.*, 2001), two new 16S rRNA-targeted oligonucleotide probes specific for sublineage I and II of the genus *Nitrospira* respectively (Table 2). For evaluating the new probes, we followed the Clone-fluorescence *in situ* hybridization (FISH) approach (Schramm *et al.*, 2002). Fluorescence *in situ* hybridization [which was performed according to Daims and colleagues (2005)] with these probes revealed that sublineage I and II *Nitrospira* were highly abundant in both samples. Hybridization of the two probes to the same cells was never observed, consistent with their specificity for the two different *Nitrospira* sublineages. *Nitrobacter* cells were not detected by FISH with probe NIT3 in either sample. The detection of coexisting, nitrite-oxidizing *Nitrospira* populations in two independent samples might be explained by at least slightly different ecological niches occupied by these organisms. The simultaneous application of the new

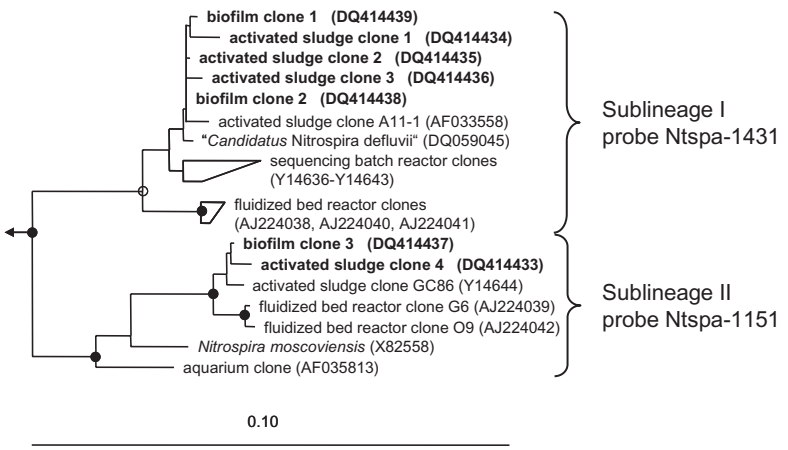


**Fig. 1.** Phylogenetic tree based on 16S rRNA genes of selected *Nitrospira*-like bacteria. Sequences obtained in this study from the biofilm and activated sludge samples are printed bold. The tree was calculated using the Maximum Likelihood algorithm implemented in the ARB software (Ludwig *et al.*, 2004) with a 50% conservation filter for the genus *Nitrospira* and a resulting number of 1158 informative positions. Black circles mark nodes with high parsimony bootstrap support (≥ 90%, 100 iterations). The bar indicates 10% estimated sequence divergence. The braces indicate the coverage of the 16S rRNA-targeted oligonucleotide probes developed in this study. The DNA sequences reported in this work have been deposited in the GenBank database (Accession Nos. DQ414433–DQ414439).

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**Table 2.** 16S rRNA-targeted oligonucleotide probes used for FISH.

| Probe name[a] | Specificity | Sequence (5′-3′) | Target site[b] | FA[c] (%) | Reference |
|---|---|---|---|---|---|
| S-*-Ntspa-1431-a-A-18 | Sublineage I of the genus *Nitrospira* | TTG GCT TGG GCG ACT TCA | 1431 | 35 | This study |
| S-*-Ntspa-1151-a-A-20 | Sublineage II of the genus *Nitrospira* | TTC TCC TGG GCA GTC TCT CC | 1151 | 35–40 | This study |
| S-G-Ntspa-0662-a-A-18[d] | Genus *Nitrospira* | GGA ATT CCG CGC TCC TCT | 662 | 35 | Daims *et al*. (2001) |
| S-G-Nbac-1035-a-A-18 (NIT3)[e] | Genus *Nitrobacter* | CCT GTG CTC CAT GCT CCG | 1035 | 40 | Wagner *et al*. (1996) |
| S-F-bAOB-1224-a-A-20 (Nso1225) | Betaproteobacterial ammonia oxidizers | CGC CAT TGT ATT ACG TGT GA | 1224 | 35 | Mobarry *et al*. (1996); Mobarry *et al*. (1997) |
| EUB-Mix | Most known *Bacteria* | 1. GCT GCC TCC CGT AGG AGT<br>2. GCA GCC ACC CGT AGG TGT<br>3. GCT GCC ACC CGT AGG TGT | 338 | 0–50 | Amann *et al*. (1990); Daims *et al*. (1999) |

**a.** According to the scheme suggested by Alm and colleagues (1996). Trivial names of widely used probes are given in parentheses.
**b.** *Escherichia coli* numbering (Brosius *et al*., 1981).
**c.** The formamide concentration in the hybridization buffer required for specific hybridization. FA, formamide.
**d.** This probe was used in equimolar amounts together with the unlabelled competitor Comp-Ntspa-0662 (Daims *et al*., 2001).
**e.** This probe was used in equimolar amounts together with the unlabelled competitor CNIT3 (Wagner *et al*., 1996).
For further details about the probes, please refer to the probeBase database (Loy *et al*., 2003).

*Nitrospira*-specific probes and probe Nso1225 targeting betaproteobacterial ammonia-oxidizing bacteria (AOB; Table 2) led to the impression that in either sample, the two *Nitrospira* populations and the AOB were localized according to specific spatial arrangement patterns. Sublineage I and II *Nitrospira* apparently clustered together with the AOB, but in the direct vicinity of AOB the density of sublineage I appeared to be higher than that of sublineage II *Nitrospira*, which seemed to maintain a certain distance to the AOB and were sometimes almost shielded from the AOB by surrounding sublineage I *Nitrospira* (Fig. 2A). We applied FISH, confocal laser scanning microscopy, and the recently developed image analysis software *daime* (Daims *et al*., 2006) to quantitatively analyse the spatial arrangement of sublineage I and II *Nitrospira* relative to AOB in the two samples. These quantitative analyses confirmed the visual impression that the arrangement of sublineage I and II *Nitrospira* was different. In the biofilm sample, pronounced co-aggregation of AOB and sublineage I *Nitrospira* was observed at distances below 50 μm relative to AOB with a maximum at 5 μm (Fig. 2D). Co-aggregation of sublineage II *Nitrospira* and AOB was also detected, but only at distances between 6 and 50 μm relative to AOB with a maximum at 30 μm (Fig. 2D). Between 0 and 6 μm, no co-aggregation of AOB and sublineage II *Nitrospira* was detected. In this distance range, the density of sublineage II *Nitrospira* was even below the density that would be expected if the populations were randomly distributed in space (Fig. 2D). The observed co-aggregation of AOB and *Nitrospira*-like bacteria is consistent with the hypothesis that AOB and *Nitrospira* are involved in a mutualistic relationship where AOB deliver the substrate for growth of *Nitrospira*, which in turn remove the nitrite that could have toxic effects on AOB (Stein and Arp, 1998). Beyond 50 μm distance to AOB, the localization pattern switched to random distribution of both *Nitrospira* populations (Fig. 2D), probably because biological interactions between these bacteria did not influence their localization over larger distances. In the activated sludge sample, the differences between the localization patterns of sublineage I and II *Nitrospira* were less pronounced (Fig. 2E). However, the probability of encountering *Nitrospira* sublineage I within a distance of 0–6 μm away from AOB was higher than for sublineage II (Fig. 2E). Statistical support for this rather subtle difference is provided by the non-overlapping confidence intervals over this distance range. The transition to random distribution of both *Nitrospira* populations was at a distance of *c*. 35 μm to AOB (Fig. 2E).

The observed arrangement of sublineage I and II *Nitrospira* relative to AOB in the biofilm might be explained by preference of the two *Nitrospira* populations for different nitrite concentrations. Ammonia-oxidizing bacteria convert ammonia to nitrite, which diffuses into the surrounding space and is consumed by *Nitrospira* colonies in the local neighbourhood of the AOB. It is tempting to speculate that the consumption of nitrite by *Nitrospira* may create in the biofilm, on a small local scale, nitrite gradients with a higher nitrite concentration close to the AOB colonies and lower concentrations at larger distances to the AOB. In the early stages of biofilm development, such gradients could develop because of the activity of any *Nitrospira* cells that grow in the vicinity of AOB. Once established, the gradients could influence the further outcome of a

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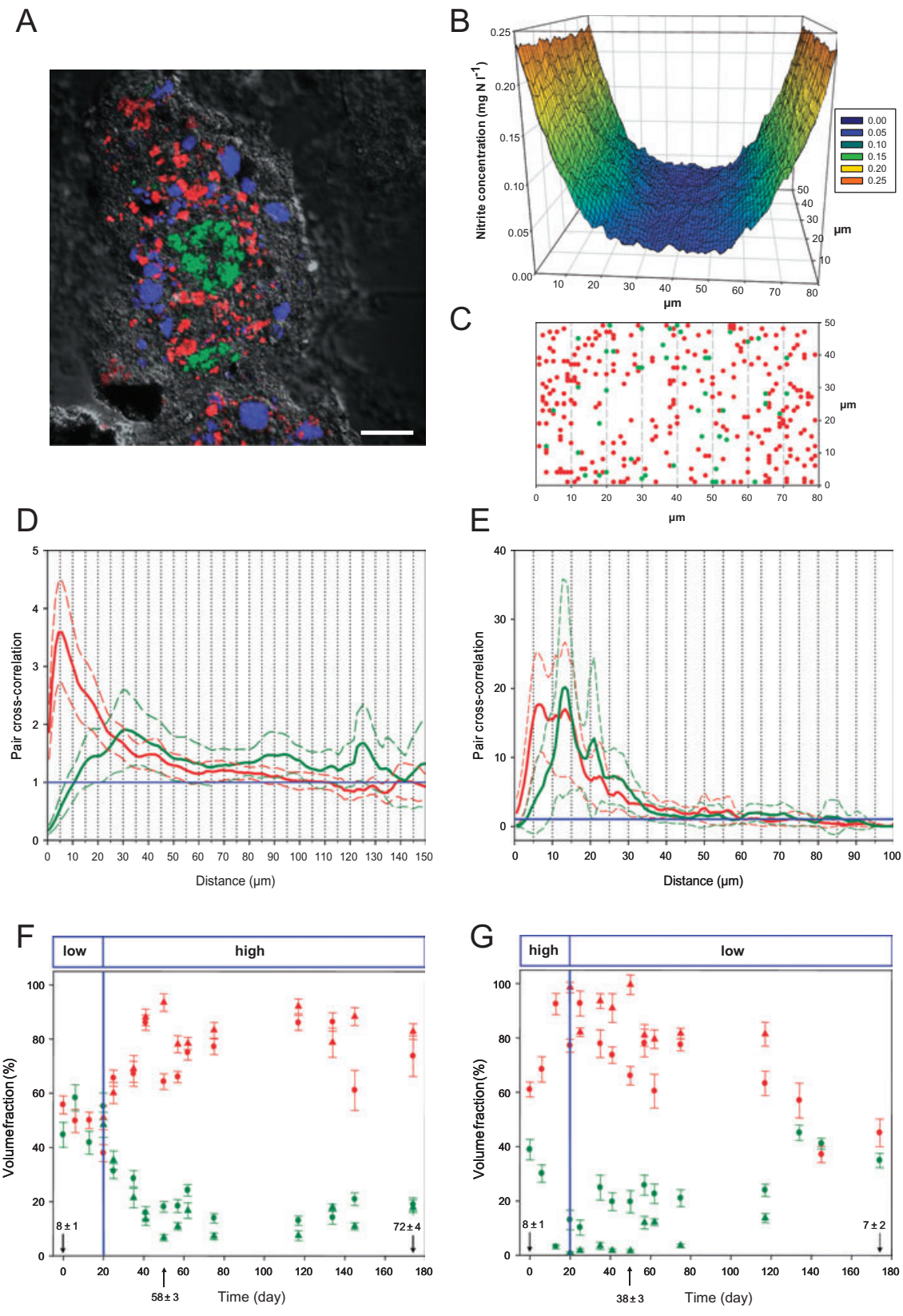


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**Fig. 2.** Localization and competition of *Nitrospira*-like bacteria detected in the biofilm and activated sludge samples.
A. Single confocal image of nitrifying bacteria in the biofilm sample stained by FISH with probes S-*-Ntspa-1431-a-A-18 (sublineage I *Nitrospira*, labelled with Cy3, red), S-*-Ntspa-1151-a-A-20 (sublineage II *Nitrospira*, labelled with FLUOS, green) and Nso1225 (AOB, labelled with Cy5, blue). The fluorescence signals are shown superimposed on a differential interference contrast image of the biofilm. Bar = 20 μm.
B. Modelled nitrite concentration gradients caused by nitrite consumption by sublineage I and II *Nitrospira* distributed between two AOB colonies. Please refer to the text and C for details of the model. The *x*–*y* plane corresponds to the 2-D domain depicted in C. The nitrite concentration is plotted on the vertical *z*-axis.
C. 2-D domain used as biofilm model for simulating nitrite consumption by *Nitrospira*. Cells of sublineage I and II *Nitrospira* are depicted by red and green dots respectively. The left and right edges mark the positions of virtual AOB colonies. Vertical dashed lines delimit $10 \times 50$ μm regions used for mapping the distribution of *Nitrospira* in the biofilm (D) to the 2-D domain (for details, please refer to the text).
D and E. Statistical analyses of the spatial arrangement of *Nitrospira*-like bacteria relative to AOB in the biofilm (D) and activated sludge (E) samples. Solid lines show the mean pair cross-correlation for AOB and sublineage I *Nitrospira* (red) or sublineage II *Nitrospira* (green), respectively, determined by analysing 21–25 confocal FISH-images per *Nitrospira* population and per sample. Values > 1 indicate co-aggregation, values < 1 indicate repulsion, and value of 1 (horizontal blue line) indicates random distribution of AOB and the respective *Nitrospira* population at the corresponding distances. Dashed lines delimit 95% confidence intervals based on the evaluated 21–25 images per sample.
F and G. Quantification of *Nitrospira*-like bacteria in activated sludge during the competition experiment (F, flasks A and B; G, flasks C and D). Markers indicate the mean biovolume fraction of sublineage I and sublineage II *Nitrospira* relative to the total biovolume of all *Nitrospira*-like bacteria, which was determined by FISH with probe S-G-Ntspa-0662-a-A-18. Red markers in F mean sublineage I, flask A (●) and B (▲); green markers in F mean sublineage II, flask A (●) and B (▲). Red markers in G mean sublineage I, flask C (●) and D (▲); green markers in G mean sublineage II, flask C (●) and D (▲). Each value was determined by analysing at least 30 image pairs; the error bars show the standard error of the mean for each measurement. Flasks B and D were accidentally not sampled between days 0 and 13, and due to failure of a pump no data are available for flask D after day 117. The values obtained for sublineages I and II do not sum up to 100% at all time points. This indicates that a yet unknown third *Nitrospira* population may have occurred in low abundance in the sample. Base mismatches at the primer and probe binding sites might explain why this population was not found in the clone libraries and was not detected by any probe below the genus level. The vertical blue lines indicate the days when the nitrite concentration was switched from low to high (F) and from high to low (G). Numbers with black arrows are the combined biovolume (in per cent) of all *Nitrospira*-like bacteria relative to the total bacterial biomass, which was determined in flasks A and C on the 3 days indicated by the arrows.

competition between different *Nitrospira* populations. Those *Nitrospira*-like bacteria, which grow faster at higher nitrite concentrations, could finally become dominant in close spatial neighbourhood to AOB. The competitive strength of these *Nitrospira* would decrease with increasing distance to the AOB. Thus, at larger distances, other *Nitrospira* populations adapted to lower nitrite concentrations could become established or even dominant, too. Based on the observed spatial distribution (Fig. 2D), sublineage I *Nitrospira* could have a selective advantage over sublineage II at higher nitrite concentrations. This hypothesis clearly depends on whether nitrite gradients could form on such a small spatial scale in a biofilm. Therefore, the consumption of nitrite by *Nitrospira* at different distances away from AOB was simulated based on a mathematical model of mass transfer and conversion in biofilms (Noguera and Picioreanu, 2004; Noguera *et al.*, 2004). In our model, the left and right edges of a 2-D $80 \times 50$ μm domain (Fig. 2C) were treated as the borders of AOB colonies. The experimentally observed spatial distribution of sublineage I and II *Nitrospira* in the biofilm was mapped to this virtual 2-D domain (Fig. 2C). For this purpose, the total amount of the *Nitrospira* populations and their local abundance at different distances relative to AOB had to be considered. The mean densities (= area fractions) of sublineage I and II *Nitrospira* ($D_{I}$ and $D_{II}$) in confocal FISH-images of the biofilm were determined. The density in one image is the ratio of the area covered by a *Nitrospira* population to the total image area. Based on the observed distribution in the biofilm (Fig. 2D), $D_{I}$ and $D_{II}$ were divided into local densities in $10 \times 50$ μm regions at increasing distances to the left and right edges of the $80 \times 50$ μm domain (Fig. 2C). Thus, area fractions of the 2-D domain covered by each *Nitrospira* population were obtained in 10 μm intervals away from these edges. Absolute cell numbers, which were needed for the simulation, were estimated by dividing these area fractions by the average area of single cells, which were measured for sublineage I or II *Nitrospira* in separate images recorded at a higher microscope magnification (data not shown). The obtained numbers of *Nitrospira* cells were randomly arranged in the respective $10 \times 50$ μm parts of the 2-D domain (Fig. 2C). The empty space in Fig. 2C represents other organisms within the biofilm (not consuming nitrite), and the biomass density of the overall biofilm was assumed to be 50 000 mg $l^{-1}$. The nitrite concentration at the left and right edges was set to a low value (0.25 mg nitrite-N $l^{-1}$), and the diffusion coefficient for nitrite within the biofilm was $1.23 \times 10^{-5}$ $cm^2$ $s^{-1}$ (Satoh *et al.*, 2003). Estimated kinetic parameters of the two uncultured *Nitrospira* populations used in the simulation are shown in Table 3. The precision of the model is limited as the exact kinetic properties of the *Nitrospira*-like bacteria are unknown and structural details of the real biofilm, including channels that could modify nutrient fluxes, were not simulated. Nevertheless, the simulation results clearly indicate that nitrite gradients can form within a few micrometres away from AOB colonies if physiologically active nitrite oxidizers are present (Fig. 2B). In addition, the nitrite gradients in the modelling results are in agreement with the aforementioned hypothesis and the quantified distribution of *Nitrospira*-like bacteria in the real

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**Table 3.** Estimated kinetic parameters of *Nitrospira*-like bacteria used for modelling local nitrite gradients in a nitrifying biofilm.

| | Max. specific growth rate[a], $\mu_{max}$ ($day^{-1}$) | Yield coefficient[b], $Y$ (g VSS (g N)$^{-1}$) | $K_M$ value for nitrite[c] (μM) |
|---|---|---|---|
| Sublineage I *Nitrospira* | 2 | 0.027 | 29 |
| Sublineage II *Nitrospira* | 0.75 | 0.027 | 10 |

**a.** The growth rate of sublineage I *Nitrospira* was estimated to be similar to the known growth rate of *N. moscoviensis* (2 $day^{-1}$; Ehrich *et al*., 1995). The growth rate of sublineage II *Nitrospira* in the biofilm was estimated to be between that of *N. moscoviensis* and *N. marina* (0.26 $day^{-1}$; Watson *et al*., 1986).
**b.** For the yield coefficient, a value from the range previously determined for *Nitrobacter* sp. (Prosser, 1989) was selected (VSS, volatile suspended solids). The maximal nitrite conversion rate, $q_{max}$, was calculated as $q_{max} = \mu_{max} \cdot Y^{-1}$.
**c.** The $K_M$ value selected for sublineage I *Nitrospira* is below known $K_M$ values of *Nitrobacter* spp. (Prosser, 1989; Hunik *et al*., 1993) according to the hypothesis that *Nitrospira* spp. are K-strategists whereas *Nitrobacter* spp. are r-strategists with higher $K_M$ values than *Nitrospira* (Schramm *et al*., 1999b). Based on microsensor measurements, 10 μM was estimated as the $K_M$ for nitrite of a mixed nitrifying community, whose active nitrite oxidizers were mainly sublineage II *Nitrospira* (Schramm *et al*., 1998; 1999b).

biofilm. At distances of 0–10 μm, where co-aggregation of sublineage I *Nitrospira* and AOB was most pronounced in the biofilm, the simulated nitrite concentration was much higher than at distances of 20–40 μm, where sublineage II *Nitrospira* co-aggregated with AOB in the biofilm (Fig. 2B–D). Simulations with 0.5 mg nitrite-N $l^{-1}$ on the edges of the 2-D domain and a biofilm density of 20 000 mg $l^{-1}$ produced similar results (data not shown). It is likely that in complex biofilms, the local abundance of *Nitrospira* is influenced not only by nitrite but also by other factors such as competition with AOB and heterotrophic bacteria for oxygen. Furthermore, Stüven and colleagues (1992) showed that hydroxylamine, which is an intermediate of the conversion of ammonia to nitrite, is released by AOB and has a strong inhibitory effect on nitrite oxidizers. Consequently, different resistance against hydroxylamine could also affect the spatial arrangement of different *Nitrospira*-like bacteria relative to AOB colonies.

In the activated sludge flocs, the differences in the spatial arrangement of sublineage I and II *Nitrospira* were still detectable but much less pronounced than in the biofilm (Fig. 2E). The constant mixing of activated sludge in a wastewater treatment plant causes shear forces, which lead to continuous floc breakage and re-aggregation (e.g. Biggs and Lant, 2000). Furthermore, the loose floc structure facilitates advective transport of solutes and gases, which counteracts the formation of gradients (Schramm *et al*., 1999a). These effects will almost certainly lead to less distinct and more dynamic localization patterns of microbial populations in sludge flocs as compared with biofilms.

### *Long-term competition experiment*

In order to test how different nitrite concentrations affect *Nitrospira* sublineages I and II, a competition experiment was performed by incubating activated sludge aliquots from the WWTP Gleisdorf in four Erlenmeyer flasks (named A–D) in buffered mineral medium ($NaHCO_3$ 20 mg $l^{-1}$, $KH_2PO_4$ 110 mg $l^{-1}$, $MgSO_4 \cdot 7H_2O$ 380 mg $l^{-1}$, $FeSO_4 \cdot 7H_2O$ 7.2 mg $l^{-1}$, ethylenediaminetetraacetic acid 10.3 mg $l^{-1}$, $ZnSO_4 \cdot 7H_2O$ 0.5 mg $l^{-1}$, $CuSO_4 \cdot 5H_2O$ 0.5 mg $l^{-1}$, $NaMoO_4 \cdot 2H_2O$ 0.5 mg $l^{-1}$, pH 7.4–7.6) amended with nitrite in low (flasks A and B) or high (flasks C and D) concentrations (i.e. two parallel incubations per nitrite concentration were carried out). Both nitrite concentrations (low: approximately 0.1 mg $NO_2^-$-N $l^{-1}$; high: approximately 5 mg $NO_2^-$-N $l^{-1}$) are in a range that selects for *Nitrospira* and against *Nitrobacter* (Wagner *et al*., 2002). The nitrite concentrations were approximately maintained by pumping small volumes of highly concentrated nitrite stock solutions (prepared in mineral medium, see above) into the flasks at appropriate flow rates and by removing accumulated excess medium (maximum 8% of the volume of the liquid in a flask) once per day. The amount of nitrite in the flasks was frequently checked by using $NO_2^-$ test strips (Merckoquant, range: 2–80 mg $l^{-1}$ nitrite, Merck, Germany) for the high concentrations and the spectrophotometric Griess assay (Promega, Wisconsin) for the low concentrations. In a second phase of the experiment, the nitrite concentrations in the flasks were switched from low to high (flasks A and B) or high to low (flasks C and D). Sufficient aeration was achieved by pumping air through porous air stones into the flasks, and the flasks were kept at room temperature in the dark during the whole experiment. Fluorescence *in situ* hybridization, confocal laser scanning microscopy and digital image analysis (Schmid *et al*., 2000; Daims *et al*., 2006) were used at different time points to determine the abundance of the two *Nitrospira* populations in the four sludge aliquots by measuring the biovolume of probe-labelled sublineage I and II *Nitrospira* relative to the total biovolume of all *Nitrospira*-like bacteria as detected by a genus *Nitrospira*-specific probe (Table 2; Fig. 2F and G).

*Nitrospira* sublineages I and II were almost equally abundant in the original activated sludge and, with slight fluctuations, also during the incubation with a low nitrite concentration in flasks A and B (Fig. 2F). However, when

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the nitrite concentration was raised in these two flasks, the abundance of sublineage I *Nitrospira* increased rapidly to 70–90% of all *Nitrospira*-like bacteria and stayed at this high level over the following 154 days, while the proportion of sublineage II *Nitrospira* decreased to 10–20% of all *Nitrospira*-like bacteria (Fig. 2F). Consistent with these findings, an inverse development of the two *Nitrospira* populations was observed in flasks C and D that might be explained by the reversed sequence of nitrite concentrations in these flasks (Fig. 2G). These results indicate that the nitrite concentration had a selective effect on the two *Nitrospira* populations. However, sublineage II *Nitrospira* were never completely eradicated although sublineage I *Nitrospira* were dominant in all flasks during the incubations with the high nitrite concentration (Fig. 2F and G). This can be attributed to the fed-batch set-up of the competition experiment: Firstly, constant biomass removal did not take place like in a continuously operated chemostat, and thus no population could be washed out. Secondly, the nitrite concentrations could not be adjusted as precisely as in a chemostat. The resulting, inevitable fluctuations of the nitrite concentrations may have enabled the coexistence of sublineage I and II *Nitrospira* during all phases of the experiment, with sublineage I being more abundant as long as the nitrite concentration was high for most of the incubation time. We can also not rule out that FISH detected metabolically inactive *Nitrospira* cells. It has been shown that nitrifying bacteria retain a high cellular ribosome content and are stained by rRNA-targeted probes during periods of starvation or chemical inhibition (Wagner *et al*., 1995; Morgenroth *et al*., 2000). This might explain the slow decrease of the relative biovolume of sublineage I *Nitrospira* in flasks C and D after day 20 (Fig. 2G).

Compared with the volume of the total bacterial biomass detectable by FISH with the bacterial probe mix (Table 2), the combined relative biovolume of both *Nitrospira* sublineages was approximately 8% in the original sludge. In all four flasks, this value increased significantly during incubation with the high nitrite concentration (Fig. 2F and G). This development was accompanied by an increase in the size of the microcolonies formed by sublineage I *Nitrospira* (data not shown), indicating that this population was enriched under these conditions. In flasks C and D, the total biovolume fraction of *Nitrospira* sank again to *c*. 7% of the total bacterial biovolume during incubation with the low nitrite concentration (Fig. 2G). Whether this change was caused solely by a decrease of *Nitrospira* or also by a simultaneous increase in the amount of heterotrophic bacteria, which may feed on organic compounds released by lysing *Nitrospira* cells (Okabe *et al*., 2005), cannot be resolved based on our data as absolute abundances were not measured.

The results of the competition experiment could be explained by a competitive advantage of sublineage I *Nitrospira* at higher nitrite concentrations. This finding correlates well with the spatial localization patterns of *Nitrospira* observed in the biofilm and sludge samples. Based on microsensor measurements, Schramm and colleagues (1999b) postulated that *Nitrospira*-like bacteria are K-strategists with a high affinity to nitrite and oxygen and reach high densities under substrate-limited conditions, while *Nitrobacter* species are r-strategists with a lower nitrite and oxygen affinity and outcompete *Nitrospira* only at higher substrate concentrations. The results obtained here allow the hypothesis that further ecological differentiation occurred within the genus *Nitrospira*, whose sublineages may occupy different positions on an imaginary scale reaching from K- to r-strategies. Accordingly, sublineage I *Nitrospira* may resemble r-strategists more than the organisms in sublineage II, and thus may outcompete them as soon as the nitrite concentration is high enough to support the required growth rate. However, additional factors such as affinity to oxygen, protozoan grazing, the presence of specific phages and the availability of organic substrates (at least some *Nitrospira*-like bacteria are mixotrophic; Daims *et al*., 2001), may also co-influence the abundance and distribution of the different sublineages under *in situ* conditions.

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# Chapter III

## Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium '*Candidatus* Nitrospira defluvii'

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# Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium '*Candidatus* Nitrospira defluvii'

**Frank Maixner,[1] Michael Wagner,[1]* Sebastian Lücker,[1] Eric Pelletier,[2] Stephan Schmitz-Esser,[1] Karin Hace,[1] Eva Spieck,[3] Robert Konrat,[4] Denis Le Paslier[2] and Holger Daims[1]**

[1]*Department für Mikrobielle Ökologie, Universität Wien, Althanstrasse 14, A-1090 Vienna, Austria.*

[2]*CEA/Genoscope CNRS-UMR 8030, 2 rue Gaston Crémieux CP 5706, 91057 Evry, France.*

[3]*Universität Hamburg, Biozentrum Klein Flottbek, Mikrobiologie, Ohnhorststr. 18, D-22609 Hamburg, Germany.*

[4]*Department für Biomolekulare Strukturchemie, Universität Wien, Campus Vienna Biocenter 5, A-1030 Vienna, Austria.*

## Summary

**Nitrite-oxidizing bacteria of the genus *Nitrospira* are ubiquitous in natural ecosystems and also in wastewater treatment plants. *Nitrospira* are members of a distinct phylum, not closely related to other nitrifiers, and no genomic sequences from this genus have been available so far. Here we applied an environmental genomics approach to sequence and assemble a 137 kbp-long genome fragment of '*Candidatus* Nitrospira defluvii', which had been enriched from activated sludge and belongs to *Nitrospira* sublineage I without isolated representatives. The annotation of this contig, which carried the 16S rRNA gene of *N. defluvii*, offered first insight into the genome of *Nitrospira*. Surprisingly, we found a gene similar to genes encoding chlorite dismutase (CLD), an enzyme degrading chlorite ($ClO_2^-$) to $Cl^-$ and $O_2$. To date, CLDs with high catalytic activity have been found only in perchlorate- and chlorate-reducing bacteria but not in nitrifiers. Heterologous expression in *E. coli* followed by enzymatic tests confirmed that this gene of *Nitrospira* encodes a highly active CLD, which is also expressed *in situ* by *Nitrospira*, indicating that this nitrite oxidizer might be involved in the bioremediation of perchlorate and chlorite. Phylogenetic analyses showed that CLD and related proteins are widely distributed among the *Bacteria* and *Archaea*, and indicated that this enzyme family appeared relatively early in evolution, has been subject to functional diversification and might play yet unknown roles in microbial metabolism.**

## Introduction

Nitrification, the microbially catalysed oxidations of ammonia to nitrite and of nitrite to nitrate, is a key process of the biogeochemical nitrogen cycle and of biological wastewater treatment. The two steps of nitrification are carried out by two distinct functional groups of chemolithotrophic prokaryotes: the ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Koops *et al*., 2003; Könneke *et al*., 2005), and the nitrite-oxidizing bacteria (NOB) (Bock and Wagner, 2001; Alawi *et al*., 2007). As all known nitrifying prokaryotes are fastidious and slow-growing organisms, their enrichment and isolation from environmental samples are difficult and most physiological studies have been performed with pure cultures of a few 'model' nitrifiers, in particular AOB related to the genus *Nitrosomonas* and NOB of the genus *Nitrobacter*. The recently published genome sequences of cultured bacterial nitrifiers (Chain *et al*., 2003; Klotz *et al*., 2006; Starkenburg *et al*., 2006; Stein *et al*., 2007) offer yet unmatched insight into the biology of these organisms. However, studies using cultivation-independent techniques have revealed a large diversity of yet uncultured and so far poorly characterized AOB, NOB and AOA in the environment (e.g. Daims *et al*., 2001a;Smith *et al*., 2001; Francis *et al*., 2005; Leininger *et al*., 2006). Our understanding of nitrification and nitrogen cycling in nature remains incomplete without more data about the ecophysiology and genomics of these uncultured nitrifiers.

The genus *Nitrospira* is one of the less intensively studied groups of NOB. This genus, which belongs to the distinct bacterial phylum *Nitrospirae* (Ehrich *et al*., 1995), is unrelated to all other known nitrifiers. Except two described species (Watson *et al*., 1986; Ehrich *et al*., 1995) and one recently isolated candidate species (Lebedeva *et al*., 2008), no members of this genus are available in

Received 7 December, 2007; accepted 22 March, 2008. *For correspondence. E-mail wagner@microbial-ecology.net; Tel. (+43) 14277 54390; Fax (+43) 14277 54389.

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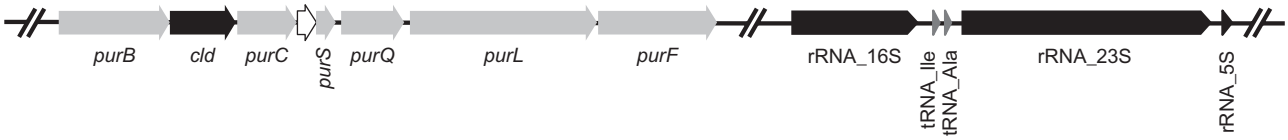


**Fig. 1.** Schematic representation of a part of the analysed genome region of *N. defluvii*. Arrows show the locations of the gene encoding chlorite dismutase (*cld*), the *pur* purine biosynthesis genes in close vicinity to *cld* and the *rrn* operon (including the spacer regions with two tRNA genes). The function of the ORF between *purC* and *purS* (white arrow) is unknown. This figure is a downsized version of Fig. S1 that shows the complete contig with all identified coding sequences.

pure culture. 16S rRNA sequence analyses showed that the genus *Nitrospira* contains several phylogenetic sublineages (Daims *et al*., 2001b), whose uncultured representatives are widely distributed in many kinds of aquatic and terrestrial habitats. Moreover, *Nitrospira* are the dominant nitrite oxidizers in wastewater treatment plants (Juretschko *et al*., 1998). Nevertheless, our understanding of the biology of *Nitrospira* is very limited compared with more easily culturable nitrifiers such as *Nitrobacter* and *Nitrosomonas* species.

During the last few years, environmental genomics approaches have dramatically influenced the way we think about the nitrogen cycle (Schleper *et al*., 2005; Hallam *et al*., 2006; Strous *et al*., 2006). In this study, we applied an environmental genomics approach to sequence a large genome fragment of the nitrite oxidizer '*Candidatus* Nitrospira defluvii' (Spieck *et al*., 2006). The analysis provided first insight into the genome of *Nitrospira* and led to the discovery of an unexpected function, which has previously not been documented in any nitrifying organism.

## Results and discussion

### *Analysis of a large genome piece of* Nitrospira

Bacterial artificial chromosome (BAC) and plasmid shotgun libraries were established from genomic DNA, which had been extracted from an enrichment of '*Candidatus* Nitrospira defluvii'. This organism was enriched from a domestic wastewater treatment plant and belongs to the phylogenetic sublineage I of the genus *Nitrospira* (Spieck *et al*., 2006), whose representatives are the dominant NOB in many wastewater treatment systems (Daims *et al*., 2001b). At the time when the metagenome libraries for this study were created, the enrichment consisted of approximately 40% of *N. defluvii* as determined by quantitative fluorescence *in situ* hybridization (FISH). No *Nitrobacter* cells were detectable by FISH in the enrichment. Despite the relatively low degree of enrichment of *N. defluvii*, it was possible to reconstruct from the obtained metagenomic sequences a 137 336 bp-long contig, which contained the complete rRNA operon of *N. defluvii* and thus clearly belonged to this bacterium (Fig. 1). The contig had an average GC-content of 58.45% and contained 153 coding sequences. No functions could be assigned to 74 open reading frames (ORFs) that shared sequence similarities only with ORFs of unknown function in other organisms (13 ORFs), or contained only short conserved motifs or structural features (two ORFs), or showed no similarity at all to any sequence in the public databases (59 ORFs). The large number of ORFs without any database matches reflects that *N. defluvii* belongs to a bacterial phylum without genome-sequenced representatives except some *Leptospirillum* strains (Tyson *et al*., 2004), which are acidophilic iron oxidizers with a different physiology than *Nitrospira*.

Functions were assigned with a relatively high degree of confidence to 59 of the remaining 79 coding sequences based on strong similarities to functionally characterized genes in other organisms. Among them was the *rrn* operon with the genes of the 16S, 23S and 5S ribosomal RNAs. The spacer region between the 16S and 23S rRNA genes contained two tRNA genes for isoleucine and alanine respectively (Fig. 1). A description of the other annotated genes is provided as supplementary online material together with a complete gene list (Table S1) and a figure showing the localization of all genes on the contig (Fig. S1).

### N. defluvii *has a gene highly similar to chlorite dismutase*

A surprising discovery on the analysed genome piece of *N. defluvii* was the gene *cld*, whose predicted product is similar in length and amino acid sequence to the enzyme chlorite dismutase (CLD) of *Dechloromonas aromatica*, *D. agitata* and *Ideonella dechloratans* (Table 1). These heterotrophic *Betaproteobacteria* are able to respire,

**Table 1.** Amino acid sequence identities between the CLD of *N. defluvii* (264 amino acids) and the CLDs of known (per)chlorate-reducing bacteria.

| Organism | Amino acid residues | Identity (%)[a] |
|---|---|---|
| *Dechloromonas aromatica* RCB | 282 | 40.5 |
| *Dechloromonas agitata* | 277 | 39.6 |
| *Ideonella dechloratans* | 285 | 38.0 |

**a.** Identity to the CLD of *N. defluvii*.

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under anaerobic conditions, by transferring electrons from organic electron donors to chlorate ($ClO_3^-$) or perchlorate ($ClO_4^-$, only *D. aromatica* and *D. agitata*) as electron acceptors (Malmqvist *et al*., 1994; Achenbach *et al*., 2001; Chaudhuri *et al*., 2002). This reaction is mediated by (per)chlorate reductases. CLD is of major importance for the (per)chlorate-reducing pathway, because the reduction of (per)chlorate yields chlorite ($ClO_2^-$), a strong oxidant with cell-damaging effects (Ueno *et al*., 2000). Chlorite is degraded by CLD in a unique and biochemically not yet fully understood reaction (van Ginkel *et al*., 1996; Hagedoorn *et al*., 2002; Eq. 1):

$$ClO_2^- \rightarrow Cl^- + O_2 \quad (1)$$

Thus (per)chlorate-reducing bacteria (PCB) depend on CLD to prevent self-toxification owing to chlorite accumulation when (per)chlorate is used as electron acceptor.

The CLD of *I. dechloratans* is located in the periplasm (Stenklo *et al*., 2001; Thorell *et al*., 2002). Accordingly, *in silico* analyses predicted that the 5′ end of gene *cld* of *Nitrospira* encodes a 26 amino acids long signal peptide for protein export into the periplasmic space. The hitherto characterized CLDs contain haem groups as cofactors (van Ginkel *et al*., 1996; Stenklo *et al*., 2001) and have a highly conserved histidine residue in the α4-helix (Fig. S2). This histidine, which has been shown to be a proximal ligand to the iron center of the haem in CLD and closely related enzymes (Hagedoorn *et al*., 2002; Ebihara *et al*., 2005), is also present in the CLD of *N. defluvii* (residue 186, Fig. S2).

As nitrifying bacteria have so far not been considered to be PCB, the discovery of *cld* in *N. defluvii* was unexpected. It raised the questions of whether the gene product really is a functional CLD, whether *N. defluvii* expresses this gene, and which function CLD might have in the metabolism of *Nitrospira*.

### *Characterization and functional validation of the CLD of* N. defluvii

To determine whether gene *cld* of *N. defluvii* encodes an enzyme with CLD activity, the gene was polymerase chain reaction (PCR)-amplified from the *N. defluvii* enrichment, cloned and heterologously expressed in *E. coli*. One reason to choose *E. coli* as expression host was the absence of any gene similar to *cld* in the *E. coli* genome. Interestingly, the transformed *E. coli* cultures became red after the expression of the recombinant protein had been induced (Fig. S3A). As liquid preparations of CLD are reddish in colour (van Ginkel *et al*., 1996), the red colour of the *E. coli* cultures was a first indicator for a successful expression of the *Nitrospira* enzyme in the phylogenetically unrelated host *E. coli*. A clear and red solution was obtained after His-tag purification of the expressed

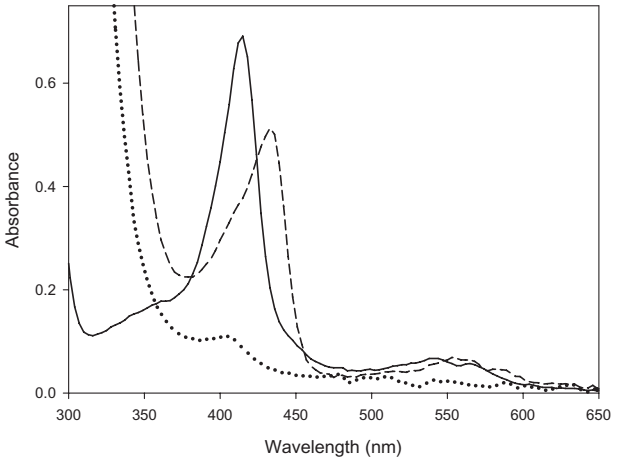


**Fig. 2.** UV/VIS spectra of the heterologously expressed CLD of *N. defluvii* immediately after purification (solid curve), in presence of 20 mM sodium dithionite as reductant (stippled curve) and after addition of 200 mM sodium chlorite (dotted curve).

protein. The CLDs of the PCB 'strain GR-1' and *I. dechloratans* are homotetramers with an apparent molecular mass of 32 or 25 kDa, respectively, per subunit (van Ginkel *et al*., 1996; Stenklo *et al*., 2001). According to denaturing gel electrophoresis, the purified protein of *Nitrospira* had an apparent molecular mass of approximately 30 kDa (including the His-tag). Thus, a single subunit of the *Nitrospira* protein is similar in mass to the subunits of previously characterized CLDs, but the number of subunits in the native enzyme of *N. defluvii* remains to be determined.

The UV/VIS spectrum of the purified protein was consistent with the spectrum of a haem enzyme and contained three distinct adsorption maxima at 415 (the Soret peak), 541 and 565 nm (Fig. 2). After reduction of the protein with sodium dithionite the three maxima shifted to 433, 553 and 583 nm respectively (Fig. 2). Highly similar spectra have been recorded with reduced CLDs of the PCB 'strain GR-1' (van Ginkel *et al*., 1996) and *I. dechloratans* (Stenklo *et al*., 2001). The haem was separable from the apoprotein by acidic butanone precipitation, indicating that the CLD of *Nitrospira* may contain a non-covalently bound a- or b-type haem like the CLDs of the (per)chlorate reducers 'strain GR-1' and *I. dechloratans* (van Ginkel *et al*., 1996; Stenklo *et al*., 2001). An alternative explanation would be that the cytochrome c maturation system of *E. coli* was not able to make the appropriate covalent attachment between the haem and the *Nitrospira* CLD. Ebihara and colleagues (2005) found a covalently bound c-type haem in the CLD-like protein of *Thermus thermophilus*, but this protein had only low CLD activity (Table 2).

We performed a first functional test by adding dissolved chlorite to a sonicated cell suspension of an *E. coli* culture, which had expressed the protein of *Nitrospira*.

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**Table 2.** Catalytic parameters of characterized enzymes with CLD activity and chlorite as substrate.

| Organism | $V_{max}$ [U(mg protein)$^{-1}$] | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) | Reference |
|---|---|---|---|---|---|
| *Nitrospira defluvii* | $1.9 \times 10^3$ | $0.96 \times 10^3$ | 15.8 | $6.1 \times 10^4$ | This study |
| 'Strain GR1' | $2.2 \times 10^3$ | $1.2 \times 10^3$ | 0.17 | $7.1 \times 10^6$ | van Ginkel *et al.* (1996) |
| *Ideonella dechloratans* | $4.3 \times 10^3$ | $1.8 \times 10^3$ | 0.26 | $6.9 \times 10^6$ | Stenklo *et al.* (2001) |
| *Thermus thermophilus*[a] | 1.6 | 0.77 | 13 | 59 | Ebihara *et al.* (2005) |

**a.** $V_{max}$ was not reported in the original publication and is estimated based on $k_{cat}$ and the molar mass, which was derived from the amino acid sequence.
*Ideonella dechloratans* and 'strain GR1' are known PCB.

According to Eq. 1, production of gas (presumably $O_2$) was observed instantly (Fig. S3B). The subsequent addition of silver nitrate confirmed that $Cl^-$ had been formed, too (Fig. S3C). In contrast, no visible gas production occurred and no $Cl^-$ was formed after addition of chlorite to a sonicated *E. coli* culture that had not expressed gene *cld* of *Nitrospira* (Fig. S3). Production of gas and $Cl^-$ were also observed when $ClO_2^-$ was added to a solution of the purified recombinant enzyme (data not shown). Thus, the heterologously expressed enzyme of *Nitrospira* clearly had the activity of CLD. In these experiments, the red colour of CLD diminished during the reaction (not shown), indicating that the enzyme was degraded owing to oxidation by excess chlorite. Similar observations were made by van Ginkel and colleagues (1996) with the CLD of the PCB 'strain GR-1'. The degradation of CLD by excess substrate also led to a dramatic reduction of the three adsorption maxima in the UV/VIS spectrum (Fig. 2) and caused the specific activity of CLD to decline in presence of higher chlorite concentrations (Fig. 3A). The temperature and pH optima of the enzyme were 25°C and pH 6.0 (Fig. S4). These optima are similar to those of the CLDs of 'strain GR-1' (30°C, pH 6.0; van Ginkel *et al.*, 1996) and *Dechlorosoma* sp. strain KJ (pH 6.0; Xu and Logan, 2003). We determined the $K_m$ value of the CLD of *N. defluvii* to be 15.8 $\pm$ 1.1 mmol $l^{-1}$ of chlorite and $V_{max}$ to be 1939 $\pm$ 47 U (mg protein)$^{-1}$ under optimal reaction conditions (Fig. 3B). Although $V_{max}$ and the turnover number ($k_{cat}$) of the *Nitrospira* enzyme are similar to the respective values of chlorite dismutases of PCB, its $K_m$ is much higher (Table 2). The high $K_m$ might be explained by suboptimal folding or missing post-translational modifications (e.g. a missing covalent attachment of the haem) of the recombinant *Nitrospira* CLD in the heterologous expression host *E. coli*, and the native enzyme may have different catalytic properties in *Nitrospira*. Alternatively, the relatively low affinity for chlorite could have evolutionary or physiological causes. The ratio $k_{cat}/K_m$, which is referred to as the performance constant, is an indicator for the reaction efficiency of an enzyme with a given substrate (Koshland, 2002). Mutations that increase the catalytic rate (i.e. increase $k_{cat}$) or the substrate affinity (i.e. decrease $K_m$) result in a better catalytic performance. Its lower performance constant indicates that the CLD of *Nitrospira* may not be adapted as perfectly as the CLDs of known PCB to the substrate chlorite (Table 2). It could still be an evolving CLD or it might have an unknown primary function that is not chlorite degradation. Ebihara and colleagues (2005) determined the 3D structure of a CLD-like protein, which has a very low performance constant with chlorite (Table 2). Because this enzyme of *Thermus thermophilus* also showed a low catalase activity, they hypothesized that it might protect the cell from damage owing to reactive oxygen intermediates (ROI) by catalysing electron transfers via its haem groups. At present we cannot exclude that the enzyme of *Nitrospira* functions *in vivo* as a scavenger of ROI, reactive nitrogen intermediates, or other toxic substances different from chlorite. Preliminary experiments were performed with $H_2O_2$, hydroxylamine, nitrite and NO as candidate substrates, but no functional clue was obtained (data not shown).

### *Expression and function of CLD in* Nitrospira

The *cld* genes and the genes coding for (per)chlorate reductases are located in close vicinity in the genomes of *I. dechloratans*, *D. agitata* and *D. aromatica* (Thorell *et al.*, 2003; Bender *et al.*, 2005), forming a functional unit for (per)chlorate reduction in these PCB. In contrast, gene *cld* of *N. defluvii* is part of a cluster that contains six other genes, which are involved in the *de novo* biosynthesis of purines, and one ORF of unknown function (Fig. 1). Thus, based on the genomic context of *cld* the functional role of CLD in *N. defluvii* is not as obvious as in the aforementioned PCB.

To determine whether *N. defluvii* actually expresses CLD, Western blot experiments were performed with CLD-specific antibodies and cell extracts from an aerobically grown nitrite-oxidizing enrichment of *N. defluvii*. This newer enrichment, which was obtained after the metagenome library construction, contained as much as 86% of *N. defluvii* (Spieck *et al.*, 2006). The Western blot revealed that *N. defluvii* expressed CLD under the applied aerobic incubation conditions (Fig. 4). The high specificity

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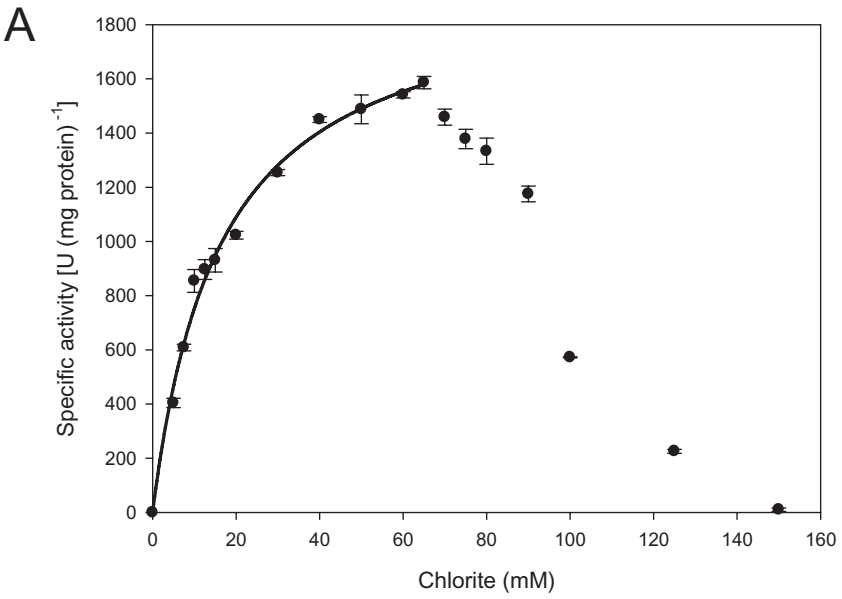


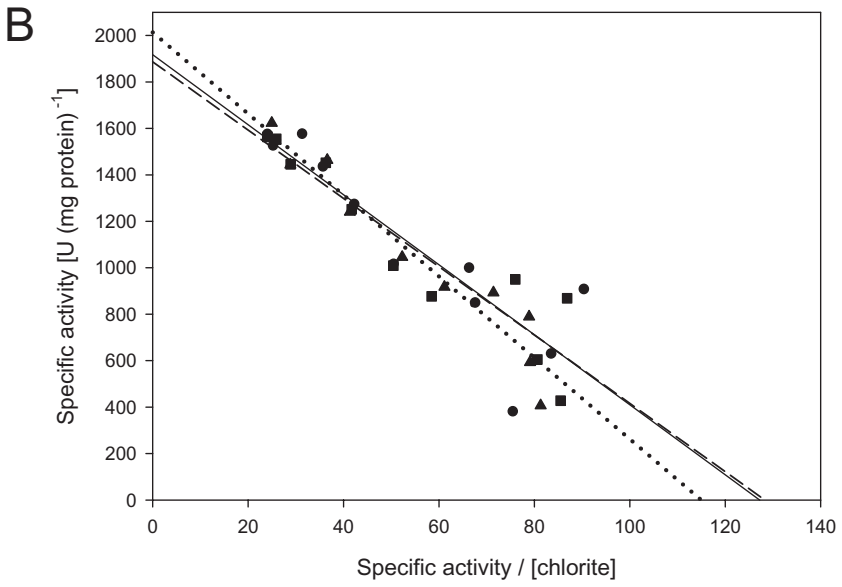


**Fig. 3.** Catalytic parameters of the recombinant CLD of *N. defluvii*.
A. Specific activity as function of the chlorite concentration. Curve fitting based on the Michaelis–Menten equation was performed on the data points between 0 and 65 mM sodium chlorite (●). This subset of the data was used to determine $K_m$ and $V_{max}$ in (B). Data points show the mean and error bars show the standard error of the mean of triplicate experiments.
B. Eadie–Hofstee plots of the data shown in (A) from 0 to 65 mM sodium chlorite. Each of the symbols (●, ▲ and ■) and regression lines (solid, stippled and dotted) depict the results of one of the triplicate experiments. The $K_m$ and $V_{max}$ values were determined based on these plots.

of the antibodies, which bound to the CLD of *Nitrospira* but not to the CLD of *D. agitata* used as a control (Fig. 4), the high abundance of *Nitrospira* in the new enrichment, and the absence of any other *cld* genes on sequence reads from the metagenomic library strongly suggest that no CLD except that of *Nitrospira* was detected in this experiment. However, at present we cannot exclude that other regions of the *N. defluvii* genome may contain additional copies of *cld*-like genes.

The expression of CLD in *N. defluvii* under aerobic conditions in a medium without (per)chlorate contradicts observations made with some previously studied PCB. In these bacteria, the expression of CLD depends on low oxygen concentrations and the presence of chlorate or perchlorate (Bender *et al.*, 2002; Chaudhuri *et al.*, 2002; O'Connor and Coates, 2002). However, there are also examples for a basal expression of CLD in presence of $O_2$ and even for a constitutive chlorate-independent expression in known PCB (Coates and Achenbach, 2004; Xu *et al.*, 2004).

Although the contig did not contain any genes of (per)chlorate reductases or related enzymes, it is tempting to speculate that *N. defluvii* might be able to gain energy via the reduction of (per)chlorate to chlorite, which

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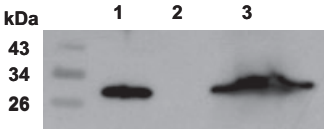


**Fig. 4.** Western blot analyses with polyclonal antibodies raised against the CLD of *N. defluvii*. Lane 1: purified *N. defluvii* CLD; lane 2: crude protein extract from *D. agitata* cells grown anaerobically in presence of chlorate; lane 3: crude protein extract from the nitrite-oxidizing enrichment of *N. defluvii*.

would subsequently be detoxified by the CLD. The electron donors for this metabolism could be simple organic molecules such as pyruvate, which is used by *Nitrospira* in wastewater treatment plants (Daims *et al*., 2001b), or even nitrite. The reduction potential ($E_0'$) of the $ClO_3^-/ClO_2^-$ redox pair is 0.709 V, that of $ClO_4^-/ClO_3^-$ is 0.788 V, and that of $NlO_2^-/NO_3^-$ is 0.43 V. Under standard conditions, the free-energy change ($\Delta G_0'$) with nitrite as electron donor is –54 kJ (mol $NO_2^-)^{-1}$ for chlorate and –61 kJ (mol $NO_2^-)^{-1}$ for perchlorate reduction (the final conversion of chlorite to $Cl^-$ and $O_2$ is not assumed to be an energy-conserving step). Nitrite oxidation with $O_2$ as electron acceptor is thermodynamically more favourable [$\Delta G_0' = -74.8$ kJ (mol $NO_2^-)^{-1}$; Cobley, 1976]. Under the assumption that the free-energy utilization efficiency of *Nitrospira* is similar to that of *Nitrobacter* (15–51%; Laudelout *et al*., 1968), a maximum of 0.6 mol ATP·(mol $NO_2^-)^{-1}$ can be produced with chlorate and 0.7 mol ATP·(mol $NO_2^-)^{-1}$ with perchlorate respectively [based on a phosphorylation potential of 44 kJ (mol ATP)$^{-1}$; Lengeler *et al*., 1999]. These values are not much lower than the ATP yield of aerobic nitrite oxidation [maximum 0.8 mol ATP·(mol $NO_2^-)^{-1}$ with the aforementioned free-energy utilization efficiency]. Thus, according to these rough estimations chlorate and perchlorate could be alternative electron acceptors for nitrite-oxidizing *Nitrospira* although growth would probably be slower than under aerobic nitrifying conditions. This reaction might be mediated by the key enzyme of nitrite oxidation, nitrite oxidoreductase (NXR). Functioning in both directions, NXR can oxidize nitrite to nitrate or reduce nitrate to nitrite (Sundermeyer-Klinger *et al*., 1984). Similar to nitrate reductase (Kucera, 2006), NXR may use chlorate instead of nitrate and reduce it to chlorite. Interestingly, the nitrite-oxidizing enzyme systems of *Nitrospira moscoviensis* and also of *Nitrobacter* can indeed oxidize nitrite with chlorate as electron acceptor (Meincke *et al*., 1992; Spieck *et al*., 1998). Accordingly, nitrite oxidation linked to chlorate reduction was observed with *Nitrobacter* pure cultures under aerobic and even under anaerobic conditions (Lees and Simpson, 1957; Hynes and Knowles, 1983). However, the nitrite oxidation rates were high for a short period only and declined soon owing to inhibition by accumulating chlorite, which was not detoxified by *Nitrobacter* (Hynes and Knowles, 1983) and destroyed cytochromes of this organism (Lees and Simpson, 1957). Thus, *Nitrobacter* is sensitive to chlorite although *cld*-like genes occur in the genomes of *Nitrobacter hamburgensis* and *N. winogradskyi* (Starkenburg *et al*., 2006). However, these genes differ from that of *N. defluvii* (Fig. 5, Fig. S2) and nothing is known about their regulation and the activities of their gene products. As *N. defluvii* has a functional CLD, chlorite produced during (per)chlorate reduction might have less adverse effects on this organism than on *Nitrobacter*.

Perchlorate, chlorate and chlorite are industrial contaminants. They are not naturally formed in significant amounts except in mineral deposits in Chile, where the perchlorate content is naturally high. However, the industrial use of perchlorate, its discharge into the environment and the application of (per)chlorate-containing fertilizers, disinfectants and bleaching agents have led to increased levels of these compounds even in areas not directly associated with industrial activities in the USA (reviewed by Coates and Achenbach, 2004) and certainly also in other countries. This might explain the ubiquity of PCB in the environment (Coates *et al*., 1999). As nitrifiers are microbial key players in most ecosystems, *Nitrospira* may occur also at contaminated sites where they are exposed to (per)chlorate and chlorite. If CLD enabled them to survive in presence of chlorite or even to use (per)chlorate as electron acceptor, *Nitrospira* would be functionally important members of (per)chlorate-degrading microbial communities and would link the nitrogen cycle to (per)chlorate bioremediation.

CLD in *Nitrospira* might have other functions not directly linked to energy conservation via (per)chlorate reduction. In hypoxic microniches, the conversion of chlorite to $Cl^-$ and $O_2$ could provide oxygen for the usual aerobic oxidation of nitrite to nitrate. If *Nitrospira* is not a (per)chlorate reducer, the chlorite could be supplied by PCB living in the same community as *Nitrospira*. A similar kind of symbiosis has been described by Coates and colleagues (1998), who found that under anaerobic conditions, chlorite degradation by PCB provided sufficient $O_2$ for hydrocarbon oxidation by an aerobic bacterium.

*Nitrospira defluvii* may also be exposed to chlorate and chlorite in nitrifying wastewater treatment plants. Most municipal and industrial wastewaters do not contain high concentrations of these compounds, but adding hypochlorite to activated sludge has been common practice as an emergency measure to fight excess growth of filamentous bacteria, which cause activated sludge bulking and foaming (Seka *et al*., 2003). Hypochlorite is a strong oxidizing agent but also decomposes to $Cl^-$ and chlorate (Adam *et al*., 1992), the latter being the substrate for the biologically catalysed production of chlorite (van Ginkel *et al*., 1995). Thus, *N. defluvii* expressing CLD may benefit from a better protection against chlorite during activated sludge chlorination.

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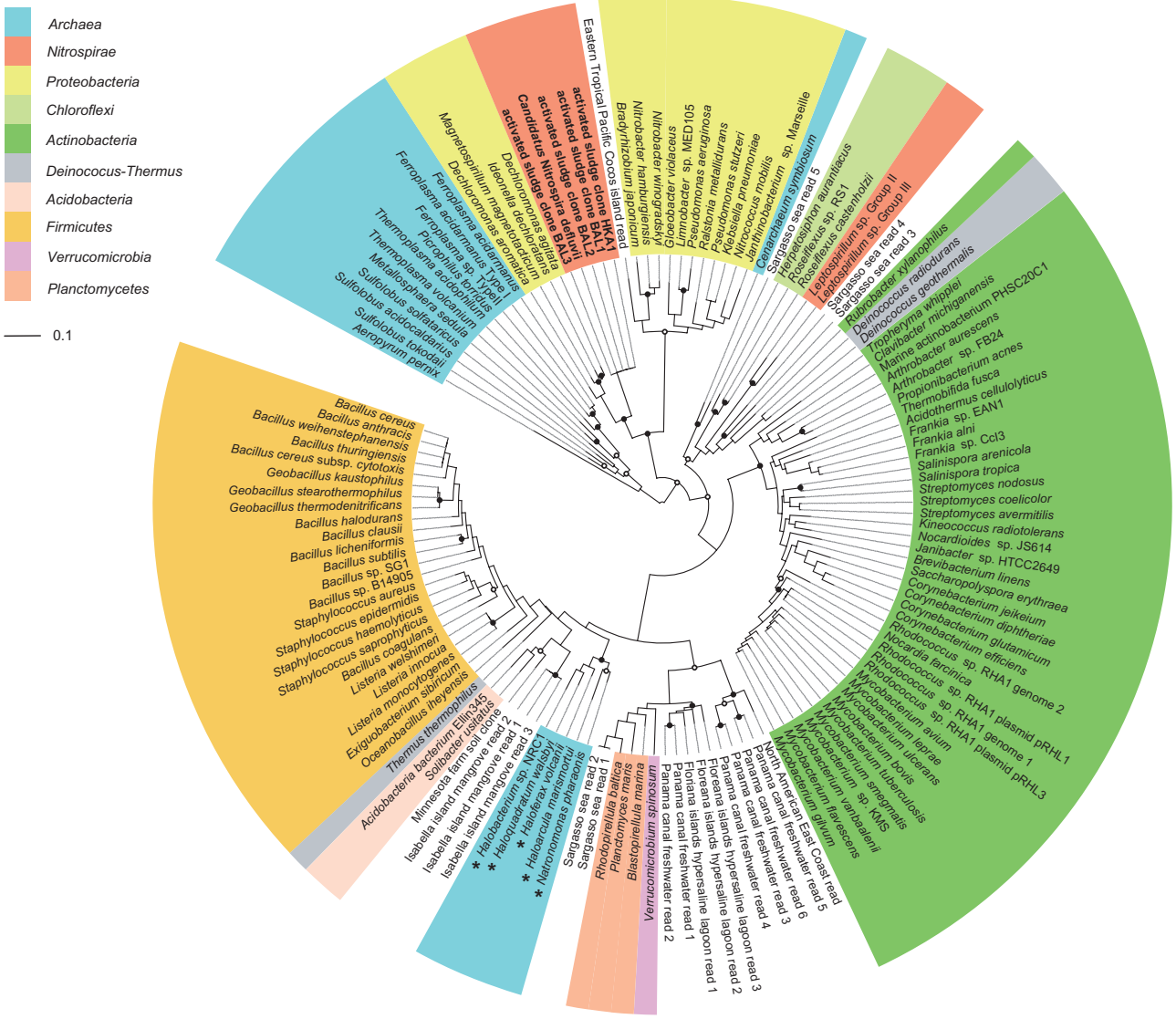


**Fig. 5.** Maximum likelihood tree based on amino acid sequences of CLD-like proteins. Sequences obtained in this study from *N. defluvii* and from nitrifying activated sludge (data not shown) are printed bold. Colours depict the affiliations of the respective organisms with bacterial and archaeal phyla according to 16S rRNA-based phylogeny. Sequences marked with an asterisk are CLD-like domains in fusion proteins. White circles represent quartet puzzling reliability values ≥70%. Black circles symbolize additional high parsimony bootstrap support (≥ 90%) based on 100 iterations. The scale bar depicts 0.1 substitutions per residue. The accession numbers of the scaffolds that contain the nucleic acid sequence reads of the environmental CLD-like proteins are listed in Table S2.

Apparently in contrast to this hypothesis, practical experience has shown that inappropriately high doses of hypochlorite can compromise nitrification in wastewater treatment plants (Seka *et al.*, 2003). However, it is not known whether AOB or NOB are inhibited and whether hypochlorite, chlorite or intermediate chlorine compounds are the main inhibitors.

### *Phylogeny and evolution of CLD-like proteins*

A database search of sequenced prokaryotic genomes and published metagenomic datasets resulted in a relatively large number of genes encoding proteins with sequence- and structural similarities to CLD ($n = 136$). As far as the source organisms are known, these genes occurred in members of nine bacterial and two archaeal phyla. A subset of the retrieved sequences constitutes the cluster of orthologous groups COG3253, which represents a family of uncharacterized proteins. For the sake of simplicity, we refer to all 136 proteins as CLD-like proteins. The range of amino acid sequence identity between the CLD-like proteins and the CLD of *N. defluvii* was rather broad (15.5–40.5%). However, the *in silico* predicted protein secondary structures were strikingly

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similar for most of the analysed sequences and the consensus structure consisted of nine helices and nine β-strands arranged in a conserved order (Fig. S2). The crystal structure of the CLD-like protein of *T. thermophilus* consists of seven α-helices, two 310-helices and 10 β-strands (Ebihara *et al*., 2005) and closely resembles the *in silico*-predicted structure (Fig. S2). Notable structural differences among the proteins seem to exist in the region between β-strands β4 and β5 (numbering according to Ebihara *et al*., 2005), where the CLD-like proteins of *Planctomycetes* contain a large insertion, and around β-strands β8 and β9. Strand β8 is replaced by a predicted helix in the CLD-like proteins of *Mycobacteria* and *Nitrobacter*, while both β8 and β9 are replaced by putative α-helices in the CLDs of *N. defluvii* and known PCB (Fig. S2). According to Ebihara and colleagues (2005), β8 and β9 are localized close to the active centre of the CLD-like protein of *T. thermophilus*, which has a very low chlorite dismutation activity (Table 2). Hence, the replacement of these β-strands by helices might be a structural hallmark of more efficient CLDs like those of *N. defluvii* and *I. dechloratans* (Fig. S2). Four residues conserved in all analysed CLD-like proteins (Fig. S2) are likely to play important roles in the catalytic mechanisms of this enzyme family. For example, the highly conserved histidine residue His-172 (homologous to His-186 in the CLD of *N. defluvii*) is the haem ligand in the enzyme of *T. thermophilus* and probably also in the CLD of *I. dechloratans* (Ebihara *et al*., 2005).

The topology of CLD-based phylogenetic trees (Fig. 5) was consistent for all treeing methods applied. It suggests that lateral transfer of *cld*-like genes has occurred not only among phyla within the *Bacteria* and *Archaea*, respectively, but also on the domain level between *Bacteria* and *Archaea*. For example, the CLD-like proteins of halophilic *Euryarchaeota* (e.g. *Halobacterium*) and of *Cenarchaeum symbiosum*, which belongs to the *Crenarchaeota*, are closer related to bacterial CLD-like proteins than to those of other *Archaea* (Fig. 5). The CLD-like proteins from the phylum *Nitrospirae* formed two non-monophyletic groups. One group contained the CLD of *N. defluvii* and was closely related to the CLDs of proteobacterial PCB, whereas the other group contained CLD-like proteins of *Leptospirillum*-related bacteria (Fig. 5). Previous phylogenetic analyses of CLDs of (per)chlorate-reducing *Proteobacteria* revealed incongruent topologies of CLD- and 16S-rRNA based trees (Bender *et al*., 2004). Our analysis includes validated CLDs and CLD-like proteins with unknown enzymatic activities and confirms that their evolutionary history most likely included lateral gene transfer events. Nevertheless, some larger groups of related organisms (according to 16S rRNA-based phylogeny) also cluster together in the CLD tree. For example, all CLD sequences of *Firmicutes* and most of *Actinobacteria* formed distinct large clusters (Fig. 5). Monophyly was observed for the CLD-like proteins of the *Firmicutes*, *Acidobacteria*, *Planctomycetes* and *Chloroflexi*, but this remains uncertain as long as the CLD-like sequences from metagenomic datasets cannot be assigned to organisms of known phylogenetic affiliation. The common ancestry of the *Planctomycetes* and *Verrucomicrobium spinosum* in the CLD-based tree corroborates the recent hypothesis that the *Planctomycetes* and the *Verrucomicrobia* belong to the same bacterial superphylum (Wagner and Horn, 2006).

Functional diversification within families of related proteins is a well-known phenomenon. A prominent example are the rhodopsins, whose functional classification into sensory rhodopsins, bacteriorhodopsins, halorhodopsins and proteorhodopsins is mirrored in phylogenetic trees (Sabehi *et al*., 2003). The main branches of the CLD-based tree could also represent groups of structurally similar enzymes with different catalytic properties. For example, the CLDs of *N. defluvii* and of known (per)chlorate-reducing *Proteobacteria*, which efficiently degrade chlorite, cluster together although *Nitrospira* and *Proteobacteria* are phylogenetically not closely related (Fig. 5). The enzyme of *T. thermophilus*, which has a low CLD activity (Table 2), falls into a different lineage in the CLD-based tree (Fig. 5). Recently, a (per)chlorate-reducing member of the *Firmicutes* was isolated, but the sequence and thus the phylogenetic affiliation of its CLD are not yet known (Balk *et al*., 2008). Interestingly, the sequences from halophilic archaea actually represent CLD-like domains fused to another protein similar to antibiotic biosynthesis monooxygenase (ABM). Bab-Dinitz and colleagues (2006) suggested several functions for these fusions, for example that the CLD-like domain might process chlorite or other substrates and thereby supply the ABM domain with $O_2$ in hypoxic environments.

The wide distribution of CLD-like proteins in the *Bacteria* and *Archaea* might imply that this enzyme family appeared relatively early in evolution and was propagated within and across phyla and domains by vertical inheritance and lateral gene transfer. However, the industrial contaminant chlorite is unlikely to have been the main substrate of the first evolved CLD-like proteins. In contrast, microbes have always been exposed to toxic byproducts of their own metabolism (e.g. free radicals, ROI), which may be targets of CLD-like proteins (see above and Ebihara *et al*., 2005). Their function in the (per)chlorate-reducing pathway may be a relatively recent specialization that impressively demonstrates how rapidly microbes can adapt to new conditions such as increasing (per)chlorate concentrations in the environment.

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## Experimental procedures

### *Characterization of the* N. defluvii *enrichment*

The enrichment of *N. defluvii* was maintained as described by Spieck and colleagues (2006). Two stages of the enrichment were used in this study. The earlier stage, which contained about 40% of *N. defluvii*, was the starting material for environmental genomics. The expression of CLD was demonstrated with a later stage that contained 86% of *N. defluvii* but had not been available when metagenome sequencing was started. The relative abundance of *N. defluvii* in the enrichments was quantified, by FISH with probes S-G-Ntspa-0662-a-A-18 (Daims *et al*., 2001b) and EUB338I-III (Amann *et al*., 1990; Daims *et al*., 1999), as described elsewhere (Schmid *et al*., 2000), and by using the image analysis software *daime* (Daims *et al*., 2006). Probe NIT3 (Wagner *et al*., 1996) was used to check for the presence of *Nitrobacter*.

### *Environmental genomics and* in silico *sequence analyses*

High molecular weight DNA was extracted in agarose plugs from a *N. defluvii* enrichment biomass pellet according to the protocol described by Strous and colleagues (2006). Following partial HindIII digestion of the DNA, a BAC library was established using vector pBeloBAC5 (Epicentre, Madison, USA) as outlined previously (Le Paslier *et al*., 2000). In addition, a shotgun randomly sheared DNA plasmid library was constructed in the low copy cloning vector pCDNA2 (Invitrogen, Carlsbad, USA). Clone picking from all libraries and bi-directional sequencing were carried out according to standard protocols. A preliminary assembly of sequence reads from the shotgun library was then performed using Phrap. Two small contigs, which contained parts of the 16S rRNA gene of *N. defluvii*, were obtained. Subsequently, BAC clones with end sequences that overlapped with one of these contigs were identified. These BAC clones were further characterized by restriction fingerprinting with HindIII to verify that they overlapped with each other and with the two small contigs. Selected overlapping BAC clones were then shotgun sequenced and assembled individually. Eventually, a new global assembly was performed to obtain the final contig.

The prediction of coding sequences on the contig and the annotation of genes were carried out by using the PEDANT (Frishman *et al*., 2001) and MaGe (Vallenet *et al*., 2006) software systems. The data collected for each identified ORF by automated searches in sequence databases and motif and domain libraries were refined by careful manual annotation. Gene functional categories were assigned according to the TIGR FAM database classification (Haft *et al*., 2001). The sequence of the contig has been deposited in the GenBank database (Accession No. EU559167).

### *Cloning and heterologous expression of CLD*

The *cld* gene of *N. defluvii* was PCR-amplified and cloned into the expression vector pET21b(+), which contains a promoter for T7 RNA polymerase and a C-terminal His-tag (Novagen, Heidelberg, Germany). Instead of extracted genomic DNA, 2 μl of the *N. defluvii* enrichment was added directly to the PCR reaction mix. Polymerase chain reaction was performed by using the Extensor Hi-Fidelity PCR enzyme mix (ABgene, Epsom, UK) and according to the protocol recommended by the manufacturer. As the gene has base mismatches to previously published primers targeting the *cld* genes of PCB (Bender *et al*., 2004), a new primer set specific for *cld* of *N. defluvii* was designed: the forward primer CLDF (5′-CGA GCG CAT ATG GCC GAT CGC GAG AAG TTA-3′) with a NdeI restriction site and the reverse primer CLDR (5′-GCG CGA GGA TCC CCC TGT GCG AAC TTT TCC AG-3′) with a BamHI restriction site. Primer CLDF binds 80 bases downstream of the 5′ end, and expression of the amplified partial gene leads to a recombinant protein that lacks the putative signal peptide. Preliminary experiments had shown that heterologous expression of the complete *cld* gene (including the signal peptide) did not yield a functional recombinant CLD. The obtained PCR-amplicon was purified with the peq GOLD Cycle-Pure-Kit (peqLab Biotechnology, Erlangen, Germany). The purified amplicon and the expression vector were digested with the restriction endonucleases NdeI and BamHI and were then ligated with T4 DNA Ligase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The expression vector with the *cld* gene was transformed by electroporation into *E. coli* XL1 Blue cells (Stratagene, Heidelberg, Germany). Sanger sequencing of 10 cloned inserts confirmed that they were identical to gene *cld* on the contig except for four single-nucleotide differences, which were not found in two or more sequences and were most likely PCR or sequencing errors (data not shown). The sequence of the cloned insert used for heterologous expression was identical to the sequence on the contig. For the heterologous expression of CLD, the expression vector with *cld* was transformed into *E. coli* strain BL21 (DE3) (Stratagene, Heidelberg, Germany). The recombinant cells were grown under agitation (225 r.p.m.) at 37°C in liquid Luria–Bertani medium (30 l of total culture volume). Following growth up to an optical density (600 nm) of 0.8, the expression of CLD was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested approximately 4 h later by centrifugation (5000 *g*, 10 min) and the reddish coloured cell pellets were stored at −20°C.

### *Purification and characterization of recombinant CLD*

Frozen cell pellets were re-suspended in 20 ml of phosphate-buffered saline (10 mM sodium phosphate, 130 mM NaCl, pH 7.2) and lysed by incubation with 10 mg $ml^{-1}$ of lysozyme (Sigma-Aldrich, St Gallen, Switzerland; 107 648 U $mg^{-1}$) for 60 min at 37°C. Cell debris was removed by two sequential centrifugation steps at 4°C (5000 *g* for 5 min and 18 000 *g* for 20 min respectively). The red supernatant was sterilized by filtration (0.2 μm pore size) and stored at 4°C until further processing. The following chromatographic purification steps were carried out by using a ÄKTA explorer chromatography system (GE Healthcare Life Sciences, Little Chalfont, UK). Cell-free protein extract was loaded onto a HisTrap HP 5 ml column (GE Healthcare Life Sciences, Little Chalfont, UK), which had been equilibrated with 10 mM sodium phosphate (pH 7.2) binding buffer. Subsequently, the column was equili-

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brated with 10 mM sodium phosphate buffer containing 20 mM imidazol (pH 7.2), washed with the same buffer plus 1 M NaCl, and equilibrated again with the imidazol buffer without NaCl. The final addition of 10 mM sodium phosphate buffer with 500 mM imidazol eluted the recombinant CLD, which formed a sharp red band in the column. Imidazol was removed by loading the eluate onto a HiPrep 26/10 Desalting Column (GE Healthcare Life Sciences, Little Chalfont, UK) and eluting with 10 mM sodium phosphate buffer (pH 7.2). To monitor the success of the purification process and to determine the apparent molecular mass of the CLD monomer, denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with a 12.5% polyacrylamide (PAA) gel and molecular weight markers (PageRuler Unstained Protein Ladder #SM0661, Fermentas, St Leon-Rot, Germany). Polyacrylamide gels were stained with Coomassie brilliantblue R250. The concentration of the purified CLD was determined by using the 2-D-Quant-Kit (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. Absorption spectra of the purified CLD were recorded by using a NanoDrop UV/VIS spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, USA).

### *Validation and reaction kinetics of CLD*

To test whether the recombinant CLD converted chlorite to $Cl^-$ and $O_2$, an *E. coli* culture expressing the CLD of *N. defluvii* was sonicated on ice to lyse the cells and 500 µl of a 200 mM $NaClO_2$ solution was added to the sonicated cell suspension. After the reaction (visible as large amounts of produced gas bubbles and foam) had stopped, 1 ml of a 200 mM $AgNO_3$ solution was added to confirm that $Cl^-$ was produced. Control experiments were performed with an *E. coli* culture that did not express the CLD of *N. defluvii*.

The rate of $Cl^-$ production by CLD was determined by measuring the concentration of $Cl^-$ with a chloride electrode (DX235-Cl connected to a Seven Multi station; Mettler-Toledo, Greifensee, Switzerland). For each measurement series, 50 ml of reaction buffer (10 mM sodium phosphate, adjusted to the respective pH) containing the respective concentration of $NaClO_2$ was stirred in a 100 ml beaker in a heated water bath at the respective temperature. After the enzymatic reaction had been started by adding 2.58 mg (250 µl) of purified CLD, the $Cl^-$ concentration was recorded every second during 1 min. Subsequently, the measured $Cl^-$ concentrations were plotted against time and the rate of $Cl^-$ formation was calculated based on the linear part of the curve. Specific enzyme activity was expressed in U (mg protein)$^{-1}$, where one unit (U) is defined as the amount of $Cl^-$ produced per minute (µmol min$^{-1}$; van Ginkel *et al.*, 1996).

In a preliminary experiment, the $NaClO_2$ concentration that brought about the highest specific activity of CLD was determined by using the optimal reaction conditions of the CLD of PCB 'strain GR-1' as reported by van Ginkel and colleagues (1996) (30°C, pH 6) and $NaClO_2$ concentrations ranging from 1 to 100 mM. This experiment was required, because CLD was quickly degraded in presence of excess $NaClO_2$. The optimal $NaClO_2$ concentration (65 mM) was then used to determine the pH and temperature optima of the enzyme. Under these optimal conditions (25°C, pH 6), another series of measurements was performed with 0–150 mM $NaClO_2$. The resulting specific activities were plotted against the $NaClO_2$ concentration, and curve fitting based on the Michaelis–Menten equation was performed with SigmaPlot version 8.0 to determine which range of $NaClO_2$ concentrations was suitable to determine $K_m$ and $V_{max}$ under optimal reaction conditions (Fig. 3A). Subsequently, Eadie–Hofstee plots over this concentration range (0–65 mM $NaClO_2$) were used to determine the catalytic parameters (Fig. 3B). All experiments were performed in triplicates. The turnover number ($k_{cat}$) was calculated by multiplying $V_{max}$ with the molar mass of one monomer of the enzyme. For this purpose, the molar mass was calculated from the amino acid sequence by using the ProtParam tool (Gasteiger *et al.*, 2005).

### *Immunological detection of CLD expressed by* N. defluvii

To determine whether *N. defluvii* expresses the *cld* gene, crude cell extracts were subjected to Western blot analyses with CLD-specific polyclonal antibodies. Biomass from a nitrite-oxidizing enrichment of *N. defluvii* was harvested by centrifugation (10 000 *g*; 20 min) and washed once in PBS. Subsequently, the biomass pellet was re-suspended in 4× SDS-PAGE loading buffer [200 mM TRIS/HCl pH 6.8, 8% (w/v) SDS, 40% (w/v) glycerol, 0.2% (w/v) bromphenol blue, 12.5% (v/v) β-mercaptoethanol], heated for 3 min at 90°C and loaded onto a SDS-PAGE gel (12.5% PAA) with molecular weight markers (PageRuler Prestained Protein Ladder #SM0671, Fermentas, St Leon-Rot, Germany). Following gel electrophoresis, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane by using the Western blot TE77 semidry-transfer unit (GE Healthcare life siences, Little Chalfont, UK) and Towbin transfer buffer according to manufacturer's instructions. The following incubations were carried out at room temperature. First, the PVDF membrane was incubated for 1 h in 5% non-fat dry milk in TBS buffer (20 mM TRIS, 150 mM NaCl, pH 7.5) to block-free binding sites. Second, the membrane was incubated for 30 min with polyclonal antibodies directed against purified CLD of *N. defluvii* (Eurogentec, Seraing, Belgium), which were diluted 1:500 in TBS plus 0.1% (v/v) Tween 20. After three washing steps in TBS-Tween buffer, the membrane was incubated for 30 min with the secondary antibody (peroxidase-conjugated goat antirabbit IgG; dianova, Hamburg, Germany), which was diluted 1:500 000 in TBS-Tween buffer. After a final washing step in TBS-Tween buffer, bound antibodies were detected by using the Western Lightning Chemoluminescence Reagent Plus Kit (Perkin Elmer Life Sciences, Milano, Italy). In all Western blot experiments, purified CLD of *N. defluvii* was used as positive control. Crude cell extracts from the PCB *D. agitata* were used as negative control to verify that the polyclonal antibodies did not bind to a closely related albeit different CLD from another bacterium than *N. defluvii*. *Dechloromonas agitata* was grown anaerobically in the medium of Bruce and colleagues (1999) containing chlorate, and the presence of active CLD was confirmed by addition of $NaClO_2$ to an aliquot of the culture, which was followed by visible $O_2$ production.

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*Phylogenetic analyses of CLD-like proteins*

Nucleic acid and amino acid sequences of CLD-like proteins were retrieved from public databases by using Pfam (Finn *et al*., 2008) and the BLAST (Altschul *et al*., 1990) search tools NCBI blastp and tblastn, IMG/M BLAST, and Camera BLAST Wizard (Markowitz *et al*., 2006; Seshadri *et al*., 2007). At first a core dataset of sequences containing the Pfam chlorite dismutase domain was collected. Subsequently, BLAST searches were performed with each of these sequences and hits were added to the dataset if (i) their length was similar, (ii) they also contained the Pfam chlorite dismutase domain, and (iii) the predicted protein secondary structure (see below) was consistent with the other CLD-like proteins. Where required, the tool ORF Finder (Wheeler *et al*., 2003) was subsequently applied to extract *cld*-like genes from sequence reads or contigs from metagenomic databases. DNA sequences were translated into amino acids by using the respective tools of the ARB software (Ludwig *et al*., 2004). Partial sequences of less than 167 amino acid residues were excluded from all following steps and analyses. A multiple alignment of the protein sequences, which was based on amino acid similarities and predicted protein secondary structures, was obtained by using PROMALS (Pei and Grishin, 2007). The automatically inferred alignment was manually refined by using the ARB sequence editor. Phylogenetic analyses were performed by applying distance-matrix, maximum-parsimony and maximum-likelihood methods: neighbour-joining (using the Dayhoff PAM 001 matrix as amino acid substitution model and the implementation in the ARB software package), FITCH (PHYLIP version 3.66 with the JTT substitution model), protein parsimony (PHYLIP version 3.66 with 100 bootstrap iterations) and protein maximum-likelihood [PHYLIP version 3.66 and PhyML (Guindon and Gascuel, 2003), both with the JTT substitution model, and TREE-PUZZLE (Strimmer and von Haeseler, 1996) with 1000 puzzling steps and the HKY substitution model]. In total, 210 alignment columns were used for phylogenetic analysis. The circular tree shown in Fig. 5 was displayed by using the iTOL online tool (Letunic and Bork, 2007).

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Illustration of the genome region of *N. defluvii* that was sequenced and analysed in this study. Arrows indicate the position and orientation of coding sequences (CDS). Colours indicate the assignment of CDS to TIGR role categories, which are listed in the bottom part of the figure. Black arrows indicate that the respective CDS shared sequence similarities only with ORFs of unknown function in other organisms, or contained only short conserved motifs or structural features. White arrows indicate that the respective CDS showed no similarity at all to any sequence in public databases. Scale bars and numbers indicate nucleotide positions.

**Fig. S2.** Alignment of selected chlorite dismutase (CLD)-like protein sequences based on conserved secondary structure motifs. Positions that are conserved in all analysed sequences of CLD-like proteins ($n = 136$) are shaded in grey. Vertical arrows point at the histidine residue that is likely to be the haem ligand in these enzymes. Each sequence is coloured according to PSIPRED (Jones, 1999) secondary structure prediction (red: α-helix, blue: β-strand). The coloured bars above the alignment show secondary structure motifs (red: α-helix, yellow: 310-helix, blue: β-strand) as detected in the crystal structure of the CLD-like protein of *Thermus thermophilus* (Ebihara *et al.*, 2005). The last column in the last row (Id. %) shows amino acid sequence identities to the CLD of *N. defluvii*.

**Fig. S3.** Functional validation of the CLD of *N. defluvii*.

A. The red-coloured *E. coli* culture, which expressed the recombinant CLD of *N. defluvii* (left test tube), and the control *E. coli* culture, which did not contain this gene (right test tube), after cell lysis by sonication but prior to addition of $NaClO_2$.

B. The same test tubes as in (A) immediately after addition of 200 mM $NaClO_2$. Formation of gas (presumably $O_2$) is visible only in the left tube, which contained the *E. coli* culture expressing the CLD of *N. defluvii*.

C. The same test tubes as in (B) after subsequent addition of 200 mM $AgNO_3$. $Ag+$ forms a white precipitate with $Cl^-$, but a yellow precipitate with $ClO_2^-$ (Holleman and Wiberg, 1985). The results shown in (B) and (C) clearly demonstrate that the CLD of *N. defluvii* has chlorite dismutase activity.

**Fig. S4.** Effects of pH (A) and temperature (B) on the specific activity of the CLD of *N. defluvii*. The pH optimum was determined in 10 mM sodium phosphate buffer solutions at 30°C. The temperature optimum was determined in 10 mM sodium phosphate buffer (pH 6.0). All experiments were performed with 65 mM chlorite. Error bars indicate the standard error of the mean ($n = 3$).

**Table S1.** List of all coding sequences identified on the analysed genome fragment of '*Candidatus* Nitrospira defluvii' and the corresponding annotations.

**Table S2.** Sources of environmental sequences of CLD-like proteins that were used for protein phylogeny (see Fig. 5).

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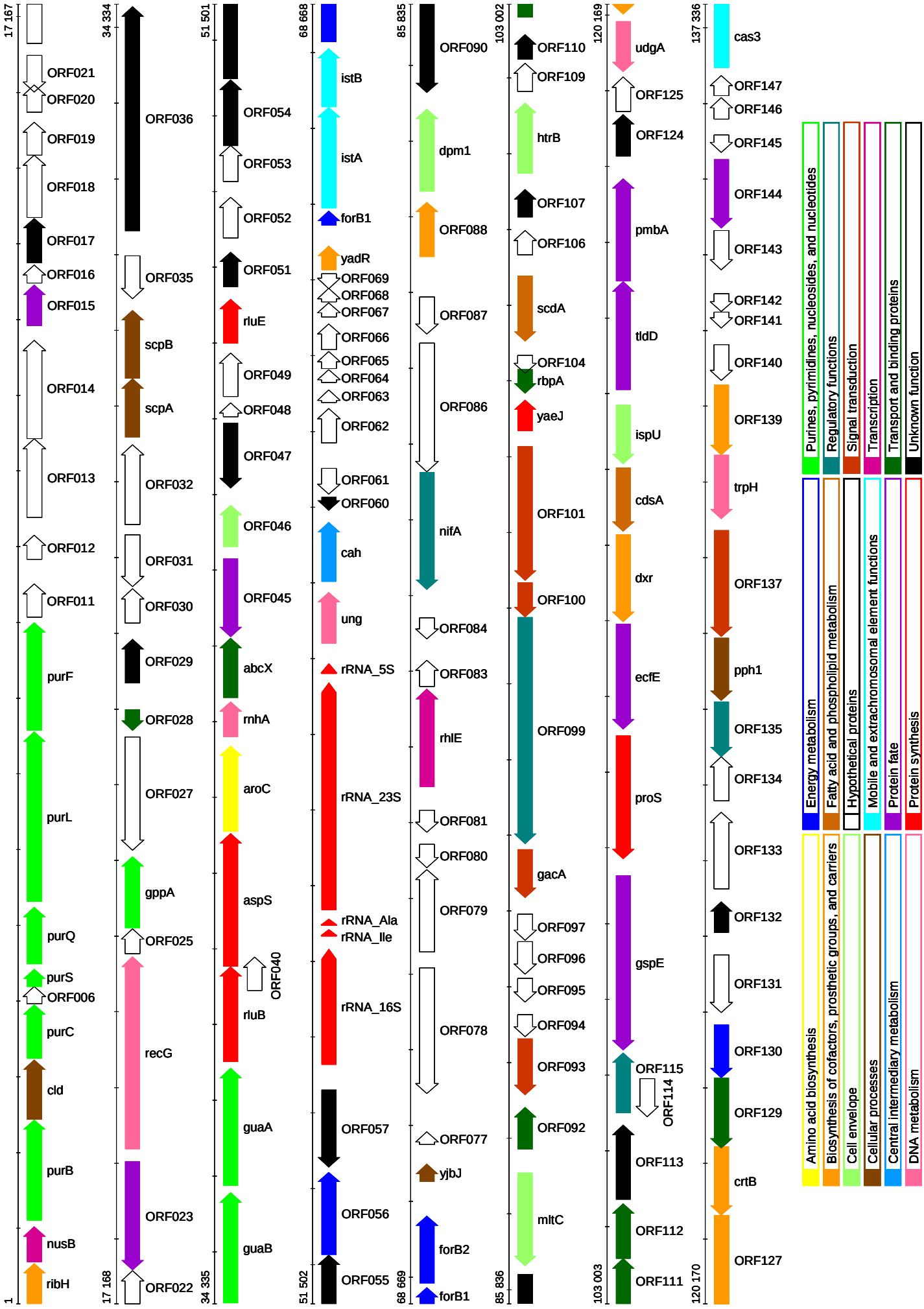
1
17 167
17 168
34 334
34 335
51 501
51 502
68 668
68 669
85 835
85 836
103 002
103 003
120 169
120 170
137 336
ribH
nusB
purB
cld
purC
purS
ORF006
purQ
purL
purF
ORF011
ORF012
ORF013
ORF014
ORF015
ORF016
ORF017
ORF018
ORF019
ORF020
ORF021
ORF022
ORF023
recG
gppA
ORF025
ORF027
ORF028
ORF029
ORF030
ORF031
ORF032
ORF035
scpA
scpB
ORF036
guaB
guaA
rluB
aspS
aroC
rnhA
abcX
ORF045
ORF046
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cah
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ORF067
ORF068
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istA
istB
forB1
forB2
yjbJ
ORF077
ORF078
ORF079
ORF080
ORF081
rhlE
ORF083
ORF084
nifA
ORF086
ORF087
ORF088
dpm1
ORF090
mltC
ORF092
ORF093
ORF094
ORF095
ORF096
ORF097
gacA
ORF099
ORF100
ORF101
yaeJ
rbpA
ORF104
ORF106
scdA
ORF107
htrB
ORF109
ORF110
ORF111
ORF112
ORF113
ORF114
ORF115
gspE
proS
ecfE
dxr
cdsA
ispU
tldD
pmbA
ORF124
ORF125
udgA
ORF127
crtB
ORF129
ORF130
ORF131
ORF132
ORF133
ORF134
ORF135
pph1
trpH
ORF137
ORF139
ORF140
ORF141
ORF142
ORF143
ORF144
ORF145
ORF146
ORF147
cas3
Amino acid biosynthesis
Biosynthesis of cofactors, prosthetic groups, and carriers
Cell envelope
Cellular processes
Central intermediary metabolism
DNA metabolism
Energy metabolism
Fatty acid and phospholipid metabolism
Hypothetical proteins
Mobile and extrachromosomal element functions
Protein fate
Protein synthesis
Purines, pyrimidines, nucleosides, and nucleotides
Regulatory functions
Signal transduction
Transcription
Transport and binding proteins
Unknown function

**Fig. S1.** Illustration of the genome region of *N. defluvii* that was sequenced and analyzed in this study. Arrows indicate the position and orientation of coding sequences (CDS). Colours indicate the assignment of CDS to TIGR role categories, which are listed in the bottom part of the figure. Black arrows indicate that the respective CDS shared sequence similarities only with ORFs of unknown function in other organisms, or contained only short conserved motifs or structural features. White arrows indicate that the respective CDS showed no similarity at all to any sequence in public databases. Scale bars and numbers indicate nucleotide positions.

**Description of the annotated genes on the contig**

Two tRNA synthetase genes were identified on the genome fragment of *N. defluvii* analyzed in this study: *aspS* for aspartyl-tRNA and *proS* for prolyl-tRNA synthetase. The contig contained genes encoding 6 of the 12 enzymes required for the *de novo* purine biosynthesis of inosine monophosphate (IMP) from 5-phosphoribosylpyrophosphate (*purB*, *purC*, *purF*, *purL*, *purQ* and *purS*) as well as both genes needed to form guanosine monophosphate from IMP (*guaA* and *guaB*). The products of three genes (*ung*, *udgA* and *recG*) are involved in DNA repair.

The gene *cah* codes for an alpha-type carbonic anhydrase (CA) that catalyzes the interconversion of $CO_2$ and $HCO_3^-$. Among the proposed roles of CA in prokaryotes are (i) the transport and concentration of $CO_2$ or $HCO_3^-$ inside the cell prior to autotrophic carbon fixation and also (ii) the removal of $CO_2$, which may increase the efficiency of decarboxylation reactions (reviewed by Smith and Ferry, 2000). As *N. defluvii* is an autotroph (Daims *et al.*, 2001; Spieck *et al.*, 2006), CA is likely to play a central role in its metabolism as it does in other autotrophic bacteria such as *Synechococcus* (Fukuzawa et al., 1992) and *Nitrobacter* (Starkenburg et al., 2006). The contig contained also two copies of gene *forB* (designated *forB1* and *forB2*), which seems to encode the beta subunit of a ferredoxin oxidoreductase similar to the pyruvate:ferredoxin oxidoreductase (POR) and 2-oxoglutarate:ferredoxin oxidoreductase (OGOR) of *Hydrogenobacter thermophilus*. Interestingly, *forB1* was interrupted by two genes, *istA* and *istB*, whose homologues in other organisms are part of IS*21*-like insertion elements and are essential for their transposition (Reimmann and Haas, 1990). The presence and location of *istA* and *istB* in *N. defluvii* show that insertion elements of the widespread IS*21* family occur in the phylum *Nitrospirae* and can cause gene inactivation in *Nitrospira*. The other copy of *forB* (*forB2*) seems to be intact. Although no other subunits of POR or OGOR were found on the contig, the *forB2* gene indicates that in *Nitrospira* one of these enzymes might be involved in pyruvate metabolism or in carbon fixation via the reductive citric acid cycle as in *H. thermophilus* (Yoon et al., 1997; Yamamoto et al., 2006). POR and OGOR are commonly considered to be oxygen sensitive and are associated with anaerobic metabolism (Campbell et al., 2006), but nitrite oxidation is an aerobic process. However, *Nitrospira*-like bacteria have been found in hypoxic regions of nitrifying biofilms (Okabe et al., 1999; Gieseke et al., 2003). Given that their other subunits are encoded elsewhere in the genome, POR or OGOR might be part of an enzymatic inventory that could help *Nitrospira* to persist under fully aerobic as well as microaerobic or even anaerobic conditions. This is the case in the facultatively anaerobic bacterium *H.*

*thermophilus*, which uses two different forms of OGOR with a different oxygen sensitivity for C-fixation in presence or absence of $O_2$ (Yamamoto et al., 2006).

The product of an exopolyphosphatase gene (*ppx*/*gppA*) might catalyze the degradation of polyphosphates, but the genome fragment did not contain any gene similar to polyphosphate kinase, the enzyme required for polyphosphate synthesis. However, polyphosphate deposits have been observed by electron microscopy in cells of two cultured *Nitrospira* species related to *N. defluvii* (Watson et al., 1986; Ehrich et al., 1995).

Several genes of different biosynthetic pathways were identified on the contig. The gene *ribH* encodes the beta chain of riboflavin synthase, an enzyme that catalyzes a key step in the synthesis of riboflavin and plays an important role for the biosynthesis of the cofactors FAD and FMN. The presence of *ribH* indicates that *N. defluvii* may be able to synthesize riboflavin (vitamin B2) and does not depend on other microorganisms (or their cell lysates) to provide this compound *in situ*. The product of gene *aroC* carries out the last step of the shikimate pathway to produce chorismate, a precursor of tryptophan, tyrosine, phenylalanine, and ubiquinone. *Nitrospira* are Gram-negative bacteria (Ehrich et al., 1995) and probably have genes required for synthesizing typical components of the Gram-negative cell envelope. Such a gene found on the contig is *htrB*, which is involved in the synthesis of lipid A, the membrane anchor region of the lipopolysaccharides in the outer membrane. Also involved in cell wall biosynthesis is *ispU* coding for undecaprenyl pyrophosphate (UPP) synthetase. UPP functions as carrier in the transport of peptidoglycan building blocks across the cytoplasmic membrane. Gene *dmp1*, which encodes a glycosyl transferase, might participate in the synthesis of cell surface polysaccharides.

Two genes on the contig may function in the biosynthesis of carotenoids: *crtB* (phyotene synthase) and *pds* (a putative phytoene dehydrogenase). *Nitrospira* is not a photosynthetic bacterium, but nevertheless may use carotenoids as protectants from oxidation damage. A particularly interesting feature is the helicase-like gene *cas3* fragment. In other bacteria this gene is associated with clustered regularly interspaced short palindromic repeats (CRISPR), which constitute an adaptive phage resistance mechanism (Barrangou et al., 2007). As *cas3* is located at the 3' end of the contig, no other CRISPR-related genes or CRISPR repeats could be found and it remains to be shown whether *Nitrospira* has a complete CRISPR system.

Other ORFs on the analyzed genome piece of *Nitrospira* may play roles, for example, in cell division, DNA/RNA and lipid metabolism, signal transduction, transport, and secretion. Please refer to Table S1 for a complete list of all annotated genes. Fig. S1 shows the localization of all genes on the contig.

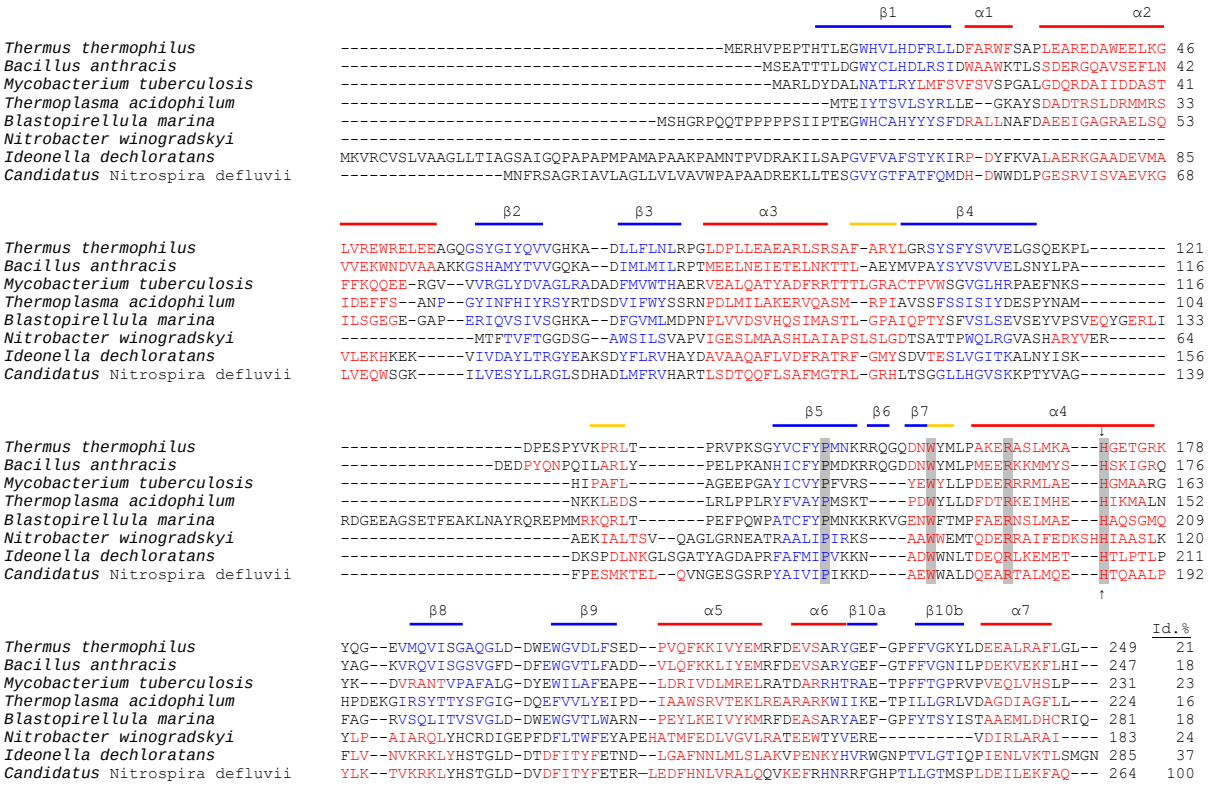


**Fig. S2.** Alignment of selected chlorite dismutase (CLD)-like protein sequences based on conserved secondary structure motifs. Positions that are conserved in all analyzed sequences of CLD-like proteins (n=136) are shaded in grey. Vertical arrows point at the histidine residue that is likely to be the heme ligand in these enzymes. Each sequence is colored according to PSIPRED (Jones, 1999) secondary structure prediction (red: alpha helix, blue: beta strand). The colored bars above the alignment show secondary structure motifs (red: alpha helix, yellow: 310-helix, blue: beta strand) as detected in the crystal structure of the CLD-like protein of *Thermus thermophilus* (Ebihara et al., 2005). The last column in the last row (Id. %) shows amino acid sequence identities to the CLD of *N. defluvii*.

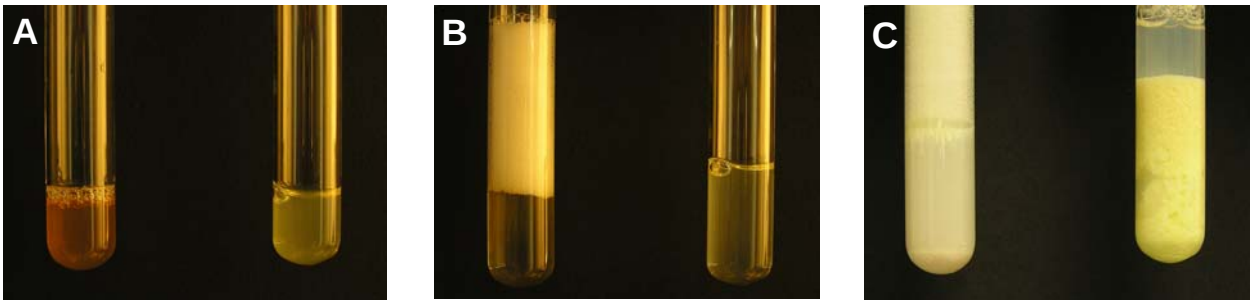


**Fig. S3.** Functional validation of the CLD of *N. defluvii*.

A. The red-coloured *E. coli* culture, which expressed the recombinant CLD of *N. defluvii* (left test tube), and the control *E. coli* culture, which did not contain this gene (right test tube), after cell lysis by sonication but prior to addition of $NaClO_2$.

B. The same test tubes as in (A) immediately after addition of 200 mM $NaClO_2$. Formation of gas (presumably $O_2$) is visible only in the left tube, which contained the *E. coli* culture expressing the CLD of *N. defluvii*.

C. The same test tubes as in (B) after subsequent addition of 200 mM $AgNO_3$. $Ag^+$ forms a white precipitate with $Cl^-$, but a yellow precipitate with $ClO_2^-$ (Holleman and Wiberg, 1985). The results shown in (B) and (C) clearly demonstrate that the CLD of *N. defluvii* has chlorite dismutase activity.

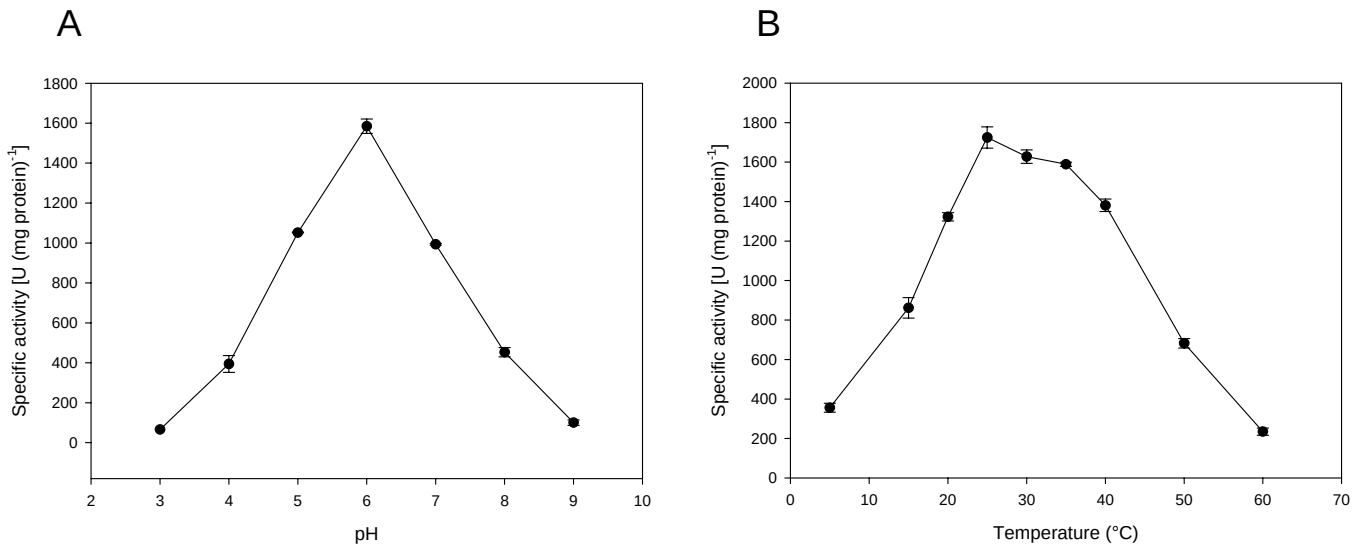


**Fig. S4.** Effects of pH (A) and temperature (B) on the specific activity of the CLD of *N. defluvii*. The pH optimum was determined in 10 mM sodium phosphate buffer solutions at 30°C. The temperature optimum was determined in 10 mM sodium phosphate buffer (pH 6.0). All experiments were performed with 65 mM chlorite. Error bars indicate the standard error of the mean (n=3).

Table S1. List of all coding sequences identified on the analyzed genome fragment of "*Candidatus* Nitrospira defluvii" and the corresponding annotations.

| Label | Begin | End | Gene | Product | ECnumber | Class | ProductType |
|---|---|---|---|---|---|---|---|
| ORF001 | 1 | 539 | ribH | Riboflavin synthase, beta chain | 2.5.1.9 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF002 | 553 | 1026 | nusB | transcription antitermination protein | | 2b : Function of strongly homologous gene | f : factor |
| ORF003 | 1103 | 2437 | purB | Adenylosuccinate lyase | 4.3.2.2 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF004 | 2434 | 3228 | cld | Chlorite dismutase | 1.13.11.49 | 1 : Function experimentally demonstrated in the studied organism | e : enzyme |
| ORF005 | 3244 | 3957 | purC | Phosphoribosylaminoimidazole-succinocarboxamide synthetase | 6.3.2.6 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF006 | 3965 | 4192 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF007 | 4189 | 4428 | purS | Phosphoribosylformylglycinamidine synthase, PurS subunit (FGAM component) | 6.3.5.3 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF008 | 4487 | 5245 | purQ | Phosphoribosylformylglycinamidine synthase, PurQ subunit (FGAM synthase I) | 6.3.5.3 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF009 | 5314 | 7560 | purL | Phosphoribosylformylglycinamidine synthase, PurL subunit (FGAM synthase II) | 6.3.5.3 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF010 | 7573 | 9003 | purF | Amidophosphoribosyltransferase | 2.4.2.14 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF011 | 9071 | 9511 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF012 | 9836 | 10153 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF013 | 10388 | 11428 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF014 | 11430 | 12725 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF015 | 12917 | 13462 | | putative thiol-disulfide oxidoreductase | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF016 | 13482 | 13721 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF017 | 13746 | 14342 | | conserved protein of unknown function with Metallo-hydrolase/oxidoreductase domain | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF018 | 14349 | 15173 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF019 | 15170 | 15607 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF020 | 15739 | 16098 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF021 | 16004 | 16492 | | membrane protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF022 | 16651 | 17607 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF023 | 17611 | 19053 | | putative TPR repeat: Peptidase M, neutral zinc metallopeptidases, zinc-binding site | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF024 | 19215 | 21755 | recG | ATP-dependent DNA helicase | 3.6.1.- | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF025 | 21794 | 22120 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF026 | 22131 | 23081 | gppA | Exopolyphosphatase | 3.6.1.11 | 2b : Function of strongly homologous gene | e : enzyme |
| ORF027 | 23162 | 24655 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF028 | 24737 | 25021 | | putative copper binding protein, Plastocyanin family | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pc : putative carrier |
| ORF029 | 25363 | 25950 | | conserved protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF030 | 26162 | 26614 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF031 | 26643 | 27323 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF032 | 27460 | 28515 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF033 | 28614 | 29393 | scpA | Segregation and condensation protein A | | 2a : Function of homologous gene experimentally demonstrated in an other organism | cp : cell process |
| ORF034 | 29390 | 30295 | scpB | putative chromosome segregation and condensation protein B | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | cp : cell process |
| ORF035 | 30445 | 31011 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF036 | 31336 | 34305 | | putative NHL repeat protein | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF037 | 34344 | 35810 | guaB | Inosine-5'-monophosphate dehydrogenase (IMP) | 1.1.1.205 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF038 | 35895 | 37451 | guaA | GMP synthetase (glutamine aminotransferase) | 6.3.5.2 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF039 | 37531 | 38793 | rluB | ribosomal large subunit pseudouridine synthase B (modular protein?) | 4.2.1.70 | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | e : enzyme |
| ORF040 | 38474 | 38914 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF041 | 38790 | 40553 | aspS | aspartyl-tRNA synthetase | 6.1.1.12 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF042 | 40572 | 41708 | aroC | chorismate synthase | 4.2.3.5 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF043 | 41823 | 42293 | rnhA | Ribonuclease H | 3.1.26.4 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF044 | 42336 | 43133 | abcX | ABC transporter, ATPase component | | 2b : Function of strongly homologous gene | t : transporter |
| ORF045 | 43143 | 44177 | | putative Trypsin-like serine protease | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF046 | 44334 | 44888 | | putative outer membrane lipoprotein precursor yeaY | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pm : putative membrane component |
| ORF047 | 45105 | 45971 | | conserved protein of unknown function, putative neutral zinc metallopeptidase | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF048 | 46050 | 46232 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF049 | 46320 | 46892 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF050 | 47022 | 47609 | rluE | Ribosomal large subunit pseudouridine synthase E | 5.4.99.12, 4.2.1.70 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF051 | 47768 | 48229 | | conserved exported protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF052 | 48409 | 48951 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF053 | 49155 | 49634 | | membrane protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF054 | 49631 | 50506 | | conserved membrane protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF055 | 50515 | 52152 | | conserved protein of unknown function (containing GH3 auxin-responsive domain) | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF056 | 52152 | 53246 | | putative Muconate cycloisomerase | 5.5.1.- | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF057 | 53304 | 54332 | | conserved exported protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| rRNA_16S | 54662 | 56193 | 16S rRNA | 16S ribosomal RNA | | 2a : Function of homologous gene experimentally demonstrated in an other organism | n : RNA |
| tRNA_Ile | 56366 | 56439 | Ile tRNA | Isoleucine tRNA | | 2a : Function of homologous gene experimentally demonstrated in an other organism | |
| tRNA_Ala | 56507 | 56579 | Ala tRNA | Alanine tRNA | | 2a : Function of homologous gene experimentally demonstrated in an other organism | |
| rRNA_23S | 56703 | 59712 | 23S rRNA | 23S ribosomal RNA | | 2a : Function of homologous gene experimentally demonstrated in an other organism | n : RNA |
| rRNA_5S | 59836 | 59952 | 5S rRNA | 5S ribosomal RNA | | 2a : Function of homologous gene experimentally demonstrated in an other organism | n : RNA |
| ORF058 | 60223 | 60903 | ung | Uracil-DNA-glycosylase | 3.2.2.- | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF059 | 61041 | 61829 | cah | Carbonic anhydrase | 4.2.1.1 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF060 | 61978 | 62160 | | conserved protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF061 | 62196 | 62537 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF062 | 62872 | 63327 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF063 | 63410 | 63571 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF064 | 63674 | 63841 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF065 | 63856 | 64080 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF066 | 64105 | 64446 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF067 | 64538 | 64726 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF068 | 64735 | 64914 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF069 | 64911 | 65108 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF070 | 65155 | 65475 | yadR | Iron-sulfur cluster assembly accessory protein | | 2b : Function of strongly homologous gene | f : factor |
| ORF071 | 65744 | 65938 | forB1-N | partial 2-oxoacid:ferredoxin oxidoreductase beta subunit (fragment N-terminal) | 1.2.7.1 | 2b : Function of strongly homologous gene | e : enzyme |
| ORF072 | 65973 | 67310 | istA | Integrase | | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF073 | 67307 | 68086 | istB | Insertion sequence IS21 putative ATP-binding protein | | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF074 | 68167 | 68889 | forB1-C | partial 2-oxoacid:ferredoxin oxidoreductase beta subunit (fragment C-terminal) | 1.2.7.1 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF075 | 68942 | 69835 | forB2 | 2-oxoacid:ferredoxin oxidoreductase beta subunit | 1.2.7.1 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF076 | 70279 | 70521 | yjbJ | putative stress response protein | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pf : putative factor |
| ORF077 | 70774 | 70944 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF078 | 71447 | 73111 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |

Table S1. List of all coding sequences identified on the analyzed genome fragment of "*Candidatus* Nitrospira defluvii" and the corresponding annotations.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ORF079 | 73318 | 74406 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF080 | 74429 | 74740 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF081 | 74897 | 75190 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF082 | 75497 | 76792 | rhlE | ATP-dependent RNA helicase | 3.6.1.- | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF083 | 76822 | 77169 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF084 | 77451 | 77732 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF085 | 78100 | 79653 | nifA | Nif-specific regulatory protein | | 2b : Function of strongly homologous gene | r : regulator |
| ORF086 | 79660 | 81360 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF087 | 81478 | 81969 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF088 | 82498 | 83223 | | putative sterol desaturase | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF089 | 83360 | 84454 | dpm1 | Dolichyl-phosphate beta-D-mannosyltransferase | 2.4.1.83 | 2b : Function of strongly homologous gene | e : enzyme |
| ORF090 | 84660 | 86231 | | conserved membrane protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF091 | 86334 | 87569 | mltC | Membrane-bound lytic murein transglycosylase C precursor | 3.2.1.- | 2b : Function of strongly homologous gene | e : enzyme |
| ORF092 | 87874 | 88437 | | putative Toluene tolerance precursor | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pt : putative transporter |
| ORF093 | 88593 | 89342 | | putative Two component transcriptional regulator, LuxR family, response regulator receiver | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pr : putative regulator |
| ORF094 | 89364 | 89654 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF095 | 89824 | 90135 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF096 | 90191 | 90622 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF097 | 90638 | 90982 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF098 | 91198 | 91842 | gacA | Response regulator (Global activator) | | 2a : Function of homologous gene experimentally demonstrated in an other organism | r : regulator |
| ORF099 | 91911 | 94907 | | Response regulator receiver and signal transduction histidine kinase (modular protein) | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pr : putative regulator |
| ORF100 | 94904 | 95365 | | Response regulator receiver (CheY-like) | | 2b : Function of strongly homologous gene | r : regulator |
| ORF101 | 95389 | 97161 | | Signal transduction histidine kinase (modular protein) | 2.7.13.3 | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF102 | 97362 | 97778 | yaeJ | Peptidyl-tRNA hydrolase domain protein | | 2b : Function of strongly homologous gene | f : factor |
| ORF103 | 97860 | 98183 | rbpA | RNA-binding protein | | 2b : Function of strongly homologous gene | f : factor |
| ORF104 | 98126 | 98365 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF105 | 98545 | 99414 | scdA | Stearoyl-CoA 9-desaturase (fragment) | 1.14.19.1 | 2b : Function of strongly homologous gene | e : enzyme |
| ORF106 | 99691 | 100014 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF107 | 100186 | 100560 | | conserved protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF108 | 100761 | 101699 | htrB | Lipid A biosynthesis lauroyl (or palmitoleoyl) acyltransferase | 2.3.1.- | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF109 | 101847 | 102221 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF110 | 102273 | 102605 | | conserved protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF111 | 102826 | 103605 | | ABC transporter permease protein | | 2b : Function of strongly homologous gene | t : transporter |
| ORF112 | 103602 | 104333 | | ABC-type transport system, ATPase component (fragment) | 3.6.3.- | 2b : Function of strongly homologous gene | t : transporter |
| ORF113 | 104381 | 105370 | | conserved protein of unknown function | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF114 | 105478 | 105981 | | protein of unknown function | | 6 : Doubtful CDS | o : ORF of unknown function |
| ORF115 | 105523 | 106323 | | TPR-repeat protein | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | o : ORF of unknown function |
| ORF116 | 106352 | 108664 | gspE | Type II secretion system protein E | | 2b : Function of strongly homologous gene | t : transporter |
| ORF117 | 108878 | 110509 | proS | Prolyl-tRNA synthetase | 6.1.1.15 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF118 | 110591 | 111982 | ecfE | Peptidase M50, putative membrane-associated zinc metallopeptidase | 3.4.24.- | 2b : Function of strongly homologous gene | e : enzyme |
| ORF119 | 112008 | 113168 | dxr | 1-deoxy-D-xylulose-5-phosphate reductoisomerase | 1.1.1.267 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF120 | 113201 | 114049 | cdsA | Phosphatidate cytidylyltransferase | 2.7.7.41 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF121 | 114093 | 114878 | ispU | Undecaprenyl pyrophosphate synthase | 2.5.1.31 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF122 | 115071 | 116507 | tldD | Peptidase U62 | | 2b : Function of strongly homologous gene | e : enzyme |
| ORF123 | 116510 | 117868 | pmbA | Peptidase U62 | | 2b : Function of strongly homologous gene | e : enzyme |
| ORF124 | 118159 | 118713 | | Phosphatidylethanolamine-binding protein | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | o : ORF of unknown function |
| ORF125 | 118750 | 119205 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF126 | 119270 | 119941 | udgA | Uracil-DNA glycosylase, family 4 | 3.2.2.- | 2a : Function of homologous gene experimentally demonstrated in an other organism | cp : cell process |
| ORF127 | 120032 | 121342 | | putative Carotene 7,8-desaturase | 1.14.99.30 | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF128 | 121339 | 122250 | crtB | Phytoene synthase | 2.5.1.- | 2b : Function of strongly homologous gene | e : enzyme |
| ORF129 | 122222 | 123154 | | ABC-type transport system, periplasmic binding protein | | 2b : Function of strongly homologous gene | t : transporter |
| ORF130 | 123151 | 123858 | | putative beta-phosphoglucomutase | 5.4.2.6 | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF131 | 124017 | 124781 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF132 | 125074 | 125487 | | UPF0047 protein | | 4 : Homologs of previously reported genes of unknown function | o : ORF of unknown function |
| ORF133 | 125650 | 126663 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF134 | 126816 | 127397 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF135 | 127390 | 128124 | | FHA domain containing protein | | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pr : putative regulator |
| ORF136 | 128134 | 128970 | pph1 | Serine/threonine protein phosphatase 2C | 3.1.3.16 | 2a : Function of homologous gene experimentally demonstrated in an other organism | e : enzyme |
| ORF137 | 128979 | 130388 | | putative serine/threonine protein kinase | 2.7.11.- | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF138 | 130535 | 131383 | trpH | Phosphoesterase (PHP family) | | 2b : Function of strongly homologous gene | e : enzyme |
| ORF139 | 131380 | 132309 | | Modular protein: putative Dihydroneopterin aldolase and SAM-dependent methyltransferases | 4.1.2.25 | 3 : Function proposed based on presence of conserved amino acid motif, structural feature or limited homology | pe : putative enzyme |
| ORF140 | 132367 | 132837 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF141 | 133060 | 133266 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF142 | 133274 | 133513 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF143 | 133827 | 134345 | | exported protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF144 | 134374 | 135285 | | Peptidase C14, Caspase-1 catalytic subunit p20 | 3.4.22.- | 2b : Function of strongly homologous gene | e : enzyme |
| ORF145 | 135376 | 135606 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF146 | 135816 | 136100 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF147 | 136129 | 136392 | | protein of unknown function | | 5 : No homology to any previously reported sequences | o : ORF of unknown function |
| ORF148 | 136492 | 137336 | cas3 | CRISPR-associated helicase Cas3 | | 2b : Function of strongly homologous gene | cp : cell process |

Table S2: Sources of environmental sequences of CLD-like proteins that were used for protein phylogeny (see Fig. 5).

| Label in Fig. 5 | Accesion no.[1] | Reference |
|---|---|---|
| Eastern tropical pacific Cocos island read | EM972568 | Rusch et al. (2007) |
| North American East cost read | EN082124 | ditto |
| Isabela island mangrove read 1 | EP183024 | ditto |
| Isabela island mangrove read 2 | EP183024 | ditto |
| Isabela island mangrove read 3 | EP183024 | ditto |
| Floreana island hypersaline lagoon read 1 | EP836070 | ditto |
| Floreana island hypersaline lagoon read 2 | EP835918 | ditto |
| Floreana island hypersaline lagoon read 3 | EQ050993 | ditto |
| Panama canal freshwater read 1 | EM410546 | ditto |
| Panama canal freshwater read 2 | EM410546 | ditto |
| Panama canal freshwater read 3 | EM490579 | ditto |
| Panama canal freshwater read 4 | EM490579 | ditto |
| Panama canal freshwater read 5 | EP707854 | ditto |
| Panama canal freshwater read 6 | EP707854 | ditto |
| Sargasso sea read 1 | EM426291 | Venter et al. (2004) |
| Sargasso sea read 2 | AACY01204540 | ditto |
| Sargasso sea read 3 | EP943272 | ditto |
| Sargasso sea read 4 | EN484981 | ditto |
| Sargasso sea read 5 | EP924593 | ditto |
| Minnesota farm soil clone | AAFX01003328 | Tringe et al. (2005) |

[1] Accession numbers refer to published scaffolds that contain the respective nucleic acid sequence reads of CLD-like proteins.

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# Chapter IV

## Nitrite oxidoreductase (Nxr) - the metabolic key enzyme of nitrite-oxidizing *Nitrospira*: Characterization of an unusual Nxr and its application as a novel functional and phylogenetic marker gene

To be submitted

# Nitrite oxidoreductase (Nxr) - the metabolic key enzyme of nitrite-oxidizing *Nitrospira*: Characterization of an unusual Nxr and its application as a novel functional and phylogenetic marker gene

Frank Maixner[1], Hanna Koch[1], Sandra Hauzmayer[1], Eva Spieck[2], Denis Le Paslier[3], Michael Wagner[1], Holger Daims[1]

Draft was not corrected by the co-authors.

[1] Department für Mikrobielle Ökologie, Universität Wien, Althanstrasse 14, A-1090 Vienna, Austria.

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

[3]CEA/Genoscope CNRS-UMR 8030, 2 rue Gaston Crémieux CP 5706, 91057 Evry, France.

## Summary

The Nxr genes of "*Candidatus* Nitrospira defluvii" were identified based on protein sequence comparisons with already known Nxr of other nitrite-oxidizing bacteria (NOB) and related nitrate/nitrite-binding enzymes of other microorganisms. The Nxr of *Nitrospira* consists of three subunits (alpha to gamma) and is present in two similar copies in the genome of *N. defluvii*. Like the Nxr forms of other characterized NOB, the *Nitrospira* enzyme belongs to the superfamily of molybdopterin-binding proteins. However, phylogenetic analyses revealed that the Nxr of *N. defluvii* differs remarkably in sequence from the Nxr of *Nitrobacter* and *Nitrococcus*, possibly reflecting key differences in enzymatic properties such as different affinities for nitrite. Also in contrast to the Nxr of *Nitrobacter*, which is localized in the cytoplasm, the *Nitrospira* Nxr seems to be located in the periplasm but attached to the cytoplasmic membrane via its gamma subunit. To prove the functionality of the detected *Nitrospira* Nxr, the transcription of the *nxrB* gene, encoding the Nxr beta subunit, was monitored in a highly enriched *N. defluvii* culture under starvation and nitrite-oxidizing conditions, respectively. In addition, the translation of Nxr was followed by the use of Nxr beta subunit-specific antibodies. Both experiments clearly showed that Nxr was expressed, and that the level of expression was much higher in presence of nitrite than during starvation.

Interestingly, *N. defluvii* cultures still contained detectable amounts of Nxr even after very long starvation periods. Thus, a stable Nxr-content may enable the organism to respond quickly to increasing nitrite concentrations without the need of energy-consuming *de novo* synthesis of Nxr.

Furthermore, a set of *Nitrospira nxrB*-specific PCR primers was designed based on the *nxrB* sequences of *N. defluvii* and evaluated with enrichments and pure cultures of other *Nitrospira* strains. These primers were used to amplify the *nxrB* genes of the cultured *Nitrospira*, which belonged to different sublineages of the genus *Nitrospira,* and of uncultured *Nitrospira* in different environmental samples. In-depth sequence analyses comparing the *nxrB*- and 16S rRNA-based phylogenies of the different sublineages of the genus *Nitrospira* showed congruent results, thus enabling the use of *nxrB* as novel phylogenetic marker. Subsequently, this new phylogenetic framework allowed the assignment of metagenomic reads to *Nitrospira*-like *nxrB* sequences.

## Introduction

In all three domains of life one driving force for evolution is the exploitation of unused niches. Particularly microbes harbor a diverse repertoire of biochemical "machineries" to take advantage of chemical gradients and different nutrients for energy generation. Prominent examples for functional diversification are certain phototrophs, which use different types of proteorhodopsin adapted to various spectral properties within their environment for light-driven energy generation (Sabehi *et al.*, 2007). The very fact that microbes are key players in all biogeochemical nutrient cycles and that they are found essentially anywhere is a testament to their metabolic innovation. Nevertheless, our current knowledge about enzymatic key enzymes involved in dissimilatory substrate turnovers is still scarce. Here we report about a novel nitrite oxdiodreductase, which catalyzes the oxidation of nitrite to nitrate, an essential step in the aerobic nitrification pathway. Nitrite-oxidizing bacteria (NOB) play a crucial role in the global biogeochemical N-cycle and also in nitrification in biological wastewater treatment. As the oxidation of nitrite to nitrate yields little free energy, all hitherto identified NOB are highly specialized, slow-growing organisms that are also very difficult to culture in artificial nutrient media. The key enzyme of nitrite oxidation is the nitrite oxidoreductase (Nxr), a complex heteromer that consists of at least two different subunits in *Nitrobacter* (Meincke *et al.*, 1992). In contrast to other key functional proteins involved in nitrification and denitrification (such as ammonia monooxygenase and nitrate reductases), our current

knowledge of Nxr is still limited. For example, yet unresolved questions are whether all known NOB harbor similar forms of Nxr and whether the Nxr subunits might be suitable as functional and also as phylogenetic markers for the detection of NOB in environmental samples. Recently, the latter question was addressed for *Nitrobacter*, whose Nxr beta subunit (NxrB) was used to reconstruct phylogenetic trees that resembled 16S rRNA-based phylogenies (Vanparys *et al.*, 2007).

Most of the few Nxr-related studies were carried out with pure cultures of *Nitrobacter* (Tanaka *et al.*, 1983, Sundermeyer-Klinger *et al.*, 1984, Meincke *et al*., 1992, Spieck *et al.*, 1996b). However, cultivation-independent approaches have revealed that different NOB, which belong to the genus *Nitrospira*, are the key NOB in wastewater treatment plants (wwtps) (Juretschko *et al.*, 1998). Moreover, 16S rRNA-based screenings have shown that *Nitrospira* are the most diverse known NOB in nature, and that these bacteria are ubiquitously distributed in a wide range of aquatic and terrestrial ecosystems (Nakamura *et al.*, 2006, Foesel *et al.*, 2008, Urich *et al.*, 2008). Thus, molecular tools to detect and identify these mostly uncultured NOB are essential for studies of nitrification in natural and engineered habitats. Ideally, such tools would allow one to simultaneously identify *Nitrospira* and monitor their nitrite-oxidizing activities *in situ*. The 16S rRNA approach combined with FISH-microautoradiography meets these requirements (Daims *et al.*, 2001), but a less time-consuming and non-radioactive approach, which would be applicable to larger numbers of samples, is still lacking.

This study emerged as a spin-off from a larger environmental genomics project, which aimed at sequencing the whole genome of a *Nitrospira* strain (*N. defluvii*) enriched from a nitrifying wwtp. Here, the goal was to identify and characterize in detail the *nxr* genes in the *N. defluvii* genome and to determine whether these genes are suitable as functional and phylogenetic markers of *Nitrospira*. In this case, *nxr* could be applied in microbial diversity surveys in a similar manner as *amoA* has been used to detect and identify ammonia oxidizers.

## Results and Discussion

### *Novel nitrite-oxidizing system in* N. defluvii

An environmental genome approach applied on a high enrichment (86% biovolume) of *N. defluvii* resulted in the first closed genome of a *Nitrospira*-like organism (for details on *N. defluvii* enrichment please refer to Spieck *et al.*, 2006). First genomic analyses supported the results of former immunological and enzymatic studies (Spieck *et al.*, 1998, Bartosch *et al.*,

1999) that the nitrite-oxidizing enzyme of *Nitrospira* displays striking differences to the well characterized nitrite oxidoreductase (Nxr) of *Nitrobacter* spp. (Sundermeyer-Klinger *et al*., 1984, Meincke *et al*., 1992). Already sequence-based comparison of both Nxr types indicated that the Nxr function might have convergently evolved in two different nitrite-oxidizing systems (Fig. 1).

Hence, assigning and annotating *N. defluvii* Nxr genes based on sequence similarity alone to already validated Nxr genes of *Nitrobacter* spp. was not trivial. In this study we rather combined five criteria considering similarities and differences of both nitrite-oxidizing systems to focus on the most parsimonious candidate genes comprising this novel nitrite-oxidizing enzyme of *N. defluvii*:

i) The Nxr of *Nitrobacter hamburgensis* (Sundermeyer-Klinger *et al*., 1984, Meincke *et al*., 1992) and *Nitrospira moscoviensis* (Spieck *et al*., 1998), a close relative to *N. defluvii*, consist of at least two subunits, the large subunit NxrA and the small subunit NxrB.

ii) Molybdenum is a important cofactor of the Nxr of *Nitrobacter* spp. (Ingledew & Halling, 1976, Sundermeyer-Klinger *et al.*, 1984, Meincke *et al.*, 1992) and the activity of the nitrite-oxidizing system of *N. moscoviensis* is dependent on the presence of molybdenum (Spieck *et al*., 1998). Therefore only molybdenum-containing oxidoreductases encoded on the genome of *N. defluvii* were shortlisted.

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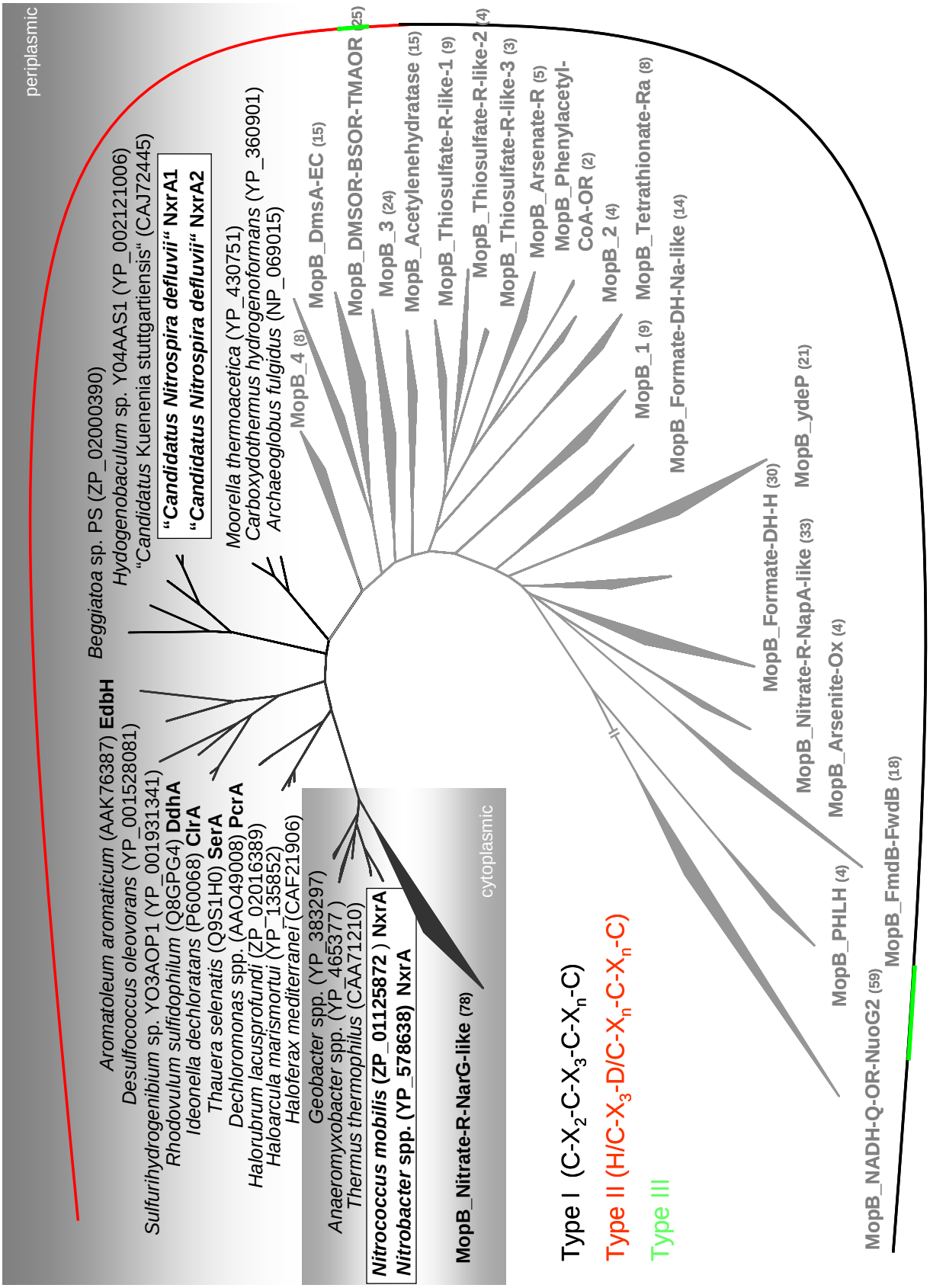
**Figure 1**: Unrooted maximum-likelihood tree (PhyML) indicating the evolutionary alliance of molyptopterin-binding (MopB) proteins of the large and heterogeneous MopB protein-superfamily (NCBI conserved domain: cl09928). Tree is based on amino acid sequences of the alpha subunits of different subfamily enzymes in the MopB superfamily. The number of sequences and in case of single species the accession number of the sequence used for tree calculation is indicated in brackets. For details for the subfamilies of the MopB superfamily please refer to Table S4. Sequences are assigned to three different MopB protein-superfamily subtypes, due to presence or absence of a specific cystein-rich motif. The colored border strip arround the tree leafs indicate the type affiliation. Sequences displayed with black tree branches share the common feature of an aspartate as molybdenum ligand and belong to the type II group of Mo-*bis*MGD (molybdopterin-guanine-dinucleotide) enzymes (Jormakka *et al*., 2004). These type II Mo-*bis*MGD enzymes can be further differentiated between periplasmic and cytoplasmic proteins (both highlighted grey). White boxes highlight nitrite oxidoreductase alpha subunit sequences (NxrA). Abbreviations for alpha subunits of validated type II group Mo-*bis*MGD enzymes: NarG, membrane bound respiratory nitrate reductase; PcrA, perchlorate reductase; SerA, selenate reductase; ClrA, chlorate reductase; DdhA, dimethyl sulphoxide reductase; EdbH, ethylbenzene dehydrogenase.


periplasmic
cytoplasmic
Aromatoleum aromaticum (AAK76387) EdbH
Desulfococcus oleovorans (YP_001528081)
Sulfurihydrogenibium sp. YO3AOP1 (YP_001931341) DdhA
Rhodovulum sulfidophilum (Q8GPG4) DdhA
Ideonella dechloratans (P60068) ClrA
Thauera selenatis (Q9S1H0) SerA
Dechloromonas spp. (AAO49008) PcrA
Haloarcula marismortui (YP_135852)
Haloferax mediterranei (CAF21906)
Geobacter spp. (YP_383297)
Anaeromyxobacter spp. (YP_465377)
Thermus thermophilus (CAA71210)
Nitrococcus mobilis (ZP_01125872) NxrA
Nitrobacter spp. (YP_578638) NxrA
MopB_Nitrate-R-NarG-like (78)
Beggiatoa sp. PS (ZP_02000390)
Hydogenobaculum sp. Y04AAS1 (YP_002121006)
"Candidatus Kuenenia stuttgartiensis" (CAJ72445)
"Candidatus Nitrospira defluvii" NxrA1
"Candidatus Nitrospira defluvii" NxrA2
Moorella thermoacetica (YP_430751)
Carboxydothermus hydrogenoformans (YP_360901)
Archaeoglobus fulgidus (NP_069015)
MopB_4 (8)
MopB_DmsA-EC (15)
MopB_DMSOR-BSOR-TMAOR (25)
MopB_3 (24)
MopB_Acetylenehydratase (15)
MopB_Thiosulfate-R-like-1 (9)
MopB_Thiosulfate-R-like-2 (4)
MopB_Thiosulfate-R-like-3 (3)
MopB_Arsenate-R (5)
MopB_Phenylacetyl-CoA-OR (2)
MopB_2 (4)
MopB_1 (9)
MopB_Tetrathionate-Ra (8)
MopB_Formate-DH-Na-like (14)
MopB_Formate-DH-H (30)
MopB_Nitrate-R-NapA-like (33)
MopB_ydeP (21)
MopB_Arsenite-Ox (4)
MopB_PHLH (4)
MopB_NADH-Q-OR-NuoG2 (59)
MopB_FmdB-FwdB (18)
Type I (C-X2-C-X3-C-Xn-C)
Type II (H/C-X3-D/C-Xn-C-Xn-C)
Type III

iii) In contrast to all other molybdenum-binding oxidoreductases found on the genome of *N. defluvii* the predicted products of the candidate genes, the large subunits NxrA1 and NxrA2, are most similar in length and amino acid sequence to Nxr large subunits of *Nitrobacter winogradskyi* Nb-255 (Starkenburg *et al.*, 2006), *N. hamburgensis* X14 (Starkenburg *et al.*, 2008) and *Nitrococcus mobilis* Nb-231 (Table S1).

iv) Immunocytochemical studies on the localization of Nxr in the cell revealed one major difference in the nitrite-oxidizing systems of *Nitrobacter* spp. and *Nitrospira* spp.. Whereas the Nxr of *Nitrobacter* is localized at the cytoplasmic face of the cell membrane and at the intracytoplasmic membranes (Spieck *et al.*, 1996a), the Nxr of *Nitrospira* is found to be membrane-associated in the periplasmic space (Spieck *et al*., 1998). This latter observation is in agreement with the *in silico* predicted twin-arginine signal peptide cleavage sites (TatP 1.0 server; Bendtsen *et al.*, 2005) in both large subunits of Nxr (NxrA1, NxrA2) of *N. defluvii*. The twin-arginine signal peptide strongly suggests an export of the Nxr complex into the periplasm via the Tat translocase system, which transports folded proteins across the cytoplasmic membrane (Lee *et al.*, 2006).

v). An additional characteristic mark distinguishing Nxr of *Nitrobacter* and *Nitrospira* has been observed in immunoblotting experiments with monoclonal antibodies raised against the alpha and beta subunit of Nxr of *N. hamburgensis*, respectively (Aamand *et al.*, 1996, Spieck *et al*., 1998, Bartosch *et al*., 1999). While the anti-alpha subunit antibody Hyb 153.2 gave a positive signal only with the NxrA of *Nitrobacter* spp. (Aamand *et al*., 1996, Bartosch *et al*., 1999), the NxrB of *Nitrobacter* and *Nitrospira* seem to share a common binding motif for the anti-beta subunit antibody Hyb 153.3 (Bartosch *et al*., 1999). The NxrB of both nitrite-oxidizing organisms could be detected by Hyb 153.3 (Spieck *et al.*, 1998, Bartosch *et al.*, 1999), although the apparent molecular masses of the recognized proteins differed in the respective organism (*Nitrobacter*: 65 kDa, *Nitrospira*: 50 kDa). The latter result is in agreement with the *in silico* predicted molar masses of the NxrB amino acid sequences using the ProtParam tool (*Nitrobacter*: 58.04 kDa, *N. defluvii*: 49.98 kDa) (Gasteiger *et al.*, 2005).

In summary, the aforementioned similarities and differences between the Nxr of *Nitrobacter* and *Nitrospira* enabled us to pinpoint the most parsimonious candidate genes for this unusual Nxr on the genome of *N. defluvii.*

*Nitrite oxidoreductase genes of* N. defluvii: *the genomic environment*

Two gene copies encoding the alpha and beta subunits of Nxr, respectively, are located in immediate vicinity on the genome of *N. defluvii.* Both *nxrAB* clusters appeared to be

organized in an operon (Fig. 2). Also other already sequenced genomes of nitrite-oxidizing organisms carry multiple nitrite oxidoreductase gene copies (Table S2). However, in contrast to *N. defluvii,* only one set of *nxrA* and *nxrB* of *Nitrobacter* or *Nitrococcus* is organised in an operon. All other copies are segregated and scattered in the respective genomes. Nevertheless, the central Nxr gene cassette is conserved in all three *Nitrobacter* genomes and encompasses in contrast to the Nxr operon of *N. defluvii* not only genes for NxrA and NxrB but also genes for several other putative accessory proteins (Starkenburg *et al.*, 2006, Starkenburg *et al.*, 2008). A unique feature of the *nxr* operons of *N. defluvii* are the transcriptional regulators (*nifA*, *NIDE3254*) located upstream of both operons (Fig. 2). The regulators possess a helix-turn-helix DNA-binding domain in the C-terminal section and a Sigma-54 interaction domain and play presumably an important role in the transcriptional activation of the respective downstream encoded Nxr genes by a conserved mechanism common to members of the enhancer binding protein family (Buck *et al.*, 2000). Beside these two similar domains in the C-terminal section, however, both regulators share no similarity at all in the N-terminal part of the protein. The gene product of *NIDE3254* has a common CheY-like response regulator receiver region at the N-terminus interacting with a yet unknown histidine kinase in a two-component signal transduction system (Stock *et al.*, 2000). The amino terminus of the *nifA*-like gene product, however, comprises a GAF domain, a motif found in signalling and sensory proteins from all domains of life (Aravind & Ponting, 1997, Ho *et al.*, 2000).

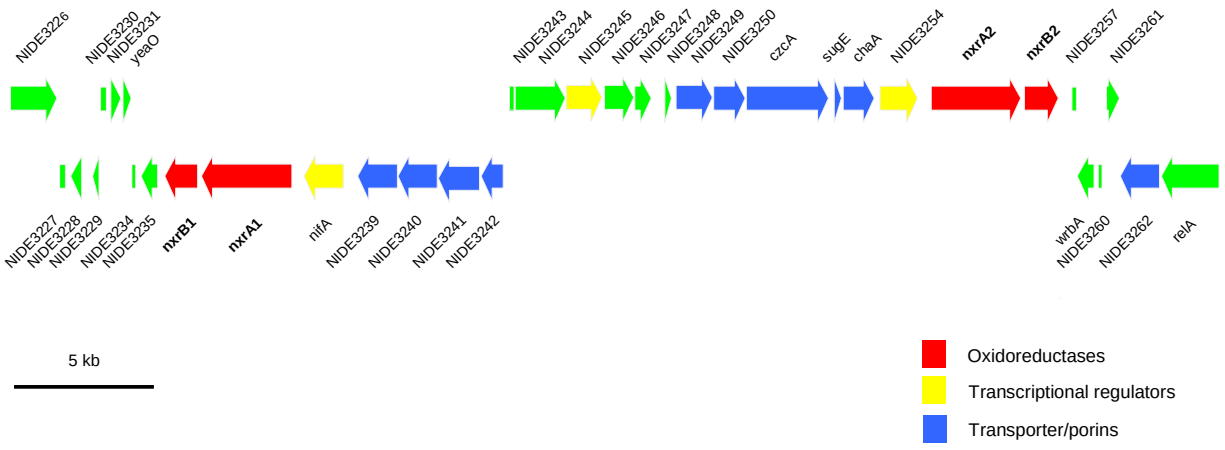



**Figure 2**: Metagenomic stretch containing two gene copies encoding the nitrite oxidoreductase alpha (*nxrA1*, *nxrA2*) and beta (*nxrB1*, *nxrB2*) subunits of *N. defluvii*. The oxidoreductase genes are displayed in red, genes for transcriptional regulators and transporters/porins are highlighted in yellow and blue, respectively. The scale bar indicates 5 kilobase pairs genomic length. For further information on the displayed annotated open reading frames please refer to table S3.

This GAF domain can bind small molecules as co-effectors such as formate or 3´, 5´cyclic guanosine monophosphate (Hopper & Bock, 1995, Anantharaman *et al.*, 2001, Kanacher *et al.*, 2002). In general, the transcriptional regulation of Nxr operons seems to be mediated at first by a Sigma-54 transcription factor and subsequently this Sigma factor interacts with one of the two transcription regulators, which harbour in addition different sensor systems to external stimuli. The strict transcriptional regulation of Nxr should enable *N. defluvii* to efficiently adapt to changing environmental conditions, which was already previously suggested by Lisa Stein and coworkers for *amoA* and *amoB* gene copies in *Nitrosomonas europaea* (Stein *et al.*, 2000). Consequently, we analyzed the gene products of both operons, whether the multiple Nxr copies were identical on amino acid level or not. While the beta subunits NxrB were identical, the catalytically active alpha subunits share only 87% amino acid identity (Table S2). This difference on the amino acid level might already indicate a functional divergence of isozymes in *N. defluvii* as it could be shown in a different study in the type II methanotroph *Methylocystis* sp. Strain SC2 (Baani & Liesack, 2008). The latter organism possesses two particulate methane monooxygenase (pMMO) isozymes with different methane oxidizing kinetics. Moreover, one pMMO type is constitutively expressed, whereas the other type is only expressed above a certain methane concentration threshold, suggesting an adaptation to fluctuating methane supply in the environment. Nevertheless, it remains to be determined whether there is a strict transcriptional regulation of one or both Nxr operons in *N. defluvii* under changing environmental conditions.

*Transcriptional analyses of* nxrB

A major goal of the transcriptional analyses of Nxr was to show, if Nxr genes were really expressed under nitrite-oxidizing conditions and hence responsible for the nitrite oxidation of *Nitrospira*, and how expression differed under nitrite limitation. Transcription of *Nitrospira*-like Nxr was demonstrated both with biomass from nitrifying activated sludge and with cells from the *N. defluvii* enrichment via RT-PCR by using primers (nxrBF916/nxrBR1237) targeting *Nitrospira* sublineage I *nxrB* sequences (Fig. 3A I, II/2). The cloned and sequenced RT-PCR products were similar to *nxrB* sequences obtained by PCR amplification from the activated sludge (Fig. 4) and identical to *nxrB* sequences of the enrichment, respectively (data not included in Fig. 4). Hence, we could show for the first time that Nxr is expressed in *Nitrospira* under nitrite-oxidizing conditions both in the natural environment (activated sludge) and in a minimal medium with nitrite as sole energy source (*N. defluvii* enrichment; Spieck *et al*., 2006). To determine *nxrB* transcription levels in *N. defluvii* during nitrite

limitation (Fig. 3A II/1) and nitrite-oxidizing conditions (Fig. 3A II/2) they were compared to the internal 16S rRNA gene pool in an approach similar to an *amoA* expression study with *Nitrosospira briensis* (Bollmann *et al.*, 2005). Thereby, we amplified in addition to *nxrB* specifically the 16S rRNA gene of *N. defluvii* from the cDNA. Interestingly, *N. defluvii* cultures still contained detectable amounts of *nxrB* mRNA even after a starvation period of 11 days and the 16S rRNA content seems to be constant under both conditions (Fig. 3A II/1). Furthermore, after nitrite addition the *nxrB* mRNA level in contrast to the 16S rRNA level even doubled, indicating a higher *nxrB* expression level during nitrite-oxidizing conditions (Fig. 3A II/2).

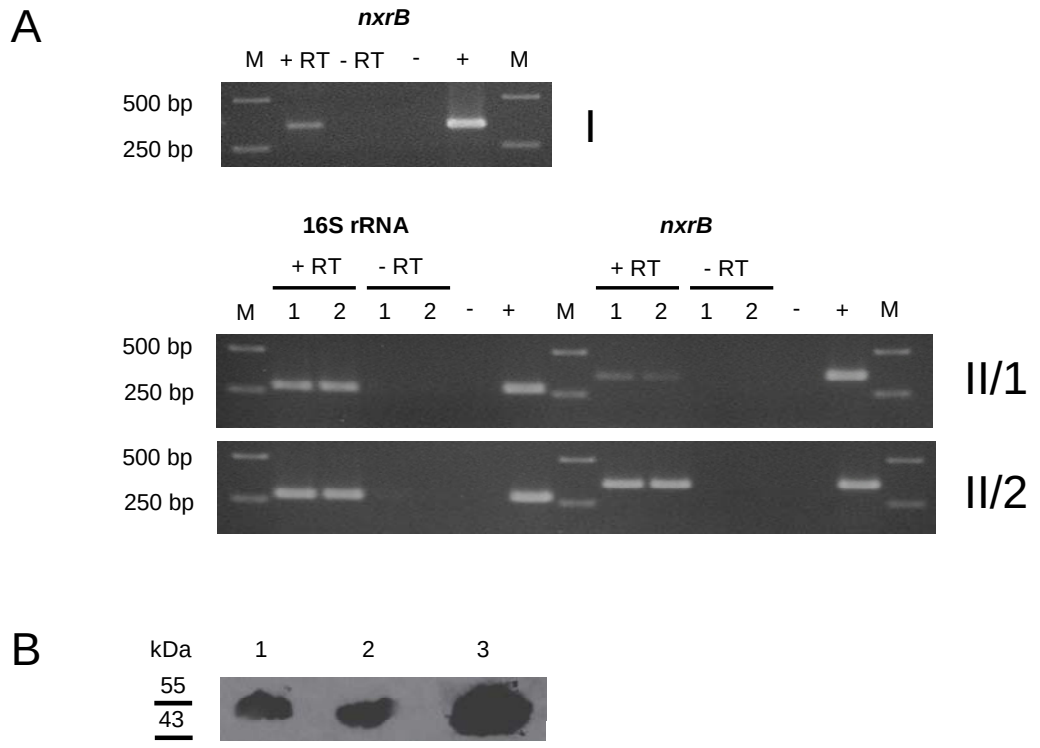



**Figure 3**: Transcriptional and Western blot analyses of *nxrB*/NxrB.

(A, I) *Nitrospira*-like *nxrB* mRNA detection in nitrifiying activated sludge. (A, II) *Nitrospira*-like 16S rRNA and *nxrB* mRNA detection in the enrichment of "*Candidatus* Nitrospira defluvii" before (II/1, 11 days starved) and after $NO_2^-$ addition (II/2, 3 days after full conversion of 300 µM $NO_2^-$). Abbreviations: M, size marker; + RT, mRNA detection via reverse transcription PCR; - RT, PCR control for DNA contamination in the RNA extract; +, positive control with cloned *nxrB* gene fragment as PCR template, -, negative control without nucleic acid; 1,2, biological replicates in experiment II.

(B) Immunological detection of NxrB in the protein fraction of numeral similar "*C.* Nitrospira defluvii" cells in different growth stages. Lane 1: *N. defluvii* NxrB + 6x HIS-Tag heterologous expressed in *E. coli*, lane 2: crude protein extract of starved (110 days) *N. defluvii* enrichment, lane 3: crude protein extract of *N. deluvii* enrichment after $NO_2^-$ addition (8 days after full conversion of 300 µM $NO_2^-$).

It remains to be determined, whether the *nxrB* presence during starvation is due to increased mRNA stability or constant mRNA supply on a low expression level. In heterotrophic bacteria it has been shown, that gene expression can be regulated at the level of mRNA stability (Mackie, 1998). The physiological state of the cell as well as the transcript secondary structure can be important determinants of variations in mRNA decay (Meyer & Schottel, 1991, Thorne & Williams, 1997, Coburn & Mackie, 1999). Nevertheless, the half-life of mRNA in heterotrophic bacteria is on average only 3 min (Takayama & Kjelleberg, 2000, Bernstein *et al.*, 2002), indicating that rather constitutive and regulated mRNA expression than mRNA stability influences the mRNA level during starvation.

Also other autotrophic nitrifiers keep a certain cell-internal RNA level during starvation, but in contrast to heterotrophs only some selected RNAs appear to be associated with the adaptation to starvation. Several studies with different ammonia-oxdizing bacteria revealed that relatively high levels of *amoCAB* mRNA, comprising the ammonia monooxygenase enzyme, and high levels of 16S rRNA were maintained during complete deprivation of ammonia (Sayavedra-Soto *et al.*, 1998, Morgenroth *et al.*, 2000, Wei *et al.*, 2004, Bollmann *et al.*, 2005, Wei *et al.*, 2006, El Sheikh & Klotz, 2008). These previous results with AOB are in agreement with the *nxrB* mRNA and 16S rRNA maintenance in *Nitrospira* observed in this study. Hence, it might be a common strategy in nitrifiers to sustain starvation periods by keeping the cell-internal content of certain RNAs high to quickly respond when substrate again becomes available.

In summary, beside this quite high mRNA level even after 11 days of starvation the comparison of the both growth phases via RT-PCR indicated a higher *nxrB* expression level under nitrite-oxidizing conditions (Fig. 3A II). Even the obvious limitations of this semi-quantitative approach (RNA extraction and cDNA synthesis efficiencies, different primer efficiencies, etc.) considered, these differences in the expression level were significant and reproducibly. In addition to the five criteria outlined in the first chapter, these results corroborate the hypothesis that the *in silico* depicted Nxr candidate genes comprise the nitrite-oxidizing system of *N. defluvii*.

*Western blot analyses of NxrB*

Already in the year 1996 Aamand and colleagues presented in their study three monoclonal antibodies (Hyb 153.1, 153.2, 153.3) raised against the Nxr of *Nitrobacter hamburgensis* (Aamand *et al.*, 1996). In follow-up studies one (Hyb 153.3) out of these three antibodies turned out to bind also specifically to a protein of *Nitrospira*-like bacteria (Spieck *et al.*, 1998,

Bartosch *et al.*, 1999). Even though the apparent molecular mass of the detected protein in *Nitrospira* was different to the mass of the recognised Nxr beta subunit of *Nitrobacter*, it was obvious that both proteins share a common binding motif for the monoclonal antibody Hyb 153.3. Thus, it was assumed that this protein represents the Nxr beta subunit of *Nitrospira.* More than one decade after the first description of this antibody we could clearly link in our study the antibody specificity with sequence data of the recognised protein in the genome of *N. defluvii*. Western blot analyses with the heterologous expressed *nxrB* gene revealed a specific binding of the antibody Hyb 153.3 with the recombinant protein (Fig. 3B).

With the reevaluated antibody Hyb 153.3 we had a closer look into the cell-internal NxrB content of starved and nitrite-oxidizing *N. defluvii* cells (Fig 3B). For this approach we used cell extracts of numerical similar *N. defluvii* cells of both growth phases. Interestingly, the Western blot results revealed that even after a starvation period of 110 days a high cell-internal NxrB content remained in *N. defluvii*. Furthermore, after reactivation of the starved cells with nitrite the per-cell NxrB content was even doubled. *N. defluvii* seems to have a certain core set of proteins, including Nxr, which enables the organism to restart its nitrite-oxidizing system relatively fast after starvation. Part of the thereby gained energy seems to be immediately used for the assembly of new Nxr.

This survival strategy is similar to the starvation adaptation employed by ammonium-oxidizing bacteria (AOB). During starvation of AOB the cell-internal AmoA content remained stable (Stein & Arp, 1998) and AmoB could even be detected after one year of ammonia starvation (Pinck *et al.*, 2001). Moreover, the potential activity of the Amo enzyme was not affected over prolonged starvation periods (Wilhelm *et al.*, 1998). In general, it can be concluded that nitrifiers posses several physiological traits such as low maintenance energy demand (Tappe *et al.*, 1999), quorum sensing (Batchelor *et al.*, 1997, Laanbroek *et al.*, 2002) or as illustrated above stable amount of both, enzymes and specific RNAs, to withstand unfavourable environmental conditions and moreover to regain their metabolic activity within a short timeframe (Geets *et al.*, 2006). Thus, in natural and engineered environments with fluctuating nutrient supply, nitrifiers such as *N. defluvii* perfectly adapted their "cellular machineries" to these changing conditions.

In the following three sections the gene products comprising the Nxr alpha, beta and gamma subunit will be described in detail, to gain first insights into the biochemical features of this unusual enzyme.

*The alpha subunit NxrA*

The large subunit of Nxr (NxrA1/NxrA2) of *N. defluvii* has two characteristic domains, an N-terminal iron-sulphur cluster [Fe-S]-binding motif and molybdopterin cofactor-binding sites. The latter domain defines NxrA according to the NCBI Conserved domain database as member of the molybdopterin-binding superfamily of proteins (cl09928) (Marchler-Bauer *et al.*, 2007), which contains all known proteins formely assigned to the DMSO reductase family (Hille, 1996). Here the term DMSO reductase family will be used in order to avoid confusions with previous studies about proteins of this family (McDevitt *et al.*, 2002, Bender *et al.*, 2005). The DMSO reductase family is a large and heterogeneous enzyme family and shares a common form of molybdenum cofactor, the bis(molybdopterin guanine dinucleotide)Mo (Mo-*bis*MGD) (Kisker *et al.*, 1997). Phylogenetic analyses with selected alpha subunits of DMSO reductase family enzymes revealed a unique and unexpected position of *N. defluvii* NxrA in the MopB superfamily tree (Fig. 1). The NxrA sequences of *Nitrospira* cluster together with membrane-bound nitrate reductase (NarG)-like sequences of `*Candidatus* Kuenenia stuttgartiensis´, *Hydrogenobaculum* sp. Y04AAS1 and *Beggiatoa* sp. PS comprising a monophyletic clade in the tree. Most apparent is the separate position of this latter described branch from the Nxr alpha subunits of *Nitrobacter* spp.. The results indicate a convergent evolution of similar enzymatic functions in two different nitrite-oxidizing systems. The *Nitrobacter* NxrAs cluster together with the NxrA of *Nitrococcus mobilis* and are closely related to the large subunit of the membrane-bound nitrate reductases (NarG) (Kirstein K., 1993). Within a few exceptions the overall tree topology is in most cases consistent with the functional affiliation of the respective sequences. Beside this functional clustering (Table S4) an additional classification based on structural variations can be applied on members of the DMSO reductase family. Three distinct DMSO reductase family types with differences in the [Fe-S]-binding motif in the alpha subunit can be distinguished (Fig. 1) (Trieber *et al.*, 1996). The majority of hitherto known DMSO reductase family enzymes, such as the perplasmic nitrate reductase (Nap), the formate dehydrogenase (Fdh) or the arsenite reductase (Aso) are members of type I enzymes defined by an cystein-rich [Fe-S]-binding motif (C-$X_2$-C-$X_3$-C-$X_n$-C) (McDevitt *et al*., 2002). In almost all type I enzymes the motif is located at the N-terminal part of the alpha subunit except for the large subunit (NuoG) of NADH-ubiquinone oxidoreductases (cd02768). In this latter enzyme family an internal domain of NuoG contains the afromentioned cystein-rich motif and shares homology to Fdh sequences supporting a common ancestor for both enzyme families (Friedrich, 1998). Similar to type I enzymes type II enzymes have also N-terminal cystein residues for [Fe-S]-binding in the alpha subunit, but

in contrast to type I enzymes three amino acid residues instead of two separate the first and the second cystein residue (Trieber *et al*., 1996). Furthermore, the first cystein residue can be replaced to a histidine ligand in some type II enzymes, such as the membrane-bound nitrate reductase (Nar) of *E. coli* (Bertero *et al.*, 2003). Furthermore, Jormarkka and co-workers proposed based on a NarG crystal structure of *E. coli* that most type II enzymes seem to share a conserved aspartate residue in the active centre acting as molybdenum ion ligand (Jormakka *et al.*, 2004). In contrast to the first two DMSO reductase family types, type III enzymes lack a cystein-rich motif. Two MopB families belong to the type III, the MopB_PHLH (cd02764) family with uncharacterized hydrogenase-like enzymes and the MopB_DMSOR_ BSOR_TMAOR (cd02769) family including the biotine sulfoxide reductase of *Rhodobacter sphaeroides* (Pollock & Barber, 1997) and the trimethylamine N-oxide reductase of *E. coli* (Weiner *et al.*, 1992).

Based on these aforementioned classifications we propose that the Nxr of *N. defluvii* is a member of type II enzymes of the DMSA reductase family considering two observations: (i) NxrA shares the molybdenum ion-binding Asp residue with most alpha subunits of type II enzyme family members (Fig S1B); (ii) The first two cysteine residues of the [Fe-S]-binding motif of NxrA of *N. defluvii* are separated by three amino acids, which is characteristic for type II enzymes (Fig. S1A). One major difference in NxrA of *Nitrospira* and related sequences to all other type II enzymes studied so far is the replacement of the second cystein residue in the binding motif with an aspartate (Fig. S1A). Since aspartate has been previously shown in a ferredoxin of the hyperthermophilic *Pyrocccus furiosus* to be a proximal ligand of the [Fe-S] (Calzolai *et al.*, 1995), we suggest to expand the type II signature motif proposed by Jormakka and colleagues (Jormakka *et al*., 2004) to the following coordination pattern: H/C-$X_3$-D/C-$X_n$-C-$X_n$-C. Beside these common characteristics, type II DMSO reductase family enzymes can be distinguished between cytoplasmic localized proteins and enzymes with a twin-arginine signal peptide transported via Tat translocase system into the periplasm (Fig. 1) (Martinez-Espinosa *et al.*, 2007).

In summary, the Nxr of *N. defluvii* is a periplasmic NarG-like type II DMSO reductase family enzyme, closely related to the NarG-like enzyme of "*C.* Kuenenia stuttgartiensis" and with only weak similarity to previously described cytoplasmic Nxr of *Nitrobacter* spp..

*The beta subunit NxrB*

NxrB (NxrB1/NxrB2) of *N. defluvii* is a member of the bacterial oxidoreductase electron transfer subunits (Berks *et al.*, 1995). Although there is no indication in the NxrB sequences

of *N. defluvii* for a signal peptide, it is very likely that NxrA and NxrB are translocated together as folded protein complex via the Tat translocase system by using a "hitchhiker" mechanism proposed by Rodrigue *et al*. (1999). Comparison of NxrA and NxrB phylogeny indicated a shared evolutionary history for the alpha and beta subunit, suggesting a tight functional alliance of both subunits (Fig. S2). Crystal structures of beta subunits closely related to the NxrB of *N. defluvii*, such as the nitrate reductase beta subunit NarH of *E. coli* (Bertero *et al*., 2003) or the beta subunit of the ethylbenzene dehydrogenase of *Aromatoleum aromaticum* (Kloer *et al.*, 2006) confirmed the presence of three [4Fe-4S] cluster (I, III, IV) and one [3Fe-4S] cluster (II) (Fig. S3B). These [Fe-S] play an important role in the intramolecular electron transfer (Blasco *et al.*, 2001). Comparative sequence analysis of NxrB and selected beta subunits of type II DMSO reductase enzymes revealed the presence of four cysteine-rich motifs for [FeS]-binding (Fig. S3A). These four [FeS] have been characterized in detail in the nitrate reductase beta subunit (NarH) of *E. coli*. EPR spectroscopy and site-directed mutagenesis (Guigliarelli *et al.*, 1996, Blasco *et al*., 2001) as well as structural analysis (Bertero *et al*., 2003, Jormakka *et al*., 2004) of NarH revealed that the [Fe-S] are organized in two pairs, each with one low- and one high-potential cluster (IV/I and III/II) (Fig. S3B). A unique feature of the NxrB of *N. defluvii* compared to all other known beta subunit sequences is the replacement of one cysteine to an aspartate residue binding presumably the low-potential [4Fe-4S] cluster IV (Fig. S3). Furthermore, two out of three cysteine residues, which additionally bind [4Fe-4S] cluster IV, are separated by an insertion of eight amino acids specific for the beta subunit sequences of *N. defluvii* and "*C.* Kuenenia stuttgartiensis" (Fig. S3). It remains to be determined whether these differences to other characterized beta subunits result in a unique mode of electron transfer.

*Gamma subunit*

The mature holoenzymes of most type II DMSO reductase family enzymes studied so far contain beside the catalytic active alpha subunit and the electron transferring beta subunit an additional gamma subunit. These gamma subunits can either have membrane-spanning helices as it is known from the nitrate reductase gamma subunit NarI from *E. coli* anchoring the alpha and beta subunit to the cytoplasma membrane (Martinez-Espinosa *et al*., 2007) or the gamma subunits can lack trans-membrane helices as seen in the soluble periplasmic ethylbenzene dehydrogenase EBDH of *A. aromaticum* (Kloer *et al*., 2006). Despite these differences in their localisation, structural and spectroscopic analysis demonstrated that all gamma subunits of type II DMSO reductase family enzymes seem to contain at least one heme b cofactor.

Whereas NarI of *E. coli* contains two heme b, only a single heme b is found in the periplasmic EBDH (Kloer *et al*., 2006), in the selenate reductase SerC of *Thauera selenatis* (Schroder *et al.*, 1997) and in the chlorate reductase ClrC of *Ideonella dechloratans* (Thorell *et al.*, 2003). Three gene products (NxrC1/C2/C3) encoded on the genome of *N. defluvii* displayed highest similarity to the aforementioned gamma subunits and to heme b binding subunits of "*C.* Kuenenia stuttgartiensis" (CAJ72454, CAJ72449), *Hydrogenobaculum* sp. Y04AAS1 (YP_002121004) and *Pyrobaculum calidifontis* (YP_001056787) (Fig.S4).

In contrast to other DMSO reductase family enzymes such as the perchlorate reductase of *Dechloromonas agitata* (Bender *et al*., 2005) or the Nxr of *Nitrobacter* spp. (Starkenburg *et al*., 2006), where the subunit encoding genes are organized in a operon structure, the three *nxrC* gene copies are separated from *nxrA* and *nxrB* and scattered in the genome of *N. defluvii*. Although all three NxrC copies are quite dissimilar to each other on the amino acid level (24-39%), together they share with other type II gamma subunits two conserved residues (Fig. S4), which were previously identified in the crystallized EBDH gamma subunit as proximal heme b ligands (Kloer *et al*., 2006). Thus, it is likely that NxrC contains at least one heme b cofactor and is part of an electron transport system involving the iron-sulphur complexes of NxrA and NxrB and the heme b in NxrC. In addition, the *in silico* predicted signal peptides in all three NxrC copies suggest a periplasmic localisation of the Nxr gamma subunit (Fig. S4).

One unique feature of NxrC3 compared to the two other NxrC copies of *N. defluvii* is the C-terminal transmembrane spanning domain (Fig. S4). Assuming that *Nitrospira moscoviensis* and *N. defluvii* have similar nitrite-oxidizing systems especially this latter finding is in aggreement with former studies with the Nxr of *N. moscoviensis*. Spieck and colleagues (1998) isolated the nitrite-oxidizing system of *N. moscoviensis* by density gradient centrifugation and separated the proteins by SDS-PAGE. The Nxr complex seems to consist of at least four major proteins with apparent molecular masses of approximately 130, 62, 46 and 29 kDa. The 130 and 46 kDa protein band might represent the alpha and beta subunit of Nxr, which have in *N. defluvii* the calculated molar masses (ProtParam) of 129 kDa and 50 kDa, respectively. Until know the 62 kDa protein band could not be asigned to any gene product of *N. defluvii*, but the apparent molecular mass of 29 kDa of the last prominent protein band of the Nxr of *N. moscoviensis* is similar to calculated molar masses of the three NxrC copies of *N. defluvii* (NxrC1: 27 kDa, NxrC2: 30 kdA, NxrC3: 26 kDa). Furthermore, the Nxr of *N. moscoviensis* exhibited its highest enzymatic activity in the membrane fraction, suggesting that the enzyme is membrane bound (Spieck *et al.*, 1998). Therefore, there has to

be an additional membrane anchoring subunit in Nxr of *Nitrospira*. Thus, NxrC3 in *N. defluvii* with the C-terminal transmembrane helix might take over this part in the nitrite-oxidizing system of *N. defluvii* (Fig S4).

*Design and evaluation of* nxrB *primer sets targeting the nitrite oxidoreductase of* Nitrospira*-like organisms*

One major goal of this study beside the characterization of the candidate genes encoding the Nxr of *N. defluvii* was the investigation whether one of these genes could serve as functional and phylogenetic marker for the genus *Nitrospira*. The most conserved of the Nxr genes, *nxrB*, was selected for primer design and evaluation (Table S2). Based on the alignment of the two nearly identical *nxrB* copies (99.9 % identity on DNA level) of *N. defluvii* (*nxrB1*, *nxrB2*) and several shotgun reads of *N. moscoviensis*, with high sequence identity to the *nxrB* of *N. defluvii,* a first *nxrB* primer pair (nxrBF14/nxrBR1239, Table 1) was designed. The sequence CAJ72448 of "*C.* Kuenenia stuttgartiensis", the closest related sequence to the *Nitrospira nxrB* in the public database, served as control to test *in silico* for unspecific primer binding.

**Table 1**: Specifications of different primer pairs developed in this study targeting *Nitrospira*-like *nxrB*.

| Name | Specificity [1] | Sequence 5´-3´ [2] | Target-site [3] | Fragment size (bp) | PCR protocol [4] |
|---|---|---|---|---|---|
| nxrBF14 | Sublineage I and II of the genus *Nitrospira* | ATA ACT GGC AAC TGG GAC GG | 14 - 33 | 1245 | 1 |
| nxrBR1239 | | TGT AGA TCG GCT CTT CGA CC | 1239 - 1258 | | |
| nxrBF19 | Beryl spring enrichment, *N. bockiana, N. marina* | TGG CAA CTG GGA CGG AAG ATG | 19 - 39 | 1239 | 2 |
| nxrBR1237 | | GTA GAT CGG CTC TTC GAC CTG | 1237 - 1257 | | |
| nxrBF169 | All known *Nitrospira*-like *nxrB* | TAC ATG TGG TGG AAC A | 169 - 184 | 485 | 3 |
| nxrBR638 | | CGG TTC TGG TCR ATC A | 638 - 653 | | |

[1] For detailed specificity please refer to SOMS Fig. S5

[2] R = A + G

[3] Positions relative to *nxrB* (NIDE3256) gene of *N. defluvii*

[4] See *Experimental procedures* for detailed description of PCR protocols

The first primer pair successfully amplified with the evaluated PCR conditions *nxrB*-like sequences (1245 bp in length) of *N. defluvii*, *N. moscoviensis* and two environmental samples (activated sludge, beech forest soil) but no amplification product was retrieved from other *Nitrospira* species (Fig. S5). For a broader coverage we designed a second primer pair (nxrBF19/nxrB1237, Table1) based on this extended dataset. Indeed, additional *nxrB*-like sequences (1239 bp in length) of *N. marina*, "*C.* N. bockiana" and from a nitrifying hot spring enrichment (Beryl spring) could be obtained (Fig. S5). Finally, the primer combination nxrBF169/nxrB638 (Table 1), designed on internal conserved regions of thitherto retrieved *Nitrospira*-like *nxrB* sequences, gave *nxrB* amplificates (485 bp in length) with all the before tested *Nitrospira*-like organisms and environmental samples. Furthermore, this latter primer pair amplified in contrast to all other primer combinations *nxrB*-like sequences of a marine sponge sample (Fig. S5). Due to its broad coverage we suggest to use the primer pair nxrBF169/nxrB638 (Table1) in environmental surveys to get a first insight into the diversity and abundance of *nxrB* genes in complex microbial communities. For in-depth phylogenetic analysis, where a higher informative content is needed additional *nxrB* amplification with the two other primer pairs (nxrBF14/nxrBR1239, nxrBF19/nxrB1237, Table1) is recommended.

*Phylogenetic analysis of* Nitrospira-*like* nxrB

A prerequisite for a suitable functional and phylogenetic maker gene is a congruent evolutionary history with the 16S rRNA gene of the respective organism. Direct comparison between 16S rRNA gene and marker gene trees indicates whether the marker gene has undergone a lateral gene transfer (LGT) among the studied organism or not. Some studies showed similar evolutionary relationships between 16S rRNA genes and marker genes, such as the alpha subunit of the ammonia monooxygenase (*amoA*) of *proteobacterial* ammonia oxidizers (Purkhold *et al.*, 2000) or the Nxr genes of *Nitrobacter* spp. (Vanparys *et al.*, 2007, Poly *et al.*, 2008). The dissimilatory sulfite reductase genes (*dsrAB*) of sulphate reducers (Klein *et al.*, 2001) or the nitrogenase reductase gene *nifH* of diazotrophs (Zehr *et al.*, 2003) on the contrary have been subjected to rare LGT, therefore aggravating the inference of phylogenetic relationships between these organisms based on their marker genes. Finally, LGT and gene duplication/divergence events in denitrification genes (*nar, nir, nor, nos*) actually resulted in a disagreement between marker gene phylogeny and 16S rRNA-based organism classification (Gregory *et al.*, 2003, Dandie *et al.*, 2007, Heylen *et al.*, 2007, Jones *et al.*, 2008). The two explicit assumptions in all these studies and also in our analysis are that

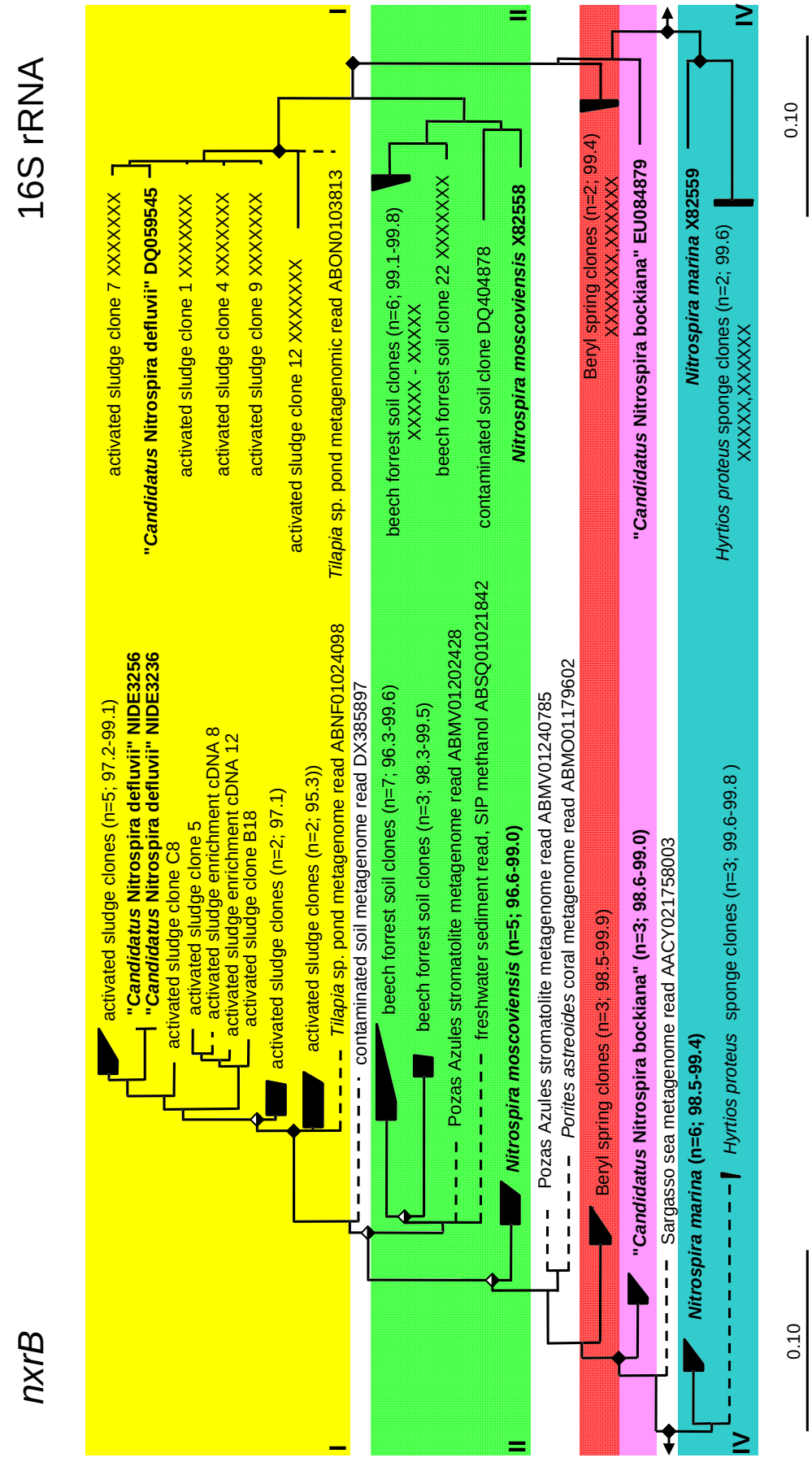

nxrB
16S rRNA
activated sludge clones (n=5; 97.2-99.1)
"Candidatus Nitrospira defluvii" NIDE3256
"Candidatus Nitrospira defluvii" NIDE3236
activated sludge clone C8
activated sludge clone 5
activated sludge enrichment cDNA 8
activated sludge enrichment cDNA 12
activated sludge clone B18
activated sludge clones (n=2; 97.1)
activated sludge clones (n=2; 95.3))
Tilapia sp. pond metagenome read ABNF01024098
contaminated soil metagenome read DX385897
beech forrest soil clones (n=7; 96.3-99.6)
beech forrest soil clones (n=3; 98.3-99.5)
Pozas Azules stromatolite metagenome read ABMV01202428
freshwater sediment read, SIP methanol ABSQ01021842
Nitrospira moscoviensis (n=5; 96.6-99.0)
Pozas Azules stromatolite metagenome read ABMV01240785
Porites astreoides coral metagenome read ABMO01179602
Beryl spring clones (n=3; 98.5-99.9)
"Candidatus Nitrospira bockiana" (n=3; 98.6-99.0)
Sargasso sea metagenome read AACY021758003
Nitrospira marina (n=6; 98.5-99.4)
Hyrtios proteus sponge clones (n=3; 99.6-99.8 )
activated sludge clone 7 XXXXXXX
"Candidatus Nitrospira defluvii" DQ059545
activated sludge clone 1 XXXXXXX
activated sludge clone 4 XXXXXXX
activated sludge clone 9 XXXXXXX
activated sludge clone 12 XXXXXXX
Tilapia sp. pond metagenomic read ABON0103813
beech forrest soil clones (n=6; 99.1-99.8)
XXXXX - XXXXX
beech forrest soil clone 22 XXXXXXX
contaminated soil clone DQ404878
Nitrospira moscoviensis X82558
Beryl spring clones (n=2; 99.4)
XXXXXXX,XXXXXX
"Candidatus Nitrospira bockiana" EU084879
Nitrospira marina X82559
Hyrtios proteus sponge clones (n=2; 99.6)
XXXXX,XXXXXX
I
II
IV
0.10
0.10

**Figure 4**: Comparison of *nxrB* and 16S rRNA gene phylogeny of nitrite-oxidizing *Nitrospira*-like bacteria. Basic tree topology is based on maximum-likelihood (fastDNAml) analysis of the respective full length nucleic acid sequences. A total of 1158 and 1197 informative positions were used in 16S rRNA gene and *nxrB* analysis, respectively. Shorter sequences were added by using the ARB Parsimony tool implemented in the ARB software package (Ludwig *et al*., 2004) without changing the overall tree topologies. Branches leading to shorter sequences are indicated in dotted lines, highlighting their unsupported affiliation. Filled and open diamonds symbolize >90% and 80-90% Parsimony bootstrap support (100 iterations) respectively. The scale bar indicates 0.1 estimated change per nucleotide. Sequences that belong to isolated or enriched *Nitrospira*-like bacteria are in boldface. In case of sequence clusters the number of sequences in the cluster and the maximal and minimal sequence similarity is indicated in brackets. Accession numbers for 16S rRNA and environmental sequences are at the end of the sequence headers. The remaining *nxrB* accession numbers are listed in the tree in Fig. S5. Consistent monophyletic groups in the *nxrB* and 16S rRNA tree are highlighted in the same color and three groups were assigned to the *Nitrospira* sublineages I, II and IV according to Daims *et al.* (2001).

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the 16S rRNA phylogeny reflects the organismal phylogeny and the 16S rRNA gene is not influenced by LGT (Woese, 1987). Comparison of trees based on *Nitrospira*-like 16S rRNA and *nxrB* sequences revealed a highly similar topology (Fig. 4). Within the few available isolated or highly enriched *Nitrospira*-like bacteria no evidence for LGT of *nxrB* could be ascertained. Nevertheless, future analysis of more *Nitrospira* enrichments or isolates is necessary to exclude LGT-events of *nxrB* in the genus *Nitrospira*. Among all analyzed *Nitrospira* only *N. defluvii* has two nearly identical *nxrB* copies. All other *Nitrospira* seem to have more than two *nxrB* copies encoded on their genome with a minimal similarity of 96.6 % in *N. moscoviensis* (Fig. 4). Excluding PCR-artefacts or sequencing errors the analyzed *Nitrospira* enrichments and isolates might consist of various strains with differences between their *nxrB* sequences and varying *nxrB* copy number. This could possibly result in false *nxrB* copy number estimates for one organism (strain). Variation in gene copy number in different strains of one species is a phenomenon already observed by comparative genomics of two *Shigella flexneri* strains (Wei *et al.*, 2003). Consequently, it could not be concluded from sequence clustering or similarity thresholds whether the *nxrB* sequences belong to one or more *Nitrospira* strains. Only other completed *Nitrospira* genomes would eventually make the *nxrB* copy number of other *Nitrospira* than *N. defluvii* assessible. This would facilitate the assignment of *nxrB* sequences to specific 16S rRNA sequences.

Beside the *nxrB* sequence analyses with the few isolated and enriched *Nitrospira*-like bacteria, we additionally screened different environmental samples for *Nitrospira*-like bacteria on the basis of the 16S rRNA gene and the *nxrB* gene, respectively. Of each analyzed sample *Nitrospira*-like 16S rRNA sequences and *nxrB* sequences could be obtained.

Phylogenetic analysis revealed a congruent positioning of these genes derived from the same sample within their phylogenetic trees at least on the sublineage level (Fig. 4).

Despite the similar overall topology some minor inconsistencies can be observed in the bifurcations within *Nitrospira* sublineage I and in branching nodes to sublineage II (Fig. 4). In sublineage I the *nxrB* sequences showed a more pronounced organisation in sub-cluster as expected by observing the 16S rRNA phylogeny. The reasons for this could be the underestimated 16S rRNA diversity of *Nitrospira*-like bacteria in the analysed activated sludge samples or the yet unknown *nxrB* copy number in other *Nitrospira* than *N. defluvii*. In sublineage II the 16S rRNA sequences cluster in a monophyletic group, whereas sublineage II *nxrB* sequences split in all applied treeing methods into two groups (*N. moscoviensis* and beech forest clones). It remains to be seen if detailed analysis with more available sequences influences the correlation of sublineage II topology of 16S rRNA gene and *nxrB* based trees. Beside these differences all other branching nodes in the trees were congruent and supported by high bootstrap values, once more highlighting the suitability of *nxrB* as functional and phylogenetic marker for the genus *Nitrospira* allowing at least an assignment of *nxrB* sequences to different *Nitrospira* sublineages (Fig. 4).

*Metagenomic* nxrB-*like sequences*

Database search of different publicly available metagenomic libraries revealed that a number of reads from the Sargasso sea (Venter *et al.*, 2004), the coral *Porites astreoides* (Wegley *et al.*, 2007), the sediment from Lake Washington (Kalyuzhnaya *et al.*, 2008), the microbial fraction of a freshwater pond and a freshwater stromatolite (Dinsdale *et al.*, 2008), and a metagenomic survey of contaminated sediments (Abulencia *et al.*, 2006) (Fig. 4) showed high sequence similarities to *nxrB* of *Nitrospira*. These *nxrB*-like sequences formed distinct branches within the *nxrB* phylogenetic tree (Fig. 4). Although their short read length hampered their phylogenetic positioning some reads clustered with *Nitrospira* sublineages whereas other reads formed new branches, which lacked their 16S rRNA gene counterpart. Two metagenomes contained in addition to *nxrB* reads *Nitrospira*-like 16S rRNA sequences. Abulencia and coworkers (2006) found a sublineage II *Nitrospira*-like 16S rRNA sequence, while comparing a conventional PCR-based SSU rRNA library with a metagenomic survey of contaminated sediments. However, the *nxrB* sequence found in this metagenomic library forms a destinct branch located between sublineage I and II (Fig. 4). The most parsimonious explanations for the different positions of both sequences in the respective trees could be either the short read length of the *nxrB* read, which hampers a coherent phylogenetic analysis,

or that the sequences originate from different organism. *Nitrospira*-like *nxrB* and 16S rRNA metagenomic reads of a freshwater pond (Dinsdale *et al*., 2008), however, cluster both in sublineage I with exact the same positioning in the respective phylogenetic tree (Fig. 4) Interestingly, this pond stocked with tilapia (cichlids) is used for waste water treatment, which corresponds with the fact that so far sublineage I almost exclusively consists of nitrifying bioreactor clones (Daims *et al*., 2001).

Summing up, *nxrB* is beyond its role as functional and phylogenetic marker in PCR-based studies a valuable informational unit for gene-centric analysis in metagenomic datasets. Gene-centric analysis describes a community from the viewpoint of its component genes (Hugenholtz & Tyson, 2008) indicating certain biochemical reactions or nutrient fluxes in the analysed environment.

**Conclusions**

Genomic and phylogenetic evidence showed that the Nxr of *N. defluvii* differs considerably from the Nxr of *Nitrobacter* spp. and *Nitrococcus mobilis*. Nevertheless, all hitherto known Nxr belong to a large and heterogeneous molybdopterin-binding enzyme superfamily and the mature holoenzyme is composed of at least three subunits (alpha to gamma).

While the alpha and beta subunit of Nxr were each encoded by two nearly identical gene copies, three genes of different gamma subunits could be allocated on the genome of *N. defluvii*. Sequence analysis with the alpha and gamma subunits indicated a periplasmatic localisation of Nxr anchored via a gamma subunit to the cytoplasmic membrane. The strict transcriptional regulation of the two *nxrAB* operons suggested a controlled gene expression dependent on different environmental conditions.

Results obtained in transcriptional and Western blot analyses indicated an Nxr up-regulation during nitrite-oxidizing conditions. Moreover, these studies revealed similar mechanisms in *N. defluvii* to cope with starvation as seen in AOB.

In-depth phylogenetic analysis of *nxrB* sequences encoding the Nxr beta subunit suggested a 16S rRNA-conform phylogeny without evidence of LGT. Hence, nxrB is a suitable functional and phylogentic marker for the genus *Nitrospira* allowing an assignment of *nxrB* at least on the *Nitrospira* sublineage level. This new marker gene extends the functional gene toolbox for monitoring activity and abundance of nitrifiers.

## Experimental procedures

*Isolates and enrichments of* Nitrospira-*like bacteria*

Different isolates and enrichments of *Nitrospira*-like bacteria were used in this study to evaluate the developed *nxrB* primer pairs. The enrichment of "*Candidatus* Nitrospira defluvii" was maintained as described by Spieck and colleagues (Spieck *et al*., 2006), the isolate *Nitrospira moscoviensis* was grown under the conditions used by Ehrich and colleagues (Ehrich *et al*., 1995). Biomass from *Nitrospira marina* (Watson *et al*., 1986), "*Candidatus* Nitrospira bockiana" (Lebedeva *et al*., 2008) and of a nitrite-oxidizing enrichment from Beryl Spring (44°,40`N 110°, 44`W; Gibbon Geyser Basin, Yellowstone National Park, USA) was kindly provided from our collaboration partner Eva Spieck in Hamburg. All isolates and enrichments were checked by flourescence *in situ* hybridization (FISH) for purity as described elsewhere (Maixner *et al*., 2008)

*Environmental samples: site description and nucleic acid isolation*

PCR-based screening for *nxrB* genes was performed in different environmental samples:
Nitrifying activated sludge was sampled on the main full-scale wastewater treatment plant (WWTP) in Vienna, Austria, receiving municipal and industrial wastewater (aerated nitrifying circulation tank, BB2_M_35). For DNA isolation samples were pelleted by centrifugation (10000g; 5min) and stored at -20°C. DNA was isolated according to Griffiths and colleagues (Griffiths *et al*., 2000). A soil sample was collected from a beech forest site next to Klausen-Leopoldsdorf, Austria. For detailed site description please refer to Kitzler and colleagues (Kitzler B., 2006). DNA was isolated with the MOBIO Power Soil™ DNA Kit (MOBIO Lab. Inc., Salana Beach, CA, USA) according to manufactures instructions. *Hyrtios proteus* sponge material was collected by scuba diving at 3m depth close to the mangrove island Twin Cays (16°48`N, 88°05`W, Belize Barrier Reef, Caribbian Sea) in January 2008. The DNA extraction protocol described by Webster and colleagues (Webster *et al*., 2008) was applied.

*(Meta)genomic data*

Nitrite oxidoreductase gene (*nxrB*) sequences of *Nitrobacter winogradskyi* (Starkenburg *et al*., 2006) and *Nitrococcus mobilis* Nb-255 (unpublished, NCBI genome project 13475), were used as query sequences to blast against the metagenomic sequence dataset of *N. defluvii*. For details to the environmental genome approach applied on an enrichment of *N. defluvii* please refer to Maixner and colleagues (Maixner *et al*., 2008). A low coverage shotgun read dataset

(unpublished data) of the genome of *N. moscoviensis* turned out to be an additional valuable resource for small *nxrB* fragments facilitating the *nxrB* primer design.

*Amplification, cloning and phylogenetic anaylsis of* 16S rRNA *and* nxrB *genes*

PCR-amplification of partial 16S rRNA genes of *Nitrospira*-like bacteria was carried out according to Maixner *et al.* (2006). Three different PCR protocols evaluated for each primer pair (see Table 1, Fig. S5) were used to amplify partial *nxrB* fragments of *Nitrospira*-like bacteria from 5-100 ng of DNA isolated from environmental samples. In case of isolated or enriched *Nitrospira* 2 µl of suspended and pre-cooked (95°C, 5min) biomass was directly added to the PCR reaction mix. For all *nxrB* primer combinations the reaction mixture with each primer at 1 pM was prepared according the manufacturer's recommendations in a total volume of 50 µl by using 8 mM $MgCl_2$ reaction buffer, 10 nmol of each deoxynucleoside triphosphate and 1.25 U of *Taq* polymerase (Fermentas, St. Leon-Rot, Germany). The programs for protocol 1 and 2 differ only in the annealing temperature and were carried out by an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at a temperature gradient ranging from 68 to 60°C (nxrBF14/nxrBR1239, protocol 1) or 59°C and 51.5 to 48°C (nxrBF19/nxrBR1237, protocol 2) for 40 s and elongation at 72°C for 90 s. Cycling was completed by a final elongation step at 72°C for 10 min. The third protocol was adapted to the shorter amplification product size and was carried out by an initial denaturation step at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at 56.2°C (nxrBF169/nxrBR638, protocol 3) for 30 s and elongation at 72°C for 60 s. Cycling was completed by a final elongation step at 72°C for 10 min. All PCR protocols were run on an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Presence and size of the amplification products were determined by agarose (1.5 %) gel electrophoreses of the reaction product. For cloning the PCR products were loaded on a low-melting preparative gel (1.5 %, Biozym Sieve GeneticPure Agarose, Biozym, Hess. Oldendorf, Germany) and amplification products with the expected size were cut out with a micro haematocrit tube (BRAND, Wertheim, Germany). Thereby retrieved gel pieces were melted at 70°C , diluted with 100 µl double-distilled water and subsequently used for cloning. Cloning, cloned sequence diversity screening with RFLP and sequencing were done as described elsewhere (Hatzenpichler *et al.*, 2008). The resulting sequence data were further phylogenetically analyzed by using the ARB software package (Ludwig *et al.*, 2004) with a comprehensive 16SrRNA and *nxrB* dataset, respectively. 16S rRNA gene sequence alignment and phylogenetic analysis was carried out according to Daims *et al*. (Daims *et al*., 2001).

Nucleic acid and amino acid sequences of NxrB-like proteins were collected from public databases using the BLAST (Altschul *et al.*, 1990) search tools NCBI blastp with the non-redundant protein sequence database and tblastn with the nucleotide collection, patent sequences and environmental samples databases. In addition to the NCBI sequence database the metagenome sequence collection databases IMG/M, CAMERA and MG-Rast (Markowitz *et al.*, 2006, Seshadri *et al.*, 2007, Meyer *et al.*, 2008) were screened for nxrB-like sequences. A core dataset of full-length sequences (> 975 bp with start and stop-codon) containing the NarY conserved domain in CDD (Marchler-Bauer *et al.*, 2007) was initially used for sequence alignment. DNA sequences were translated into amino acids by using the Perform Translation tool in the ARB software (Ludwig *et al.*, 2004). The NxrB sequence alignment was automatically inferred with the ClustalW protein alignment program implemented in the ARB software package and afterwards manually refined by using the ARB sequence editor. The four conserved FeS-cluster-binding sites characteristic for the beta subunits of type II Mo-*bis*MGD enzymes were used as starting points for the manual refinement of the sequence alignment. Finally, partial environmental sequences highly similar to *nxrB* sequences of *Nitrospira*-like organism were aligned against the core alignment of full-length sequences. All phylogenetic analyses were performed with nucleic acid sequences. Therefore the nucleic acid sequences were realigned according to the corresponding amino acid sequences with the respective tool in the ARB software. Phylogenetic analysis were performed by applying distance-matrix, maximum-parsimony and maximum-likelihood methods implemented in the ARB software package: neighbour-joining (using the Felsenstein algorithm for nucleic acid correction), DNA parsimony (PHYLIP version 3.66 with 100 bootstrap iterations) and DNA maximum-likelihood [fastDNAml, PhyML (Guindon & Gascuel, 2003) with the HKY substitution model and TREE-PUZZLE (Strimmer & von Haeseler, 1996) with 1000 puzzling steps and the HKY substitution model]. In total 1197 alignment columns were used for phylogenetic analysis. To determine the basic tree topology all full length sequences were processed by the maximum-likelihood method fastDNAml and all shorter sequences were added subsequently by use of the ARB_PARSIMONY tool implemented in the ARB program (Ludwig *et al.*, 2004) without changing the overall tree topology.

*RT-PCR of* nxrB *mRNA of* Nitrospira-*like bacteria*

Biomass from a nitrifying activated sludge and cells from the *N. defluvii* enrichment (both starved and induced) were harvested by centrifugation and stored at -80°C until further processing. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, USA) according to

the protocol recommended by the manufacturer and with the same modifications described in Hatzenpichler *et al.* (2008). After DNA digestion with DNase (Fermentas, St. Leon-Rot, Germany), reverse transcription of 3µg total RNA of each treatment was carried out by using the RevertAID first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer`s instructions. The reverse primer Ntspa1158R (Maixner *et al*., 2006), specific for the 16S rRNA gene of the genus Nitrospira and nxrBR1237 (see Table 1) targeting the *nxrB* gene were used for cDNA synthesis. For cDNA amplification the reaction mixture with the primer combinations 616F/Ntspa1158R (Maixner *et al*., 2006) for the 16S rRNA gene and nxrBF916 (5`-GAG CAG GTG GCG CTC CCG C-3`) /nxrB1237R (see Table 1) for the *nxrB* gene, respectively was prepared according the manufacturer's recommendations in a total volume of 50 µl by using 8 mM MgCl2 reaction buffer and 1.25 U of *Taq* polymerase (Fermentas, St. Leon-Rot, Germany). Thermal cycling for both primer combinations was carried out by an initial denaturation step at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 40 s and elongation at 72°C for 60 s. Cycling was completed by an final elongation step at 72°C for 10 min.

*Cloning and heterologous expression of NxrB*

The *nxrB* gene of *N. defluvii* was amplified by using the High Fidelity PCR enzyme mix (Fermentas, St. Leon-Roth, Germany) according to the protocol recommended by the manufacturer and cloned into the expression vector pET21b(+), which contains a promoter for T7 RNA polymerase and a C-terminal His-tag (Novagen, Heidelberg, Germany). Instead of extracted genomic DNA, 2 µl of precooked *N. defluvii* enrichment were added directly to the PCR reaction mix. The forward primer NXRV2 (5`- CGA GCG CAT ATG CCA GAA GTC TAT AAC TGG -3`) containing an NdeI restriction site upstream of the start codon and the reverse primer NXRR (5`- TTA CGA GAA TTC CCC AGC CAG TTC ACG CGC TC -3`) with an EcoRI restriction site were used in the reaction. Thermal cycling was carried out by an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56.4°C for 40 s and elongation at 68°C for 90 s. Cycling was completed by an final elongation step at 68°C for 10 min. The resulting amplification product was cloned into the cloning vector pCR-XL-TOPO by using the TOPO XL cloning kit (Invitrogen, Carlsbad, USA). The cloned amplicon and the expression vector pET 21b(+) were digested with the restriction endonucleases NdeI and EcoRI and were then ligated with T4 DNA Ligase (Invitrogen, Carlsbad, USA) according to the manufacturer`s protocol. The expression vector with the *nxrB* gene was transformed by electroporation into E. coli XL1 blue cells

(Stratagene, Heidelberg, Germany). Sanger sequencing confirmed that the cloned insert used for heterologous expression was identical to the sequence of the genome. For heterologous expression of NxrB, the expression vector pET21b(+) with the *nxrB* gene was transformed into E. coli BL21 (DE3) cells (Stratagene, Heidelberg, Germany). The recombinant cells were grown at 37°C under agitation (225 r.p.m.) in liquid Luria-Bertani media. After growth up to an optical density (600 nm) of 0.8, the expression of NxrB was induced by adding isopropyl-β-D-thiogalactopyranosidase (IPTG) to a final concentration of 1mM. Cells were harvested approximately 4h later by centrifugation (5000 g, 10 min) and the cell pellets were stored at -20°C.

*Immunological detection of NxrB of* N. defluvii

Western blot analysis was applied on crude cell extracts of a defined number of *N. defluvii* cells with the NxrB-specific monoclonal antibodies Hyb 153-3 (Aamand *et al*., 1996) to monitor the per cell amount of NxrB in *N. defluvii* after nitrite limitation and induction with nitrite, respectively. Biomass of *N. defluvii* enrichment was collected in the aforementioned growth stages (limited, induced). In order to disintegrate the tight cell assembly of *N. defluvii* for quantification of absolute cell numbers, the biomass was bead beaten with the Fastprep Bead-beater (BIO 101, Vista; using only the big sphere) at level 4 for 5 seconds. The disjoint biomass was harvested by centrifugation (10000 g, 20 min) and the pellet was re-suspended in 1x phosphate-buffered saline (1xPBS). The cell suspension was used one half each for immunological detection (stored at 4°C) and to determine the cell concentration of *N. defluvii* in the suspension by direct visual cell counting. For this purpose the latter cell fraction was fixed with ice cold 4% paraformaldehyde (3 vol. PFA solution, 1vol. sample) for 1h at RT and filtered onto a polycarbonate filter (pore size 0.2 µm; diameter 47 mm; type GTTP; Millipore, Bedford, USA). Subsequently, the filters were washed two times with 1xPBS and double-distilled water; air dried and stored at -20°C prior to fluorescence in situ hybridization (FISH). FISH of fixed cells on membrane filters was performed according to the protocol of Glöckner and colleagues (Glöckner *et al*., 1996). Altogether three Cy3-labelled probes [Ntspa1431 (Maixner *et al*., 2006), Ntspa662 and Ntspa712 (Daims *et al*., 2001)] targeting all *N. defluvii* in the enrichment were used simultaneously in one FISH experiment to increase the signal to noise ratio. In each experiment 28 pictures were recorded with a CLSM (LSM 510 Meta, Zeiss, Oberkochen, Germany) and the fluorescent cells in each image were counted (approximately 100 cells per image). To account in the calculation of the initial cell concentration for the cell-loss during FISH the median biovolume area before and after FISH

needed to be determined. Therefore, filter pieces before and after FISH were embedded in a 1:500 dilution of Sybr Green II (Cambrex, Rockland, USA) in Citiflour (Citifluor Ltd., Leicester, United Kingdom). 17 pictures of the stained biomass were recorded for each experiment and the median biovolume area was obtained by using the digital image analysis program *daime* (Daims *et al.*, 2006). The ratio of the median biovolume area before and after FISH resulted in the so called FISH-loss factor. Finally, the cell concentration in the original suspension was calculated from the average number of cells per image, the known area of each image (in $\mu m^2$), the known area of the filter, the FISH-loss factor, the filtered volume of the diluted suspension, and the dilution factor. In regard to the determined cell concentration a defined volume of the original suspension was taken to end up with equal amounts of starved and induced *N. defluvii* cells in the biomass. The biomass of *N. defluvii* was harvested by centrifugation (10000g; 20 min), the biomass pellet was re-suspended in 5 x Lysis buffer [7 M urea, 2 M thiourea, 20 mg/ml amberlite, 4% (w/v) CHAPS, 40 mM TRIS, 2% (v/v) IPG-buffer, 0.2% (w/v) bromphenol blue, 1% (w/v) DDT, 10% (w/v) glycerol], heated for 3 min at 90°C and loaded onto a SDS-PAGE gel (12.5% PAA) with molecular weight marker (PageRuler Prestained Protein Ladder #SM0671, Fermentas, St. Leon-Rot, Germany). All subsequent steps for the immunological detection of NxrB were performed as described by Maixner and colleagues (Maixner *et al.*, 2008) with the following modifications. The polyvinylidene fluoride (PVDF) membrane was incubated for 30 min with the monoclonal antibody Hyb 153.3, (Aamand *et al.*, 1996) which was diluted 1:1000 in TBS buffer (20 mM TRIS, 150 mM NaCl, pH 7,5) plus 0.1% (v/v) Tween 20. The secondary antibody (peroxidase-conjugated goat antimouse IgG; dianova, Hamburg, Germany) was diluted 1:5000 in TBS-Tween buffer. In the Western blot experiments crude cell extracts of recombinant *E.coli* BL21 (DE3) cells after heterologuos expression of NxrB were used as positive control. There was no unspecific binding of Hyb 153-3 against crude cell extract of non-recombinant *E. coli* BL21 (DE3) observed (data not shown).

*Phylogenetic analysis of the large nitrite oxidoreductase subunit (NxrA) of* N. defuvii *in a molybdopterin-binding (MopB) protein-superfamily tree*

By using the query tool in the Conserved Domain database (Marchler-Bauer *et al.*, 2007), the NxrA sequences of *N.defluvii* could be assigned to the Molybdopterin-Binding (MopB) superfamily of proteins, due to their similar domain architecture. Sequences of all subfamilies of the large MopB enzyme superfamily (cl09928) were collected by using the protein domain hierarchy viewer and editor CDTree (CDTree 3.1, (Marchler-Bauer *et al.*, 2007). Dependent

on the sequence number in one subfamily at least 2 sequences (see table S4) per subfamily were selected for phylogenetic analysis to cover the subfamily sequence diversity. Additional NxrA-like sequences closely related to the NxrA sequences of *N. defluvii*, *Nitrobacter hamburgiensis* X14 and *Nitrococcus mobilis* Nb-255 were retrieved from public databases using the BLAST (Altschul *et al*., 1990) search tool NCBI blastp with the non-redundant protein sequence database. The alignment for in total 432 sequences was automatically inferred with the ClustalW protein alignment program implemented in the ARB software package and afterwards manually refined using the ARB sequence editor. Phylogenetic analyses were performed by applying distance-matrix, maximum-parsimony and maximum-likelihood methods implemented in the ARB software package: neighbour-joining (using the Dayhoff PAM 001 matrix as amino acid substitution model), protein parsimony (PHYLIP version 3.66 with 100 bootstrap iterations) and DNA maximum-likelihood [PhyML (Guindon & Gascuel, 2003) with the JTT substitution model]. In total 680 alignment columns were used for phylogenetic analysis.

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**A**

```
Archeoglobus fulgidus          (NP_069015)  37 GEIKTVR----SVCSPNCTGA--CGFDALVYNGRIETLTQAADYPEPEYN----------PRGCLRGQSMM  91
C. hydrogenoformans            (YP_360901)  41 REVKFVR----TTCSPNCTSA--CGIKAMVVDGQIKALFPTNDYPDPEYN----------PRGCLRGISFI  96
Moorella thermoacetica         (YP_430751)  68 KEVKYVR----TTCSPNCTLA--CGIRAMVVDGQIKALLPSNDYPEPEYG----------PRGCLRGLSFI 122
"C. Nitrospira defluvii" NxrA2 (NIDE3256)  59 RYDSSFT----WVCSPNDTHA--CRIRAFVRNGVVMRVEQNYDHQTYEDLYGNRGTFAHNPRMCLKGFTFH 123
"C. Nitrospira defluvii" NxrA1 (NIDE3237)  60 RYDSSFT----WCCSPNDTHG--CRVRAFVRNGVVMRVEQNYDHQTYEDLYGNRGTFAHNPRMCLKGFTFH 124
"C. Kuenenia stuttgartiensis   (CAJ72445)  58 QYDRTFT----YCCSPNDTHA--CRIRAFVRNNVMMRVEQNYDHQNYSDLYGNKATRNWNPRMCLKGYTFH 122
Hydrogenobaculum sp.           (YP_002121006) 55 KPDETTV----ILCNPNDTHG--CYLNAYVKNGIITRLEPTYKYGDATDIYGIKASHRWEPRCCNKGLALV 119
Beggiatoa sp.                  (ZP_02000390)  48 HYDSSFT----FLCAPNDTHN--CLLRGYVKNGVVTRIAPTYGYQKAKDLDGNQSTQRWDPRCCQKGLALV 112
Aromatoleum aromaticum EdbH    (YP_158333)  73 KWDKVN---WGS--HLNICWPQGSCKFYVYVRNGIVWREEQAAQTPACNVD-----YVDYNPLGCQKGSAFN 134
Desulfococcus oleovorans       (YP_001528081) 60 TWDKVT---YGT--HLVDCYPGN-CLWRVYSKDGVVFREEQAAKYPVIDPS-----GPDFNPRGCQKGASYS 120
Sulfurihydrogenibium sp.       (YP_001931341) 57 AYTYGKKEEHGTAYHCVNCQGN--CAWDVWVQNGIVTRENQAANYPQINPK-----IPDANPRGCNKGVQHS 121
Rhodovulum sulfidophilum DdhA  (Q8GPG4)     57 TWDY-----VGKAAHCINCLGN--CAFDIYVKDGIVIREEQLAKYPQISPD-----IPDANPRGCQKGAIHS 116
Ideonella dechloratans ClrA    (P60068)     60 TWDS-----VGVMTHSNGCVAG--CAWNVFVKNGIPMREEQISKYPQL-PG-----IPDMNPRGCQKGAVYC 118
Thauera selenatis SerA         (Q9S1H0)     64 TWDS-----TGFTIHSNGCVAG--CAWRVFVKNGVPMREEQVSEYPQL-PG-----VPDMNPRGCQKGAVYC 122
Dechloromonas agitata PcrA     (AAO49008)   51 SWDKKTR-----GAHLINCTGA--CPHFVYTKDGVVIREEQSKDIPPMPN-----IPELNPRGCNKGECAH 109
Haloarcula marismortui         (YP_135852)  70 DWDSVAR-----STHSVNCTGS--CSWNVYVKDGQVWREEQAGDYPTFDES-----LPDPNPRGCQKGACYT 129
Nitrococcus mobilis            (ZP_01125872) 24 QHDNIFR-----STHGVNCTGG--CSWAIYVKDGIITWEMQQTDYPLLGRGEGGRGIPPYEPRGCQRGISAS  88
Nitrobacter hamburgiensis      (YP_578638)  24 SHDNVFR-----STHGVNCTGG--CSWAIYVKDGIITWEMQQTDYPLLER-----SLPPYEPRGCQRGISAS  83
Escherichia coli NarG          (1Q16_A)     41 QHDKIVR-----STHGVNCTGS--CSWKIYVKNGLVTWETQQTDYPRTRP-----DLPNHEPRGCPRGASYS 100
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H/CxxxD/Cx(n)Cx(n)C

**B**

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                                                                                    L.aa   Id.%
Archeoglobus fulgidus          (NP_069015) 155 VHKGAMMRLAS----------------------MFGWSALHGYTMNGDLPAFWSQTFGVQTEEFE 197  896   22
C. hydrogenoformans            (YP_360901) 161 VHKGAMVALAT----------------------LAGWTLHHAYDLNGDLPMFWPQTFGVQTEELE 203  853   21
Moorella thermoacetica         (YP_430751) 186 VQKGAWIALAT----------------------MAGWSLIHPYDQNGDLPMFWPQTFGVQTEELE 228  876   21
"C. Nitrospira defluvii" NxrA2 (NIDE3256) 235 MGKHANTRFNNCVLPLLDSWIRKVNPDQAQGGRYWNNYTWHGDQDPSQPWWNGTQNCDVD 294 1147  100
"C. Nitrospira defluvii" NxrA1 (NIDE3237) 236 VGKMMNTRFNGGCLPLLDSWIRKVDAEKAQGGKYYSNYTWHGDQDPSHPFWNGTQNCDVD 295 1146   87
"C. Kuenenia stuttgartiensis   (CAJ72445) 234 IGKYGMYRFNN-CLAIVDAHNRGVGPDQALGGRNWSNYTWHGDQAPGHPFSHGLQTSDVD 292 1148   58
Hydrogenobaculum sp.           (YP_002121006) 230 LRYVGQYRMSN-MMALLDSNIRKVEPDKALGGVGWDNYSEHTDLPPAHTLVTGQQTVDFD 289 1154   37
Beggiatoa sp.                  (ZP_02000390) 223 TRVFAQYREAN-AMALLDDKIRGKGTD-SLGGRGFDNYSWHTDLPPGHPMVTGQQTVDFD 281 1138   39
Aromatoleum aromaticum EdbH    (YP_158333) 198 IAWGAGFRMTY----------------------LMDGVSPDINVDIGDTYMGAFHTFGKMHMGYS 240  976   19
Desulfococcus oleovorans       (YP_001528081) 184 VHVMAVHRLMV----------------------SLGATVLDLDSTIGDFNRGIYETFGKFMFMDS 226  993   17
Sulfurihydrogenibium sp.       (YP_001931341) 185 ARAASGKRLGT----------------------LLGAVRPYIASYVGDMFPGVSLVYGEGNIGFS 227  962   19
Rhodovulum sulfidophilum DdhA  (Q8GPG4) 180 MRMAAPYRFAS----------------------LVGGVQLDIFTDVGDLNTGAHLAYGNALESFT 222  910   17
Ideonella dechloratans ClrA    (P60068) 180 ITNTAYTRMTK----------------------LLGAISPDATSMTGDLYTGIQTVRVPASTVST 222  914   19
Thauera selenatis SerA         (Q9S1H0) 184 ITSAGYSRLAN----------------------LIGAIKPDVSSMTGDLYPGIQTVRMPARTVST 226  918   20
Dechloromonas agitata PcrA     (AAO49008) 173 VSFSAGHRFAH----------------------YIGAHTHTFFDWYSDHPTGQTQTCGVQGDSAE 215  927   21
Haloarcula marismortui         (YP_135852) 193 VSFASGSRLVN----------------------LLGGVSHSFYDWYSDLPPGQPITWGTQTDNAE 235  952   21
Nitrococcus mobilis            (ZP_01125872) 186 LSYAAGSRFLQ----------------------LFGGVNMSFYDWYADLPNSFPEIWGDQTDVCE 228 1218   17
Nitrobacter hamburgiensis      (YP_578638) 181 LSFAAGTRFLS----------------------LMGGSLMSFYDWYADLPTSFPEIWGDQTDVCE 223 1214   16
Escherichia coli NarG          (1Q16_A)    198 VSYASGARYLS----------------------LIGGTCLSFYDWYCDLPPASPQTWGEQTDVPE 240 1247   16
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**Figure S1**: Alignments of metal-coordinating regions of alpha subunits of molybdopterin binding (MopB) proteins which belong to the type II group of Mo-*bis*MGD (molybdopterin-guanine-dinucleotide) enzymes (Jormarkka *et al*., 2004). Accession numbers of the sequences are indicated in brackets. Positions conserved in all analyzed sequences have red characters. (A) Iron-sulfur [FeS]-binding center. [FeS]-binding residues are highlighted. A new signature motif for type II Mo-*bis*MGD enzymes is displayed in the box below the alignment. (B) Molybdenum (Mo) ligand binding site. The conserved aspartate residue, highlighted in blue, coordinates the Mo ion in the active center. The last two columns show the overall length of the sequences in amino acids (L. aa) and amino acid sequence identities (Id. %) to the NxrA2 of *N. defluvii.*

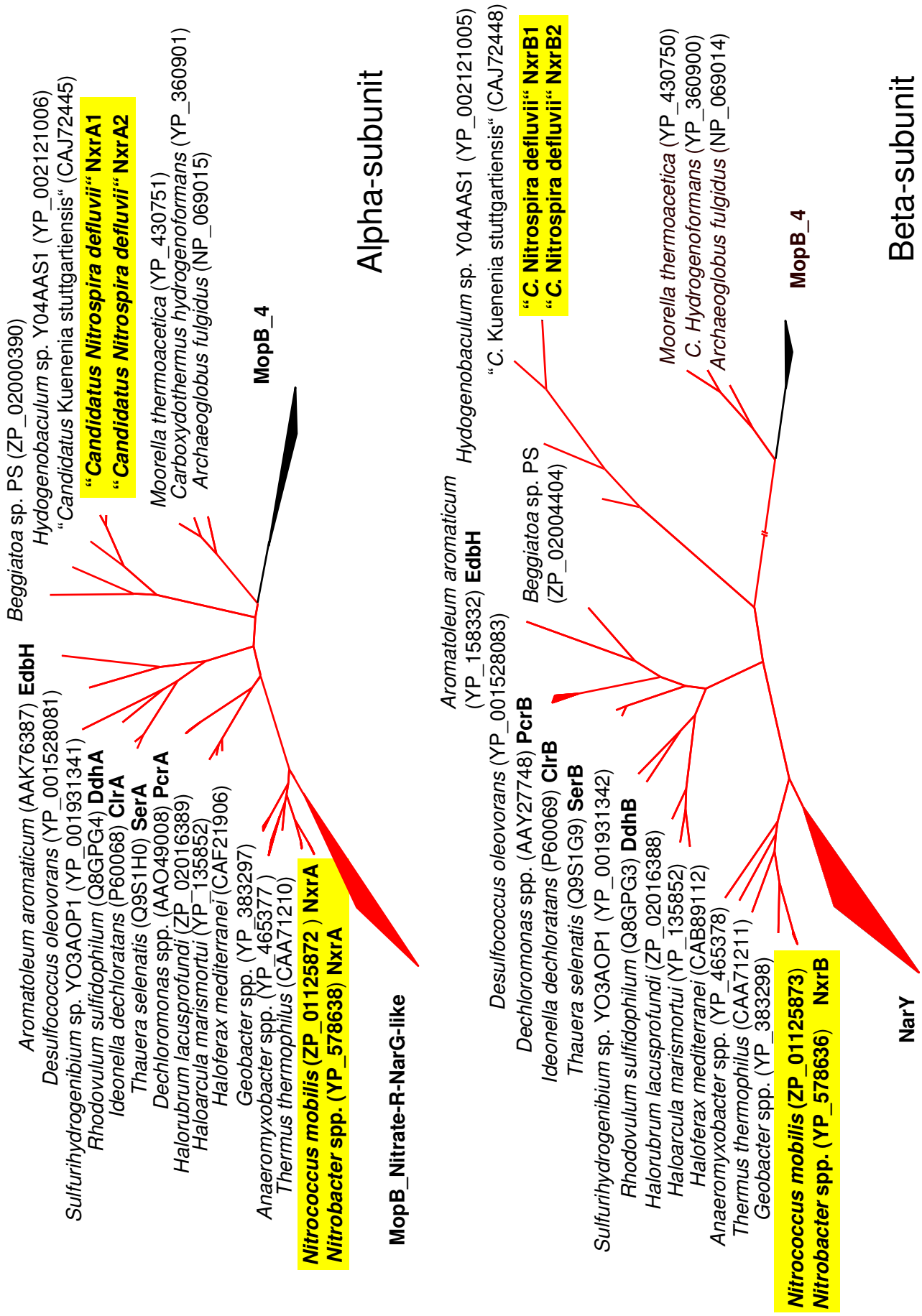
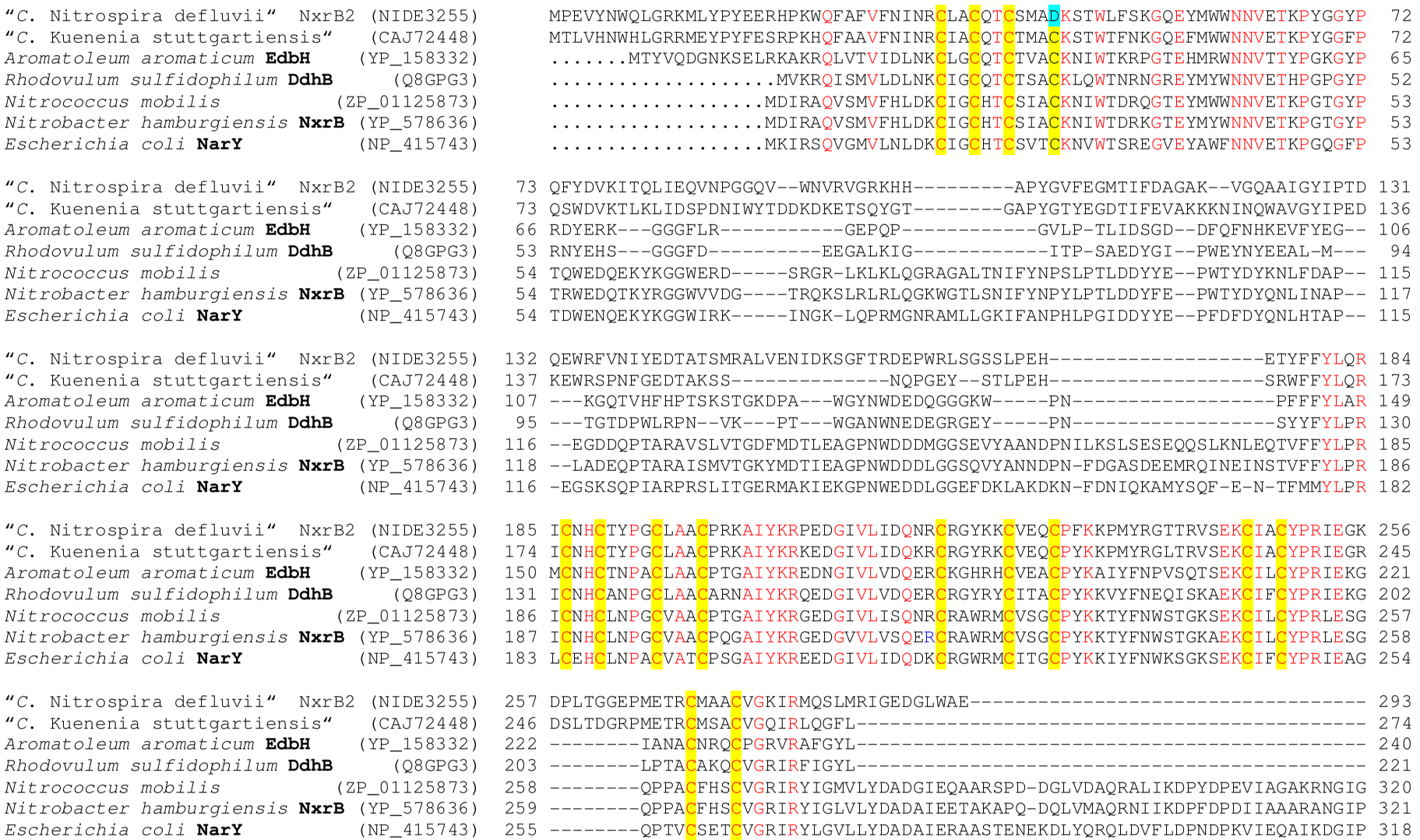

Alpha-subunit
Aromatoleum aromaticum (AAK76387) EdbH
Desulfococcus oleovorans (YP_001528081)
Sulfurihydrogenibium sp. YO3AOP1 (YP_001931341)
Rhodovulum sulfidophilum (Q8GPG4) DdhA
Ideonella dechloratans (P60068) ClrA
Thauera selenatis (Q9S1H0) SerA
Dechloromonas spp. (AAO49008) PcrA
Haloarcula marismortui (YP_135852)
Haloferax mediterranei (CAF21906)
Geobacter spp. (YP_383297)
Anaeromyxobacter spp. (YP_465377 )
Thermus thermophilus (CAA71210)
Nitrococcus mobilis (ZP_01125872 ) NxrA
Nitrobacter spp. (YP_578638) NxrA
MopB_Nitrate-R-NarG-like
Beggiatoa sp. PS (ZP_02000390)
Hydogenobaculum sp. Y04AAS1 (YP_002121006)
"Candidatus Kuenenia stuttgartiensis" (CAJ72445)
"Candidatus Nitrospira defluvii" NxrA1
"Candidatus Nitrospira defluvii" NxrA2
Moorella thermoacetica (YP_430751)
Carboxydothermus hydrogenoformans (YP_360901)
Archaeoglobus fulgidus (NP_069015)
MopB_4
Beta-subunit
Aromatoleum aromaticum (YP_158332) EdbH
Desulfococcus oleovorans (YP_001528083)
Dechloromonas spp. (AAY27748) PcrB
Ideonella dechloratans (P60069) ClrB
Thauera selenatis (Q9S1G9) SerB
Sulfurihydrogenibium sp. YO3AOP1 (YP_001931342)
Rhodovulum sulfidophilum (Q8GPG3) DdhB
Halorubrum lacusprofundi (ZP_02016388)
Haloarcula marismortui (YP_135852)
Haloferax mediterranei (CAB89112)
Anaeromyxobacter spp. (YP_465378)
Thermus thermophilus (CAA71211)
Geobacter spp. (YP_383298)
Nitrococcus mobilis (ZP_01125873)
Nitrobacter spp. (YP_578636) NxrB
NarY
Hydogenobaculum sp. Y04AAS1 (YP_002121005)
"C. Kuenenia stuttgartiensis" (CAJ72448)
Beggiatoa sp. PS (ZP_02004404)
"C. Nitrospira defluvii" NxrB1
"C. Nitrospira defluvii" NxrB2
Moorella thermoacetica (YP_430750)
C. Hydrogenoformans (YP_360900)
Archaeoglobus fulgidus (NP_069014)
MopB_4

**Figure S2**: Comparison of amino acid tree topologies of the alpha- and beta-subunit of type II group of selected Mo-*bis*MGD (molybdopterin-guanine-dinucleotide) enzymes. Sequences of the alpha-subunit displayed with red tree branches share the common feature of an aspartate as molybdenum ligand. Corresponding beta-subunit sequences are also highlighted with red tree branches. Yellow boxes highlight nitrite oxidoreductases (NxrA). The MopB_4 enzyme subfamily acts as outgroup. The tree calculations were performed using the maximum-likelyhood algorithm (PhyML) implemented in the ARB software package (Ludwig *et al.* 2004). A total of 480 and 429 informative positions were used in the alpha- and beta-subunit analysis, respectively. The accession numbers of the sequences used for tree calculation are indicated in brackets. For the abbreviations please refer to Fig. 1. The beta subunit abbreviations only differ in the last character from the listed alpha subunits.

**A**


"C. Nitrospira defluvii" NxrB2 (NIDE3255)
"C. Kuenenia stuttgartiensis" (CAJ72448)
Aromatoleum aromaticum EdbH (YP_158332)
Rhodovulum sulfidophilum DdhB (Q8GPG3)
Nitrococcus mobilis (ZP_01125873)
Nitrobacter hamburgiensis NxrB (YP_578636)
Escherichia coli NarY (NP_415743)

```
"C. Nitrospira defluvii"  NxrB2 (NIDE3255)  294 ------DRWHPLYYTIRVEQVALPLYPQWGTEPNGYYIPPRHAPRGYNRQMFGPG----------------- 342
"C. Kuenenia stuttgartiensis"   (CAJ72448)  275 ----DDNPKNPITWLIRHQKIALPLYPQFGTEPNIYYIPPRWAPRAYLRQMFGPG----------------- 323
Aromatoleum aromaticum EdbH     (YP_158332)  241 -----DDTTSHVHKLVKKWKVALPLHAEYGTGPNIYYVPP--MGARGFGEDGEIT----------------- 288
Rhodovulum sulfidophilum DdhB      (Q8GPG3)  222 -----DDEAGPVHLLVERYKVAIPLHPEWGTKPSVFYVPP--LAPPRIGDDGEPT----------------- 269
Nitrococcus mobilis          (ZP_01125873)  321 DAVVTAAQNSPVYKFVKRWGLALPLHPEFRTMPMLFYVPPLLPVIAAIRDGQYETTAGMETAPADETGPNLN 392
Nitrobacter hamburgiensis NxrB (YP_578636)  322 DSKIEAAQKSPVYQFVKKWGIALPLHPEFRTLPMLFYVPPLGPVLAKVENGVYDN-----IASESRLGPLMS 388
Escherichia coli NarY           (NP_415743)  319 LSVIEAAQQSPVYKMAMEWKLALPLHPEYRTLPMVWYVPPLSPIQSAADAGEL--------------GSNGI 376

"C. Nitrospira defluvii"  NxrB2 (NIDE3255)  343 --VDNAIEKYLVP-------SRELLAVLQLWRASQQIVFRYDVIPGPKV--------------FETQIHGKRF 392
"C. Kuenenia stuttgartiensis"   (CAJ72448)  324 --VDEAIEKFMVP-------SRELLAVMSLFRMTQTIVYEYKIEEGPKV--------------FETEIHGKKF 373
Aromatoleum aromaticum EdbH     (YP_158332)  289 --DKTRIPLDVLEGLFG----PEVKRVLAVLHTERENMRA----------------------GRGSELMDL 331
Rhodovulum sulfidophilum DdhB      (Q8GPG3)  270 --EETRVPLAYLKELFG----EAVVPALETLKTERAKKQS----------------------GAESELMDT 312
Nitrococcus mobilis          (ZP_01125873)  393 TVDKARIPMQYMASLFTAGNVDKIKHVYHKLVAVRMYMRSEQVGDQKEEDV-QEALRRADLTPEQCISIFEL 463
Nitrobacter hamburgiensis NxrB (YP_578636)  389 SLERSRIPLRYMASLLSGGNEDIIRDVYKKLVAVRVYMRSKKVKDIPEEEV-QRALAEGKTTAAEVEAIWRL 459
Escherichia coli NarY           (NP_415743)  377 -LPDVESLRIPVQYLANLLTAGDTKPVLRAL-KRMLAMRHYKRAETVDGKVDTRALEEVGLTEAQAQEMYRY 446
                                                                                                                      Id.%
"C. Nitrospira defluvii"  NxrB2 (NIDE3255)  393 DMYNDTVLGFNKSGKE-VARIQVEEPIYIRPAERVNWL..............................  429   100
"C. Kuenenia stuttgartiensis"   (CAJ72448)  374 TMYNDTVIGFGEDGKE-VVRTTVEEPIHIRPDKHYNSI..............................  410    69
Aromatoleum aromaticum EdbH     (YP_158332)  332 LISKKWSDRFGGFTNDPLTQS.....................................................  352    31
Rhodovulum sulfidophilum DdhB      (Q8GPG3)  313 LIGYRHPEMFKLS.....................................................  325    36
Nitrococcus mobilis          (ZP_01125873)  464 TSLPTFEERFVVPPMERERAMDGELPPLDPYSR-----KAAGGFGFRDAPARRF..............  512    32
Nitrobacter hamburgiensis NxrB (YP_578636)  460 TSLPTFEERFVVPPMERETAVDSLFPQLDPVSRNYPVRKGEVGVGFHTDPARGP..............  513    30
Escherichia coli NarY           (NP_415743)  447 LAIANYEDRFVVPSSHRELAREA-FPEKNGCGFTFGDGCHGSDTKFNLFNSRRIDAIDVTSKTEPHP  512    28
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**B**

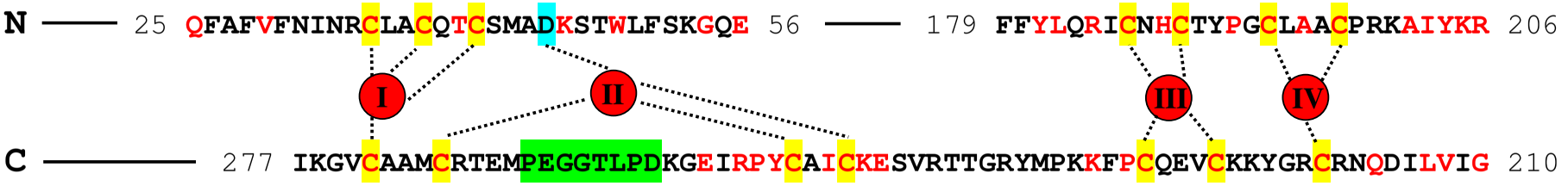

**Figure S3**: (A) Alignment of selected beta subunits of type II Mo-*bis*MGD enzymes. Accession numbers of the sequences are indicated in brackets. Residues conserved in all displayed sequences appear red. [FeS] coordinating residues are highlighted in yellow. The last column in the last row shows amino acid sequence identities (Id. %) to NxrB2 of *N. defluvii*. (B) Sketch of the possible [FeS] cluster coordination in NxrB of *N. defluvii.* Sequence stretches were taken from (A) and stringed together from the N- to the C-terminus. [FeS] coordinating residues are highlighted in yellow and blue. A sequence specific insertion is highlighted in green. The red filled circles represent the four [FeS] cluster.

```
"C. Nitrospira defluvii" NxrC1 (NIDE3271) ----------MRRMTSVHTSRTRLLT-LLSSLCVLALILGGLGIPIV 36
"C. Nitrospira defluvii" NxrC2 (NIDE3278) ----------MRLVQTRNKRLVFGILFSALIVGIMLTIGQVPLA 34
"C. Nitrospira defluvii" NxrC3 (NIDE0904) ----------MARVRRPGTSWWVGAVGLVGLCVAAQVQPAYS 32
"C. Kuenenia stuttgartiensis" (CAJ72454) KQVKCFNCHGEEGRGNGPLTVALQTQWGMPYRARDLTQSWNYKGGSTAGDIYRTISTGFN 240
"C. Kuenenia stuttgartiensis" (CAJ72449) ----------MKKFYRLLGSSSVALLGCLFLSVALCIAEEAEGVKGVA 38
Hydrogenobaculum sp. (YP_002121004) ----------MNRYLLFSLLIFTSMSLSFG 20
Aromatoleum aromaticum EdbC (YP_158331) ----------MKAKRVPGG 9
Thauera selenatis SerC (Q9S1G7) ----------MRTSSMMKRMAAMSLAAAAAWATGAAAAADGA-PAA 35
Ideonella dechloratans ClrC (P60000) ----------MKTNILVKRMAVIGLAVAAACTGAAAAQGAVPQA 35
Pyrobaculum calidifontis (YP_001056787) ----------MMGGRLLHGAVLAIAVVLAALGVILITAQ 29

"C. Nitrospira defluvii" NxrC1 (NIDE3271) SS----------EGMIIRAQALSGDIPTAPEDPLWQKIAP 66
"C. Nitrospira defluvii" NxrC2 (NIDE3278) VS----------QPVTIPVKAIKGVIPMDGANPIWEGVPG 64
"C. Nitrospira defluvii" NxrC3 (NIDE0904) QQ----------SVSVRAALVKGSLPADDPTAAWATAAV 62
"C. Kuenenia stuttgartiensis" (CAJ72454) ETPMGSYKETLSDEERWSLAHYVESLSKKMASDVVVKVKLLEEGELPDHPADEVWGLAAP 300
"C. Kuenenia stuttgartiensis" (CAJ72449) EEELTP----------AKEVLNVKYMQIDVPAHITVGALEGAFKNAEG 76
Hydrogenobaculum sp. (YP_002121004) QE----------LISKSVEGEIPLNPMADIWNKAPS 46
Aromatoleum aromaticum EdbC (YP_158331) KE----------LLLDLDAPIWAGAESTTFEMFPT 34
Thauera selenatis SerC (Q9S1G7) QR----------TIQVLSVKGG-DAASPQAAVWKKAPT 62
Ideonella dechloratans ClrC (P60000) QR----------IIRVLSVAGG-DAASPQAAVWKKAPT 62
Pyrobaculum calidifontis (YP_001056787) AP----------TVVVKYVNGNIPLDPAAPVWP--TP 54

"C. Nitrospira defluvii" NxrC1 (NIDE3271) MTLPLSGQVITRPVWPEPTAHA-LAVRAVHNGTDIAFLLEWQDNTKND-RLT-PGTFRDG 123
"C. Nitrospira defluvii" NxrC2 (NIDE3278) VIIPLSGQTITTPMHPNISVKS-VFVKAVSNGKDLGLRLDWSDQTKNDTAIG-PQDFRDQ 122
"C. Nitrospira defluvii" NxrC3 (NIDE0904) SEFPMSPQVHWPVRITDVTAKS-VKVRGLHDGTTVAIMVEYTDPSEDPD-----DAAALE 116
"C. Kuenenia stuttgartiensis" (CAJ72454) LEIPLAGQIVAHPRLWEPAIDS-MMIRGLYNDREIAFLVEWDDRTNLQEDIF-RDAVSLQ 358
"C. Kuenenia stuttgartiensis" (CAJ72449) VQVKLQKQDKAFP--NGGGSVNSAEIKAIHDGITIYFQVIWDDATDNKQAIA-TQEFRDG 133
Hydrogenobaculum sp. (YP_002121004) TTIQLSGQAIVAPMDLNPATKS-ITVKSIHNSKYIAFLLSWSDPHPSTFRVN--DKFSDA 103
Aromatoleum aromaticum EdbC (YP_158331) PLVMVKEVSPFLALSEGHGVIKRLDVAALHNGSMIALRLKWAS-EKHDKIVD-LNSFVDG 92
Thauera selenatis SerC (Q9S1G7) GQVALQTAFPGHASIVGTALTQQMTAQAVRAGDRLFVRLAWRDATANTEIKD-TDQFVDG 121
Ideonella dechloratans ClrC (P60000) TQVTLLTAFPGHISIVGTAATQKLAAQAVRASGRLFVRLAWSDRTANTVMKD-TDQFLDG 121
Pyrobaculum calidifontis (YP_001056787) IEVPLTSQQLTYPLPAAAETRS-VYVSAVYNATHIAFLLTWQDSTRDVATPGGLDVFPDA 113
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"C. Nitrospira defluvii" NxrC1 (NIDE3271) VALGLPLG-----DAPAFFCMGQLDHYINIWHWKADWQSDIDRRASRTTEKDKAAS--- 174
"C. Nitrospira defluvii" NxrC2 (NIDE3278) AAVMFPVNT---AGAPPFQCMGQSGGTVNIWRWNAEWQKDLGKDSAGMWDVDDQYPGIF 178
"C. Nitrospira defluvii" NxrC3 (NIDE0904) FMVGDTKA-----HFAH-GQPMAQEGGPVNIWYWK---------------------- 146
"C. Kuenenia stuttgartiensis" (CAJ72454) FPVKIPES-----LKKP-YFGMGDSGGVVNLWQWKACWKEGFGSIAE---------- 399
"C. Kuenenia stuttgartiensis" (CAJ72449) AALMFPLGKITISPEEPFSPRMGDRQKPVNLWHWKADWEADLLATGGIEECPARYPN--- 190
Hydrogenobaculum sp. (YP_002121004) VALEVPLK-----PDNTTPITMGGNGKEVVILQWAAYRQYNITKGFADIPKIEPYYYTG- 157
Aromatoleum aromaticum EdbC (YP_158331) VGAMFPVA-----RGAQAVTMGATGRPVNAWYWKAN---------- 123
Thauera selenatis SerC (Q9S1G7) AAVQFPVN-----GKDTTLAFMGDPDNPVNVWHWR---------- 151
Ideonella dechloratans ClrC (P60000) AAVEFPVN-----GKVATLPFMGDPVNVVNVWHWR---------- 151
Pyrobaculum calidifontis (YP_001056787) VAIQFPVS-----RAQLPYICMGTVDNPVNIIYWKAGVGVENLVAGAGYG---------- 158

"C. Nitrospira defluvii" NxrC1 (NIDE3271) -----GEPRRFEVIPR---------------------RASSVEDLIGGGFSTLTTKE 205
"C. Nitrospira defluvii" NxrC2 (NIDE3278) WDYYFEEPAGGVTYPDRIGRSLGPFNSGIWSGNIMSDPTLRVSSVEDLNANGFSTLTTQA 238
"C. Nitrospira defluvii" NxrC3 (NIDE0904) ----------------------------NKDGKGADLGAKGFGTLKPHG 167
"C. Kuenenia stuttgartiensis" (CAJ72454) ----------------------------MPANEPGEIVELNAKGFKKISIQS 423
"C. Kuenenia stuttgartiensis" (CAJ72449) --MHDDFSTNPHSVNYHKGVIQSAAELSGGYAAHNLLSLPRGRAVEDLNAEGFGTLTSQD 248
Hydrogenobaculum sp. (YP_002121004) -----YIYPQAKPPYAYPKDWNNKYAQDYVGGKVPYWKNTRDTSVIEFIADGYTTTTWRK 212
Aromatoleum aromaticum EdbC (YP_158331) ----------------------------ANEPMEIVAEGFSAVRRMK 142
Thauera selenatis SerC (Q9S1G7) ----------------------------ADGRTRNLVAKGFGTATPVP 171
Ideonella dechloratans ClrC (P60000) ----------------------------ADGRTLNLLAKGFGTSTPVP 171
Pyrobaculum calidifontis (YP_001056787) ----------------------------LSPQQREALGLQATPTSPVELLP 181

"C. Nitrospira defluvii" NxrC1 (NIDE3271) KQGRVQG-----KATWKDG-----TWRVVMRRPLSSEEQENEAKLIPGRIQAVSFAVWNG 255
"C. Nitrospira defluvii" NxrC2 (NIDE3278) HQDVIGNGVWEPSGSLKGGGYTGPTWRVVVKRSLETS-DANDTQFKAGASVPIAFAIWDG 297
"C. Nitrospira defluvii" NxrC3 (NIDE0904) ----------HQDVKAKGVYQGGVWKVVFSRTLNTEHVAEDTQFQPGQFASIAFAVWDG 216
"C. Kuenenia stuttgartiensis" (CAJ72454) PE----------NQDVMGEGLHDNGRWKVVFKRPLKTEDGKKDIQFEIGKLIPIAFAVWDG 474
"C. Kuenenia stuttgartiensis" (CAJ72449) ----------HQDVDGCSKFENKKWTVVFCRSLNTG-DPLDVQFVPGESTYFNMAVWNG 296
Hydrogenobaculum sp. (YP_002121004) ----------HQEAKGVGVYKNGKWYVEIIRPFNIK-GALNPEWGPNSKTFIDFAVWQG 260
Aromatoleum aromaticum EdbC (YP_158331) DKAG----------SDLKAVAQHRNGEWNVILCRSMATG--DGLAKLQAGGSSKIAFAVWSG 192
Thauera selenatis SerC (Q9S1G7) ----------AEGLRSTATRTRDGWEVVISRPLRVK-AEEGADLQGRRTMPIAFAAWDG 219
Ideonella dechloratans ClrC (P60000) ----------TEDLRSASVRTGDGWEVVLSRPLRVK-AEEGANLQGRRTMPIGFAAWDG 219
Pyrobaculum calidifontis (YP_001056787) QN----------AQVVQAYTTYAGGRWQVVIVRPLASV---HPLMSSLASDFSAAFATWDG 229
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"C. Nitrospira defluvii" NxrC1 (NIDE3271) ENKERN------GQKAIA-PWFQLALDPVTKT---------------------------- 280
"C. Nitrospira defluvii" NxrC2 (NIDE3278) SNIERN------GMKALS-TWFTLKMP----------------------------------- 317
"C. Nitrospira defluvii" NxrC3 (NIDE0904) KKMETGQPKEKGSEKAISSWWYFRADAPPDYSPYMYALLAVALALGFELVLVRRLKKGS- 275
"C. Kuenenia stuttgartiensis"  (CAJ72454) SNGDAG------SQKSVS-SWYYILLEKPMPKTVFVYVLVAVVMGASVELWFLARLRRFP 528
"C. Kuenenia stuttgartiensis"  (CAJ72449) DREDRN------GQKNISIQWHPLSLERIAWQ---------------------------- 322
Hydrogenobaculum sp.       (YP_002121004) YAKERD------SRKAISYAWIPLIIEGK---------------------------- 283
Aromatoleum aromaticum EdbC (YP_158331)  GNAERS------GRKSYSGEFVDFEILK---------------------------- 214
Thauera selenatis SerC         (Q9S1G7)  ENQERD------GLKAVTMEWWQLNF---------------------------- 239
Ideonella dechloratans C1rC    (P60000)  ENQERD------GLKAVTMEWWQLRF---------------------------- 239
Pyrobaculum calidifontis   (YP_001056787) GKGERG------GLKA-TSGWISFTLERPAAPARVETVTQAAPTTVTATQIVETSPTWAW 283

                                                                                  Id.%  Id.%  Id.%
                                                                                  NxrC1 NxrC2 NxrC3
"C. Nitrospira defluvii" NxrC1 (NIDE3271) ---------------------------- 280       100    39    24
"C. Nitrospira defluvii" NxrC2 (NIDE3278) ---------------------------- 317        39   100    24
"C. Nitrospira defluvii" NxrC3 (NIDE0904) ---------------------------- 275        24    24   100
"C. Kuenenia stuttgartiensis"  (CAJ72454) PKSEEEDQ------------------- 535        25    26    34
"C. Kuenenia stuttgartiensis"  (CAJ72449) ---------------------------- 322        26    28    22
Hydrogenobaculum sp.       (YP_002121004) ---------------------------- 283        30    25    22
Aromatoleum aromaticum EdbC (YP_158331)  ---------------------------- 214        23    25    19
Thauera selenatis SerC         (Q9S1G7)  ---------------------------- 239        27    27    24
Ideonella dechloratans C1rC    (P60000)  ---------------------------- 239        27    27    27
Pyrobaculum calidifontis   (YP_001056787) VVIGVLIAVIAILLGLAFRRK 303        30    30    22
```

**Figure S4**: ClustalW alignment of selected gamma subunits of type II Mo-*bis*MGD enzymes. Sequences display the first NCBI Blast hits for the three NxrC copies of *N. defluvii*. Accession numbers of the sequences are indicated in brackets. Residues conserved in all displayed sequences appear red. Heme b coordinating residues are highlighted with an asterisk. Transmembrane topologies and signal peptides predicted with the Phobius server (Kall *et al*., 2004) are highlighted with green and blue, respectively. The sequence CAJ72454 was truncated at the N-terminus. Therefore the predicted signal peptide is not displayed. The last columns in the last row shows amino acid sequence identities (Id. %) to the three NxrC copies of *N. defluvii*.

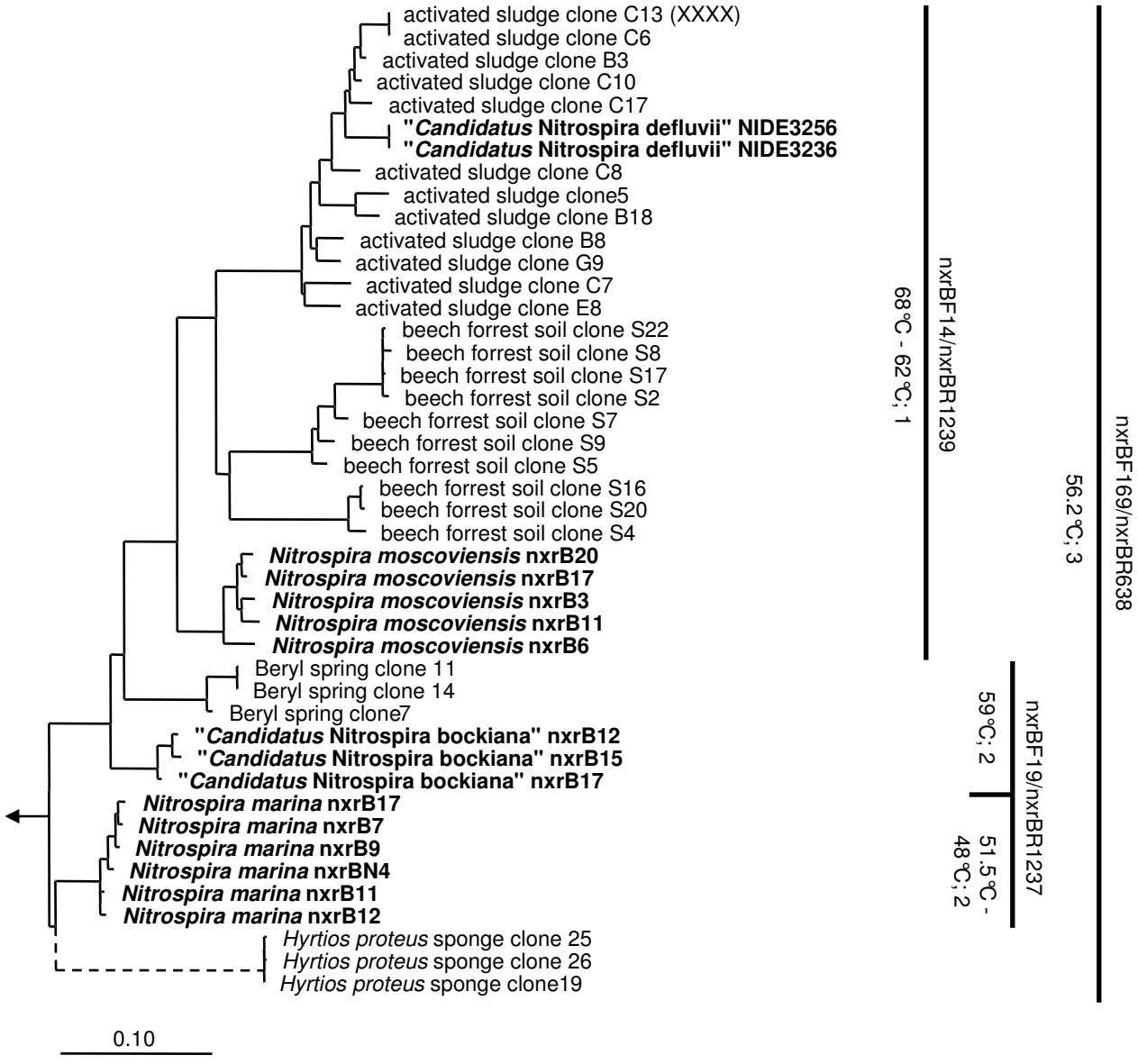
activated sludge clone C13 (XXXX)
activated sludge clone C6
activated sludge clone B3
activated sludge clone C10
activated sludge clone C17
"Candidatus Nitrospira defluvii" NIDE3256
"Candidatus Nitrospira defluvii" NIDE3236
activated sludge clone C8
activated sludge clone5
activated sludge clone B18
activated sludge clone B8
activated sludge clone G9
activated sludge clone C7
activated sludge clone E8
beech forrest soil clone S22
beech forrest soil clone S8
beech forrest soil clone S17
beech forrest soil clone S2
beech forrest soil clone S7
beech forrest soil clone S9
beech forrest soil clone S5
beech forrest soil clone S16
beech forrest soil clone S20
beech forrest soil clone S4
Nitrospira moscoviensis nxrB20
Nitrospira moscoviensis nxrB17
Nitrospira moscoviensis nxrB3
Nitrospira moscoviensis nxrB11
Nitrospira moscoviensis nxrB6
Beryl spring clone 11
Beryl spring clone 14
Beryl spring clone7
"Candidatus Nitrospira bockiana" nxrB12
"Candidatus Nitrospira bockiana" nxrB15
"Candidatus Nitrospira bockiana" nxrB17
Nitrospira marina nxrB17
Nitrospira marina nxrB7
Nitrospira marina nxrB9
Nitrospira marina nxrBN4
Nitrospira marina nxrB11
Nitrospira marina nxrB12
Hyrtios proteus sponge clone 25
Hyrtios proteus sponge clone 26
Hyrtios proteus sponge clone19
0.10
nxrBF14/nxrBR1239
68°C - 62°C; 1
nxrBF19/nxrBR1237
59°C; 2
51.5°C - 48°C; 2
56.2°C; 3
nxrBF169/nxrBR638

**Figure S5**: Overview tree displaying all *Nitrospira*-like *nxrB* sequences derived in this study. The maximum-likelyhood *nxrB* tree in Fig. 4 provided the basis tree. The displayed groups in Fig. 4 have been ungrouped and all environmental metagenome and cDNA sequences were removed in the ARB software package (Ludwig *et al*., 2004) without changing the overall tree topology. The scale bar indicates 0.1 estimated change per nucleotide. Sequences that belong to isolated or enriched *Nitrospira*-like bacteria are in boldface. Accession numbers of the sequences in the tree are indicted in brackets. Vertical bars on the right display the specificity of the *nxrB* primer pairs listed in Table 1. Each bar is superimposed with the primer pair names. Beneath the bar the optimal annealing temperatures and PCR protocol (for details see *Experimental Procedures*) is listed.

**Table S1**: Amino acid sequence identities between the two NxrA proteins of *N. defluvii* (NxrA1: 1146aa; NxrA2: 1147aa) and the NxrAs of known sequenced nitrite-oxidizing bacteria.

| Organism | nxrA genes | Amino acid residues | Identity aa (%)[a] | Reference |
|---|---|---|---|---|
| *Nitrobacter winogradskyi* Nb-255 | Nwi_0774 | 1214 | 20.6 (21.3) | (Starkenburg *et al.*, 2006) |
| | Nwi_2068 | 1214 | 22.5 (22.8) | |
| *Nitrobacter hamburgiensis* X14 | Nham_0951 | 1214 | 23.1 (24.3) | (Starkenburg *et al.*, 2008) |
| | Nham_2961 | 1229 | 23.9 (23.9) | |
| | Nham_3449 | 1214 | 22.5 (23.3) | |
| *Nitrococcus mobilis* Nb-231 | NB231_00610 | 1220 | 22.7 (23.3) | (Waterbury,J. unpublished) |
| | NB231_16083 | 1218 | 20.6 (22.9) | |

[a] Identity to the NxrA1 (NxrA2) of *N. defluvii*.

**Table S2**: Copy number and amino acid identity of NxrA, NxrB and NxrC proteins of known sequenced nitrite-oxidizing bacteria

| Organism | nxrA copies | Identity aa (%) | nxrB copies | Identity aa (%) | nxrC copies | Identity aa (%) |
|---|---|---|---|---|---|---|
| *Nitrobacter winogradskyi* Nb-255 | 2 | 94 | 2 | 97 | 1 | |
| *Nitrobacter hamburgiensis* X14 | 3 | 94 - 92 | 2 | 97 | 1 | |
| *Nitrococcus mobilis* Nb-231 | 2 | 89 | 1 | | 1 | |
| "*Candidatus* Nitrospira defluvii" | 2 | 87 | 2 | 100 | 3 | 39-24 |

**Table S3**: List of all coding sequences of the genome fragment of *N. defluvii* displayed in Fig.2 and their corresponding annotations.

| Gene | Length (aa) | Product |
|---|---|---|
| NIDE3226 | 1818 | putative Oligoendopeptidase F |
| NIDE3227 | 246 | protein of unknown function |
| NIDE3228 | 441 | putative Response regulator, CheY-like |
| NIDE3229 | 264 | protein of unknown function |
| NIDE3230 | 243 | protein of unknown function |
| NIDE3231 | 426 | protein of unknown function, contains CBS domain pair |
| yeaO | 363 | conserved protein of unknown function DUF488 |
| NIDE3234 | 174 | protein of unknown function |
| NIDE3235 | 657 | exported protein of unknown function, contains CHASE3 domain |
| nxrB1 | 1290 | putative Nitrate oxidoreductase, beta subunit |
| nxrA1 | 3441 | putative Nitrate oxidoreductase, alpha subunit |
| nifA | 1521 | Nif-specific regulatory protein NifA |
| NIDE3239 | 1548 | putative Multidrug resistance protein A (modular protein), EmrA-like |
| NIDE3240 | 1509 | putative Multidrug resistance outer membrane protein, TolC-like |
| NIDE3241 | 1599 | putative Multidrug resistance protein B, EmrB-like |
| NIDE3242 | 882 | putative Nitrite transporter NirC |

| | | |
|---|---|---|
| NIDE3243 | 198 | protein of unknown function |
| NIDE3244 | 1956 | putative Sensory histidine kinase |
| NIDE3245 | 1392 | Sigma-54 dependent transcriptional regulator |
| NIDE3246 | 1143 | putative Histidine kinase |
| NIDE3247 | 645 | Response regulator, LuxR family |
| NIDE3248 | 285 | protein of unknown function |
| NIDE3249 | 1443 | putative Heavy metal efflux system, outer membrane lipoprotein, CzcC-like |
| NIDE3250 | 1233 | putative Heavy metal efflux system, membrane fusion protein, CzcB-like |
| czcA | 3132 | Heavy metal efflux pump, CzcA family |
| sugE | 315 | Quaternary ammonium compound-resistance protein SugE |
| chaA | 1188 | Calcium/proton antiporter |
| NIDE3254 | 1422 | Sigma-54 dependent transcriptional regulator |
| nxrA2 | 3444 | putative Nitrate oxidoreductase, alpha subunit |
| nxrB2 | 1290 | putative Nitrate oxidoreductase, beta subunit |
| NIDE3257 | 207 | putative Bacterioferritin-associated ferredoxin |
| wrbA | 633 | NAD(P)H:quinone oxidoreductase WrbA |
| NIDE3260 | 186 | protein of unknown function |
| NIDE3261 | 513 | conserved protein of unknown function DUF192 |
| NIDE3262 | 1512 | putative Small-conductance mechanosensitive channel with cAMP-binding domain (modular protein) |
| relA | 2184 | GTP pyrophosphokinase |

**Table S4**: Characteristics of the subfamilies of the MopB superfamily enzymes displayed in Fig. 1

| Subfamily Name | CDD number[1] | Subfamily description | Number of sequences used for tree calculation |
|---|---|---|---|
| MopB_Nitrate-R-NarG-like | cd02750 | Respiratory nitrate reductase A (NarGHI), alpha chain (NarG) and related proteins | 78 |
| MopB_4 | cd02765 | No characterized representative | 8 |
| MopB_DmsA-EC | cd02770 | Anaerobic dimethylsulfoxide reductase (DMSOR) of Escherichia coli and other related DMSOR-like enzymes | 15 |
| MopB_DMSOR-BSOR-TMAOR | cd02769 | Dimethylsulfoxide reductase (DMSOR), biotin sulfoxide reductase (BSOR), trimethylamine N-oxide reductase (TMAOR) and other related proteins | 25 |
| MopB_3 | cd02766 | No characterized representative | 24 |
| MopB_Acetylene-hydratase | cd02759 | Acetylene hydratase (Ahy) and other related proteins | 15 |
| MopB_Thiosulfate-R-like-1/2/3 | cd02755 | Thiosulfate-, sulfur-, and polysulfide-reductases, and other | 9/4/3 |

| | | | |
|---|---|---|---|
| | | related proteins | |
| MopB_Arsenate-R | cd02757 | Respiratory arsenate reductase, As(V), catalytic subunit (ArrA) and other related proteins | 5 |
| MopB_Phenylacetyl-CoA-OR | cd02760 | Phenylacetyl-CoA:acceptor oxidoreductase, large subunit (PadB2), and other related proteins | 2 |
| MopB_2 | cd02763 | No characterized representative | 4 |
| MopB_Tetrathionate-Ra | cd02758 | Tetrathionate reductase, subunit A, (TtrA) and other related proteins | 8 |
| MopB_1 | cd02762 | No characterized representative | 9 |
| MopB_Formate-DH-Na-like | cd02752 | Formate dehydrogenase N, alpha subunit | 14 |
| MopB_ydeP | cd02767 | No characterized representative | 21 |
| MopB_Formate-DH-H | cd02753 | Formate dehydrogenase H | 30 |
| MopB_Nitrate-R-NapA-like | cd02754 | Nitrate reductases, NapA, NasA, and NarB | 33 |
| MopB_Arsenite-Ox | cd02756 | Arsenite oxidase | 4 |
| MopB_FmdB-FwdB | cd02761 | Molybdenum/tungsten formylmethanofuran dehydrogenases, subunit B | 18 |
| MopB_PHLH | cd02764 | uncharacterized putative hydrogenase-like homologs (PHLH) of molybdopterin binding (MopB) proteins | 4 |
| MopB_NADH-Q-OR-NuoG2 | cd02768 | The NuoG/Nad11/75-kDa subunit (second domain) of the NADH-quinone oxidoreductase (NADH-Q-OR)/respiratory complex I/NADH dehydrogenase-1 (NDH-1) | 59 |

[1] NCBI Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) number

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# Chapter V

## Summary/Zusammenfassung

## Summary

Nitrification is a key process of the biogeochemical nitrogen cycle in natural and engineered (e.g. wastewater treatment plants) habitats. The two steps of aerobic nitrification, the oxidation of ammonia to nitrite and subsequently from nitrite to nitrate, are catalysed by two distinct functional groups of chemolithotrophic prokaryotes: the ammonia-oxidizing bacteria (AOB) and archaea (AOA), and the nitrite-oxidizing bacteria (NOB). So far, most genomic and physiological data originate from a few "model" nitrifiers, in particular AOB related to the genus *Nitrosomonas* and NOB of the genus *Nitrobacter*. Among the fastidious and slow-growing nitrifiers these two organisms stand in the forefront of isolated microorganisms. However, cultivation-independent approaches have revealed a huge diversity of poorly characterized AOB, AOA and NOB in a variety of ecosystems. Moreover, most of these yet uncultivated nitrifiers seem to represent nitrifying key players in different environments. Hence, our current understanding of nitrification and the biogeochemical nitrogen cycle is far from being complete.

This thesis mainly focused on the genus *Nitrospira*, one of the aforementioned important but yet less intensively studied groups of NOB. *Nitrospira*-like bacteria are the dominant NOB in wastewater treatment plants and the most diverse known NOB in nature, being widespread in various aquatic and terrestrial habitats. However, ecophysiological and genomic data of these mostly uncultured NOB are either scarce or lacking. Molecular and, for the first time metagenomic data retrieved in the course of this thesis brought forward novel previously unknown ecophysiological traits of these conspicuous NOB.

In the first part of this thesis, niche differentiation between two coexisting uncultured populations of *Nitrospira*-like bacteria in nitrifying biofilm and activated sludge samples were analyzed. Fluorescence *in situ* hybridization (FISH) and subsequent quantitative image analysis provided indication of a different spatial arrangement of members of two phylogentic sublineages within the genus *Nitrospira* relative to AOB microcolonies. The obtained spatial distribution pattern suggested a niche differentiation of the coexisting *Nitrospira* populations with respect to their preferred concentrations of nitrite. Supporting evidence for this hypothesis was obtained by monitoring the population dynamics of these *Nitrospira*-like bacteria during a long-term competition experiment: high nitrite concentration selected for one sublineage whereas the other sublineage was suppressed under these conditions. In addition mathematical modelling of nitrite consumption predicted steep nitrite gradients within biofilms which would allow the co-occurrence of different *Nitrospira* populations

adapted to low and high nitrite concentrations, respectively. Thus, the results obtained in this study confirm the hypothesis that ecological differentiation occurred within the genus *Nitrospira*.

Within the second part of this thesis the successful enrichment of a *Nitrospira*-like bacterium enabled us to launch an environmental genomic approach aimed towards sequencing large genome fragments and ultimately the complete genome of this organism, which was provisionally classified as "*Candidatus* Nitrospira defluvii". The analysis of a 137 kbp-long fragment, harbouring the ribosomal RNA of *N. defluvii*, revealed the presence of an open reading frame with high sequence similarity to chlorite dismutase (Cld) genes. This enzyme, which has yet been described only for (per)chlorate-reducing *Proteobacteria*, transforms toxic chlorite to chloride and oxygen. The biochemical activity of the non-proteobacterial Cld of *N. defluvii* was confirmed by heterologous expression of the intact enzyme in *E. coli* and subsequent incubation with chlorite. The presence of Cld in the genome of a nitrifying organism is surprising because these bacteria were not known to be involved in (per)chlorate degradation. Nevertheless, given that (per)chlorate and chlorite are widely used as disinfectants or bleaching agents and are by-products of various industrial processes, it is tempting to speculate that *N. defluvii* might link the bioremediation of these compounds with nitrogen turnover at contaminated sites.

Finally, the last chapter of this thesis describes for the first time in detail the metabolic key enzyme of nitrite oxidizing *Nitrospira*, the nitrite oxidoreductase (Nxr). The goal was to identify the *nxr* genes in the already closed *N. defluvii* genome and to determine whether these genes are suitable as functional and phylogenetic markers of *Nitrospira*. The mature Nxr holoenzyme is composed of at least three subunits (alpha to gamma) encoded on the genome of *N. defluvii*. Interestingly, sequence analysis revealed remarkable differences between Nxr subunits of *N. defluvii* and Nxr subunits of other known NOB, such as *Nitrobacter* and *Nitrococcus*. Furthermore, in contrast to the Nxr of latter NOB, which are anchored to the cytoplasmic face of the cell membrane, the *Nitrospira* Nxr is located in the periplasm. Transcriptional analysis of the detected *Nitrospira* Nxr genes clearly indicated a higher expression level in presence of nitrite than during starvation. Moreover, both *nxr* mRNA and Nxr protein could be detected even after a long starvation period. During starvation slow-growing nitrifiers possess stable enzymes and RNAs to quickly respond to nutrient pulses without energy consuming *de novo* synthesis of their cellular "machineries". Additionally, comparative phylogenetic analysis between *Nitrospira* 16S rRNA and *nxrB* gene sequences, the gene encoding the Nxr beta subunit, revealed a congruent evolutionary history without

any sign of lateral gene transfer. Thus, *nxrB* is the first functional and phylogenetic marker for the genus *Nitrospira* and the new *nxrB*-specific primer set extends the molecular toolbox for monitoring activity and abundance of NOB.

Taken together, all three chapters of this thesis deeply extend our current knowledge of the ecology and physiology of nitrite-oxidizing *Nitrospira*-like bacteria. Combining novel molecular methods with metagenomic data allowed in-depth characterization of this slow-growing, mostly uncultured NOB. The presented results in this thesis provide a sound basis for already ongoing new research topics, such as the crystallization of Cld and the heterologous expression of Nxr.

## Zusammenfassung

Ein Kernprozess des biogeochemischen Stickstoffzyklus in natürlichen Lebensräumen und technischen Anlagen (z. B. Kläranlagen) ist die Nitrifikation. Dieser aerobe Prozess untergliedert sich in zwei Stufen, der oxidativen Umwandlung von Ammoniak zu Nitrit bzw. von Nitrit zu Nitrat. Zwei verschiedene funktionelle Gruppen chemolithotropher Mikroorganismen sind dabei katalytisch an der Stoffumsetzung beteiligt: die Ammoniak oxidierenden Bakterien (AOB) bzw. Archaeen (AOA) und die Nitrit oxidierenden Bakterien (NOB). Unter den anspruchsvollen, langsam wachsenden nitrifizierenden Bakterien galten bislang besonders AOB der Gattung *Nitrosomonas* und NOB der Gattung *Nitrobacter* als die Modellorganismen, über die die meisten physiologischen Werte und Genomdaten existieren. Kultivierungsunabhängige Ansätze jedoch haben ergeben dass in den unterschiedlichsten Ökosystemen eine Vielzahl uncharakterisierter AOB, AOA und NOB vorkommen, die darüber hinaus eine Schlüsselrolle in der Nitrifikation in diesen Habitaten einnehmen. Unser bisheriges Verständnis über Nitrifikation und dem biogeochemischen Stickstoffzyklus ist daher nur lückenhaft und es bedarf einer genaueren Untersuchung dieser nicht kultivierbaren nitrifizierenden Mikroorganismen um tiefere Erkenntnisse über diesen Schlüsselprozess in der Umwelt zu erlangen.

Ein Hauptaugenmerk in dieser Doktorarbeit wurde auf NOB der Gattung *Nitrospira* gelegt. Diese wenig untersuchte Gruppe von Bakterien ist maßgeblich an der Stickstoffeleminierung in Kläranlagen beteiligt und ist darüber hinaus weit verbreitet in den unterschiedlichsten aquatischen und terrestrischen Lebensräumen. Bisher ist jedoch wenig bekannt über die Ökophysiologie dieser meist unkultivierbaren NOB und es gab auch keine Genomdaten der Gattung *Nitrospira*. In dieser Arbeit konnte zum ersten Mal auf Metagenomsequenzen dieser Bakteriengruppe zurückgegriffen werden und zusammen mit dem Einsatz verschiedener molekularbiologischer Techniken erschlossen sich bis dato unbekannte ökophysiologische Charakteristika dieser besonderen NOB.

Der erste Teil dieser Arbeit handelt von zwei verschiedenen *Nitrospira* Populationen, die gemeinsam in nitrifizierenden Biofilmen oder in Belebtschlamm in Kläranlagen auftreten, dort aber unterschiedliche ökologische Nischen besetzen. Der gekoppelte Einsatz von Fluoreszenz *in situ* Hybridisierung (FISH) und anschließender quantitativer Bildanalyse ergab erste Hinweise für eine unterschiedliche räumliche Anordnung beider Populationen gegenüber AOB Mikrokolonien. Dieses räumliche Verteilungsmuster lässt auf eine Nischendifferenzierung beider *Nitrospira* Populationen bezüglich ihrer Präferenz für

verschiedene Nitritkonzentrationen schließen. Unterstützt wurde diese Hypothese zum einem durch Erkenntnisse, die aus der Dynamik der *Nitrospira* Populationen in einen Langzeitversuch gewonnen wurden: hohe Nitritkonzentrationen wirkten selektiv auf eine Population wobei das Wachstum der anderen Population unter diesen Bedingungen unterdrückt wurde. Zum anderem konnte ein mathematisches Modell zeigen, dass durch konstanten Nitritverbrauch mittels NOB starke Nitritgradienten innerhalb von Biofilmen auftreten können, was wiederum ein gemeinsames Auftreten verschiedener *Nitrospira* Populationen mit unterschiedlicher Nitritaffinität ermöglichen würde. Ökologische Differenzierung tritt folglich nicht nur bei verschiedenen NOB sondern auch bei Mikroorganismen innerhalb der Gattung *Nitrospira* auf.

Im zweiten Abschnitt dieser Doktorarbeit ermöglichte eine hohe Anreicherung eines Vertreters der Gattung *Nitrospira*, "*Candidatus* Nitrospira defluvii", Metagenomanalysen mit diesem NOB durchzuführen. Dabei wurde auf einem Sequenzfragment mit 137 kbp Länge neben der ribosomalen RNS von *N. defluvii* ein „open reading frame" mit hoher Sequenzähnlichkeit zu Genen, die für Chloritdismutasen (Cld) kodieren, entdeckt. Es handelt sich dabei um ein Enzym dass die Umwandlung von giftigem Chlorit zu Chlorid und Sauerstoff katalysiert und bis dato nur in (Per)chlorat-reduzierenden *Proteobakterien* beschrieben wurde. Die Cld von *N. defluvii* wurde erfolgreich in *E. coli* überexpremiert und das gewonnene rekombinante Enzym zeigte hohe Aktivität nach Zugabe von Chlorit. Da bisher NOB mit dem Abbau von (Per)chlorat nicht in Verbindung gebracht wurden ist es einerseits erstaunlich das *N. defluvii* dieses Enzym besitzt. Andererseits werden gerade (Per)chlorat und Chlorit häufig als Desinfektions- oder als Bleichmittel eingesetzt und sind das Beiprodukt verschiedenster industrieller Prozesse. Somit ist es gut möglich, dass *N. defluvii* den Abbau dieser Komponenten mit der Nitrifizierung an kontaminierten Standorten koppelt.

Das letzte Kapitel dieser Arbeit beschreibt im Detail die Nitritoxidoreduktase (Nxr), das metabolische Schlüsselenzym der Nitrit-oxidierenden *Nitrospiren*. Ziel war es zu einem Nxr Gene im Genom von *N. defluvii* zu entschlüsseln und darüber hinaus festzustellen ob eines dieser Gene als funktioneller und phylogenetischer Marker verwendet werden kann. Der Nxr Enzymkomplex besteht aus mindestens drei Untereinheiten (Alpha, Beta, Gamma). Interessanterweise besteht nur eine geringe Sequenzähnlichkeit zwischen den Nxr Untereinheiten von *N. defluvii* und den Untereinheiten anderer bekannter NOBs (*Nitrobacter* und *Nitrococcus*). Ein weiteres Unterscheidungsmerkmal stellt die zelluläre Lokalisation der NXR bei den verschiedenen NOBs dar. Während sich die NXR von *Nitrobacter* und

*Nitrococcus* im Cytoplasma befindet, ragt die Nxr von *N. defluvii* in das Periplasma. Allen NOBs ist aber gemeinsam, dass sie die Nxr über die Gamma-Untereinheit an der Cytoplasmamembran verankern. Transkriptionsanalysen der *Nitrospiren* Nxr Gene haben des Weiteren ergeben, dass der Expressionslevel der Nxr unter Nitrit-oxidierenden Bedingungen deutlich über dem bei Hungerperioden liegt, wobei die Halbwertezeit der Nxr mRNS und des Nxr Enzyms in den Zeiten ohne Energiezufuhr ungewöhnlich hoch ist. Langsam wachsende nitrifizierende Mikroorganismen scheinen während Hungerperioden einen basalen Satz an stabilen Enzymen und RNS zu behalten, der es ihnen ermöglicht ohne Proteinneusynthese rasch auf Nährstoffeinträge zu reagieren. Neben der Expressionsanalyse lag ein Schwerpunkt dieser Studie in der vergleichenden phylogenetischen Analyse von *Nitrospiren* 16S rRNS Genen und Nxr Genen. Es stellte sich heraus, dass die *nxrB*-Phylogenie (Gen der Nxr Beta-Untereinheit) der Phylogenie der 16S rRNS folgt und es bis jetzt keinen Hinweis für lateralen Gentransfer für dieses Nxr Gen gibt. Unter dieser Voraussetzung bildet *nxrB* den ersten funktionellen und phylogenetischen Marker für die Gattung *Nitrospira*. Die in dieser Arbeit entwickelten *nxrB* spezifischen Primer erweitern somit die molekularen Möglichkeiten, Aktivität und Vorkommen dieser NOB in unterschiedlichen Habitaten zu untersuchen.

Insgesamt erweitern alle drei Kapitel dieser Doktorarbeit unser bisheriges Verständnis über die Ökophysiologie Nitrit-oxidierender *Nitrospiren*. Gerade erst durch die Kopplung neuer molekularer Methoden mit ersten Metagenomdaten wurde eine detaillierte Beschreibung dieser langsam wachsenden, meist unkultivierbaren NOB möglich. Darüber hinaus bilden die in dieser Arbeit vorgelegten Ergebnisse bereits die Grundlage für aktuelle Forschungsthemen, wie z.B. die Kristallisation der Cld oder die heterologe Expression der Nxr.

# Appendix

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# Selective enrichment and molecular characterization of a previously uncultured *Nitrospira*-like bacterium from activated sludge

**Eva Spieck,[1]* Christine Hartwig,[1] Iris McCormack,[1] Frank Maixner,[2] Michael Wagner,[2] André Lipski[3] and Holger Daims[2]**

[1]*Universität Hamburg, Biozentrum Klein Flottbek, Mikrobiologie, Ohnhorststr. 18, D-22609 Hamburg, Germany.*

[2]*Department für Mikrobielle Ökologie, Universität Wien, Althanstr. 14, A-1090 Vienna, Austria.*

[3]*Universität Osnabrück, Abteilung Mikrobiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany.*

## Summary

**Previously uncultured nitrite-oxidizing bacteria affiliated to the genus *Nitrospira* have for the first time been successfully enriched from activated sludge from a municipal wastewater treatment plant. During the enrichment procedure, the abundance of the *Nitrospira*-like bacteria increased to approximately 86% of the total bacterial population. This high degree of purification was achieved by a novel enrichment protocol, which exploits physiological features of *Nitrospira*-like bacteria and includes the selective repression of coexisting *Nitrobacter* cells and heterotrophic contaminants by application of ampicillin in a final concentration of 50 μg ml$^{-1}$. The enrichment process was monitored by electron microscopy, fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes and fatty acid profiling. Phylogenetic analysis of 16S rRNA gene sequences revealed that the enriched bacteria represent a novel *Nitrospira* species closely related to uncultured *Nitrospira*-like bacteria previously found in wastewater treatment plants and nitrifying bioreactors. The enriched strain is provisionally classified as '*Candidatus* Nitrospira defluvii'.**

## Introduction

Aerobic chemolithoautotrophic nitrification is a two-step process that involves two distinct functional groups of bacteria: ammonia-oxidizing bacteria (AOB), which transform ammonia to nitrite, and nitrite-oxidizing bacteria (NOB), which further oxidize nitrite to nitrate. Based on morphological characteristics, NOB have been divided into four genera. This classification also reflects the phylogenetic diversity of NOB: *Nitrobacter* and *Nitrococcus* belong to the *Alpha-* and *Gammaproteobacteria*, respectively, whereas *Nitrospina* is provisionally assigned to the *Deltaproteobacteria*. The genus *Nitrospira* is more distantly related to the other known NOB, because it is part of the deep-branching bacterial phylum *Nitrospira.* It so far contains only two described and cultured species, *Nitrospira moscoviensis* isolated from an urban heating system in Moscow (Ehrich *et al*., 1995) and *Nitrospira marina*, which was obtained from Atlantic Ocean water samples (Watson *et al*., 1986). Besides morphological and phylogenetic characterization, the fatty acid profiles of NOB offer a high potential for differentiation of new isolates and enrichment cultures, because the phylogenetic heterogeneity of these bacteria is reflected in genus-specific profiles (Lipski *et al*., 2001).

Until recently, most insight into the ecology and biochemistry of NOB were based on experiments done with pure *Nitrobacter* cultures. However, cultivation-independent molecular methods (Wagner *et al*., 1996; Burrell *et al*., 1998; Hovanec *et al*., 1998; Juretschko *et al*., 1998) and immunological techniques (Bartosch *et al*., 1999) have revealed that uncultured bacteria related to the genus *Nitrospira* and not *Nitrobacter* are the dominant NOB in aquaria, nitrifying laboratory-scale reactors, and full-scale wastewater treatment plants. In subsequent studies, similar *Nitrospira*-like bacteria were also detected in different soils (Bartosch *et al*., 2002), marine sponge tissue (Hentschel *et al*., 2002), the Australian Nullarbor caves (Holmes *et al*., 2001) and recently in hot springs (Lebedeva *et al*. 2005). Hence it has become clear that nitrification in natural and engineered ecosystems cannot be understood without a detailed insight into the ecophysiology of these novel uncultured nitrite oxidizers. The first insight into their physiological properties was achieved by applying cultivation-independent molecular approaches (Okabe *et al*., 1999; Schramm *et al*., 1999; Daims *et al*., 2001a; Wagner *et al*., 2002), but detailed biochemical and genome-level analyses of *Nitrospira*-like bacteria would require high enrichments or pure cultures.

Received 19 May, 2005; accepted 18 July, 2005. *For correspondence. E-mail spieck@mikrobiologie.uni-hamburg.de; Tel. (+49) 40 42816 424; Fax (+49) 40 42816 254.

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The initial developments in this direction were made by Bartosch and colleagues (1999), who found that *Nitrospira*-like bacteria could be selectively enriched from activated sludge in mixotrophic medium with a low nitrite concentration (3 mM) and that *Nitrobacter* overgrew *Nitrospira*-like bacteria in media with a higher nitrite content (30 mM). However, a dense enrichment consisting almost exclusively of *Nitrospira*-like bacteria was not obtained. Whereas most investigations have focused on the phylogenetic diversity of *Nitrospira* (Daims *et al*., 2001a), less attention has been paid on developing methods to culture these fastidious and slow-growing organisms.

In this study, a new strategy for the selective enrichment of *Nitrospira*-like bacteria from environmental samples has been developed and successfully applied on nitrifying activated sludge from a full-scale wastewater treatment plant. The protocol specifically exploits known biological features of *Nitrospira*-like bacteria and combines cultivation techniques with molecular and chemotaxonomic methods used to monitor the enrichment process. Based on this approach, the hitherto highest enrichment of previously uncultured *Nitrospira*-like bacteria from activated sludge was achieved. The novel bacterium, which was enriched to approximately 86%, was clearly separated from the two species *N. moscoviensis* and *N. marina* and has provisionally been classified as '*Candidatus* Nitrospira defluvii'.

## Results

### *Abundance and characterization of nitrifying bacteria in the activated sludge samples*

The concentrations of dissolved inorganic nitrogen compounds in the bulk liquid of the activated sludge at the time of sampling (Table 1) were in the range usually observed in municipal wastewater treatment plants. The measured conversion rates of ammonia and nitrite (Table 1) confirmed the presence of metabolically active nitrifying bacteria. Cell numbers in the original sludge samples were estimated by using the MPN (most-probable-number) technique with 10.0 mM $NH_4Cl$ for the AOB, and 10.0, 3.0 and 0.3 mM $NaNO_2$ for the NOB in the cultivation medium. Cell counts gave values of $4.0 \times 10^5$ to $7.6 \times 10^6$ cells $ml^{-1}$ for the AOB and $2.5 \times 10^5$ to $1.5 \times 10^6$ cells $ml^{-1}$ for the NOB with 10.0 mM $NaNO_2$. Higher cell numbers of $3.3–4.9 \times 10^6$ cells $ml^{-1}$ were obtained when the nitrite concentration was reduced to 3 mM $NaNO_2$. Incubation with 0.3 mM $NaNO_2$ yielded cell numbers similar to those of the experiments with 3 mM $NaNO_2$.

The activated sludge was further analysed by electron microscopy in order to identify the nitrifying bacteria based on morphological and ultrastructural criteria. One abundant bacterial population resembled *Nitrosomonas*-like AOB (data not shown) while microcolonies of another frequently encountered population resembled *Nitrospira*-like bacteria (Bartosch *et al*., 1999). Cells exhibiting structural features of the known *Nitrobacter* species were not detected in the original material.

### *Enrichment of* Nitrospira*-like bacteria*

In general, serial dilution is the method of choice for isolation of nitrifying bacteria using positive tubes of the highest dilution as inoculum for the enrichment cultures (Bock and Koops, 1992). The enrichment of *Nitrospira*-like bacteria from activated sludge was successful after serial dilution in Erlenmeyer flasks instead of small glass tubes, which were used in earlier studies to enrich other nitrifiers. In accordance to Both and colleagues (1992), different nitrite concentrations were applied to discriminate different NOB. The two cultured *Nitrospira* species are known to be nitrite-sensitive and are inhibited by nitrite concentrations above 6 mM (Watson *et al*., 1986) or 15 mM (Ehrich *et al*., 1995). The primary cultures completely oxidized 3 mM $NaNO_2$ within 2 weeks. The consumed nitrite was regularly replaced to obtain a sufficiently high cell density for the working steps in Fig. 1. The most diluted (1:100) primary culture that showed nitrite-oxidizing activity served as starting material for the further enrichment procedure. The increasing abundance of *Nitrospira*-like bacteria was accompanied by the formation of characteristic microcolonies, which aggregated to small, loose flocs that were visible to the naked eye. The density of single

**Table 1.** Concentrations of inorganic nitrogen and activities of nitrifying bacteria in activated sludge samples from the wastewater treatment plant at Dradenau in Hamburg, Germany.

| Date of sampling | $NH_4^+$ (mg $l^{-1}$) | $NO_2^-$ (mg $l^{-1}$) | $NO_3^-$ (mg $l^{-1}$) | Activity of AOB (μmol $NH_4^+$ $h^{-1}$ $ml^{-1}$) | Activity of NOB (μmol $NO_2^-$ $h^{-1}$ $ml^{-1}$) |
|---|---|---|---|---|---|
| 11 November 1997 | 8.5 | 0.7 | n.d. | 2.4 | 1.5 |
| 15 January 1998 | 8.9 | 0.7 | 5.2 | 10.3 | 3.2 |
| 5 February 1998 | n.d. | 1.7 | 9.5 | 5.5 | 1.1 |

In this plant, nitrification and denitrification are combined in the same, partially aerated activated sludge tanks. Ammonia or nitrate concentrations below the detection limit can be explained by the time and location of sampling in temporarily nitrifying or denitrifying zones respectively.
n.d., not detectable.

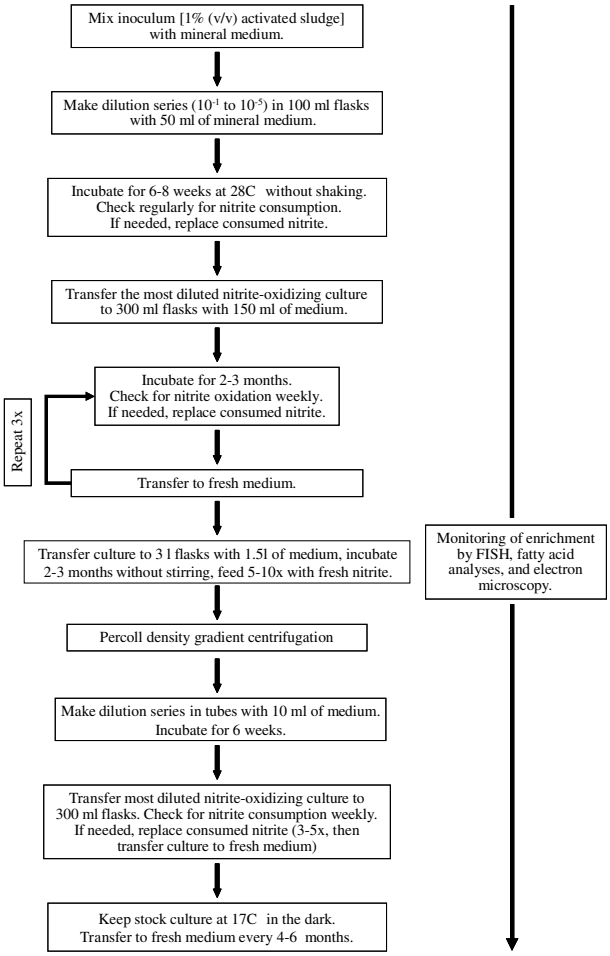


**Fig. 1.** Flow chart of the procedure developed to enrich *Nitrospira*-like bacteria. Every step was performed in mineral medium with 3 mM $NaNO_2$. All media, except in the 3 l bottles and the dilution series, were supplemented with ampicillin (50 μg $ml^{-1}$). One per cent (v/v) inoculum was used to transfer cultures.

planktonic cells and small cell aggregates never became high enough to cause turbidity of the medium. The typical brownish colour of NOB became visible after concentrating the enriched biomass by centrifugation. In *Nitrobacter* the brownish colour belongs to FeS proteins that are part of the β-subunit of the nitrite oxidoreductase (Kirstein and Bock, 1993). In *N. moscoviensis* an unstable haem was detected in the β-subunit of the nitrite-oxidizing system (Spieck *et al*., 1998).

### *Inhibition of* Nitrobacter *and heterotrophic organisms*

Various treatments with antibiotics were tested to determine whether the growth of *Nitrobacter* and heterotrophic bacteria could be suppressed without compromising *Nitrospira*-like bacteria. Tetracycline and streptomycin were not suitable as these antibiotic substances caused a decrease of *Nitrospira*-like bacteria. In contrast, the fraction of *Nitrospira*-like bacteria in the enrichments became larger when 50 μg $ml^{-1}$ ampicillin was added to the medium. The size of the largest, macroscopically visible microcolonies increased approximately by a factor of 10 (data not shown). Microscopic observation confirmed that repeated application of ampicillin also inhibited growth of *Nitrobacter*. However, higher concentrations of ampicillin (100 or 200 μg $ml^{-1}$) suppressed the growth of *Nitrospira*-like bacteria, too.

### *Morphology of enriched* Nitrospira*-like bacteria*

The structure of the large cell aggregates of *Nitrospira*-like bacteria detected by fluorescence *in situ* hybridization (FISH) was similar to that observed in activated sludge in earlier studies (Juretschko *et al*., 1998; Bartosch *et al*., 1999; Daims *et al*., 2001a). They consisted of small groups of cells with a spherical appearance, separated by cell-free channels and voids of different sizes (Fig. 2A). Electron microscopy confirmed that these *Nitrospira* clusters were relatively loose aggregates of short, slightly curved cells that were frequently organized in tetrads embedded in extracellular polymeric substances (EPS) (Fig. 3A). Interestingly, the shape and arrangement of the *Nitrospira* cells changed significantly after prolonged incubation (about 2 months) with repeated addition of fresh substrate to the cultures. Following nitrate accumulation and increasing cell density, the microcolonies disintegrated (Fig. 2B) and the length of the cells increased (Fig. 3B and C). Their irregular appearance now resembled that of *N. moscoviensis*, which tends to form star-like extensions of the outer membrane (Ehrich *et al*., 1995). Likewise, longer periods of starvation led to the appearance of relatively long, curved rods with one turn, which occurred mainly as free-living planktonic cells (Fig. 3D). This morphological change to the characteristic spiral cell shape could be accelerated in stirred or aged cultures and was reversible when starved cultures were transferred into fresh mineral medium containing nitrite.

### *Purification by Percoll density gradient centrifugation*

The separation of *Nitrospira*-like bacteria from other microorganisms was further improved by Percoll density gradient centrifugation of highly enriched cultures, where a relatively large fraction of the target NOB occurred as single planktonic cells. Fresh cultures were not suitable for this technique because most *Nitrospira* cells were embedded in large aggregates that included also other microorganisms (Fig. 2A) and thereby prevented physical cell isolation. After centrifugation of a concentrated culture, *Nitrospira*-like bacteria formed a brownish coloured band in the upper part of the centrifugation tube. This fraction was harvested and used as inoculum for new

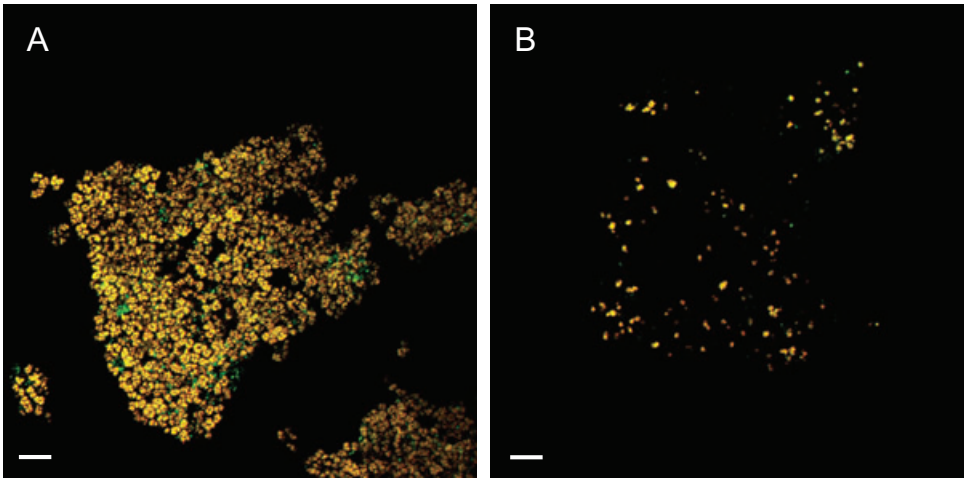


**Fig. 2.** Epifluorescence micrographs of the nitrite-oxidizing enrichment culture obtained from activated sludge.
A. Aggregate of *Nitrospira*-like bacteria labelled by FISH with *Nitrospira*-specific probe S-G-Ntspa-0662-a-A-18 (red) and the EUB338 probe mix (green). *Nitrospira* cells, which were detected by the *Nitrospira*-specific probe and by probe EUB338-I, appear yellow. Contaminants appear green. Bar = 10 μm.
B. Stationary-phase aggregate of enriched *Nitrospira*-like bacteria in the process of disintegration. Fluorescence *in situ* hybridization (FISH) was performed with the same probes as in (A).

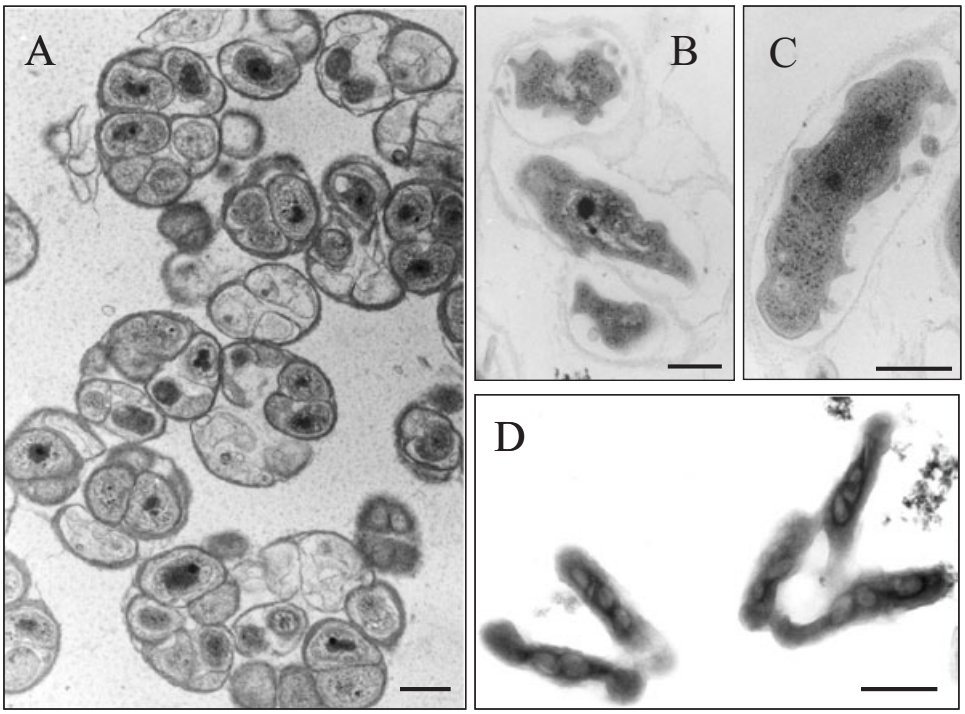


**Fig. 3.** Electron micrographs of enriched *Nitrospira*-like bacteria derived from activated sludge.
A. Ultrathin section of a microcolony as shown in Fig. 2A. Short cells are surrounded by dense layers of EPS. Bar = 1 μm.
B and C. Ultrathin sections of *Nitrospira* cell aggregates undergoing the transition to planktonic lifestyle similar to the colony shown in Fig. 2B. The EPS matrix is disappearing and the cells are more elongated than in (A). Bars = 0.25 μm.
D. Electron micrograph of planktonic *Nitrospira*-like bacteria after disintegration of microcolonies. Compared with (A) the cell morphology has changed considerably. Bar = 0.5 μm.

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dilution series in mineral nitrite medium. In these series, growth of *Nitrospira*-like bacteria was detected up to dilutions of $10^{-7}$ to $10^{-9}$.

Fluorescence *in situ* hybridization (FISH) analysis demonstrated that the enrichment we obtained with the protocol described here consisted of 86 ± 3% of *Nitrospira*-like bacteria. After repeated ampicillin treatment, application of the Percoll technique and subsequent dilution, only very few other bacterial cells were detectable by FISH (Fig. 2A and B). No probe-conferred fluorescence was observed when the biomass was hybridized to the *Nitrobacter*-specific probe S-G-Nbac-1035-a-A-18. Accordingly, cells with the typical *Nitrobacter* morphology were not detected by electron microscopy of the enriched biomass.

### *Phylogeny of the enriched* Nitrospira*-like bacteria*

Comparative analysis of 16S rRNA sequences confirmed that the enriched NOB were related, but not identical to *N. moscoviensis* and *N. marina*. Six out of 10 16S rRNA sequences, which were retrieved from the enrichment, were related to the genus *Nitrospira*. These six sequences fell regardless of the applied treeing method into sublineage I (Daims *et al*., 2001a) of the genus *Nitrospira* (Fig. 4) and were highly similar (97.7–99.6%) to other environmentally obtained 16S rRNA sequences in this sublineage. Although differences between some of the retrieved sequences were found in a few alignment columns (99.7–100% sequence similarity of the six *Nitrospira*-like sequences with each other), no common patterns shared by two or more of the sequences could be identified. The observed differences were most likely caused by occasional polymerase chain reaction (PCR) or sequencing errors.

### *Fatty acid profiles*

The enrichment of *Nitrospira*-like organisms was additionally tracked by the analysis of the fatty acid profiles of the cultures (Table 2). The first enrichment culture in 1999 was dominated by the long-chain fatty acid *cis*-11-octadecenoic acid which is the major fatty acid of the *Alphaproteobacteria* including the genus *Nitrobacter* (Lipski *et al*., 2001). In the course of the further enrichment procedure *cis*-11-hexadecenoic acid was a stable and dominating compound of the cultures, increasing to about 42% in 2004. This shift reflects the enrichment of *Nitrospira*-like bacteria as among all known genera of NOB *cis*-11-hexadecenoic acid is present only in the genus *Nitrospira* as a major lipid (Lipski *et al*., 2001).

Moreover, the lipid profiles of the enrichment cultures showed significant differences to the profiles of the two described species of this genus. *Nitrospira moscoviensis* is characterized by a unique branched-chain compound, 11-methyl-hexadecanoic acid, which was not detected in this enrichment culture. The second species *N. marina*

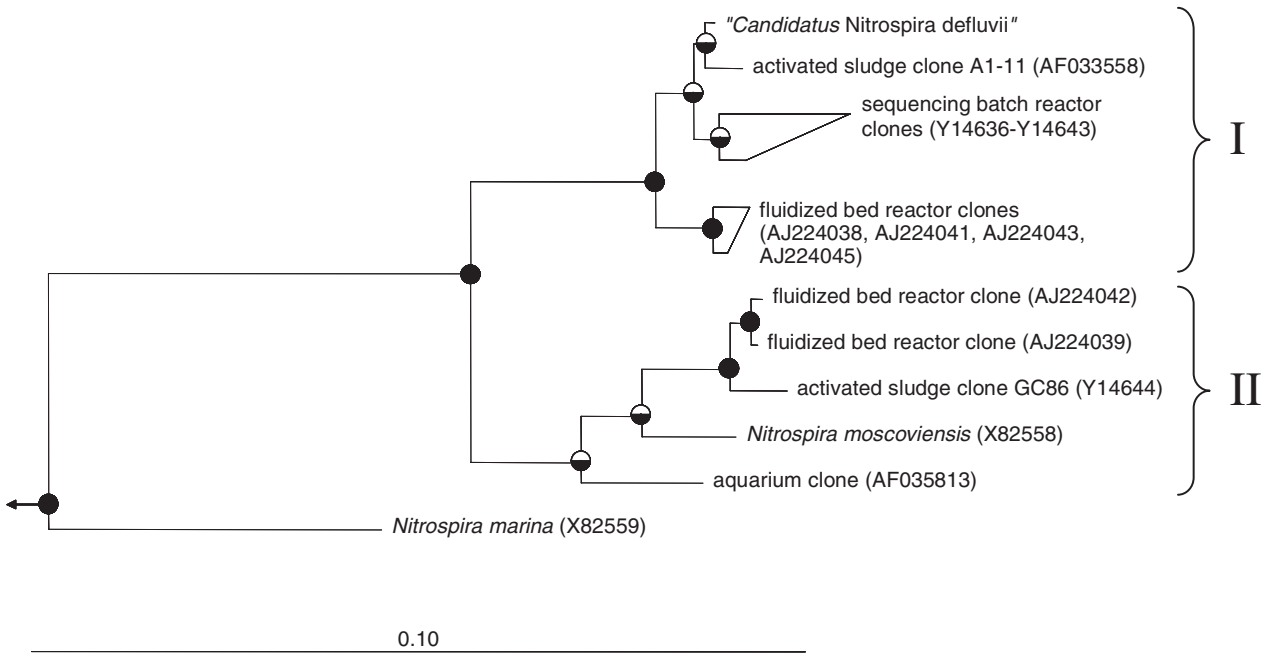


**Fig. 4.** Phylogenetic tree based on maximum likelihood analysis of the 16S rRNA sequences of '*Candidatus* Nitrospira defluvii', of the two described species *N. moscoviensis* and *N. marina*, and of some uncultured *Nitrospira*-like bacteria detected in nitrifying bioreactors and aquarium filters in earlier studies. All analysed sequences are longer than 1400 nucleotides. Black semicircles on tree nodes symbolize high support by quartet puzzling reliability values ≥ 70%. Black full circles indicate additional high parsimony bootstrap support (≥ 90%) based on 100 iterations. The brackets delimit main sublineages (numbered I and II) of the genus *Nitrospira*. The scale bar indicates 0.1 estimated change per nucleotide.

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**Table 2.** Fatty acid profiles of the enriched *Nitrospira*-like bacteria.

| Date of culture | November 1999 | May 2001 | October 2004 |
|---|---|---|---|
| Fatty acids (%) | | | |
| 8:0 | | | 0.5 |
| 12:0 | 2.5 | 1.0 | 0.8 |
| 14:0 | 8.5 | 12.1 | 7.4 |
| ECL[a] 14.429 | | 1.0 | |
| 15:0 iso | 4.7 | 5.9 | 2.1 |
| 15:0 anteiso | | 2.3 | 0.8 |
| 15:0 | | 0.5 | 0.4 |
| 14:0 3OH | | | 0.7 |
| 16:0 iso | | 1.2 | 1.1 |
| 16:1 cis 7 | | 2.9 | 3.0 |
| 16:1 cis 9 | 3.3 | 2.1 | 1.3 |
| 16:1 cis 11 | 14.2 | 33.6 | 42.2 |
| 16:0 | 18.1 | 16.9 | 15.8 |
| 15:0 iso 3OH | | 1.0 | |
| 16:0 10-methyl | | | 0.6 |
| 17:0 anteiso | | 0.5 | 0.4 |
| 17:0 cyclo 9–10 | | 6.6 | 6.7 |
| 17:0 | | | 0.5 |
| ECL[a] 17.122 | | 1.0 | |
| 16:0 3OH | 3.7 | 2.9 | 2.6 |
| ECL[a] 17.567 | | | 1.9 |
| 18:1 cis 9 | | 0.5 | 1.4 |
| 18:1 cis 11 | 34.5 | 1.4 | 5.1 |
| 18:0 | 3.3 | 0.7 | 3.0 |
| 18:1 cis 11 11-methyl | 2.5 | | |
| 17:0 iso 3OH | | 1.8 | |
| ECL[a] 18.762 | | 1.2 | |
| 19:0 cyclo 11–12 | 4.8 | 0.9 | 1.8 |
| ECL[a] 19.685 | | 1.4 | |

**a.** For unknown fatty acids the equivalent chain length (ECL) is given. Culture volume, 1.5 l.

has major amounts of 7-*cis*-hexadecenoic acid, which was present only in small amounts in the enrichment cultures derived from activated sludge. These data confirmed the results of the 16S rRNA sequence analyses that the *Nitrospira*-like cells are related to but not identical with the known species of the genus *Nitrospira*. Minor amounts of fatty acids so far not assigned to the genus *Nitrospira* indicated the presence of several groups of accompanying microorganisms. These are iso/anteiso-branched-chain fatty acids and cyclopropyl lipids, which when summed together account for about 12% of probably non-*Nitrospira*-related lipids in the enrichment culture from 2004.

## Discussion

This work describes the first selective enrichment of a member of *Nitrospira* sublineage I, which is of major importance for nitrogen removal in wastewater treatment plants but does not contain cultured representatives. The abundance of these organisms in nitrifying wastewater treatment plants usually amounts for 1–10% of the total bacterial population (Daims *et al*., 2001b; Juretschko *et al*., 2002).

The activated sludge biomass was diluted in Erlenmeyer flasks in order to reduce the density of heterotrophic bacteria and organic material. Preliminary experiments have shown that growth of *Nitrospira* derived from activated sludge was enhanced in Erlenmeyer flasks supplying a large surface for the medium that facilitates oxygen diffusion in comparison with tubes. Low nitrite concentrations in the mineral medium and regular dilutions of the cultures effectively promoted *Nitrospira* during the enrichment process. This was important as the fatty acid profiles from the early stages of the enrichment suggested that *Nitrobacter* cells were also present. Schramm and colleagues (1999) postulated, based on microsensor and FISH data, that *Nitrospira*-like bacteria have a higher affinity for nitrite than *Nitrobacter.* Reactor experiments performed with different nitrite concentrations in the influent lend additional weight to this hypothesis (Wagner *et al*., 2002). Under the assumption that nitrite concentration is the major factor controlling the competition between *Nitrospira* and *Nitrobacter* and based on models of r- and K-selection in microbial ecology (Andrews and Harris, 1986), *Nitrospira*-like bacteria can thus be considered as K-strategists while *Nitrobacter* species are r-strategists, which depend on relatively high nitrite concentrations and will be outcompeted by *Nitrospira*-like bacteria if nitrite is the growth-limiting factor.

A newly discovered property of *Nitrospira*-like bacteria, which was exploited during the enrichment procedure, is their resistance to ampicillin applied in a concentration apparently harmful to *Nitrobacter* and contaminating heterotrophic bacteria. The higher resistance of *Nitrospira* against ampicillin might be explained by the formation of dense microcolonies embedded in EPS that serve as diffusion barrier for dissolved substances. Furthermore, Foley and Gilbert (1996) suggested that observed resistance of microbial biofilms against antibiotics could be due to a high binding capacity of charged molecules and degradation by extracellular enzymes.

The inhibition of heterotrophs was important for the enrichment even though the standard mineral medium did not contain any organic carbon source. Extracellular exopolymeric substances and soluble organic compounds produced by nitrifying bacteria are utilized by heterotrophic organisms (Rittmann *et al*., 1994; Kindaichi *et al*., 2004) and most likely served as carbon and energy sources for contaminating bacteria in the enrichments. Further separation of *Nitrospira*-like bacteria was achieved by Percoll density gradient centrifugation of stationary-phase cultures, which contained many planktonic *Nitrospira* cells (Fig. 3D). It is tempting to speculate that the switch from growth in clusters while nutrients are plentiful (Figs 2A and 3A) to a planktonic lifestyle when resources become limited (Fig. 3D) is an efficient strategy to escape adverse conditions and to colonize new habi-

tats. Variable cell morphology in adaptation to changing growth conditions may be a common feature of the genus *Nitrospira* as this phenomenon was also reported for *N. moscoviensis* (Ehrich *et al*., 1995) and *N. marina* (Watson *et al*., 1986).

The application of FISH combined with microautoradiography (FISH-MAR; Lee *et al*., 1999) on uncultured *Nitrospira*-like bacteria in nitrifying biofilm provided evidence that these NOB take up and assimilate pyruvate (Daims *et al*., 2001a). According to Watson and colleagues (1986) *N. marina* grows better in the presence of pyruvate than in pure nitrite mineral medium. Therefore, in order to achieve faster growth and higher yield of *Nitrospira*-like bacteria, some of the enrichments obtained in this study were supplied with small amounts of pyruvate (0.5 mM). Although this substrate stimulated growth of *Nitrospira*, the enhanced growth of heterotrophs proved the addition of pyruvate unsuitable for obtaining larger amounts of highly enriched *Nitrospira* biomass.

*Nitrospira*-like bacteria are fastidious and slow-growing organisms, which are easily overgrown by other bacteria under suboptimal conditions. Therefore, the cultures were monitored by molecular, chemotaxonomic and microscopical methods to determine which factors enhanced or inhibited the enrichment of *Nitrospira*. This strategy allowed selection of the best enrichments for the next steps of the procedure and led to the development of a protocol that can be used in future to enrich or even isolate other *Nitrospira*-like bacteria or similarly recalcitrant nitrifiers from environmental samples (Fig. 1). By the application of ampicillin further high enrichments of *Nitrospira*-like bacteria were obtained from several other habitats (E. Spieck, unpublished results). It is reasonable to believe that the accelerated collection of new members of these formerly 'uncultivated' bacteria will disclose increasing phylogenetic, physiological and ecological diversity.

Based on phylogenetic analyses of 16S rRNA gene sequences, the genus *Nitrospira* has been divided into four sublineages (Daims *et al*., 2001a). The genus hosts two described species, which belong to sublineage II (*N. moscoviensis*) and IV (*N. marina*) respectively. In contrast, the *Nitrospira*-like bacterium enriched in this study is a representative of sublineage I, which so far contained only 16S rRNA sequences of uncultured *Nitrospira*-like bacteria found in nitrifying wastewater treatment plants and bioreactors (Daims *et al*., 2001a). This organism is clearly different from *N. moscoviensis* and *N. marina* (Table 3). The similarity between the 16S rRNA sequences of these species and the enriched organism is 94.1% (*N. moscoviensis*) and 88.3% (*N. marina*). According to Stackebrandt and Goebel (1994), it is unlikely that two organisms with 16S rRNA sequence similarity below 97% have more than 70% DNA similarity and belong to the same species. Furthermore, Keswani and Whitman (2001) showed that even if two organisms have as much as 98.6% 16S rRNA sequence similarity there is still a probability of 99% that they represent different species. Phenotypically, the *Nitrospira*-like culture can be differentiated from the two representatives of sublineage II and IV by its unique lipid pattern dominated by 11-*cis*-hexadecenoic acid and not by 11-methyl-hexadecanoic acid and 7-*cis*-hexadecenoic acid respectively. Based on the results of this study we propose, according to Murray and Schleifer (1994) and Murray and Stackebrandt (1995), provisional classification of the novel *Nitrospira*-like bacterium as '*Candidatus* Nitrospira defluvii'. The short description of '*Candidatus* Nitrospira defluvii' is as follows: phylogenetically related to the genus *Nitrospira*; not isolated; Gram-negative; major fatty acids are 11-*cis*-hexadecenoic acid and hexadeacanoic acid; short curved rods in microcolonies with a diameter of 0.5–1.2 μm or long spiral-shaped rods with one to four turns, 0.2–0.4 μm in width and length of 0.7–1.7 μm; basis of assignment, 16S rRNA gene sequences (Accession No. DQ059545) and detection by the genus-specific oligonucleotide probe complementary to helix 23 of the 16S rRNA S-G-Ntspa-0662-a-A-18 (5′-AGAGGAGCG CGGAAUUCC-3′); cell aggregates or free-living plank-

**Table 3.** Comparable characteristics of '*Candidatus* Nitrospira defluvii' enriched in this study, *N. moscoviensis* and *N. marina*.

| | '*Candidatus* Nitrospira defluvii' | *N. moscoviensis* | *N. marina* |
|---|---|---|---|
| Cell morphology | Short, slightly curved cells or spiral-shaped rods | Irregularly shaped cells or spiral-shaped rods | 'Comma-shaped' cells or spiral-shaped rods |
| Size | 0.2–0.4 μm × 0.7–1.7 μm | 0.2–0.4 μm × 0.9–2.2 μm | 0.3–0.4 μm × 0.8–1.0 μm |
| Turns | 1–4 | 1–3 | 1–12 |
| Tendency to aggregate | Strong | Present | Weak |
| Temperature for cultivation | 28°C | 37°C | 28°C |
| Medium | Non-marine | Non-marine | Marine |
| Phylogenetic affiliation in genus *Nitrospira* | Sublineage I | Sublineage II | Sublineage IV |
| Dominant lipids | 16:1 cis 11<br>16:0 | 16:1 cis 11<br>16:0<br>16:0 11-methyl[a] | 16:1 cis 7<br>16:1 cis 11<br>16:0[a] |

**a.** As reported by Lipski and colleagues (2001).

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tonic cells; aerobic chemolithoautotrophic nitrite oxidizer, mesophilic; utilizes pyruvate (this study).

*Nitrospira*-like bacteria, and not members of the genus *Nitrobacter*, are mainly responsible for nitrite oxidation in most wastewater treatment systems and also in many natural habitats. As no pure cultures or high enrichments of the widespread sublineage I of this genus were available, our knowledge of the physiology of these organisms is scarce. The enrichment obtained in this study will make it possible to conduct encompassing physiological experiments that could not be performed with environmental samples containing lower amounts of *Nitrospira*-like bacteria, and it provides the biomass for an environmental genomics approach launched recently in order to investigate the genomic make-up and biology of these and related *Nitrospira*-like bacteria.

## Experimental procedures

### *Activated sludge samples*

The samples used as primary inoculum for the enrichment of *Nitrospira*-like bacteria were taken between November 1997 and December 1998 from the nitrification stage of the municipal wastewater treatment plant in Köhlbrandhöft/Dradenau, Hamburg, Germany ($1.8 \times 10^6$ population equivalents). The activated sludge had a pH of 7.7–8.1 and a temperature of 18–20°C. The chemical demand for oxygen was in the range of 295–325 mg $ml^{-1}$.

### *Chemical analyses*

Concentrations of ammonium were measured by high-performance liquid chromatography (HPLC) according to Corbin (1984). Nitrite and nitrate concentrations were determined by ion-pair chromatography with a Hypersil ODS C18 column ($125 \times 4.6$ mm) (Meincke *et al*., 1992) followed by UV detection in an automated system (Kontron, Eching, Germany).

### *Quantification of AOB and NOB*

Most-probable-number (MPN) analyses were performed as described by Mansch and Bock (1998) to estimate the abundance of culturable AOB and NOB in activated sludge samples. Aliquots of sludge containing nitrifying bacteria were sonicated for 3 min and dilution series were prepared in 0.9% NaCl up to $10^{-7}$. Three replicates per dilution were inoculated with 0.1 ml aliquots using 10 ml glass tubes containing nitrite mineral medium (Ehrich *et al*., 1995) with a final volume of 5 ml per tube. The nitrite concentration in the medium was adjusted to 10.0, 3.0 or 0.3 mM in parallel dilution series. For AOB mineral medium according to Krümmel and Harms (1982) with 10 mM $NH_4Cl$ was prepared. The diluted samples were incubated for 6 weeks without agitation in the dark at 28°C. Formation or consumption of nitrite detected by the Griess–Ilosavay spot test (Schmidt and Belser, 1982) indicated growth of AOB and NOB respectively. Statistical calculations for the estimation of the MPN were performed in accordance to Alexander (1982).

### *Determination of nitrification activity*

In order to measure the turnover of ammonia and nitrite, 5 ml of activated sludge was transferred into 45 ml of mineral medium amended with 5 mM $NH_4Cl$ or 5 mM $NaNO_2$ respectively. The sludge samples were protected from light and incubated under agitation (120 r.p.m.) at 28°C. The consumption of ammonia and nitrite was monitored during a period of 6 h.

### *Enrichment of* Nitrospira*-like bacteria*

In contrast to the protocol followed by Bartosch and colleagues (1999), *Nitrospira*-like bacteria were enriched in pure mineral medium (Ehrich *et al*., 1995), which contained only 3 mM $NaNO_2$ as energy source and no organic carbon in order to suppress growth of heterotrophic bacteria. The enrichment was started in 100 ml Erlenmeyer flasks filled with 50 ml of the medium and 1% (v/v) activated sludge. The flasks were incubated at 28°C in the dark without agitation. Nitrite consumption was frequently measured to identify cultures containing actively growing NOB. These enrichments were supplied with sterile nitrite solution every 3–5 days to a final concentration of 3 mM $NaNO_2$ respectively. After approximately 6 weeks, 1.5 ml aliquots of the enrichments were transferred into 300 ml flasks filled with 150 ml of fresh nitrite medium. Three different antibiotics were added to the enrichments in order to suppress growth of nitrite-oxidizing *Nitrobacter* cells and heterotrophic bacteria. Filter-sterilized stock solutions of ampicillin, tetracycline or streptomycin were added to the medium to final concentrations of 50–200 μg $ml^{-1}$ (ampicillin), 10–25 μg $ml^{-1}$ (tetracycline) and 50–100 μg $ml^{-1}$ (streptomycin). Cultures with a high cell density were grown in 3 l flasks and were used to separate *Nitrospira*-like bacteria from contaminants by Percoll density gradient centrifugation and subsequent serial dilutions as described by Ehrich and colleagues (1995).

### *Electron microscopy*

For observation by electron microscopy, cells were fixed with 2.5% (v/v) glutaraldehyde and 2% (w/v) osmium tetroxide and embedded in Epon 812 (Serva) according to a previously published protocol (Ehrich *et al*., 1995). The embedded samples were sectioned, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined in a transmission electron microscope (Model 420, Philips). Cell morphology was visualized by staining the bacteria with uranyl acetate as described by Spieck and colleagues (1996).

### *Fluorescence* in situ *hybridization*

Aliquots of the enrichments were prepared for FISH by paraformaldehyde fixation as described by Amann (1995). Fixed biomass was spotted onto microscope slides and FISH was performed according to the protocol detailed by Manz

and colleagues (1992) and Daims and colleagues (2005). The applied oligonucleotide probes were S-G-Ntspa-0662-a-A-18 specific for the genus *Nitrospira* (Daims *et al*., 2001a), S-*-Ntspa-0712-a-A-21 targeting the phylum *Nitrospira* (Daims *et al*., 2001a), S-G-Nbac-1035-a-A-18 specific for the genus *Nitrobacter* (= NIT3; Wagner *et al*., 1996), and the EUB probe mix that detects most known *Bacteria* (Amann *et al*., 1990; Daims *et al*., 1999). The probes were 5′ labelled with either the dye FLUOS [5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester] or the sulfoindocyanine dye Cy3. The *Nitrospira*- and *Nitrobacter*-specific probes were used together with their competitor oligonucleotides as described by Daims and colleagues (2001a) and Wagner and colleagues (1996) respectively. Labelled probes and unlabelled competitors were obtained from Thermo Hybaid (Interactiva Division, Ulm, Germany). Probe-conferred fluorescence was recorded with a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Oberkochen, Germany) by using an Ar ion laser to detect FLUOS (488 nm excitation wavelength) and an HeNe laser (543 nm) to detect Cy3 respectively.

### *Quantification of* Nitrospira

The fraction of *Nitrospira*-like bacteria in the enrichments, based on the total bacterial population (autotrophs plus heterotrophs), was determined as relative biovolume by quantitative FISH, confocal laser scanning microscopy and digital image analysis according to a protocol described elsewhere (Schmid *et al*., 2000) and by using DAIME, an image analysis program developed at the University of Vienna.

### *Cloning and phylogenetic analysis of 16S rRNA genes*

Bacterial 16S rRNA genes were PCR amplified, cloned and sequenced as described by Juretschko and colleagues (1998) 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. The obtained sequences were aligned and phylogenetically analysed by using the ARB program (Ludwig *et al*., 2004) according to Daims and colleagues (2001a).

### *Fatty acid profiles*

Biomass for fatty acid extraction was obtained from large-scale enrichments grown in 1.5 l of medium in 3 l Erlenmeyer flasks. Cultures were incubated without stirring for 2–3 months and regularly fed with sterile nitrite solution. When dense flocs of *Nitrospira*-like bacteria had developed, cells were harvested by centrifugation, washed in 0.9% (w/v) NaCl and stored at −20°C. Saponification of the cell pellets with 15% NaOH in 50% methanol, acid methylation with 6 N HCl in 50% methanol and extraction of fatty acid methyl esters (FAMEs) were performed as described by Sasser (1990). The FAME extracts were analysed using a Hewlett–Packard model 6890 gas chromatograph equipped with a 5% phenyl methyl silicone capillary column and a flame ionization detector. The identity of fatty acids was verified by GC-MS with 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. The chromatographic conditions were used as described previously (Lipski and Altendorf, 1997). The positions of hydroxy, methyl, cyclopropene groups and double bonds were given from the carboxyl group of the fatty acid molecule according to the recommendations of the IUPAC-IUB Commission on biochemical nomenclature (CBN) 1977.

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# 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. Daims[3] and E. Spieck[2]

Correspondence
E. Spieck
spieck@mikrobiologie.uni-hamburg.de

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

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

[3]Department 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 *Nitrospina* 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* (Garrity & 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 defluvii' (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).

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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 | *Nitrospira*-like cells + *Nitrobacter*-like cells + different contaminants |
| | | | 16S rRNA partial gene sequence | '*Candidatus* Nitrospira bockiana' + *Nitrospira moscoviensis*-like |
| | | | FISH | '*Candidatus* Nitrospira bockiana' + *N. moscoviensis*-like |
| | | | Purity test in complex medium | Several types of contaminants |
| Incubation at elevated temperature | Enrichment 4 | 45 °C | Electron microscopy, ultrathin sections | *Nitrospira*-like cells (no *Nitrobacter*) |
| | | | FISH | '*Candidatus* Nitrospira bockiana' (no *N. moscoviensis*-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: *Nitrospira*-like + Gram-positive contaminant |
| | | | DGGE with subsequent 16S rRNA partial gene sequence | 2 bands corresponding to '*Candidatus* Nitrospira bockiana' and *Nocardioides* 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 *Nitrospira* cells |
| | | | DGGE with subsequent 16S rRNA partial gene sequence | Only one band, corresponding to '*Candidatus* Nitrospira bockiana' |
| | | | FISH | '*Candidatus* Nitrospira bockiana' - no *N. moscoviensis*-like |
| | | | Purity test in complex medium | No contaminant |

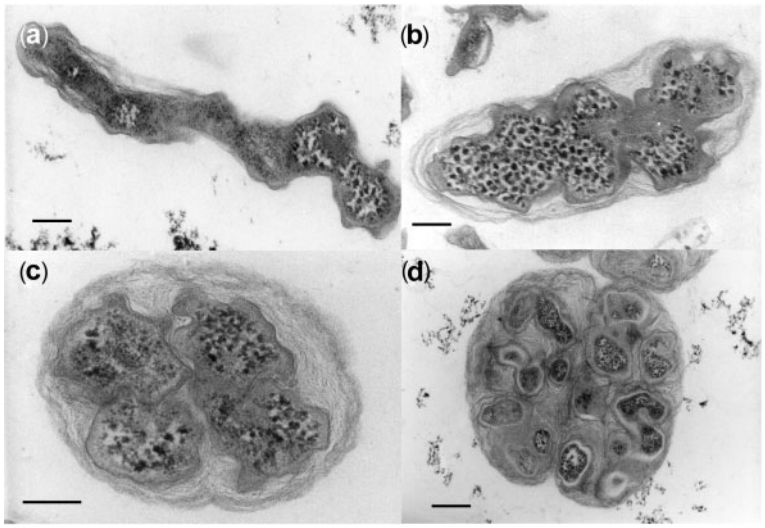


**Fig. 1.** Electron micrographs of ultrathin sections of cells of '*Candidatus* Nitrospira bockiana' derived from the Moscow heating system (enrichment 3) showing the different stages in the formation of microcolonies. (a) Single planktonic cell. (b) Secretion of EPS accompanied by a decrease in cell 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

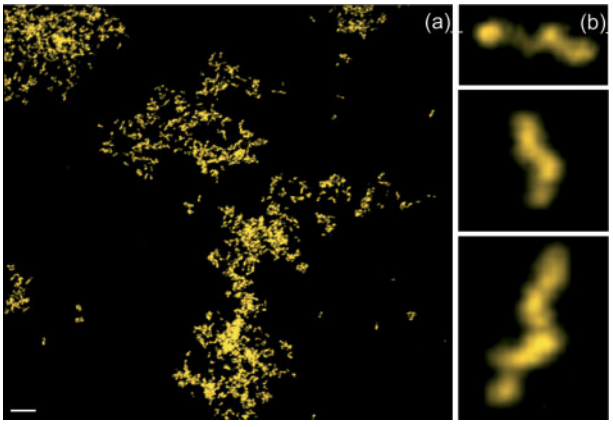


**Fig. 2.** (a) Detection of '*Candidatus* Nitrospira bockiana' in the final isolate by FISH with probes S-G-Ntspa-0662-a-A-18 (red) and the EUB338 probe mix (green). *Nitrospira* cells appear yellow because of hybridization to all probes. Note the absence of any other bacteria. The contrast of this 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), 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.

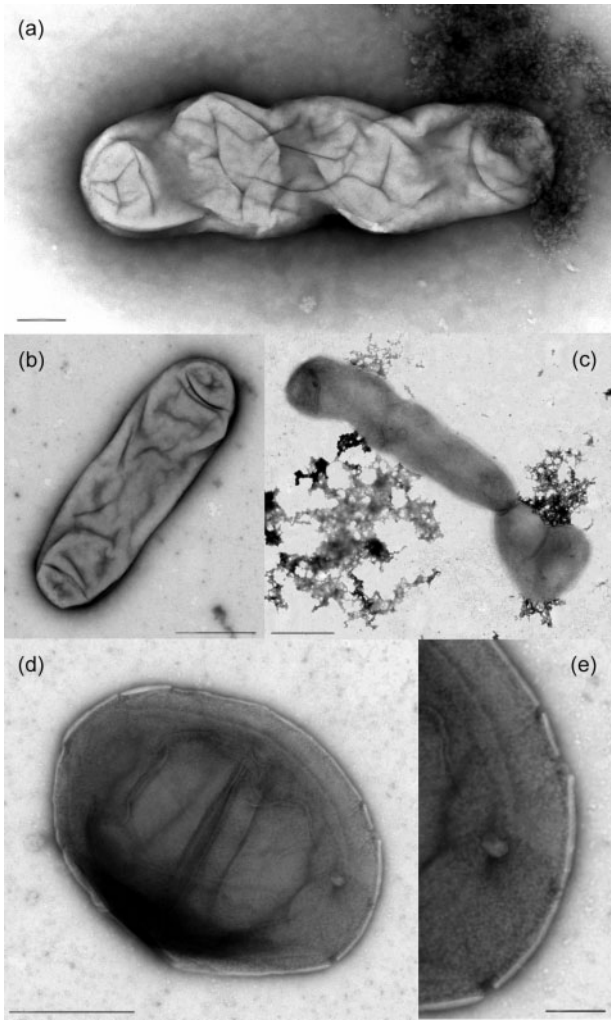


**Fig. 3.** Overall shape of '*Candidatus* Nitrospira bockiana', stained with uranyl acetate. (a) Spiral rod; (b) long straight rod; (c) unequal fission; (d) coccoid cell; (e) section of (d) showing membrane-bound particles in detail. Bars, 0.2 μm (a); 0.5 μm (b–d); 0.1 μm (e).

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

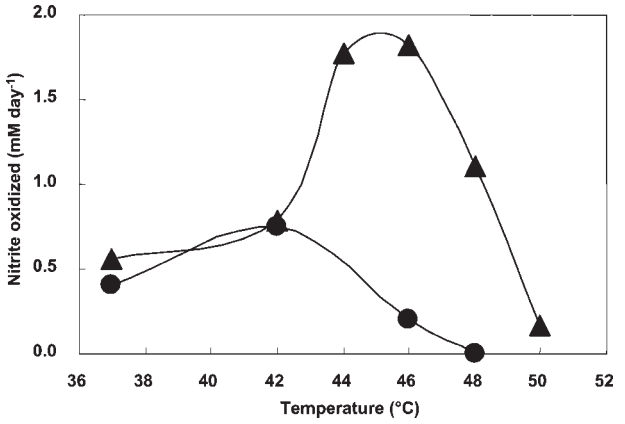


**Fig. 4.** Temperature optima for nitrite consumption by enrichment 5 and '*Candidatus* Nitrospira bockiana'. ▲, enrichment 5; inoculum was taken from a pre-culture grown at 45 °C. Nitrite consumption was measured between day 3 and 4 with 5 mM as substrate concentration. ●, final isolate; inoculum was 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 *Nocardiodaceae*, a member of the phylum *Actinomycetales* (Yoon *et al.*, 2005). The *Nocardioides* sp. was Gram-positive, aerobic, rod-shaped (0.4–0.7 × 0.5–1.3 μm) or coccoid (0.7 μm 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) | 0.3–0.6 × 1.0–2.5 or 0.9 × 0.9 | 0.2–0.4 × 0.7–1.7 | 0.2–0.4 × 0.9–2.2 | 0.3–0.4 × 0.8–1.0 |
| 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 *Nitrospira* | Novel sublineage | Sublineage I | Sublineage II | Sublineage IV |
| Dominant lipids | 16 : 1*cis*7, 16 : 0, 16 : 0 11 methyl | 16 : 1*cis*11, 16 : 0 | 16 : 1*cis*11, 16 : 0, 16 : 0 11 methyl | 16 : 1*cis*7, 16 : 1*cis*11, 16 : 0 |
| Tolerance against nitrite (mM) | 26–30*, 18† | 20–25 | 15 | 6 |

*Enrichment 5.
†Final isolate.

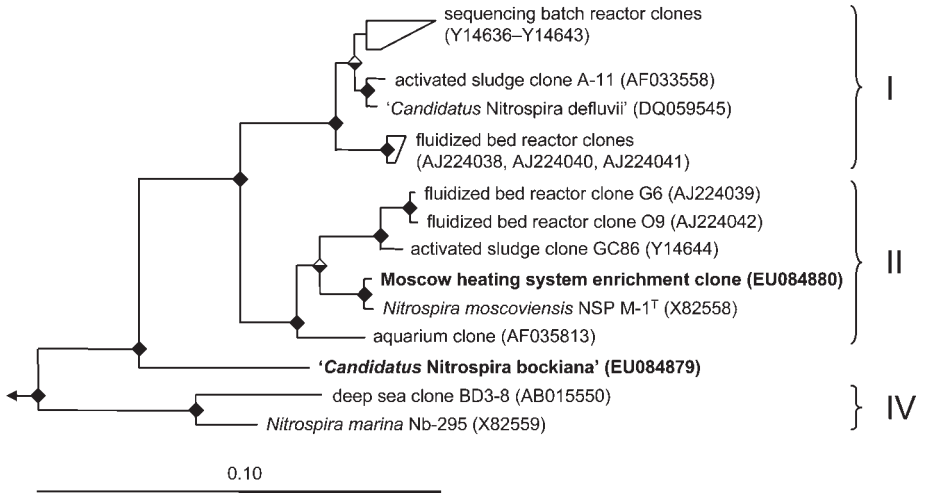


**Fig. 5.** Phylogenetic affiliation of '*Candidatus* Nitrospira bockiana'. The phylogenetic tree is based on 16S rRNA genes of selected *Nitrospira*-like bacteria. Sequences of '*Candidatus* Nitrospira bockiana' and of another *Nitrospira*-like bacterium, which was found only in the early enrichment, 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 $\geqslant$70 %. Filled diamonds symbolize additional high parsimony bootstrap support ($\geqslant$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* amd *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 *Nocardioides* 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 *Nocardioides* 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 inequal 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*cis*7. Neither pyruvate nor yeast extract stimulates nitrite oxidation. Organotrophic growth is not observed.

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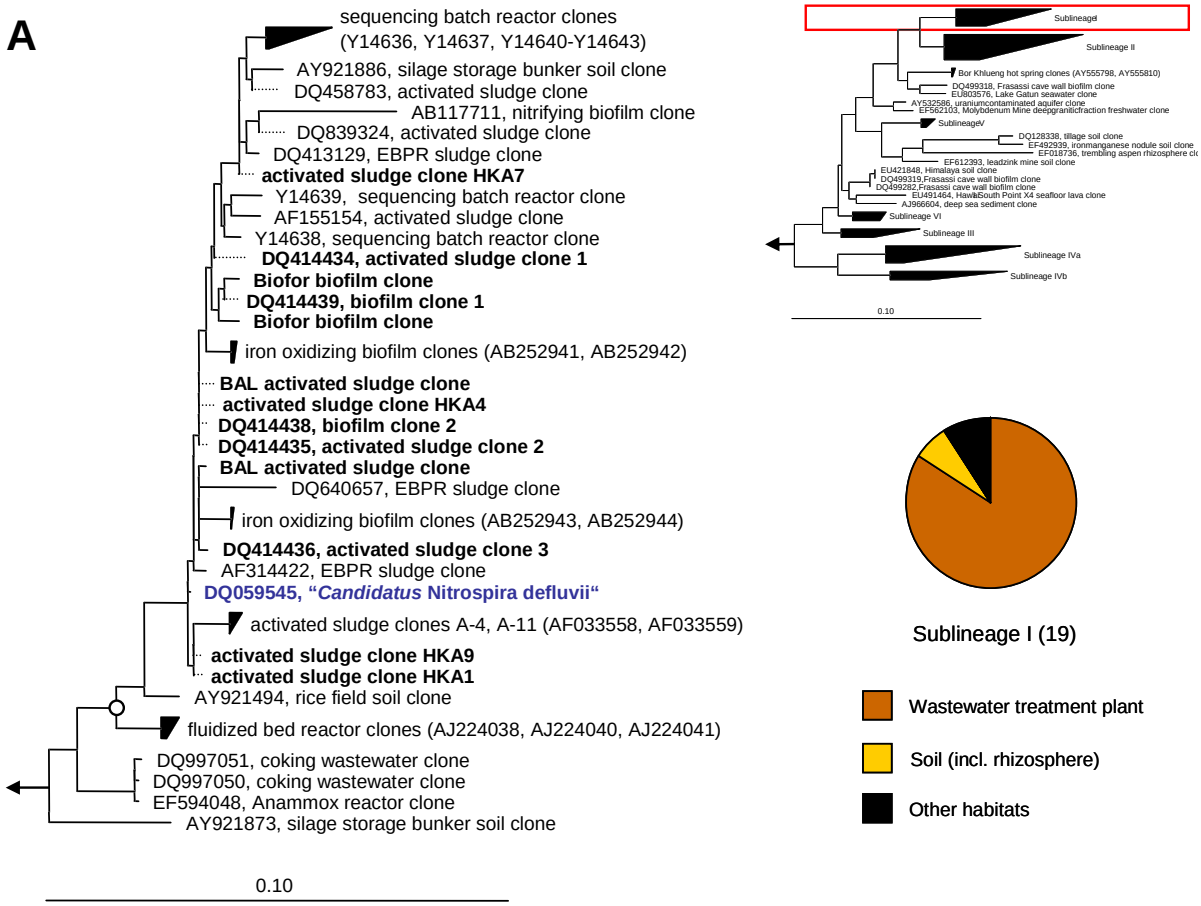
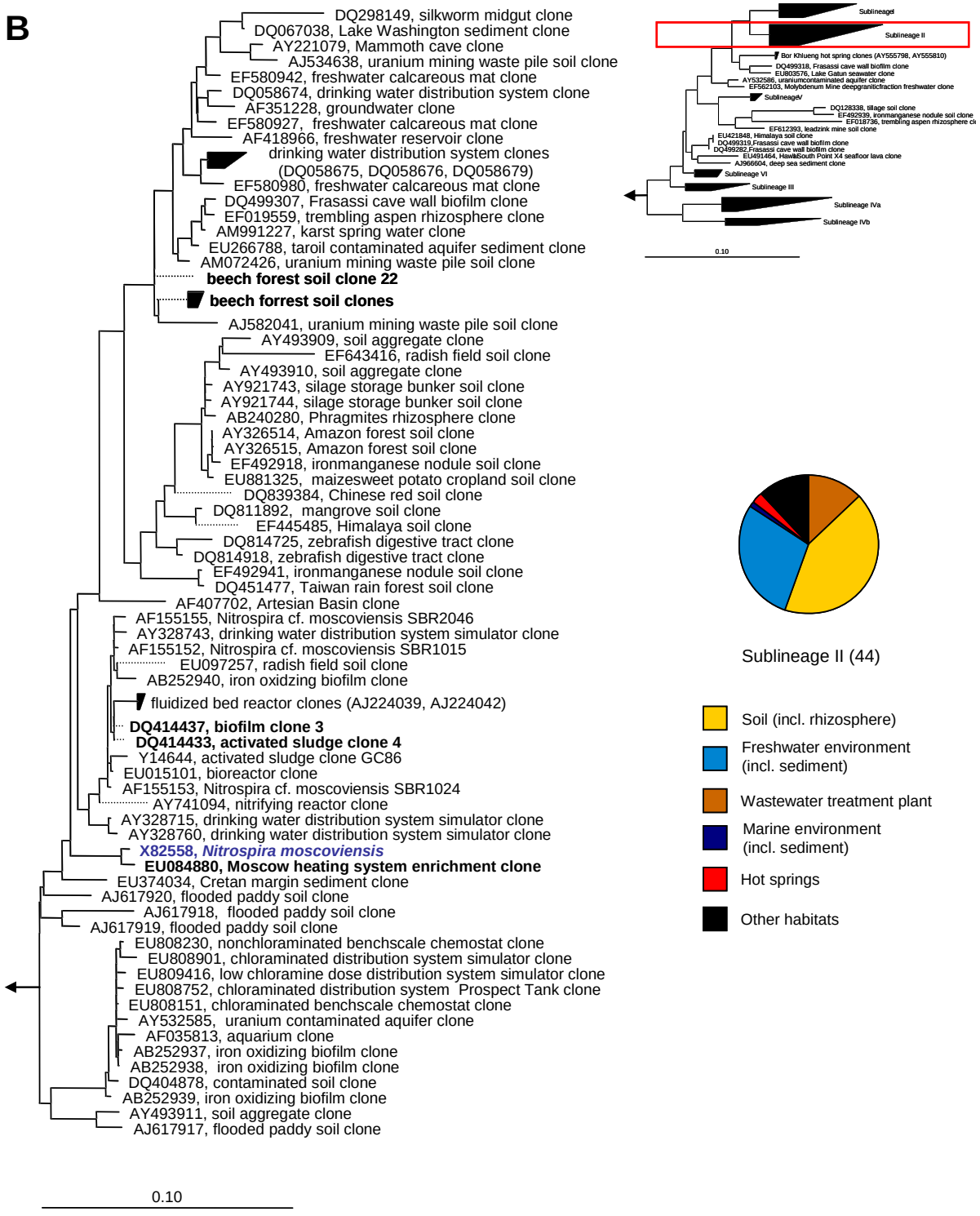
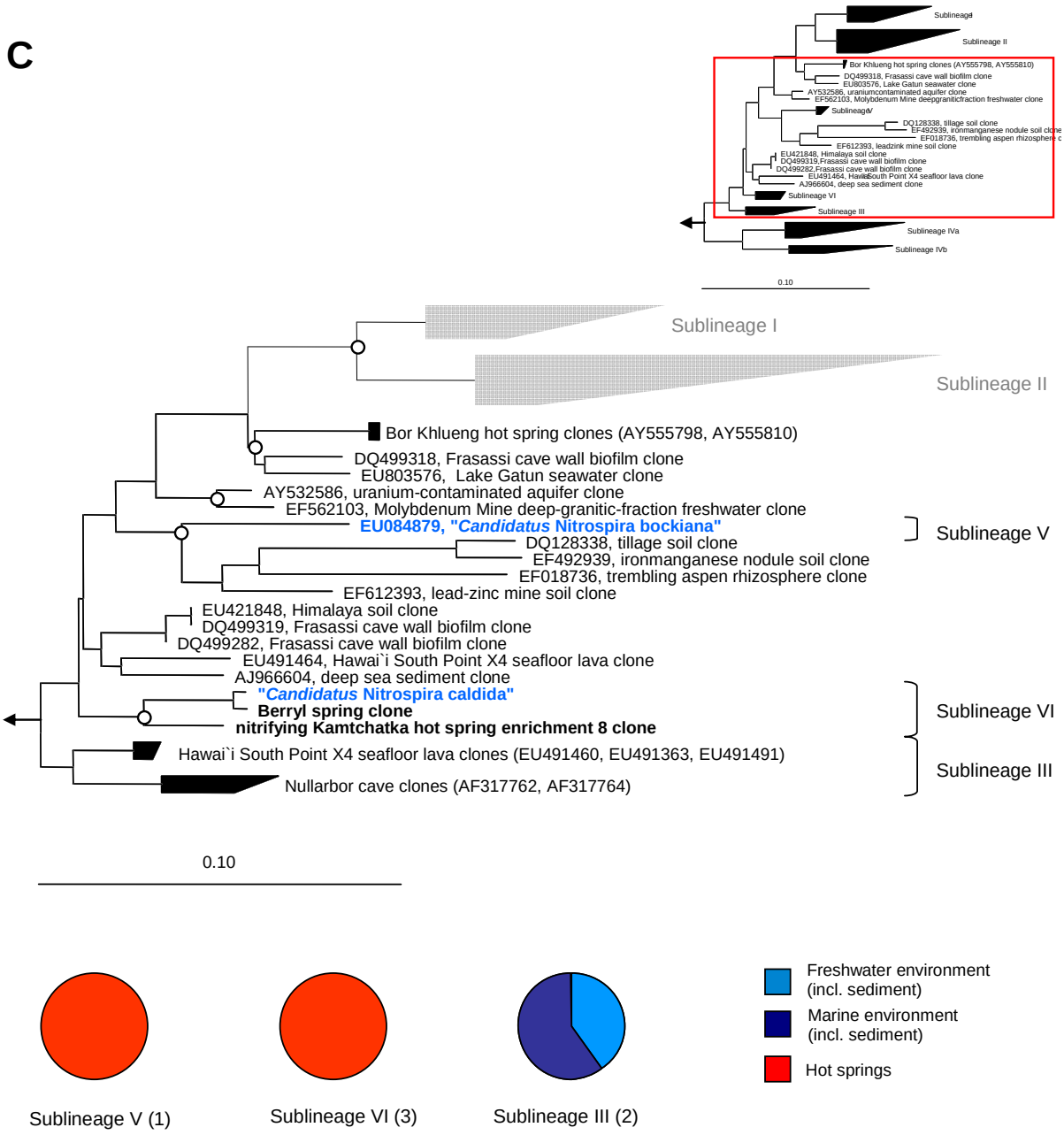
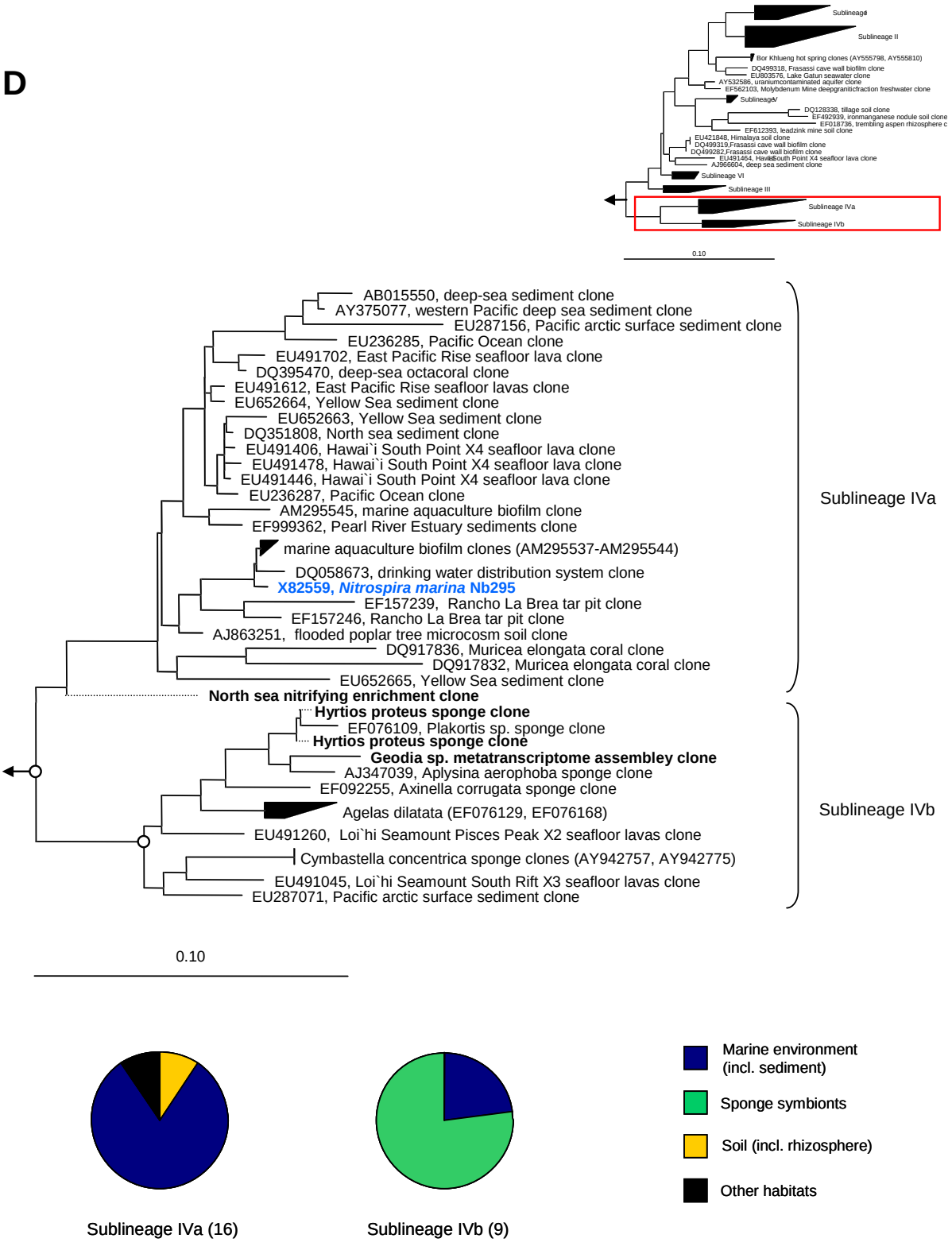



**Appendix - figure 1:** Phylogenetic tree (subdivided into parts A-D) based on 16S rRNA sequences of selected *Nitrospira*-like bacteria displaying their affiliation to different sublineages in the genus *Nitrospira*. Sequences obtained in this study are printed bold. So far enriched and isolated *Nitrospira*-like bacteria are highlighted in blue. The subdivided tree-parts shown in A-D give a detailed view on the phylogeny within the genus Nitrospira, which is already shown in general in figure 5 in the general introduction. The sublineage currently displayed in detail is highlighted with a red frame in the overview-tree in the upper right corner. The tree was calculated using the Maximum Likelihood algorithm implemented in the ARB software (Ludwig *et al.*, 2004) with a 50% conservation filter for the genus *Nitrospira*. The basic tree toplogy was determined with sequences longer than 1300 nucleotides. Shorter Sequences, between 1000 to 1300 nucleotides in length, were added with the ARB_Parsimony tool of the ARB software package without changing the overall tree topology. The branches leading to these shorter sequences are indicated with dotted lines. Beside the sequences originating from this thesis all other sequences derived from the SILVA rRNA database project (SSU database, version 96), using only non-chimeric sequences (Pintail_quality ≥ 50 %) with an alignment quality ≥ 50 % (Pruesse *et al.*, 2007). The partially filled circles at the tree nodes represent quartet puzzling reliability values ≥ 70 % and filled circles symbolize additional high parsimony bootstrap support (≥ 90 %) based on 100 iterations. The bar indicates 10% estimated sequence divergence.
Pie charts display the environmental distribution pattern of *Nitrospira*-sublineages. For each sublineage, the origin (isolation source) of each 16S rRNA sequence belonging to this sublineage was determined and subsequently assigned to one of the categories (e.g. wastewater treatment plant) listed below the pie chart. In the pie chart the proportion of each category for each sublineage is shown. The number of different sampling sites (studies) is given in parenthesis.


B
DQ298149, silkworm midgut clone
DQ067038, Lake Washington sediment clone
AY221079, Mammoth cave clone
AJ534638, uranium mining waste pile soil clone
EF580942, freshwater calcareous mat clone
DQ058674, drinking water distribution system clone
AF351228, groundwater clone
EF580927, freshwater calcareous mat clone
AF418966, freshwater reservoir clone
drinking water distribution system clones
(DQ058675, DQ058676, DQ058679)
EF580980, freshwater calcareous mat clone
DQ499307, Frasassi cave wall biofilm clone
EF019559, trembling aspen rhizosphere clone
AM991227, karst spring water clone
EU266788, taroil contaminated aquifer sediment clone
AM072426, uranium mining waste pile soil clone
beech forest soil clone 22
beech forrest soil clones
AJ582041, uranium mining waste pile soil clone
AY493909, soil aggregate clone
EF643416, radish field soil clone
AY493910, soil aggregate clone
AY921743, silage storage bunker soil clone
AY921744, silage storage bunker soil clone
AB240280, Phragmites rhizosphere clone
AY326514, Amazon forest soil clone
AY326515, Amazon forest soil clone
EF492918, ironmanganese nodule soil clone
EU881325, maizesweet potato cropland soil clone
DQ839384, Chinese red soil clone
DQ811892, mangrove soil clone
EF445485, Himalaya soil clone
DQ814725, zebrafish digestive tract clone
DQ814918, zebrafish digestive tract clone
EF492941, ironmanganese nodule soil clone
DQ451477, Taiwan rain forest soil clone
AF407702, Artesian Basin clone
AF155155, Nitrospira cf. moscoviensis SBR2046
AY328743, drinking water distribution system simulator clone
AF155152, Nitrospira cf. moscoviensis SBR1015
EU097257, radish field soil clone
AB252940, iron oxidzing biofilm clone
fluidized bed reactor clones (AJ224039, AJ224042)
DQ414437, biofilm clone 3
DQ414433, activated sludge clone 4
Y14644, activated sludge clone GC86
EU015101, bioreactor clone
AF155153, Nitrospira cf. moscoviensis SBR1024
AY741094, nitrifying reactor clone
AY328715, drinking water distribution system simulator clone
AY328760, drinking water distribution system simulator clone
X82558, Nitrospira moscoviensis
EU084880, Moscow heating system enrichment clone
EU374034, Cretan margin sediment clone
AJ617920, flooded paddy soil clone
AJ617918, flooded paddy soil clone
AJ617919, flooded paddy soil clone
EU808230, nonchloraminated benchscale chemostat clone
EU808901, chloraminated distribution system simulator clone
EU809416, low chloramine dose distribution system simulator clone
EU808752, chloraminated distribution system Prospect Tank clone
EU808151, chloraminated benchscale chemostat clone
AY532585, uranium contaminated aquifer clone
AF035813, aquarium clone
AB252937, iron oxidizing biofilm clone
AB252938, iron oxidizing biofilm clone
DQ404878, contaminated soil clone
AB252939, iron oxidizing biofilm clone
AY493911, soil aggregate clone
AJ617917, flooded paddy soil clone
0.10
Sublineage I
Sublineage II
Bor Khlueng hot spring clones (AY555798, AY555810)
DQ499318, Frasassi cave wall biofilm clone
EU803576, Lake Gatun seawater clone
AY532586, uranium contaminated aquifer clone
EF562103, Molybdenum Mine deepgraniticfraction freshwater clone
Sublineage V
DQ128338, tillage soil clone
EF492939, ironmanganese nodule soil clone
EF018736, trembling aspen rhizosphere clone
EF612393, leadzinc mine soil clone
EU421848, Himalaya soil clone
DQ499319,Frasassi cave wall biofilm clone
DQ499282,Frasassi cave wall biofilm clone
EU491464, HawaiiSouth Point X4 seafloor lava clone
AJ966604, deep sea sediment clone
Sublineage VI
Sublineage III
Sublineage IVa
Sublineage IVb
0.10
Sublineage II (44)
Soil (incl. rhizosphere)
Freshwater environment (incl. sediment)
Wastewater treatment plant
Marine environment (incl. sediment)
Hot springs
Other habitats


C
Sublineage I
Sublineage II
Bor Khlueng hot spring clones (AY555798, AY555810)
DQ499318, Frasassi cave wall biofilm clone
EU803576, Lake Gatun seawater clone
AY532586, uranium-contaminated aquifer clone
EF562103, Molybdenum Mine deep-granitic-fraction freshwater clone
EU084879, "Candidatus Nitrospira bockiana"
DQ128338, tillage soil clone
EF492939, ironmanganese nodule soil clone
EF018736, trembling aspen rhizosphere clone
EF612393, lead-zinc mine soil clone
EU421848, Himalaya soil clone
DQ499319, Frasassi cave wall biofilm clone
DQ499282, Frasassi cave wall biofilm clone
EU491464, Hawai`i South Point X4 seafloor lava clone
AJ966604, deep sea sediment clone
"Candidatus Nitrospira caldida"
Berryl spring clone
nitrifying Kamtchatka hot spring enrichment 8 clone
Hawai`i South Point X4 seafloor lava clones (EU491460, EU491363, EU491491)
Nullarbor cave clones (AF317762, AF317764)
Sublineage V
Sublineage VI
Sublineage III
0.10
Sublineage V (1)
Sublineage VI (3)
Sublineage III (2)
Freshwater environment (incl. sediment)
Marine environment (incl. sediment)
Hot springs


D
Sublineage I
Sublineage II
Bor Khlueng hot spring clones (AY555798, AY555810)
DQ499318, Frasassi cave wall biofilm clone
EU803576, Lake Gatun seawater clone
AY532586, uraniumcontaminated aquifer clone
EF562103, Molybdenum Mine deepgranitic fraction freshwater clone
Sublineage V
DQ128338, tillage soil clone
EF492939, ironmanganese nodule soil clone
EF018736, trembling aspen rhizosphere c
EF612393, leadzink mine soil clone
EU421848, Himalaya soil clone
DQ499319,Frasassi cave wall biofilm clone
DQ499282,Frasassi cave wall biofilm clone
EU491464, Hawai`i South Point X4 seafloor lava clone
AJ966604, deep sea sediment clone
Sublineage VI
Sublineage III
Sublineage IVa
Sublineage IVb
0.10
AB015550, deep-sea sediment clone
AY375077, western Pacific deep sea sediment clone
EU287156, Pacific arctic surface sediment clone
EU236285, Pacific Ocean clone
EU491702, East Pacific Rise seafloor lava clone
DQ395470, deep-sea octacoral clone
EU491612, East Pacific Rise seafloor lavas clone
EU652664, Yellow Sea sediment clone
EU652663, Yellow Sea sediment clone
DQ351808, North sea sediment clone
EU491406, Hawai`i South Point X4 seafloor lava clone
EU491478, Hawai`i South Point X4 seafloor lava clone
EU491446, Hawai`i South Point X4 seafloor lava clone
EU236287, Pacific Ocean clone
AM295545, marine aquaculture biofilm clone
EF999362, Pearl River Estuary sediments clone
marine aquaculture biofilm clones (AM295537-AM295544)
DQ058673, drinking water distribution system clone
X82559, Nitrospira marina Nb295
EF157239, Rancho La Brea tar pit clone
EF157246, Rancho La Brea tar pit clone
AJ863251, flooded poplar tree microcosm soil clone
DQ917836, Muricea elongata coral clone
DQ917832, Muricea elongata coral clone
EU652665, Yellow Sea sediment clone
North sea nitrifying enrichment clone
Hyrtios proteus sponge clone
EF076109, Plakortis sp. sponge clone
Hyrtios proteus sponge clone
Geodia sp. metatranscriptome assembley clone
AJ347039, Aplysina aerophoba sponge clone
EF092255, Axinella corrugata sponge clone
Agelas dilatata (EF076129, EF076168)
EU491260, Loi`hi Seamount Pisces Peak X2 seafloor lavas clone
Cymbastella concentrica sponge clones (AY942757, AY942775)
EU491045, Loi`hi Seamount South Rift X3 seafloor lavas clone
EU287071, Pacific arctic surface sediment clone
Sublineage IVa
Sublineage IVb
0.10
Marine environment (incl. sediment)
Sponge symbionts
Soil (incl. rhizosphere)
Other habitats
Sublineage IVa (16)
Sublineage IVb (9)

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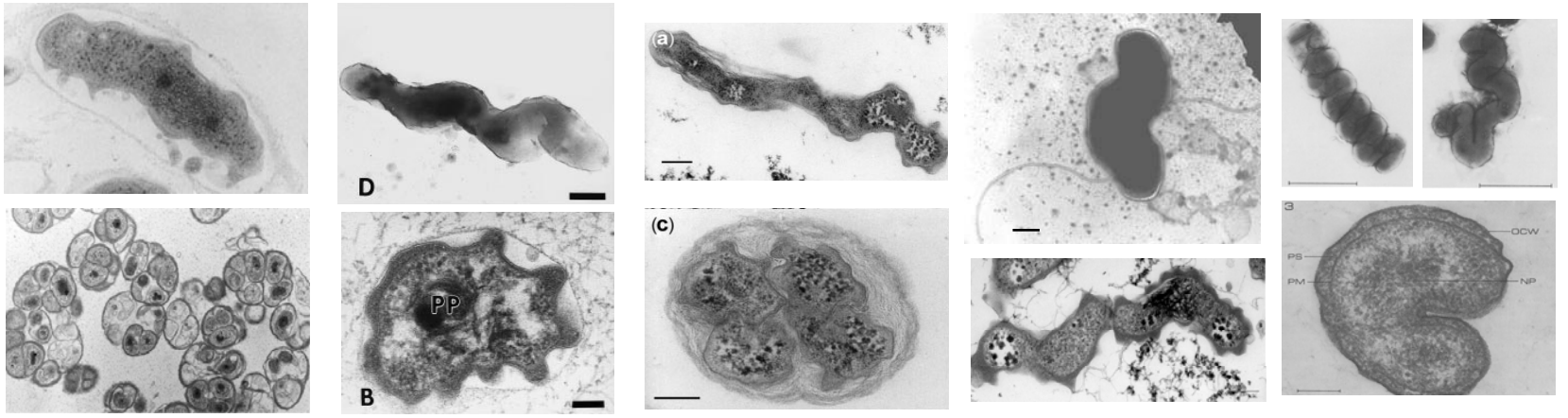
| | ***'Candidatus* Nitrospira defluvii'** | ***Nitrospira moscoviensis*** | ***'Candidatus* Nitrospira bockiana'** | ***'Candidatus* Nitrospira calida'** | ***Nitrospira marina*** |
|---|---|---|---|---|---|
| Isolation source | activated sludge, Dradenau, Hamburg | iron pipe, Moscow heating system | internal corrosion deposits ,steel pipeline, Moscow heating system | microbial mat of Gorjachinsk hot spring in the Baikal rift zone | Gulf of Main, Atlantic ocean (206m depth) |
| Optimum growth temperature (°C) | 28-32 | 39 | 42 | 46-50 | 28 |
| Tolerance against nitrite(mM) | 20-25 | 15 | 18 | 6 | 6 |
| Dominant lipids | 16:1 cis 11<br>16:0 | 16:1 cis 11<br>16:0<br>16:0 11 methyl | 16:1 cis 7<br>16:0<br>16:0 11 methyl | | 16:1 cis 7<br>16:1 cis 11<br>16:0 |
| Cell morphology | short, slightly curved cells or spiral-shaped rods | irregularly shaped cells or spiral-shaped rods | spirals, curved and straight rods or coccoid cells | spirals, curved and straight rods | "comma-shaped" cells or spiral-shaped rods |
| Cell size | 0.2-0.4 µm x 0.7-1.7 µm | 0.2-0.4 µm x 0.9-2.2 µm | 0.3-0.6 µm x 1.0-2.5 µm or 0.9 x 0.9 µm | 0.3-0.5 µm x 1.0-2.2 µm | 0.3-0.4 µm x0.8-1.0 µm |
| Aggregate formation | strong | present | present 15 | present | weak |
| Reference | (Spieck *et al.*, 2006) | (Ehrich *et al.*,1995) | (Lebedeva *et al.*, 2008) | (unpublished) | (Watson *et al.*, 1986) |



**Appendix - table 1:** Comparable characteristics of so far isolated and enriched *Nitrospira*-like bacteria.

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**Tutor and lecturer of the "International FISH-course" (2004, 2006, 2007, 2008), Department of Microbial Ecology, University of Vienna, Austria**

## List of poster presentations

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## Curriculum vitae

| | |
|---|---|
| **Name:** | Frank Michael Maixner |
| **Date of birth:** | 26.08.1977 |
| **Place of birth:** | Freising, Germany |
| **Citizenship:** | German |
| **Family status:** | Married to Erika Lercher,<br>Two children, Franziska and Maria |
| **Education:** | |
| **1984-1988** | Primary school, Miesbach, Germany |
| **1988-1997** | Grammar school, Miesbach, Germany<br>Graduation with Abitur 1997 |
| **1997-1998** | "Freiwilliges soziales Jahr", m&I Fachklinik Bad Heilbrunn, Germany |
| **1998-2004** | Studies at the Technical University of München (TUM), Germany,<br>Main subject: Microbiology<br>Minor subjects: Medical Virology,<br>Limnology<br>Diploma Thesis at the Department of Microbial Ecology, Faculty of Life Sciences, University of Vienna, Austria, with the title:<br>"Diversität und Genomik ausgewählter stickstoffelemenierender Bakterien aus Abwasseranlagen."<br><br>Dipl.-Biol. Univ. graduation, April 2004. |
| **2004-2009** | Ph.D studies at the Department of Microbial Ecology, Faculty of Life Sciences, University of Vienna, Austria,<br>supervised by Prof. Dr. Michael Wagner |