A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria

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Edited by Edward F. DeLong, Massachusetts Institute of Technology, Cambridge, MA, and approved June 18, 2010 (received for review March 25, 2010)

Nitrospira **are barely studied and mostly uncultured nitrite-oxidizing bacteria, which are, according to molecular data, among the most diverse and widespread nitrifiers in natural ecosystems and biological wastewater treatment. Here, environmental genomics was used to reconstruct the complete genome of "*Candidatus* Nitrospira defluvii" from an activated sludge enrichment culture. On the basis of this first-deciphered *Nitrospira* genome and of experimental data, we show that *Ca*. N. defluvii differs dramatically from other known nitrite oxidizers in the key enzyme nitrite oxidoreductase (NXR), in the composition of the respiratory chain, and in the pathway used for autotrophic carbon fixation, suggesting multiple independent evolution of chemolithoautotrophic nitrite oxidation. Adaptations of *Ca*. N. defluvii to substrate-limited conditions include an unusual periplasmic NXR, which is constitutively expressed, and pathways for the transport, oxidation, and assimilation of simple organic compounds that allow a mixotrophic lifestyle. The reverse tricarboxylic acid cycle as the pathway for CO_2 fixation and the lack of most classical defense mechanisms against oxidative stress suggest that *Nitrospira* evolved from microaerophilic or even anaerobic ancestors. Unexpectedly, comparative genomic analyses indicate functionally significant lateral gene-transfer events between the genus *Nitrospira* and anaerobic ammonium-oxidizing planctomycetes, which share highly similar forms of NXR and other proteins reflecting that two key processes of the nitrogen cycle are evolutionarily connected.**

environmental genomics | nitrification | Nitrospirae

Nitrification, the microbially catalyzed sequential oxidation of ammonia via nitrite to nitrate, is a key process of the biogeochemical nitrogen cycle and of biological wastewater treatment. The second step of nitrification is carried out by chemolithoautotrophic nitrite-oxidizing bacteria (NOB), which are phylogenetically heterogeneous (1) and occur in a wide range of aquatic and terrestrial ecosystems. Most studies on the physiology of NOB used pure cultures of *Nitrobacter*, which belong to the *Alphaproteobacteria* (1), and complete genome sequences from NOB are available for three *Nitrobacter* strains (2, 3) and the marine gammaproteobacterium *Nitrococcus mobilis* (GenBank accession no. NZ_AAOF00000000). However, cultivation-independent molecular methods revealed that *Nitrospira*, forming a deeply branching lineage in the bacterial phylum Nitrospirae (4), are by far the most diverse and abundant NOB (5). In addition to their wide distribution in natural habitats such as soils (6), sediments (7), the oceans (8), and hot springs (9), members of the genus *Nitrospira* are the predominant NOB in wastewater treatment plants (5) and thus belong to the microorganisms most relevant for biotechnology.

The immense ecological and technical significance of *Nitrospira* contrasts with our scarce knowledge about these bacteria. As the majority of *Nitrospira* are uncultured, and the available cultures are difficult to maintain, only a few studies have addressed their ecology and physiology (e.g., 5, 10, 11). Furthermore, except for one 137-kbp contig (12), genomic sequences from *Nitrospira* have not been obtained yet. This situation has been highly unsatisfactory because deeper insight into the biology of these elusive NOB is crucial for a better understanding of nitrogen cycling in natural and engineered systems.

Recently, a *Nitrospira* strain was enriched from activated sludge and partly characterized (13). This organism, tentatively named "*Candidatus* Nitrospira defluvii," belongs to *Nitrospira* sublineage I, which is most important for sewage treatment (5) but has no representative in pure culture. Here, the complete genome of *Ca*. N. defluvii was reconstructed from a metagenomic library of the enrichment. More than two decades after *Nitrospira* were discovered (8), we provide an analysis of a *Nitrospira* genome with previously unmatched insight into the biology of *Nitrospira*, show striking differences in key metabolic pathways between *Nitrospira* and other NOB, and change the current perception on the evolution of NO_2^- oxidation.

Results and Discussion

Genome Reconstruction. Quantitative FISH has shown that the NO_2^--oxidizing enrichment consisted of 86% of *Ca*. N. defluvii and did not contain other known NOB (13). The complete genome of *Ca*. N. defluvii was reconstructed from this enrichment by an environmental genomics approach similar to that used for inferring the genome sequence of the anaerobic ammonium-oxidizing bacterium ("anammox" organism) "*Candidatus* Kuenenia stuttgartiensis" (14). The completeness and correct assembly of the *Nitrospira* genome was indicated by the retrieval of all 63 clusters of orthologous groups (COGs) of proteins, which are present in all genomes in the current COG database (Fig. S1), by lack of suspicious redundancy in gene content, and by the presence of all essential genes in key biosynthetic pathways. The low frequency of single nucleotide polymorphisms (about one per 500 kbp) strongly suggests that the enrichment culture contained only one *Nitrospira* strain. Key features of the genome are summarized in Table S1 and Fig. S1. About 30% of the predicted coding sequences (CDS) have

Author contributions: S.L., M.W., D.L.P., and H.D. designed research; S.L., F.M., H.K., B.V., and J.S.S.D. performed research; T.R. and E.S. contributed new reagents/analytic tools; S.L., F.M., E.P., T.R., and H.D. analyzed data; and S.L., M.W., and H.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. FP929003).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1003860107/-/DCSupplemental.

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no homologs in other organisms, reflecting the distant relationship of *Nitrospira* to other bacteria and the lack of genome sequences from the genus *Nitrospira* in public databases. Furthermore, only two lineages within the phylum Nitrospirae have been explored on a genomic level. The closest genome-sequenced relatives of *Ca*. N. defluvii belong to the genus *Leptospirillum* and are aerobic acidophilic iron oxidizers (15–17). In addition, the genome sequence of the anaerobic sulfate reducer *Thermodesulfovibrio yellowstonii* (GenBank accession no. NC_011296), also belonging to the Nitrospirae, is publicly available.

Nitrite Oxidation and Energy Metabolism. The key enzyme for NO_2^- oxidation by NOB is nitrite oxidoreductase (NXR), which shuttles two electrons per oxidized NO_2^- into the electron transport chain. In *Nitrobacter*, NXR is an iron-sulfur molybdoprotein (18) located at the inner cell membrane and at the intracytoplasmic membranes (ICM). The reaction catalyzed by this NXR is reversible, so that the enzyme also reduces NO_3^- with electrons derived from organic compounds. Depending on the applied purification method, this NXR was found to consist of two (18) or three subunits with a supposed $\alpha_2\beta_2\gamma_1$ stoichiometry (19). The α-subunit (NxrA) is thought to contain the substrate-binding site with the molybdopterin cofactor (Mo-co) (18, 19), whereas the β-subunit (NxrB) with [Fe-S] clusters probably channels electrons from the α- to the γ-subunit or directly to the membrane-integral electron transport chain (20).

Nitrospira are Gram-negative bacteria lacking ICM (8). Although no NO_3^--reducing activity has been demonstrated yet for their nitrite-oxidizing system, the term NXR is used here to be consistent with established terminology (2). The first insight into the nature of the *Nitrospira* NXR was obtained by studying a pure culture of *Nitrospira moscoviensis* (21). Four major proteins were detected in membrane fractions showing a high NO_2^--oxidizing activity in vitro. Antibodies originally raised against NxrB of *Nitrobacter* bound to one of these proteins, which was designated the NxrB of *N. moscoviensis* (21). Another protein with an apparent molecular mass of 130 kDa resembled the NxrA of *Nitrobacter* (115–130 kDa). The other two proteins were not further characterized. The NXR of *N. moscoviensis* was also shown to contain molybdenum and to be located at the inner cell membrane, where it faces the periplasmic space (21).

The genome of *Ca*. N. defluvii was screened for CDS with a predicted molecular mass resembling the NxrA and NxrB of *N. moscoviensis* and similarity to known NO_2^-/NO_3^--binding molybdoenzymes, such as the NXR of *Nitrobacter* or bacterial nitrate reductases (NARs). Two candidates were identified for each NxrA and NxrB (Table S2). The genes are colocalized in two clusters (*nxrA1B1* and *nxrA2B2*), which are separated by 17 other CDS from each other. The amino acid identities are 86.6% for the two NxrA and 100% for the two NxrB copies (the *nxrB* genes are identical except for a synonymous single-base substitution). NxrA1 and NxrA2 contain binding motifs for one [Fe-S] cluster and for molybdenum, which are indicative of the type II group in the dimethyl sulfoxide (DMSO) reductase family of Mo-co–binding enzymes (*SI Results* and Fig. S2 *A* and *B*). Five residues, which are conserved in the α-subunits of NARs and in the NxrA of *Nitrobacter* and *Nitrococcus*, have been proposed to interact with NO_2^-/NO_3^- or to affect the conformation of the substrate entry channel (22). Except for one threonine, which is replaced by asparagine (Fig. S2*B*), these residues are conserved in both NxrA copies of *Ca*. N. defluvii, suggesting that the α-subunit contains the substrate-binding site. Consistent with the periplasmic orientation of NXR in *N. moscoviensis* (21), NxrA1 and NxrA2 of *Ca*. N. defluvii contain an N-terminal twin-arginine motif for export via the twin-arginine protein translocation (Tat) pathway.

Both NxrB copies of *Ca*. N. defluvii lack a predicted signal peptide, but may be cotranslocated with NxrA into the periplasm by a "hitchhiker" mechanism as proposed for the β-subunits of other periplasmic Mo-co–binding enzymes (e.g., ref. 23). Four cysteine-rich binding motifs for [Fe-S] clusters, which occur also in NxrB of *Nitrobacter* and *Nitrococcus*, were identified (Fig. S2 *C* and *D*). Homologous [Fe-S] clusters mediate intramolecular electron transfer in nitrate reductase A of *Escherichia coli* (24).

All NxrA and NxrB copies of *Ca*. N. defluvii lack transmembrane helices, although NXR is membrane-associated in *Nitrospira* (21). Theoretically, the α/β-complex might cluster with a membrane-bound terminal oxidase that receives electrons from NXR. However, other enzymes in the DMSO reductase family contain an additional membrane-integral γ-subunit, which is the membrane anchor of the holoenzyme and channels electrons between the β-subunit and the electron transport chain via one or two hemes (25). Four proteins encoded by *Ca*. N. defluvii could be heme-containing subunits of NXR (Table S2). Each has one transmembrane domain and an N-terminal signal peptide for translocation via the Sec pathway. The largest candidate (66.7 kDa) is a *c*-type cytochrome with two predicted heme-binding sites. The other three proteins are smaller (29.7–34.3 kDa) and remotely similar to the γ-subunit of chlorate reductase, which contains one *b*-type heme (26). These genes are not in direct proximity of the *nxrAB* clusters, but the predicted molecular masses of their products resemble the two uncharacterized major proteins from *N. moscoviensis* membrane extracts (62 and 29 kDa) (21). Their biological functions and the exact composition of NXR await experimental clarification.

The sequenced *Nitrobacter* genomes encode a peptidyl-prolyl *cis-trans* isomerase (NxrX) proposed to assist in the folding of NXR (2, 3). *Ca*. N. defluvii lacks a homolog of NxrX, but one CDS is similar to chaperones involved in the assembly of other DMSO reductase-family enzymes (26). It is located directly upstream of one putative membrane-integral NXR subunit (Table S2) and could play a role in NXR maturation.

On the basis of biochemical (21) and genomic data for *Nitrospira*, a membrane-bound periplasmic NXR that consists of at least two subunits is proposed (Fig. 1). High-potential electrons from NO_2^- are probably transferred to cytochrome (cyt.) *c* as in *Nitrobacter* (19) and then to a terminal cyt. *c* oxidase (Fig. 1). In *Nitrobacter*, the terminal oxidase is of the *aa3* type (3). The lack of detectable cyt. *a* in *Nitrospira* cultures (4, 8) and of genes coding for *a*-type cytochromes in *Ca*. N. defluvii implies that *Nitrospira* possess a different type of terminal oxidase. Intriguingly, the genome does not encode any known heme-copper oxidase, which could transfer electrons from cyt. *c* to O_2. However, *Ca*. N. defluvii has a heterodimeric cyt. *bd* quinol oxidase (genes *cydA* and *cydB*; Table S2) that could receive electrons derived from low-potential donors, such as organic carbon, via the quinol pool (Fig. 1). The genome contains four additional CDS that resemble the CydA subunit of cyt. *bd* oxidases, but can be distinguished from the canonical proteins by phylogenetic analysis (Fig. S3*A*). We refer to these uncharacterized proteins as putative "cyt. *bd*-like oxidases." They contain 14 predicted transmembrane helices and several histidines that may serve as heme ligands (Fig. S3*B*). Interestingly, one of these CDS (Nide0901) also contains a putative copper (Cu_B)-binding site (Fig. S3*B*). This motif is characteristic for the binuclear center of heme-copper cyt. *c* oxidases, and it is thus tempting to speculate that Nide0901 could replace the lacking canonical heme-copper oxidases in *Nitrospira* (Fig. 1). The proposed function of Nide0901 as terminal oxidase gains further support from transcriptional analysis. High levels of *nide0901* mRNA were detected in the presence of the electron donor NO_2^- and the terminal electron acceptor O_2, whereas the transcription of this gene decreased markedly in the absence of these substrates (Fig. S3*D*). An alternative to a membrane-bound terminal oxidase would be a soluble cytoplasmic O_2 reductase, but this is not supported by the genomic data.

The genome-based model of energy metabolism in *Ca*. N. defluvii composes a branched respiratory chain for NO_2^- oxidation for the use of low-potential electron donors such as organic substrates and for reverse electron transport (Fig. 1). In addition, two copper-containing nitrite reductases (NirK; Table S2) were identified. NirK forms NO from NO_2^- in denitrifying organisms, including other nitrifiers (e.g., 27). Although denitrification by *Ca*. N. defluvii has not been experimentally

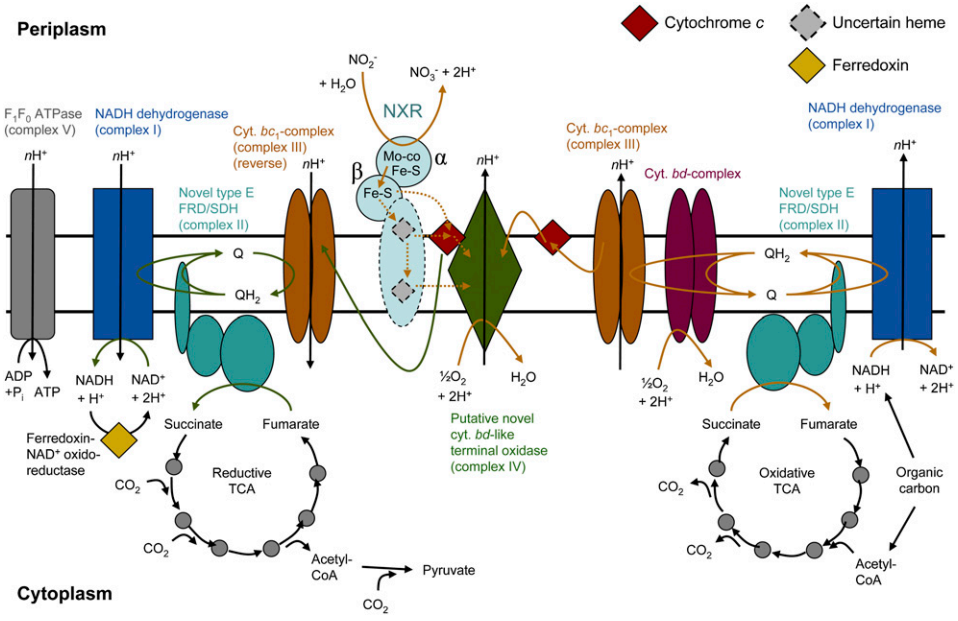

Fig. 1. Genome-based model of energy metabolism in *Ca*. N. defluvii. Orange arrows indicate electron flow in the oxidative branches of the electron transport chain; green arrows indicate reverse electron transport from NO_2^- to NAD^+. Dashed black lines point out that the membrane-integral subunit of NXR is uncertain. Dashed orange arrows show hypothetical possibilities for electron flow from NXR to the putative cyt. *c*-oxidase. *n*H^+ indicates that the number of translocated protons is unknown because the H^+/e^- ratio of the respective complexes has not been determined for *Nitrospira*. FRD, fumarate reductase; SDH, succinate dehydrogenase. See Table S2 for a list of the involved proteins.

demonstrated, the *nirK* genes indicate that this organism may denitrify NO_2^-, for example, by using organic substrates as the electron donor. If NXR works reversibly in *Nitrospira*, denitrification could also start from NO_3^-. Other denitrification genes were not found. In *Nitrobacter*, NO may function in reverse electron transport (28) and in electron flux regulation (27). It remains unclear whether NO plays similar physiological roles in *Nitrospira*.

Expression of NXR. To test whether NO_2^- induces the expression of NXR, RNA was extracted from enrichment biomass during starvation in NO_2^--free medium and after addition of NO_2^-, and *nxrB* mRNA was analyzed by reverse transcription (RT)–PCR. Interestingly, a low level of *nxrB* mRNA was detected after starvation for 11 d in NO_2^--free medium (Fig. S2*E*). Addition of NO_2^- led to an increased transcription of *nxrB*, whereas the level of 16S rRNA from *Ca*. N. defluvii did not change markedly (Fig. S2*E*). NxrB protein was detected even after starvation in NO_2^--free medium for 110 d, and its level increased markedly upon addition of NO_2^- (Fig. S2*E*). These results support the annotation of NXR. The constitutive expression of NXR should enable *Ca*. N. defluvii to use NO_2^-, whose concentration usually is low and fluctuates in natural habitats, immediately after this energy source becomes available.

Autotrophy. NOB of the genus *Nitrobacter* (2) and, on the basis of genomic data, also *Nitrococcus* use the Calvin–Benson–Bassham (CBB) cycle for CO_2 fixation. The key enzymes of this pathway are ribulose-1,5-bisphosphate carboxylase (RubisCO) and ribulose-5-phosphate kinase. *Nitrospira* also grow chemolithoautotrophically on NO_2^- and CO_2 (4, 13), but their pathway for CO_2 fixation was not identified previously. *Ca*. N. defluvii encodes a form IV RubisCO-like protein (Fig. S4*A*) lacking functional key residues of canonical RubisCO (Fig. S4*B*). In *Bacillus subtilis*, a form IV RubisCO-like protein has no bona fide carboxylating activity (29). The absence of other genes similar to RubisCO and of ribulose-5-phosphate kinase suggests that the CBB cycle does not operate in *Ca*. N. defluvii. Instead, all genes of the reductive tricarboxylic acid (rTCA) cycle are present, including the key enzymes ATP-citrate lyase and 2-oxoglutarate:ferredoxin oxidoreductase (OGOR), and also pyruvate:ferredoxin oxidoreductase (POR) (Table S2 and *SI Results*).

Operation of the rTCA cycle in *Ca*. N. defluvii was confirmed by the small carbon isotopic fractionation factor (ε) between biomass and CO_2 of 2–6‰ (Table S3), typical for the rTCA cycle (30). Furthermore, the abundant (~80% of all fatty acids) and characteristic straight-chain fatty acid for *Ca*. N. defluvii, $C_{16:1}$ ω5 (13), was 3–6‰ enriched relative to the biomass, whereas isoprenoid lipids were ~4‰ depleted (Table S3). This trend of more enriched straight-chain lipids is unusual for almost all carbon fixation pathways except for the rTCA cycle (31).

As POR and OGOR generally are O_2-sensitive enzymes (32), the rTCA cycle is found mainly in anaerobic organisms, and its presence in an aerobic nitrifier seems surprising. However, this pathway is functional in some microaerophilic autotrophs such as *Hydrogenobacter thermophilus* (33), and it was identified in *Leptospirillum* genomes (16, 17). *H. thermophilus* has two isozymes of OGOR, a two-subunit enzyme needed under anoxic conditions and a more O_2-tolerant unique five-subunit form, which supports mainly aerobic growth (34), and it also has an unusual five-subunit POR (35). Highly similar five-subunit OGOR and POR in *Ca*. N. defluvii (*SI Results*) and *Leptospirillum* (16) may allow the rTCA cycle to function in these aerobic members of the Nitrospirae phylum. Thus, on the basis of genomic and isotopic data, *Nitrospira* fix CO_2 via the rTCA cycle and represent the only nitrifier for which this pathway has been detected.

Use of Organic Substrates. *Ca*. N. defluvii and *Nitrospira marina* benefit from simple organic compounds in nitrite media (8, 13), and uncultured *Nitrospira* in sewage plants take up pyruvate (5). However, it is unknown whether *Nitrospira* use organic substrates only as carbon sources or also for energy generation. Interestingly, the *Ca*. N. defluvii genome encodes pathways for the catabolic degradation and for the assimilation of acetate, pyruvate, and formate (Fig. S5 and *SI Results*), and candidate genes were found for the degradation of branched amino acids. As the Embden–Meyerhof–Parnas pathway is complete, *Ca*. N. defluvii should be able to metabolize hexose sugars. This is consistent with carbon being stored as glycogen (*SI Results*). Two of the three sequenced *Nitrobacter* genomes also contain the complete glycolysis pathway (3), but growth of *Nitrobacter* on sugars has not been reported. Whether *Ca*. N. defluvii can take up and use sugars should depend mainly on functional sugar transport systems. The genome indeed contains putative sugar transporters (Table S1), but their function remains to be determined.

The oxidative tricarboxylic acid (oTCA) cycle shares most enzymes with the rTCA cycle except for citrate synthase and the 2-oxoglutarate dehydrogenase complex (ODH). *Ca*. N. defluvii encodes citrate synthase but apparently lacks ODH, which may,

however, be replaced by OGOR (Table S2 and *SI Results*). A complete oTCA cycle was reported for *Nitrobacter* (28), indicating that this pathway is not unusual in NOB.

Purely heterotrophic growth of *Nitrospira* has not been observed yet. However, if all potentially involved genes are functional, *Ca*. N. defluvii benefits from a mixotrophic lifestyle using organic compounds from sewage in addition to NO_2^- and CO_2.

Stress Response and Defense. *Ca*. N. defluvii is exposed to a plethora of potentially toxic substances in sewage. Accordingly, the genome encodes multidrug efflux systems and transporters for heavy metals, organic solvents, and antimicrobials (Table S1), and it contains genes for cyanate and arsenic resistance (Table S2 and *SI Results*). As shown previously (12), *Ca*. N. defluvii has a functional chlorite dismutase that could degrade ClO_2^- in polluted environments, in chlorinated activated sludge, or in the proximity of chlorate-reducing microbes. Most intriguingly, *Ca*. N. defluvii lacks key genes for protection from reactive oxygen species (ROS) present in most aerobic organisms. No catalase, superoxide dismutase, or superoxide reductase was found. Two cyt. *c* peroxidases and several thioredoxin-dependent peroxiredoxins could function as H_2O_2 scavengers (Table S2 and *SI Results*). Protection from O_2^- and H_2O_2 might be conferred by manganese [Mn(II)] (36). Indeed, the required permease for manganese import was identified in the genome. Bacterioferritin and carotenoids (Table S2) could also contribute to protection from radicals and ROS. Moreover, the intracellular O_2 level could be kept low by the canonical cyt. *bd* oxidase. Homologs in other organisms have a high affinity to O_2 and contribute to oxidative stress protection (37). Growth of *Nitrospira* in biofilms and flocs (e.g., ref. 13) could offer additional protection from ambient O_2.

Ca. N. defluvii carries one region of clustered, regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (*cas*) genes for phage defense (38). The CRISPR repeats of *Ca*. N. defluvii show no sequence similarity to those of *Leptospirillum* groups II and III, which also differ in their Cas proteins (17), suggesting that this defense mechanism was independently acquired by different members of the Nitrospirae phylum.

Ecophysiology and Evolutionary History of *Nitrospira*. The NXRs of *Nitrobacter*, *Nitrococcus*, and *Nitrospira* differ in their subcellular localization and phylogenetic position within the DMSO reductase family. The NXRs of *Nitrobacter* and *Nitrococcus* are closely related to NARs. They are associated with the cytoplasmic membrane and ICM with the active site facing the cytoplasm (39). The unique NXR of *Nitrospira* does not cluster with the NARs (Fig. 2). It is also attached to the cytoplasmic membrane, but is oriented toward the periplasmic space (21; this study). The periplasmic orientation should be energetically advantageous because proton release by NO_2^- oxidation in the periplasm and concomitant proton consumption by O_2 reduction in the cytoplasm contribute to the membrane potential (Fig. 1). Furthermore, only a cytoplasmic NXR requires the transport of NO_2^- and NO_3^- in opposite directions across the inner membrane. Accordingly, putative NO_2^-/NO_3^- transporters are found in all sequenced *Nitrobacter* genomes (3) and in *Nitrococcus*. Their substrate affinities and turnover rates could be limiting factors for NO_2^- oxidation by these NOB. This and the catalytic properties of NXR could explain the relatively high apparent $K_m(NO_2^-)$ value of *Nitrobacter* (11). In contrast, the predicted NO_2^- and NO_3^- transporters of *Ca*. N. defluvii (Table S1) most likely play no role in nitrite oxidation but are required only for nitrogen assimilation and resistance against excess nitrite (*SI Results*).

Consistent with the predicted advantages of their periplasmic NXR, *Nitrospira* are better adapted to low NO_2^- concentrations (10, 11), which also were key to the selection against coexisting *Nitrobacter* during enrichment (13). As NO_2^- rarely accumulates in natural environments, the highly efficient use of this substrate most likely is a main reason for the competitive success and wide natural distribution of *Nitrospira*.

The use of different key enzymes and pathways (e.g., CO_2 fixation) by *Nitrospira* in contrast to the proteobacterial NOB *Nitrobacter* and *Nitrococcus* suggests that chemolithoautotrophic NO_2^- oxidation evolved independently in these lineages. On the basis of the close phylogenetic affiliation of *Nitrobacter* and *Nitrococcus* to phototrophic *Proteobacteria*, which also possess ICM, Teske et al. (1) hypothesized that these NOB were derived from phototrophic ancestors. Indeed, a recently isolated anaerobic phototroph, which uses NO_2^- as the electron donor, is closely related to *Nitrococcus* (40). A cytoplasmically oriented NXR would probably be no disadvantage for phototrophic NOB where the membrane potential is sustained mainly by light-driven cyclic electron flow. The orientation of NXR may not easily be reversed, because it intimately affects the interaction with downstream components of the electron transport chain. Hence, the conservation of a cytoplasmic NXR during the transition from phototrophy to chemolithotrophy could explain the orientation of NXR in *Nitrobacter* and *Nitrococcus*. In contrast and consistent with the absence of ICM in *Nitrospira*, no phototrophic relative of *Nitrospira* is known and we hypothesize that the capability to gain energy from NO_2^- oxidation has evolved in this lineage from an anaerobic nonphototrophic ancestor. An anaerobic or microaerophilic origin of *Nitrospira* would be consistent with the rTCA cycle, the presence of the anaerobic cobalamin biosynthesis pathway (Table S2), and the lack of classic defense mechanisms against ROS. Additional support for this hypothesis stems from estimating genus divergence times within the Nitrospirae phylum by using 16S rRNA as the molecular clock (*SI Results*). Extant *Nitrospira* are active at low dissolved O_2 levels in bioreactors and might still prefer hypoxic conditions (41).

Intriguingly, comparative genomics revealed an unexpected evolutionary link between *Nitrospira* and anammox organisms. For example, the closest homolog of the NXR of *Ca*. N. defluvii was found in *Ca*. K. stuttgartiensis (Fig. 2). NO_2^- oxidation is an integral step of the anammox metabolism where it replenishes the electron transport system (14), and this NXR-like protein is the only candidate for a NO_2^--oxidizing enzyme in the *Kuenenia* genome. Its α-subunit contains the signature residues of NO_2^-/NO_3^--binding molybdoenzymes (22) (Fig. S2 *A* and *B*). The NXRs of *Nitrospira* and *Kuenenia* are highly similar (amino acid identities are 57.4–57.7% for the α-subunit and 62.5% for the β-subunit) and form a monophyletic lineage in the tree of type II enzymes *of* the DMSO reductase family (Fig. 2). In addition, both *Ca*. N. defluvii and *Ca*. K. stuttgartiensis have the putative chaperone for NXR assembly in analogy to NxrX of *Nitrobacter*. *Ca*. K. stuttgartiensis also has a putative cyt. *bd*-like oxidase, which is the closest relative of the four cyt. *bd*-like oxidases of *Ca*. N. defluvii (Fig. S3*A*). Interestingly, its gene is located in close proximity to *nxrA*, *nxrB*, two putative membrane subunits of NXR, and the chaperone in the *Kuenenia* genome (Fig. 3). The same region contains a monoheme cyt. *c*-like protein and three proteins of unknown function, which also have highly similar homologs in *Ca*. N. defluvii (Fig. 3). Thus, both organisms share a set of highly similar proteins that function in NO_2^- oxidation and probably in electron transport and respiration, and these genes are clustered as a small metabolic island in the anammox genome. As anammox organisms are planctomycetes and consequently not closely related to the Nitrospirae (14), these observations are strongly indicative of a horizontal gene transfer (HGT) that established NXR and the other proteins in both lineages. Consistent with the fundamental importance of the transferred genes for the basic metabolism of *Nitrospira* and anammox, this HGT apparently occurred early during the evolution of these lineages as no remarkable deviation in GC content or codon use of the respective genes was observed in either organism.

To explore further the influence of vertical gene transfer and HGT on the evolutionary history of *Ca*. N. defluvii, we calculated phylogenies for each protein of *Ca*. N. defluvii and identified the organism encoding the respective most closely related homolog (Fig. S6). Most remarkably, in this analysis *Ca*. K. stuttgartiensis was the single organism that shared the highest number of closest

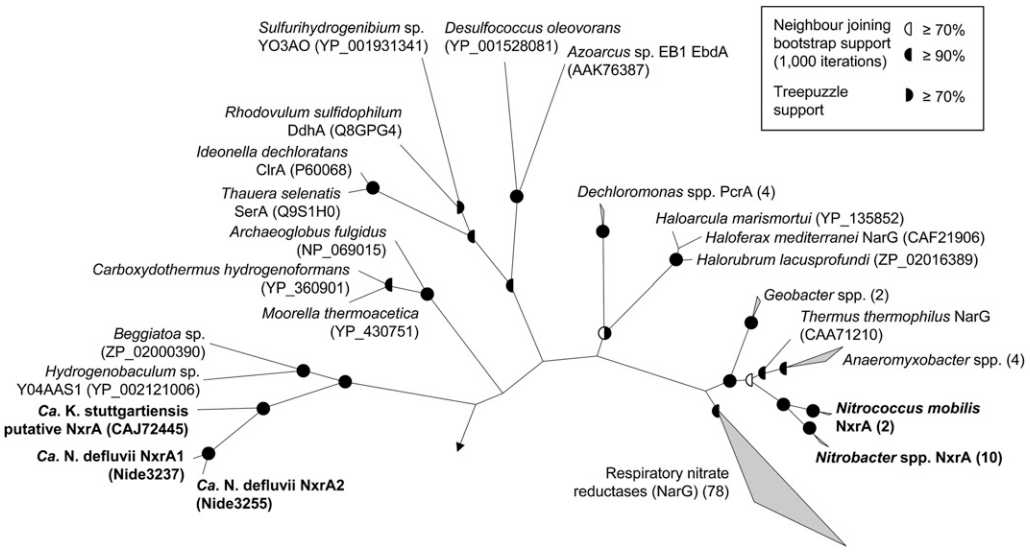

Fig. 2. Maximum-likelihood tree showing the phylogenetic positioning of selected type II enzymes of the DMSO reductase family. For phylogenetic analysis of the catalytic (α) subunits, 1,308 amino acid positions were considered. Names of validated enzymes are indicated: Nxr, nitrite oxidoreductase; Nar, 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.

homologs (71 hits) with *Ca*. N. defluvii and thus exchanged, compared with all other organisms for which genome sequences are available, the most genes with *Nitrospira* via HGT. Surprisingly, the 71 hits even exceed the number of best hits with members of the Nitrospirae phylum, namely *Thermodesulfovibrio* (67 hits) and different *Leptospirillum* strains (39–66 hits). These findings illustrate a surprisingly small set of the most closely related homologs in the Nitrospirae, most likely reflecting the dramatically different ecological niches inhabited by the genera affiliated with this phylum.

Taken together, the metagenome sequence of *Ca*. N. defluvii revealed that this globally important nitrite oxidizer differs fundamentally in its enzymatic repertoire (unusual NXR and putative terminal oxidase) and metabolic pathways (rTCA for autotrophy) from all other known nitrifiers, but strikingly exploits almost the same gene repertoire for NO_2^- oxidation as the anammox organism *Ca*. K. stuttgartiensis. The unique genomic features of *Nitrospira* have already provided some well-supported hypotheses for its competitive success in most nitrifying ecosystems and suggest that *Nitrospira* are well adapted to hypoxic environmental niches where nitrite oxidation has rarely been studied until now. From an applied perspective, the lack of common protection mechanisms against oxidative stress in *Nitrospira* implies that

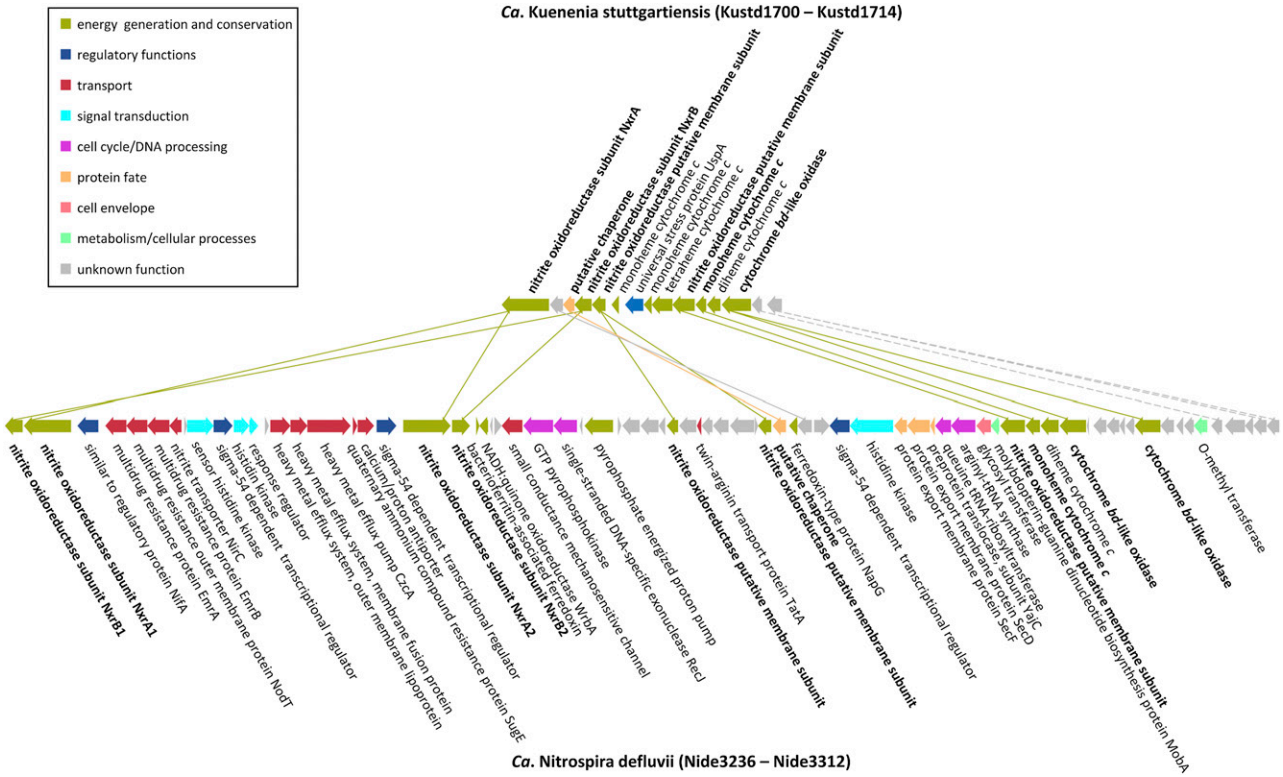

Fig. 3. Schematic of the genomic regions in *Ca*. K. stuttgartiensis and *Ca*. N. defluvii, which contain shared genes coding for NXR, putative cyt. *bd*-like oxidases and electron carriers, and proteins of unknown function. Solid lines connect genes that are the closest homologs on the basis of protein phylogeny. Their predicted functions are in boldface. Dashed lines connect similar genes that are not the closest relatives in the respective phylogenetic protein trees. Predicted CDS and connecting lines are colored according to functional classes. CDS and intergenic regions are drawn to scale.

a good aeration control is crucial for maintaining stable and active populations of these organisms in engineered systems.

Materials and Methods

Genomic Sequencing and Annotation. Metagenome sequencing and the reconstruction of the whole *Ca*. N. defluvii genome were carried out by Genoscope (*SI Materials and Methods*). The MaGe software system (42) was used for the prediction, automatic annotation, and manual annotation refinement of all CDS as described in *SI Materials and Methods*.

Phylogenetic Analyses. Amino acid sequences of type II DMSO reductase-family enzymes, of RubisCO and RubisCO-like proteins, and of cyt. *bd* and cyt. *bd*-like oxidases were aligned, and phylogenetic trees were computed by using ARB (43). For the calculation of phylogenetic trees for each protein in the proteome, PhyloGenie (44) was used. For details, see *SI Materials and Methods*.

Expression Analysis of NxrB and the Putative Terminal Cyt. *c* Oxidase (Nide0901). *Ca*. N. defluvii enrichment biomass was incubated in mineral media with or without NO_2^- and, for Nide0901, also under oxic or anoxic conditions as described in *SI Materials and Methods*. Following total RNA extraction, 16S rRNA of *Nitrospira* and *nxrB* or *nide0901* transcripts were detected by RT–PCR (*SI Materials and Methods*). Translation of NxrB was shown by Western blotting with a monoclonal antibody that binds to the NxrB of *Nitrospira* (21) (*SI Materials and Methods*).

Stable Carbon Isotopic Fractionation. The isotopic fraction of *Ca*. N. defluvii was measured following methods published earlier (45) (*SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank Lisa Stein for analyses of *nirK* genes and Peter Bottomley, Jim Hemp, Jim Prosser and Andreas Schramm for helpful discussions. Christiane Dorninger, Alexander Galushko, Christian Baranyi, Jan Dolinšek, Patrick Tischler, Irene Rijpstra, and Michiel Kienhuis are acknowledged for technical support. This work was supported by the Vienna Science and Technology Fund (Wiener Wissenschafts-, Forschungs-, und Technologiefonds, Grant LS 216 to H.D., S.L., and F.M. and by Grant LS09-40 to H.D., H.K., and S.L.), the Austrian Research Fund (Fonds zur Förderung der Wissenschaftlichen Forschung, Grant S10002-B17 to H.D., M.W., S.L., and F.M.), and the German Research Foundation (Deutsche Forschungsgemeinschaft, Grant SP 667/3-1 to E.S.).

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