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

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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 (Kₛ = 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 CO₂, 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 CO₂ 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
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 *Nitrosira* 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 N₂ 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* (Purkholt 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 N₂O from sewage treatment plants. Nitrite is considered the main factor for N₂O formation (Kampschreur et al., 2008), and the activity of NOB decreases the nitrite concentrations and consequently the N₂O 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 *Nitrosira*. 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 KH₂PO₄ and 0.3 mM CaCl₂ dissolved in distilled water. This solution was dispersed in serum bottles (ratio 1:5 of liquid to air headspace), which were closed with rubber stoppers. Following injection of CO₂ 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⁻¹ of trace metal solution (Pfenning and Lippert, 1966), 1 mM MgSO₄, 5 mM NH₄HCO₃ and 20–50 mM KNO₂. The medium for mixotrophic cultures was also amended with 40 mM NaHCO₃. The pH was maintained at 6.9–7.4 by adding either NH₄HCO₃ or CO₂. 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 DAME (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 °C, flow rate 0.6 ml min⁻¹, eluent 1.5 mM H₃PO₄). 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 (Lück 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 mg of ammonia (Vejmelmekova et al., 2011). The objective was to convert NH₄HCO₃ to NH₄NO₃ and CO₂ during a study on nitrogen recovery from sewage (Vejmelmekova 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 Nitrosonomas eutropha (Vejmelmekova 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 NaHCO₃/CO₂ yielded enrichments of Nitrobacter. However, a temperature increase to 37 °C and replacement of the buffer by NH₄HCO₃/CO₂ resulted in a nitrite-oxidizing culture dominated by the lancet-shaped cells (Figure 1). The AOB were residing in flocs, they could be removed by short centrifugation steps during the enrichment procedure despite the addition of NH₄HCO₃. 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 ‘Nitrolance-tus hollandicus strain Lb’.

N. hollandicus aerobically grew as a chemolithoautotrophic nitrite oxidizer in culture media containing NO₂⁻ as the sole energy source and HCO₃⁻/CO₂ 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 Laudenlout, 1966) and the genome of ‘Ca. Nitrospira defluvi’ encodes a formate dehydrogenase and a formate transporter (Lück 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 H₂, thiosulfate and CO at various concentrations and with
different O₂ 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 NO₂⁻/CO ratio for *N. hollandicus* was 5–20 mM, but the organism displayed a high tolerance to NO₂⁻/CO (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 di-amino 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 C20 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.

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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 (Starkenburg *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; Lücke *et al.*, 2010). NXR is a membrane-associated and contains a subunit (NxrA) with the catalytic site and a subunit (NxrB) that channels electrons derived from nitrite to downstream components of the respiratory chain (Kirstein and Bock, 1993). Most likely, a subunit (NxrC) functions as a membrane anchor that may be involved in electron transport (Lücke *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 dissipatory 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 $a_3$ type (Supplementary Table S4). This short respiratory chain, including a canonical F$_{0}$F$_{1}$ ATPase, appears to be highly similar to that of *Nitrobacter* (Speick 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* (Sundemeyer-Klingler *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).
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-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 $K_r(NO_2^-)$, 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, Spi Eck et al., 1996). The lack of ICM in N. hollandicus leads to a smaller surface of the nitrite-oxidizing membranes in this

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.
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’ (Lu¨cker et al., 2010) and it faces the periplasmic space (Spieck et al., 1998; Lu¨cker et al., 2010). The phylogeny of NXR from the genus *Nitrospira* 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 (Lu¨cker et al., 2010). Accordingly, *Nitrospira* and *Nitrosophaera* are much better adapted to lower nitrite concentrations than most NOB (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 molybdenopterin-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 $K_{M(NO_2^-)}$ 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 *Nitrooccus* 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 *Rhodopsseudomonas* and *Thiocapsa*, which are closely affiliated with *Nitrobacter* and *Nitrooccus*, respectively (Schott et al., 2010). The NXR of *Nitrobacter* and *Nitrooccus* 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 *Nitrooccus*, 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 *Nitrooccus* 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 *Nitrooccus* 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-depndent 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 *Nitrooccus* (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 H₂ 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 H₂ 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, Nitrooccus 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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