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ORIGINAL ARTICLE

Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum *Chloroflexi*

Dimitry Y Sorokin[1,2], Sebastian Lücker[3], Dana Vejmelkova[2,4], Nadezhda A Kostrikina[1], Robbert Kleerebezem[2], W Irene C Rijpstra[5], Jaap S Sinninghe Damsté[5], Denis Le Paslier[6,7,8], Gerard Muyzer[2,9], Michael Wagner[3], Mark CM van Loosdrecht[2] and Holger Daims[3]

[1]*Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia;* [2]*Department of Biotechnology, Delft University of Technology, Delft, The Netherlands;* [3]*Department of Microbial Ecology, Ecology Centre, University of Vienna, Vienna, Austria;* [4]*Department of Water Technology and Environmental Engineering, ICT Prague, Prague, Czech Republic;* [5]*Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, Den Burg, The Netherlands;* [6]*Commissariat à l'Energie Atomique, Genoscope, Evry, France;* [7]*Centre National de la recherche Scientifique, Evry, France and* [8]*Université d'Evry Val d'Essonne, Evry, France*

Nitrite-oxidizing bacteria (NOB) catalyze the second step of nitrification, a major process of the biogeochemical nitrogen cycle, but the recognized diversity of this guild is surprisingly low and only two bacterial phyla contain known NOB. Here, we report on the discovery of a chemolithoautotrophic nitrite oxidizer that belongs to the widespread phylum *Chloroflexi* not previously known to contain any nitrifying organism. This organism, named *Nitrolancetus hollandicus*, was isolated from a nitrifying reactor. Its tolerance to a broad temperature range (25–63 °C) and low affinity for nitrite ($K_s = 1$ mM), a complex layered cell envelope that stains Gram positive, and uncommon membrane lipids composed of 1,2-diols distinguish *N. hollandicus* from all other known nitrite oxidizers. *N. hollandicus* grows on nitrite and CO_2, and is able to use formate as a source of energy and carbon. Genome sequencing and analysis of *N. hollandicus* revealed the presence of all genes required for CO_2 fixation by the Calvin cycle and a nitrite oxidoreductase (NXR) similar to the NXR forms of the proteobacterial nitrite oxidizers, *Nitrobacter* and *Nitrococcus*. Comparative genomic analysis of the *nxr* loci unexpectedly indicated functionally important lateral gene transfer events between *Nitrolancetus* and other NOB carrying a cytoplasmic NXR, suggesting that horizontal transfer of the NXR module was a major driver for the spread of the capability to gain energy from nitrite oxidation during bacterial evolution. The surprising discovery of *N. hollandicus* significantly extends the known diversity of nitrifying organisms and likely will have implications for future research on nitrification in natural and engineered ecosystems.

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Introduction

Chemolithoautotrophic nitrite-oxidizing bacteria (NOB) gain energy from the oxidation of nitrite to nitrate, which is the second step of nitrification and thus represents a major biogeochemical process in aquatic and terrestrial ecosystems. Under oxic conditions, nitrite oxidation by NOB is the principal source of nitrate, which constitutes about 88% of the fixed nitrogen in the oceans (Gruber, 2004) and is the predominant inorganic nitrogen form in aerated soils (Dechorgnat *et al.*, 2011). The only other microbial process known to contribute to the global nitrate pool is anaerobic ammonium oxidation ('anammox') that forms nitrate via anaerobic nitrite oxidation (Strous *et al.*, 2006). Nitrate is a major nitrogen source for many organisms and an important electron acceptor for anaerobic respiration. In addition to their crucial function in natural

Correspondence: H Daims, Department of Microbial Ecology, Ecology Centre, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.
E-mail: daims@microbial-ecology.net
[9]Present address: Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, 1090 GE Amsterdam, The Netherlands.
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ecosystems, NOB are important to mankind as they contribute to nitrogen loss in fertilized soils, but are essential for N-removal from sewage in most biological wastewater treatment plants. Winogradsky (1892) isolated the first NOB from the genus *Nitrobacter* in 1892. Since then the number of newly isolated NOB has been very limited owing to difficulties with growing NOB under laboratory conditions. Consequently, our understanding of the physiological and genomic properties of this important microbial group is restricted to a few representatives. For example, the Genomes Online Database (Kyrpides, 1999) lists only eight genome projects for NOB as opposed to 727 just for *Escherichia coli* (status of February 2012). All currently known NOB belong either to the *Proteobacteria* (Teske *et al.*, 1994) or to the genus *Nitrospira* of the phylum *Nitrospirae* (Ehrich *et al.*, 1995); the phylogenetic affiliation of the marine NOB genus *Nitrospina* with the *Deltaproteobacteria* or a distinct phylum remains uncertain (Teske *et al.*, 1994; Schloss and Handelsman, 2004). Occasionally, new NOB enrichments or isolates are reported (for example, Lebedeva *et al.*, 2011), but these strains usually belong to one of the already established groups. The only exceptions in the past 25 years were the description of *Nitrotoga* (Alawi *et al.*, 2007) as a new nitrite-oxidizing genus in the *Betaproteobacteria* and the discovery of anoxygenic phototrophic NOB from the *Alpha-* and *Gammaproteobacteria* (Griffin *et al.*, 2007; Schott *et al.*, 2010). Thus, the diversity of NOB still appears to be restricted to a few phylogenetic groups, most of which are *Proteobacteria*. In contrast to other N-cycle processes, such as denitrification (Shapleigh, 2006) or N_2 fixation (Zehr *et al.*, 2003), there has been no sign that nitrite oxidation became established in a larger number of microbial phyla in the course of evolution. This was also thought about the first step of nitrification, the oxidation of ammonia to nitrite, as all known ammonia-oxidizing bacteria (AOB) were either *Beta-* or *Gammaproteobacteria* (Purkhold *et al.*, 2000). This picture changed drastically with the discovery of ammonia-oxidizing archaea (Könneke *et al.*, 2005) and their dominance in marine (Wuchter *et al.*, 2006) and terrestrial (Leininger *et al.*, 2006) ecosystems. In analogy, several nitrite oxidizers may have not been identified yet. Recognizing the natural diversity of NOB is essential for assessing the consequences of the increased human discharge of nitrogen, in the form of fertilizers and sewage (Canfield *et al.*, 2010), on N-cycling in natural and engineered ecosystems. A better understanding of NOB is also important for reducing the release of the greenhouse gas N_2O from sewage treatment plants. Nitrite is considered the main factor for N_2O formation (Kampschreur *et al.*, 2008), and the activity of NOB decreases the nitrite concentrations and consequently the N_2O emissions. Moreover, knowing the diversity and genomic makeup of NOB is crucial for reconstructing the evolution of nitrite oxidation as part of a major biogeochemical cycle on Earth.

Here we report on the discovery and physiological, as well as genomic, characterization of a thermotolerant nitrite-oxidizing bacterium from a bioreactor that showed high nitrite-oxidizing activity, but did not contain functionally significant amounts of known NOB. This nitrite oxidizer, belonging to a deep-branching lineage of the *Chloroflexi*, is the only identified nitrifier from this bacterial phylum and represents the only known non-proteobacterial nitrite oxidizer besides the genus *Nitrospira*. Owing to its distinct phylogenetic affiliation and physiological traits, this bacterium was overlooked in past research on nitrification.

Materials and methods

Cultivation conditions

Nitrolancetus hollandicus was cultured in mineral medium that contained 1.1 mM KH_2PO_4 and 0.3 mM $CaCl_2$ dissolved in distilled water. This solution was dispensed in serum bottles (ratio 1:5 of liquid to air headspace), which were closed with rubber stoppers. Following injection of CO_2 into the gas phase to a final concentration of 10% (v/v), the bottles were sterilized at 120 °C for 20 min. After sterilization, the medium was supplemented with 1 ml l^{-1} of trace metal solution (Pfennig and Lippert, 1966), 1 mM $MgSO_4$, 5 mM NH_4HCO_3 and 20–50 mM KNO_2. The medium for mixotrophic cultures was also amended with 40 mM NaHCOO. The pH was maintained at 6.9–7.4 by adding either NH_4HCO_3 or CO_2. The bottles were incubated on a rotary shaker under agitation (100–120 r.p.m) at 37 °C. The solid medium was prepared by 1:1 mixing of double-strength liquid medium and 4% washed Noble agar at 50 °C.

Microscopy and FISH

Phase contrast micrographs of *N. hollandicus* cultures were recorded with a Zeiss Axioplan 2 imaging microscope. Details on electron microscopy are provided in Supplementary Text S1. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes was carried out according to the protocol described elsewhere (Daims *et al.*, 2005) using the probes listed in Supplementary Table S2. New 16S rRNA-targeted oligonucleotide probes specific for *N. hollandicus* were designed using the software ARB (Ludwig *et al.*, 2004), and the hybridization conditions were optimized by using the software DAIME (Daims *et al.*, 2006) as described elsewhere (Daims *et al.*, 2005). Confocal FISH images were recorded using a Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany). The biovolume fraction of *N. hollandicus* in activated sludge was measured by digital analysis of confocal FISH

images by using the program DAIME as described previously (Daims and Wagner, 2007).

Chemical analyses
Nitrite was determined colorimetrically by the diazotation reaction-based assay (Griess-Romijn van Eck, 1966) and nitrate by the salicylate method (Bhandari and Simlat, 1986). Formate was measured by HPLC (high performance liquid chromatography) anionic chromatography using an Aminex HPx-87H column (300×7.8 mm, $t = 60\,^{\circ}C$, flow rate $0.6\,ml\,min^{-1}$, eluent 1.5 mM H_3PO_4). Cell protein concentrations were analyzed by the Lowry method (Lowry *et al.*, 1951) after cell lysis in 1 M NaOH. For details of other physiological and chemical analyses, please refer to Supplementary Text S1.

Genomics and phylogeny
High-molecular-weight genomic DNA was extracted from a *N. hollandicus* culture by the Hexadecyltrimethylammonium bromide (CTAB) method as recommended by the DOE Joint Genome Institute (JGI, http://my.jgi.doe.gov/general/index.html), with the only modification that the phenol:chloroform: isoamyl alcohol extraction was performed before the chloroform:isoamyl alcohol purification step in order to avoid carryover of residual phenol. Subsequently, genomic sequences were obtained using GS FLX Titanium (Roche, Basel, Switzerland) and Illumina (Illumina, San Diego, CA, USA) sequencing technologies. One 1/4 picotiter plate Titanium run with a 3-kb paired-end library yielded 199 240 sequence reads that were assembled using the software Newbler (Roche). The 25 258 338 sequence reads (36 bp) obtained from a 1/8 Illumina sequencing run were used to correct Titanium sequencing errors. Contigs were arranged in scaffolds that were ordered by size. The MaGe software system (Vallenet *et al.*, 2006) was used for the automatic prediction and annotation of coding sequences (CDS). The annotation of all CDS in key pathways, including those for nitrite oxidation, respiration and carbon fixation, was manually refined by using the respective tools of MaGe as described in detail elsewhere (Lücker *et al.*, 2010). For details on *nxrA* gene sequencing and phylogenetic analyses, please refer to Supplementary Text S1.

Sequences from *N. hollandicus* have been deposited in the GenBank database (16S rRNA gene: accession no. JQ345500; *nxrA* genes: accession no. JQ279817 to JQ279820; genome sequence: accession no. CAGS01000001 to CAGS01000736).

Results

Isolation and physiological characterization
A laboratory-scale bioreactor was operated at 35 °C to achieve partial nitrification of digestor effluent containing up to 428 mM of ammonia (Vejmelkova *et al.*, 2011). The objective was to convert NH_4HCO_3 to NH_4NO_3 and CO_2 during a study on nitrogen recovery from sewage (Vejmelkova *et al.*, 2011). As desired, the reactor showed high ammonia- and also nitrite-oxidizing activities. Fluorescence *in situ* hybridization revealed that this activated sludge was dominated by cell aggregates of AOB, which were related to *Nitrosomonas europaea and Nitrosomonas eutropha* (Vejmelkova *et al.*, 2011), but with the exception of a small number of *Nitrobacter* cells no known NOB were detected. Interestingly, however, microscopic observation of the sludge revealed a high density of large, conspicuous cells whose morphology resembled the tip of a lancet. Serial dilution of sludge aliquots and incubation at 30 °C in nitrite-containing liquid mineral medium buffered with $NaHCO_3/CO_2$ yielded enrichments of *Nitrobacter*. However, a temperature increase to 37 °C and replacement of the buffer by NH_4HCO_3/CO_2 resulted in a nitrite-oxidizing culture dominated by the lancet-shaped cells (Figure 1). As the AOB were residing in flocs, they could be removed by short centrifugation steps during the enrichment procedure despite the addition of NH_4HCO_3. The lancet-shaped organism was highly enriched after serial dilutions of this culture, and finally a pure culture was obtained (Supplementary Text S1). The isolated bacterium was designated '*Nitrolancetus hollandicus* strain Lb'.

N. hollandicus aerobically grew as a chemolithoautotrophic nitrite oxidizer in culture media containing NO_2^- as the sole energy source and HCO_3^-/CO_2 as the sole carbon source (Figure 2, Supplementary Table S1). However, in contrast to all other known NOB, *N. hollandicus* was not able to assimilate nitrogen from nitrite and required ammonium for growth. Several organic substrates (acetate, pyruvate, lactate, propionate, butyrate, ethanol, glycerol, glucose, fructose and yeast extract) added to the medium either had no detectable effect or inhibited nitrite oxidation and growth. However, when newly incubated in media containing both nitrite and formate, *N. hollandicus* first consumed most of the nitrite and then started to utilize formate (Supplementary Figure S1a). The sequential use of nitrite and formate resembled diauxy, but the ability to use formate depended on preincubation of the culture with nitrite. This might be explained by the nitrite-dependent expression of genes also needed for the respiration of formate. The use of formate is not uncommon among NOB, as *Nitrobacter* is known to grow on formate (Van Gool and Laudelout, 1966) and the genome of '*Ca.* Nitrospira defluvii' encodes a formate dehydrogenase and a formate transporter (Lücker *et al.*, 2010). Nevertheless, the biochemical details of formate use may differ substantially between these species (Supplementary Figure S2 and Supplementary Text S1). No growth of *N. hollandicus* was observed with other inorganic electron donors such as H_2, thiosulfate and CO at various concentrations and with

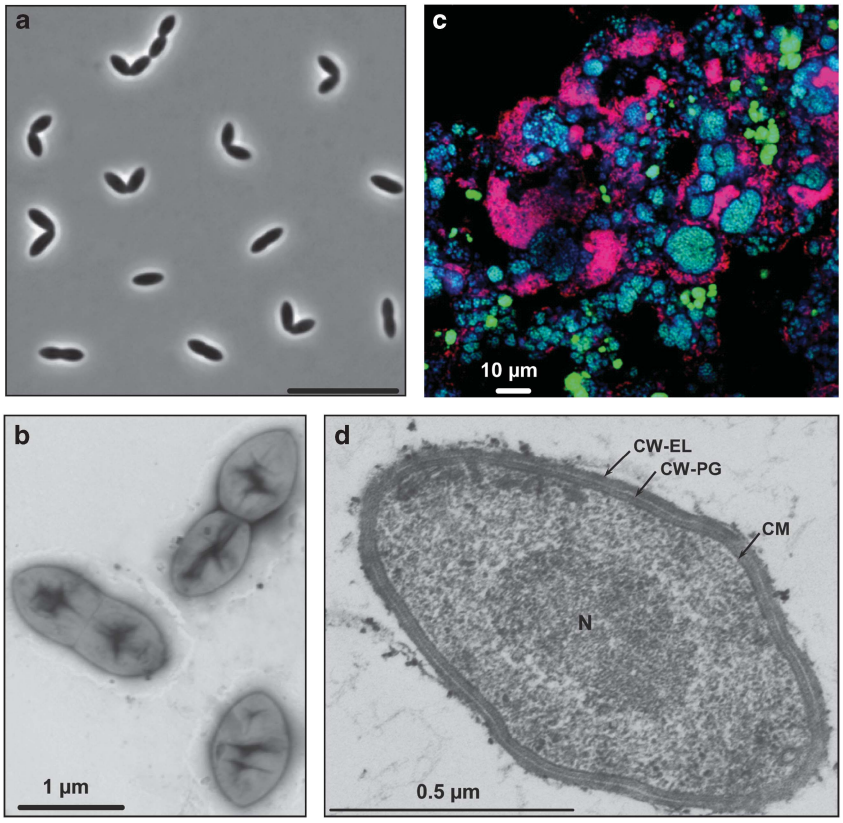

Figure 1 Morphology and *in situ* detection of *N. hollandicus*. (**a**) Phase contrast micrograph of an autotrophically grown pure culture. (**b**) Transmission electron micrograph of cells from the same culture as in panel a. (**c**) *In situ* detection of *N. hollandicus* and AOB in activated sludge from the bioreactor that was the source of the isolated *N. hollandicus* strain. The populations were detected by FISH using the probes Ntlc804 (specific for *N. hollandicus*), Cluster6a192 (specific for AOB of the *N. oligotropha* lineage) and the EUB338 probe mix targeting most *Bacteria*. Cells of *N. hollandicus* appear magenta, AOB green-blue and other bacteria dark blue. (**d**) Thin section electron micrograph of a cell from a culture that was grown mixotrophically in medium containing NO_2^-, HCO_3/CO_2 and formate. The layered cell envelope is clearly visible. N, nucleoide; CW-EL, external layer of the cell wall; CW-PG, peptidoglycane; CM, cytoplasmic membrane.

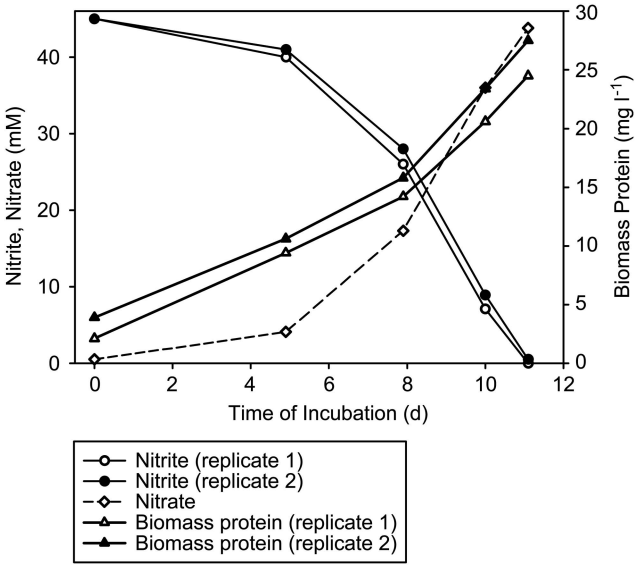

Figure 2 Growth of *N. hollandicus* in mineral medium containing NO_2^- as the sole energy source. NO_2^- was stoichiometrically oxidized to NO_3^- while biomass protein increased. Results of two replicate experiments are shown. Nitrate was measured only in one experiment.

different O_2 levels (2–20%) in the gas phase. Additional tests showed that CO was also not consumed by nongrowing cultures, but 5% CO in the gas phase did not inhibit growth on nitrite. Under anoxic conditions, small amounts of nitrate were reduced to nitrite in the presence of formate as electron donor, but the culture did not grow.

The optimal NO_2^- concentration range for *N. hollandicus* was 5–20 mM, but the organism displayed a high tolerance to NO_2^- (up to 75 mM) and ammonium (>200 mM) at pH 7–7.5. *N. hollandicus* is a thermotolerant nitrite oxidizer with a growth temperature optimum and maximum at 40 and 46 °C, respectively. Nitrite-oxidizing activity was observed even up to 63 °C with an optimal range from 35 to 55 °C (Supplementary Figure S1b). The organism grew at pH values from 6.2 to 8.3, with the optimal range being pH 6.8–7.5 (Supplementary Figure S1c).

Phylogeny and in situ detection

Phylogenetic analyses based on 16S rRNA gene sequences revealed that *N. hollandicus* is affiliated with the thermophilic heterotrophs *Sphaerobacter*

thermophilus and *Thermomicrobium roseum* (Figure 3) in the deep-branching class *Thermomicrobia* of the bacterial phylum *Chloroflexi* (Hugenholtz and Stackebrandt, 2004). To confirm that the isolated organism was identical to the lancet-shaped cells in the source bioreactor, a specific 16S rRNA-targeted oligonucleotide probe (Supplementary Table S2) was designed based on the 16S rRNA gene sequence obtained from the culture. In FISH experiments, this probe hybridized to the *N. hollandicus* culture and to the lancet-shaped bacteria in the reactor biomass (Figure 1c). The same cells were simultaneously detected by probes targeting the phylum *Chloroflexi*, confirming their phylogenetic affiliation with this line of descent. FISH also demonstrated a tight spatial clustering of *N. hollandicus* with AOB in the sludge flocs (Figure 1c) similar to the colocalization frequently reported to occur between AOB and other NOB (Schramm *et al.*, 1999; Maixner *et al.*, 2006), which reflects the mutualistic symbiosis between the two guilds of nitrifiers (Stein and Arp, 1998). *N. hollandicus* was a dominant population representing 34% of the total bacterial biovolume in the bioreactor. Thus, this highly abundant organism most likely caused the strong nitrite-oxidizing activity of the biomass (Vejmelkova *et al.*, 2011) rather than the low numbers of *Nitrobacter* cells, which were sporadically detected by FISH in the same samples.

Cell morphology, ultrastructure and chemical composition

N. hollandicus cells are relatively large (1 to 1.2 × 2 to 4 μm), non-motile, rod- (lancet-) shaped and occur as single cells, pairs or short chains with individual cells attached at their poles (Figure 1). The cells stained Gram-positive and their envelope comprised a thin electron-dense peptidoglycan layer and several outer layers that may consist of carbohydrates or protein (Figure 1d). Complex cell envelope structures with multiple layers are not uncommon among *Chloroflexi*, although they lack an outer membrane as found in Gram-negative bacteria (Sutcliffe, 2011). Meso-diaminopimelic acid, as characteristic for the cell wall of Gram-negative bacteria, was not detected by chemical analysis of peptidoglycan from *N. hollandicus*. Instead, the diamino acids ornithine and lysine were present, suggesting that the peptidoglycan composition of *N. hollandicus* resembles that of Gram-positive bacteria. The predominant membrane lipids were uncommon and were composed of C_{20} alkyl 1,2-diols with a polar head group (sugar or phosphorus-sugar moiety) attached to the first OH group, whereas the second OH group was esterified with a fatty acid (predominantly 12 methyl-octadecanoic acid) (Supplementary Figure S3). Similar glycolipids have only been identified in thermophilic *Chloroflexi* including *Thermomicrobium*, a close relative of *Nitrolancetus* (Figure 3) and *Roseiflexus castenholzii*, as well as in *Thermus* (Pond *et al.*, 1986; Wait *et al.*, 1997; van ver Meer *et al.*, 2002). Alkyl 1,2-diol lipids also occur in hot spring microbial mats (van ver Meer *et al.*, 2002), but a specific role for the adaptation to high temperatures is debatable (Wait *et al.*, 1997). Thin sections revealed the absence of intracytoplasmic membranes (ICM) that are found in *Nitrobacter* and *Nitrococcus* (Figure 1d).

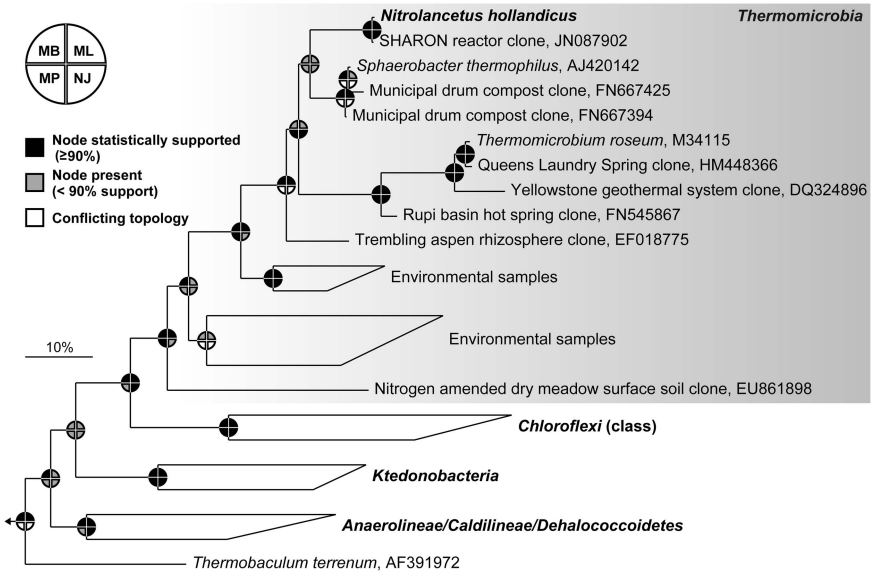

Figure 3 Phylogenetic affiliation of *N. hollandicus*. 16S rRNA-based Bayesian inference tree (s.d. = 0.009202) showing the affiliation of *N. hollandicus* (boldface) with the class *Thermomicrobia* (gray box) of the phylum *Chloroflexi*. Pie charts indicate statistical support of nodes based on bootstrap analysis or Bayesian inference, respectively. Conflicting topologies among the different methods are also indicated. Representatives of the phylum *Actinobacteria* were used as outgroup. The scale bar represents 10% estimated sequence divergence. MB, Bayesian inference; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining.

Genomic analysis

The genome of *N. hollandicus* strain Lb was partially reconstructed by high-throughput sequencing. The assembled sequence comprised 3 999 304 bp in 736 contigs organized on 42 scaffolds (Supplementary Table S3). Thirty-nine of the 40 universally occurring clusters of orthologous groups used by MLTreeMap (Stark *et al.*, 2010) and tRNAs for all 20 amino acids were represented, suggesting that the obtained sequences cover a major part of the *N. hollandicus* genome (Supplementary Table S3).

All known NOB oxidize nitrite by the enzyme NXR, which belongs to the type II group in the dimethyl sulfoxide reductase family of molybdopterin-cofactor-binding enzymes (Meincke *et al.*, 1992; Lücker *et al.*, 2010). NXR is membrane associated and contains an α subunit (NxrA) with the catalytic site and a β subunit (NxrB) that channels electrons derived from nitrite to downstream components of the respiratory chain (Kirstein and Bock, 1993). Most likely, a γ subunit (NxrC) functions as a membrane anchor that may be involved in electron transport (Lücker *et al.*, 2010). The genome sequence of *N. hollandicus* contains four highly similar (90.3–95.8% protein sequence identity) CDS for NxrA as fragments on contig boundaries. We obtained the full sequences of these genes by PCR, cloning and Sanger sequencing. Only one CDS was found for each of the other NXR subunits. Three *nxrA* genes form a cluster also containing a two-component response regulator. The fourth *nxrA* copy is directly upstream of *nxrB*, a NarJ homolog, and *nxrC* (Supplementary Figure S4a), suggesting that these genes represent a functional unit. The genomic localization of this *nxrA* copy was confirmed by PCR and Sanger sequencing. NarJ is the δ subunit of dissimilatory nitrate reductase (NAR) and inserts the molybdenum cofactor into the α subunit (Blasco *et al.*, 1998). It may have a similar role in NXR biosynthesis (Starkenburg *et al.*, 2006). Neither NxrA nor NxrB contained predicted signal peptides for protein secretion, indicating a cytoplasmic localization of these subunits.

Electrons derived from NO_2^- most likely are accepted by cytochrome *a* or *c* and then transferred to a terminal cytochrome *c* oxidase, which in *N. hollandicus* is of the aa_3 type (Supplementary Table S4). This short respiratory chain, including a canonical F_1F_0 ATPase, appears to be highly similar to that of *Nitrobacter* (Spieck and Bock, 2005). Genome annotation also revealed complexes I–III (Supplementary Table S4) that are not required for nitrite oxidation, but may function in the reverse flow of electrons from nitrite to NAD^+ or in energy conservation by the oxidation of organic compounds such as formate. The presence of a [NiFe]-hydrogenase, which contains the signature residues of group 1 (H_2 uptake) hydrogenases (Vignais and Billoud, 2007), implies that H_2 might serve as an electron donor, although the use of H_2 was not observed in our cultivation-based assays.

N. hollandicus possesses an ammonia transporter of the Amt type and a nitrite/nitrate transporter (NarK). The latter is needed for nitrite import into the cell, because NXR is cytoplasmic, and for the export of nitrate. An urea transporter was identified, but no urea catabolic genes were found in the assembled contigs. Further, while an assimilatory nitrate reductase (NasC) is encoded in the genome, no known assimilatory nitrite reductase was found. One CDS similar to ferredoxin-dependent nitrite or sulfite reductase (NirA/Sir) is present, but the gene product, based on the genomic context, most probably functions as Sir (Supplementary Table S4). Cytochrome *c* nitrite reductase (NrfA) is also absent, indicating that *N. hollandicus* is unable to reduce nitrite to ammonia and thus depends on ammonia as N-source. This would explain the observed requirement of the culture for ammonium. Alternatively, ammonia might have gene regulatory functions in *N. hollandicus* and thus be indispensable. *N. hollandicus* appears to lack genes for denitrification (Supplementary Text S1). Solely nitrate reduction to nitrite was observed (see above) and may be explained by NXR working in reverse as in *Nitrobacter* (Sundermeyer-Klinger *et al.*, 1984) or by the activity of the assimilatory nitrate reductase NasC. However, the genome of *N. hollandicus* has not been completely sequenced yet, and the remaining parts may contain the missing genes for nitrite reduction to ammonia and denitrification.

The only autotrophic carbon fixation pathway present in the genome of *N. hollandicus* is the Calvin–Benson–Bassham cycle (Figure 4 and Supplementary Table S4). This pathway is highly unusual within the *Chloroflexi* where only the genus *Oscillochloris* is known to use the Calvin cycle, whereas other autotrophic members of this phylum use the 3-hydroxypropionate cycle for CO_2 fixation (Berg, 2011). The ribulose-1,5-bisphosphate carboxylase (RubisCO) of *N. hollandicus* belongs to a distinct phylogenetic lineage that is related to the red form I RubisCO and also contains the RubisCO of *Oscillochloris trichoides* (Supplementary Figure S4e).

The presence of a formate transporter and a molybdopterin-dependent formate dehydrogenase (Figure 4 and Supplementary Table S4) is consistent with the utilization of formate by the culture (Supplementary Figure S1a). Only few other putative transporters for organic substrates, including amino acids, were identified in the genome (Figure 4). *N. hollandicus* encodes the complete oxidative tricarboxylic acid cycle that provides building blocks for biosynthesis, but may also be involved in the degradation of storage compounds. Indeed, the glycolytic pathway and gluconeogenesis are present, as well as genes for the production and utilization of glycogen deposits (Supplementary Table S4).

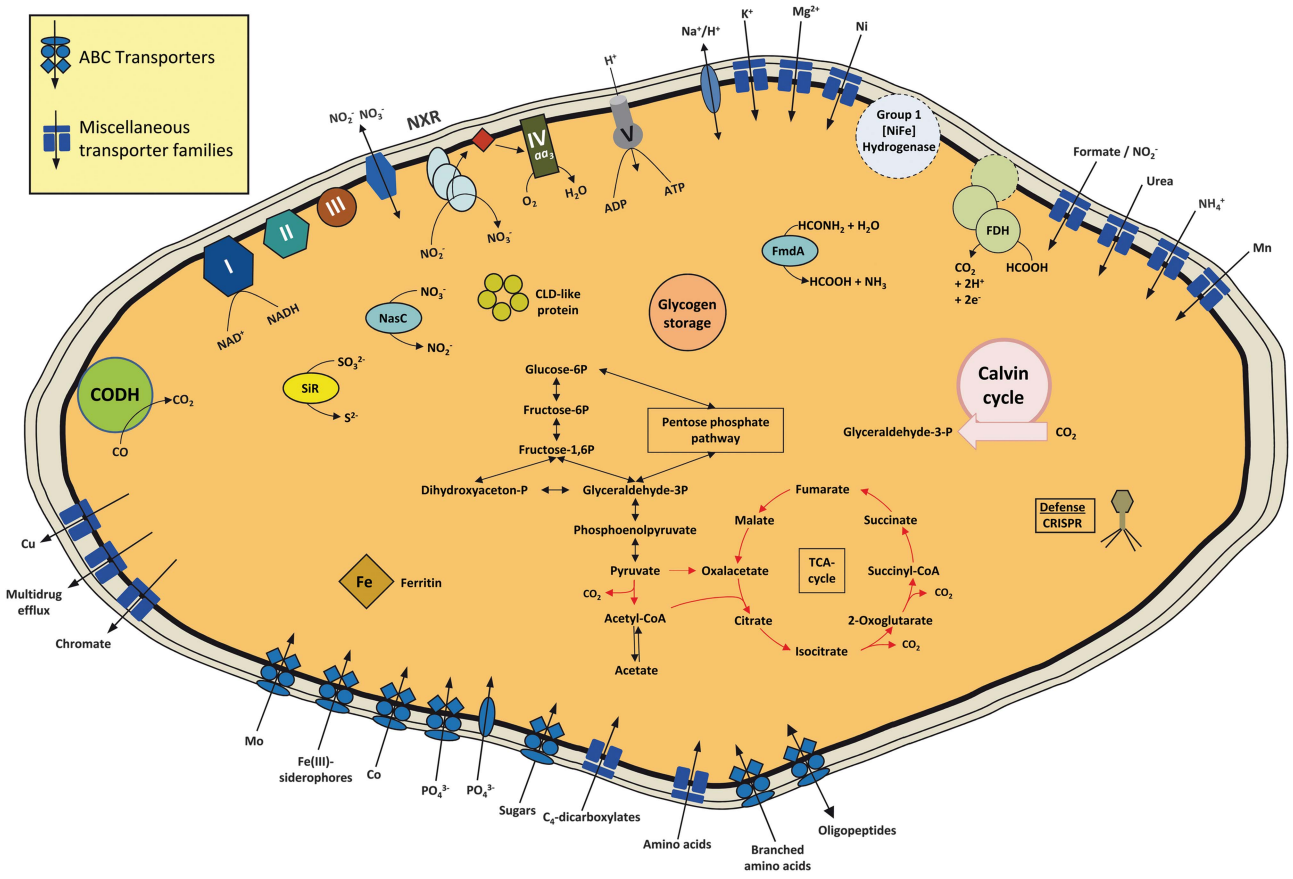

Figure 4 Cell metabolic cartoon constructed from the annotation of the *N. hollandicus* genome. Enzyme complexes of the electron transport chain are labeled by Roman numerals. CLD, chlorite dismutase; FDH, formate dehydrogenase; FmdA, formamide amidohydrolase; TCA-cycle, tricarboxylic acid cycle; CRISPR, clustered, regularly interspaced short palindromic repeats.

Discussion

Discovery of N. hollandicus

N. hollandicus is the only known nitrite oxidizer not affiliated to the *Proteobacteria* or *Nitrospirae* phyla and demonstrates that the capability to gain energy from nitrite oxidation occurs also within a third bacterial phylum, the *Chloroflexi*. Until now, the *Chloroflexi* were not known to contain any nitrifier, and this phylum thus had no role in nitrification research. *Chloroflexi* are physiologically diverse and abundant in habitats as different as hot springs, freshwater lakes and rivers, activated sludge, sediments, and marine sponges (Juretschko *et al.*, 2002; Hanada and Pierson, 2006; Taylor *et al.*, 2007). Nitrification activity has been detected in many of these systems, and it appears possible that *Chloroflexi*, either *Nitrolancetus*-like bacteria or other representatives, contribute to this process along with previously known nitrifiers. This leads to the interesting question of whether other yet unknown nitrite oxidizers might be dwelling in nature. Intriguingly, published molecular data indicate that such organisms exist (Supplementary Text S1).

Ecophysiology of Nitrolancetus and other NOB

All presently known NOB can be assigned to one of two groups that reflect different ecological strategies. One group consists of *r*-strategists (Andrews and Harris, 1986) that require relatively high nitrite concentrations and grow relatively fast as compared with the other group containing slow-growing K-strategists, which outcompete the *r*-strategists in habitats with low nitrite concentrations (Schramm *et al.*, 1999). The physiological properties of most *Nitrobacter* strains and of *Nitrococcus* (Watson and Waterbury, 1971; Bartosch *et al.*, 1999) (Supplementary Table S1) indicate that these NOB represent *r*-strategists (Schramm *et al.*, 1999). According to its high $K_s(NO_2^-)$, *N. hollandicus* also belongs to this physiological group, although the observed growth rates of the pure culture were relatively low compared with those of *Nitrobacter* and *Nitrococcus* (Supplementary Table S1). The ICM of *Nitrobacter* and *Nitrococcus* contain NXR and are part of the nitrite-oxidizing machinery in these NOB (for example, Spieck *et al.*, 1996). The lack of ICM in *N. hollandicus* leads to a smaller surface of the nitrite-oxidizing membranes in this

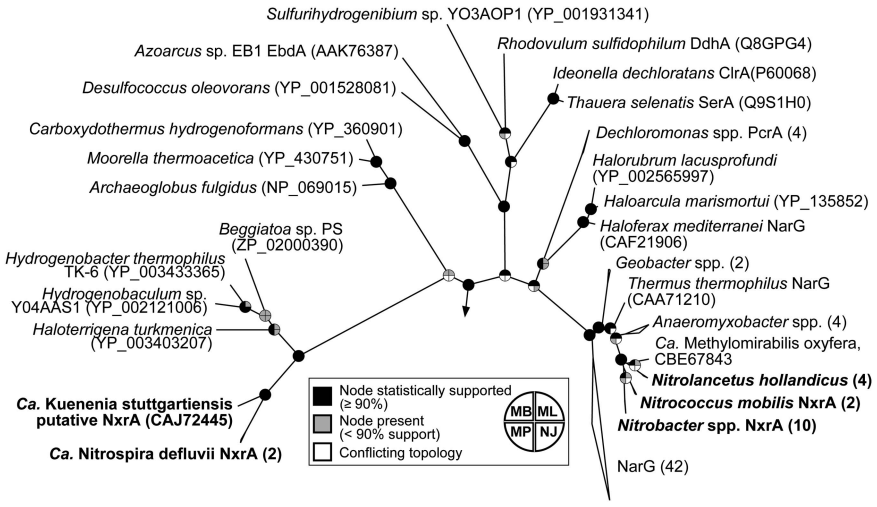

Figure 5 Phylogeny of NXR and related enzymes, based on the α subunit. Maximum likelihood tree showing the phylogenetic positioning of selected type II enzymes of the dimethyl sulfoxide reductase family, based on the catalytic (α) subunits. For the analysis 1102 amino acid positions were considered. Names of validated enzymes are indicated (Nxr, boldface; Nar, membrane-bound respiratory nitrate reductase; Pcr, perchlorate reductase; Ebd, ethylbenzene dehydrogenase; Ddh, dimethylsulfide dehydrogenase; Clr, chlorate reductase; Ser, selenate reductase). Parentheses contain the number of sequences within a group or the accession number, respectively. Pie charts indicate statistical support of nodes based on bootstrap analysis or Bayesian inference, respectively. More distantly related molybdoenzymes were used as outgroup.

organism and thus may explain the lower growth rates. The NXR forms of *Nitrobacter*, *Nitrococcus* and *Nitrolancetus* are closely related to each other and to the dissimilatory nitrate reductases (NARs) (Figure 5). The α-subunit NxrA, containing the substrate-binding site, is oriented toward the cytoplasmic side of the cytoplasmic membrane (and of the ICM) in *Nitrobacter* and *Nitroccocus* (Spieck and Bock, 2005), and probably also in *Nitrolancetus* because of the lack of any signal peptide for protein secretion. Thus, known NOB with this cytoplasmic form of NXR consistently require high nitrite concentrations to be competitive. In contrast, the NXR of *Nitrospira* forms a distinct phylogenetic lineage together with NXR of the anammox organism '*Ca*. Kuenenia stuttgartiensis' (Lücker *et al.*, 2010) (Figure 5) and it faces the periplasmic space (Spieck *et al.*, 1998; Lücker *et al.*, 2010). The phylogeny of NXR from the genus *Nitrospina* remains unknown because of lacking sequences, but this enzyme is known to be periplasmic as well (Spieck and Bock, 2005). Periplasmic forms of NXR are thought to be more efficient, because more proton-motive force should be generated per oxidized nitrite and no nitrite/nitrate transport across the cytoplasmic membrane is needed (Lücker *et al.*, 2010). Accordingly, *Nitrospira* and *Nitrospina* are much better adapted to lower nitrite concentrations than most *Nitrobacter*, *Nitrococcus* and *Nitrolancetus* (Watson and Waterbury, 1971; Bartosch *et al.*, 1999; Schramm *et al.*, 1999) (Supplementary Table S1). On the basis of the properties of characterized NOB, the cellular localization of NXR and its phylogenetic placement within the molybdopterin-cofactor-binding enzymes (Figure 5 and Supplementary Figure S4b) seem to be useful indicators for predicting the ecological strategies of nitrite oxidizers.

The only previously known thermotolerant NOB belong to the genus *Nitrospira* (Lebedeva *et al.*, 2011). These organisms are inactive below 37 °C, are inhibited already by nitrite concentrations of 6 mM and do not utilize formate or other organic compounds. In contrast, *N. hollandicus* tolerates a broad temperature range (Supplementary Figure S1b), as well as high nitrite concentrations, and can grow mixotrophically on nitrite and formate (Supplementary Figure S1a). These physiological properties distinguish *N. hollandicus* from all other known NOB. An additional unique feature is the apparent dependence of *N. hollandicus* on ammonia as N-source for growth. This may explain why *N. hollandicus* was not enriched or isolated in previous studies, because mineral media usually applied for the cultivation of NOB contain only trace amounts of ammonia, added as ammonium molybdate (Ehrich *et al.*, 1995). The growth requirements of *N. hollandicus* match the conditions in the source bioreactor operated at 35 °C with high concentrations of ammonium. On the contrary, most conventional nitrifying wastewater treatment plants are operated at lower temperatures and low ammonia and nitrite concentrations in the bulk liquid. These conditions are optimal for K-strategists such as *Nitrospira* (Daims *et al.*, 2001), but select against NOB requiring higher nitrite levels (Schramm *et al.*, 1999). Thus, owing to its high $K_s(NO_2^-)$ of 1 mM (Supplementary Table S1) and its relatively high temperature optimum, the functional importance of *N. hollandicus* in such conventional wastewater

treatment plants is unlikely. Indeed, by screening several full-scale wastewater treatment plants and laboratory-scale reactors using specific PCR and FISH, we did not detect *Nitrolancetus* except in the reactor that was the source of isolation. From a technical perspective, however, *N. hollandicus* will likely become relevant with the introduction of increased biogas production and with the treatment of the resulting ammonium-rich digestate. Interestingly, a screening of publicly available 16S rRNA gene sequences retrieved the sequence of a close relative to *N. hollandicus*, which had been obtained from a SHARON reactor in Korea (Figure 3). The high temperatures (30–40 °C) and short sludge retention times in such reactors favor the growth of AOB over that of NOB such as *Nitrobacter* and *Nitrospira*, thus enabling partial nitrification of ammonia to nitrite. The effluent is highly suitable for cost-efficient N-removal by anammox bacteria (van Dongen *et al.*, 2001). *Nitrolancetus*-like NOB, which persist under these conditions, would disturb the process by oxidizing nitrite to nitrate. Under this premise, knowledge of *N. hollandicus* and its ecophysiology could be extremely helpful for process design and optimization.

On the basis of the observed properties of *N. hollandicus*, the natural habitat of these NOB is likely to be characterized by elevated temperatures and high ammonia and nitrite concentrations. Such conditions may be found, for instance, in piles of decomposing plant biomass or dung. Consistently, uncultured relatives of *N. hollandicus* and *S. thermophilus* have been detected in municipal compost (Figure 3).

Evolutionary history of NOB

As *Nitrobacter* and *Nitrococcus* form ICM and are related to phototrophic *Proteobacteria* also containing ICM, a phototrophic origin was suggested for these NOB (Teske *et al.*, 1994). This hypothesis gained strong support from the recent isolation of extant phototrophic NOB in the genera *Rhodopseudomonas* and *Thiocapsa*, which are closely affiliated with *Nitrobacter* and *Nitrococcus*, respectively (Schott *et al.*, 2010). The NXR of *Nitrobacter* and *Nitrococcus* is oriented toward the cytoplasm (see also above). In contrast, genome analysis revealed an evolutionary link between *Nitrospira* and anammox organisms (planctomycetes) that likely involved horizontal gene transfer of a periplasmic NXR (not closely related to the NXR of *Nitrobacter* and *Nitrococcus*, Figure 5 and Supplementary Figure S4b) and other proteins involved in nitrite oxidation and electron transport (Lücker *et al.*, 2010). These lines of evidence suggested two independent lines of evolution of nitrite oxidation, exemplified by the different forms of NXR, in *Nitrobacter* and *Nitrococcus* versus *Nitrospira* and anammox (Lücker *et al.*, 2010). The discovery of *Nitrolancetus* adds more complexity to this picture, because this member of the *Chloroflexi* clearly did not evolve from the same ancestors as any of the other known NOB. The affiliation of *N. hollandicus* with the *Chloroflexi* is supported not only by 16S rRNA phylogeny (Figure 3) but also by its distinct lipid composition, phylogenetic analysis of 39 universally occurring clusters of orthologous groups (Supplementary Figure S4f) and by numerous highly similar sequence matches between the *N. hollandicus* contig sequences and the genomes of *Sphaerobacter* and *Thermomicrobium* (Supplementary Figure S4g). Although several other *Chloroflexi* are anoxygenic phototrophs (Bryant and Frigaard, 2006), *N. hollandicus* is not closely related to these organisms (Figure 3), has no remnants of a photosynthetic apparatus in its genome, and its next known relatives are chemoorganoheterotrophs. Thus, a direct phototrophic origin of nitrite oxidation in *N. hollandicus* appears unlikely. However, the cytoplasmic NXR of *N. hollandicus* is closely related to the NXR of *Nitrobacter* and *Nitrococcus* despite the large phylogenetic distance between the *Chloroflexi* and *Proteobacteria* (Figure 5 and Supplementary Figure S4b). Interestingly, a similar NXR-like enzyme is found in the anaerobic, nitrite-dependent methane oxidizer '*Candidatus* Methylomirabilis oxyfera' that belongs to the distinct bacterial phylum NC10 (Ettwig *et al.*, 2010). The high similarity of NXR in these organisms (Figure 5 and Supplementary Figure S4a and S4b) indicates an unexpected evolutionary link. The most parsimonious explanation is that horizontal gene transfer established the cytoplasmically oriented NXR in ancestors of these very distantly related bacteria. This hypothesis gains support from analyses of the genes upstream and downstream of *nxr* (Supplementary Text S1 and Supplementary Figures S4a, S4c and d). Whether this evolutionary scenario supports or contrasts a phototrophic origin of nitrite oxidation in *Nitrobacter* and *Nitrococcus* (Teske *et al.*, 1994) is a fascinating question that might be better illuminated once first sequences of *nxr* loci from the proteobacterial phototrophic NOB (Schott *et al.*, 2010) become available.

As the presence of NXR in *N. hollandicus* may result from horizontal gene transfer, it is tempting to speculate about the lifestyle of its ancestor before the *nxr* gene cluster was acquired. Although utilization of CO or H_2 was not observed, the genome of *N. hollandicus* contains the required genes (see above and Supplementary Text S1). Thus, it appears possible that the ancestor used CO or H_2 as energy source. Extant relatives of *N. hollandicus* might then still be able to utilize these substrates. Indeed, *T. roseum* can oxidize CO and, similar to *N. hollandicus*, it has genes coding for a [NiFe]-hydrogenase (Wu *et al.*, 2009).

Intriguingly, the two known NXR forms are not restricted to NOB but occur in functionally different N-cycle bacteria. The periplasmic form of *Nitrospira* is present in anammox organisms, which also

oxidize nitrite (Strous *et al*., 2006; Lücker *et al*., 2010). The NXR type of *Nitrobacter*, *Nitrococcus* and *Nitrolancetus* is shared with *Ca*. M. oxyfera where it may function reversibly and reduce nitrate to nitrite (Ettwig *et al*., 2010). Hence, NXR is a beautiful example for the exchange and functional adaptation of genetic modules as a key motif in the evolution of the N-cycle (Klotz and Stein, 2008).

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