

Complete Genome Sequence of *Nitrobacter hamburgensis* X14 and Comparative Genomic Analysis of Species within the Genus *Nitrobacter*^{∇†}

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The alphaproteobacterium *Nitrobacter hamburgensis* X14 is a gram-negative facultative chemolithoautotroph that conserves energy from the oxidation of nitrite to nitrate. Sequencing and analysis of the *Nitrobacter hamburgensis* X14 genome revealed four replicons comprised of one chromosome (4.4 Mbp) and three plasmids (294, 188, and 121 kbp). Over 20% of the genome is composed of pseudogenes and paralogs. Whole-genome comparisons were conducted between *N. hamburgensis* and the finished and draft genome sequences of *Nitrobacter winogradskyi* and *Nitrobacter* sp. strain Nb-311A, respectively. Most of the plasmid-borne genes were unique to *N. hamburgensis* and encode a variety of functions (central metabolism, energy conservation, conjugation, and heavy metal resistance), yet ~21 kb of a ~28-kb “autotrophic” island on the largest plasmid was conserved in the chromosomes of *Nitrobacter winogradskyi* Nb-255 and *Nitrobacter* sp. strain Nb-311A. The *N. hamburgensis* chromosome also harbors many unique genes, including those for heme-copper oxidases, cytochrome *b*₅₆₁, and putative pathways for the catabolism of aromatic, organic, and one-carbon compounds, which help verify and extend its mixotrophic potential. A *Nitrobacter* “subcore” genome was also constructed by removing homologs found in strains of the closest evolutionary relatives, *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*. Among the *Nitrobacter* subcore inventory (116 genes), copies of genes or gene clusters for nitrite oxidoreductase (NXR), cytochromes associated with a dissimilatory nitrite reductase (NirK), PII-like regulators, and polysaccharide formation were identified. Many of the subcore genes have diverged significantly from, or have origins outside, the alphaproteobacterial lineage and may indicate some of the unique genetic requirements for nitrite oxidation in *Nitrobacter*.

Nitrification is a two-step process by which ammonia is converted to nitrate via nitrite. Nitrification plays a key role in transformation of fertilizer nitrogen in agricultural systems and is a key component of nitrogen removal in wastewater treatment. Production of soluble inorganic nitrogen by nitrification can lead to the contamination and eutrophication of terrestrial and aquatic ecosystems, while the gaseous products of nitrifier denitrification, nitric oxide (NO) and nitrous oxide (N₂O), destroy stratospheric ozone and greatly contribute to global warming (26, 54, 61). Nitrite-oxidizing bacteria (NOB) participate in the process of nitrification by converting nitrite (NO₂⁻), the end product of ammonia oxidation, into nitrate (NO₃⁻) according to the following reaction; NO₂⁻ + H₂O → NO₃⁻ + 2H⁺ + 2e⁻. Nitrite also functions as an electron donor for the reduction of NAD⁺ via reverse electron flow as well as for the generation of ATP by oxidative phosphorylation (17).

Although several phylogenetically distinct genera (*Nitrospira*, *Nitrobacter*, *Nitrococcus*, and *Nitrospina*) carry out NO₂⁻ oxidation, most of what is known about the physiology and biochemistry of NOB has been derived from studies of the genus *Nitrobacter*. As facultative chemolithoautotrophs, *Nitrobacter* spp. oxidize nitrite and fix carbon dioxide (CO₂) via the Calvin-Benson-Basham pathway. However, they also have the ability to assimilate a narrow range of simple organic carbon (C) compounds (pyruvate, acetate, α-ketoglutarate, and glycerol) in the absence of nitrite (3, 52). The effectiveness of organotrophy varies among members of the genus *Nitrobacter*. For example, in *Nitrobacter winogradskyi*, the highest growth rates are achieved using nitrite as an energy source, yet organic carbon additions to nitrite-containing cultures can increase the final growth yield and protein content after nitrite is depleted (52). In contrast, the highest growth rates reported for *Nitrobacter hamburgensis* X14 are achieved in media containing both nitrite and organic carbon (6, 7, 56). The genetic and full enzymatic bases for these differing phenotypes have not been explored.

Nitrobacter is a member of the family *Bradyrhizobiaceae* and is closely related (97 to 98% identity of 16S rRNA) to the *Bradyrhizobium* and *Rhodopseudomonas* genera. *Rhodopseudo-*

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monas palustris can grow either chemotrophically or phototrophically and is arguably one of the most metabolically versatile bacteria known (38), and the plant symbiont *Bradyrhizobium japonicum* has one of the largest prokaryote genomes. However, neither of these close *Nitrobacter* relatives has the ability to use nitrite as an energy source. The availability of genome sequences from these three genera has provided an opportunity to gain insight into the basis of what distinguishes the “metabolically limited” *Nitrobacter* from its close “physiologically versatile” relatives and has led to a better understanding of the physiological requirements for nitrite oxidation.

Here we present an analysis of the genome sequence from *N. hamburgensis* X14 and a comparative analysis of the genomes within the genus *Nitrobacter*. Included in our *Nitrobacter* comparative analysis are the genomes of *Nitrobacter winogradskyi* Nb-255 and *Nitrobacter* sp. strain Nb-311A (NB311A). Curiously, NB311A was isolated near the west coast of central Africa and grows in seawater (B. Ward, unpublished results), yet its 16S rRNA gene sequence is 100% identical to that of the soil isolate *N. winogradskyi*. *N. winogradskyi* was previously sequenced and analyzed (53), and a draft sequence of NB311A was recently made available, for which no published reports currently exist. In addition, we included in our analysis five complete genomes from *Rhodopseudomonas palustris* and three from *Bradyrhizobium japonicum*, which are also available in public databases. Placed in the context of these *Bradyrhizobiaceae* members, the genetic framework for determining the metabolic variations and similarities that exist within *Nitrobacter* is analyzed and the genomic basis of lithoautotrophy versus trophic flexibility among closely related bacteria is explored.

MATERIALS AND METHODS

Construction, sequencing, and assembly. Genomic DNA from *N. hamburgensis* X14 was isolated; purified; sheared into approximately 3-kb, 8-kb, and 40-kb fragments; and ligated into the pUC18, pMCL200, and pCC1Fos cloning vectors, respectively. After amplification, double-ended plasmid sequencing reactions were performed at the DOE Joint Genome Institute using ABI 3730xl DNA analyzers and MegaBACE 4500 genetic analyzers as previously described (9, 16; <http://www.jgi.doe.gov/>).

Processing of sequence traces, base calling, and assessment of data quality were performed with PHRED and PHRAP (14, 15) at an estimated error rate of <2.5 bp per Mbp sequenced. After quality control of the 66,403 total initial reads of the draft sequence, 58,661 sequences were assembled, producing an average of 11-fold genome coverage. The reads were assembled into 52 high-quality draft sequence contigs, visualized with CONSED (23), and linked into 18 larger scaffolds using paired-end sequence information. Gaps between linked contigs were closed either by walking on gap-spanning clones or with PCR products generated from genomic DNA, while physical (or uncaptured) gaps were closed by combinatorial PCR. Sequence finishing and polishing added 2,360 reads, and final quality assessment of the completed genome was completed as previously described (9).

Genome analysis and annotation. Automated gene modeling for *N. hamburgensis* was completed by combining results from the Critica, Generation, and Glimmer modeling packages. Automated annotation was produced by searching all predicted peptides against TIGRFAMs (HMMPFam trusted cutoffs), PRIAM (rpsblast 1e–30 cutoff), PFAM (HMMPFam trusted cutoffs), InterPro (interproscan default cutoffs), and COGs (rpsblast 1e–10 cutoff). Proteins that failed to return a definitive result with the aforementioned profile searches were annotated on the basis of BLASTP searches against the KEGG and Swissprot-TREMBL (1e–5 cutoff) peptide databases. In the manual curation phase, the protein set was also searched against GenBank’s nonredundant database and the PROSITE, EcoCYC, and MetaCYC databases to further assess function. Gross truncations of peptides ($n = 171$), base insertions or deletions that produced frameshifts ($n = 153$), or internal stop codons ($n = 23$) that resulted in coding

sequences (CDSs) that were $\leq 80\%$ of the best BLASTP match were considered pseudogenes.

NB311A was isolated by Stan Watson in 1968 from surface waters in the tropical Eastern Atlantic Ocean off the coast of West Africa (J. Waterbury, personal communication). The draft genome sequence of NB311A was downloaded from a public database at the Venter Institute (<https://research.venterlinstitute.org/moore/>) and reannotated using the same criteria as for *N. hamburgensis* and *N. winogradskyi* to facilitate an accurate comparative analysis.

Cross genomic analysis. Orthologous and paralogous groups were determined using OrthoMCL version 1.4. A peptide database of 11,704 sequences of the CDS translations from *Nitrobacter hamburgensis* X14 (accession no. CP000319), *Nitrobacter winogradskyi* Nb-255 (CP000114), and *Nitrobacter* sp. strain NB311A (AAMY01000000) was assembled. To determine which CDS translations were conserved in all three species, an all-versus-all analysis was performed using BLASTP (BLASTALL 2.2.13) with seg, with an E value threshold of 1×10^{-5} . The results were processed by OrthoMCL (mcl-06-21) using an inflation factor of 1.5. The output of OrthoMCL was parsed to separate orthologous and paralogous groups. Duplicated genes/paralogs were counted as a single “gene type.” The clustered orthologous/paralogous data set displayed an average E value of 1×10^{-113} and an average identity of 86%. NCBI Taxplot software (<http://www.ncbi.nlm.nih.gov/sutils/taxik2.cgi?isbact=1>) was used to compare the similarity between the conserved proteins in the three *Nitrobacter* genomes using *N. winogradskyi* as the query genome with the cutoff set at 10.

***Nitrobacter* core and subcore construction.** The OrthoMCL output was filtered to produce a list of ortholog/paralog groups which contained genes from all three *Nitrobacter* species. The resulting list is the core set of genes shared by the three *Nitrobacter* species. The *Nitrobacter* core genes or CDSs were extracted into a separate data set and were subjected to BLASTP searches ($1e-10$ and $1e-20$ cutoffs) against a database consisting of *Bradyrhizobium japonicum* (GenBank/EMBL/DBJ accession numbers BA000040, CP000494, and CU234118) and *Rhodopseudomonas palustris* (BX571963, CP000250, CP000283, CP000301, and CP000463) sequences (46111 peptides). Core sequences without a match ($\geq 1e-10$) were designated the *Nitrobacter*-specific subcore. The subcore was curated manually to remove the remaining peptides ($n = 7$) that were still found to be conserved in *B. japonicum* or *R. palustris* that were not filtered out by the $e-10$ cutoff. Additional manual searches for conserved gene clusters added seven peptides to the subcore using the following criteria: (i) the peptides were present in the $1e-20$ subcore list, (ii) the peptides were adjacent to a peptide(s) in the $1e-10$ database, and (iii) the peptide sequence identity to similar peptides in the *R. palustris*/*B. japonicum* database was $<50\%$.

Nucleotide sequence accession numbers. The sequence and annotation of the complete *N. hamburgensis* X14 chromosome are available at GenBank/EMBL/DBJ using accession number CP000319. The sequences of the *N. hamburgensis* plasmids pPB13, pPB12, and pPB11 are available as plasmid 1, plasmid 2, and plasmid 3, using accession numbers CP000320, CP000321, and CP000322, respectively.

RESULTS AND DISCUSSION

Genome overview. The *N. hamburgensis* X14 genome consists of a 4,406,967-bp chromosome (61.7% G+C) and three plasmids: pPB11 (121,408 bp, 61.7% G+C), pPB12 (188,318 bp, 61.2% G+C), and pPB13 (294,829 bp, 60.4% G+C). Based on BLAST analysis, *N. hamburgensis* shares more total CDSs with NB311A (1,434) than with *N. winogradskyi* (974). In contrast, when conserved proteins were analyzed for similarity, 76% of the proteins in *N. winogradskyi* were more similar to those of NB311A than to those of *N. hamburgensis* (S. R. Starkenburg, unpublished results). This analysis is consistent with the fact that *N. winogradskyi* and NB311A have identical 16S rRNA gene sequences that are approximately 98% identical to the *N. hamburgensis* 16S rRNA gene.

The genome of *N. hamburgensis* is much larger than those of the other two *Nitrobacter* species, containing ~ 1.6 and 0.9 Mbp more genetic material than *N. winogradskyi* and NB311A, respectively (Table 1). Several features of the *N. hamburgensis* genome account for its relatively large size. First, a disproportionately large number of pseudogenes and paralogs were

TABLE 1. General genome characteristics

Strain	Origin	% 16S rRNA gene identity ^a	No. of chromosome bases	% GC	No. of:					
					Total genes	Genes without predicted function	Pseudogenes	Paralogs	Paralog groups	Plasmids
<i>N. hamburgensis</i> X14	Soil	98	4,406,967	61.61	4,716	1,848	347	634	251	3 ^b
<i>N. winogradskyi</i> Nb-255	Soil	100	3,402,093	62.05	3,118	993	21	283	74	0
<i>Nitrobacter</i> sp. strain Nb-311A ^c	Marine	100	~4,105,362	62	4,256	1,461	ND ^d	478	143	ND

^a 16S rRNA identity relative to *N. winogradskyi* Nb-255.

^b pPB13, 294,829 bp; pPB12, 188,318 bp; pPB11, 121,408 bp.

^c Draft sequence.

^d ND, not determined (an accurate count of the pseudogenes or the presence of plasmids was not possible from the unfinished, draft sequence of NB311A).

identified, which account for 20% of the genome. 347 CDSs (~8% of the sequence space) were identified as pseudogenes in *N. hamburgensis*, compared to 21 pseudogenes in *N. winogradskyi* (pseudogene annotation was not completed for the draft genome of *Nitrobacter* sp. strain NB311A). *N. hamburgensis* also has the largest number of paralogs (634 genes in 251 groups), approximately 30% more than NB311A and over twice the number found in *N. winogradskyi*. Second, the ma-

ajority of the genes carried on the *N. hamburgensis* plasmids do not have orthologs in the *N. winogradskyi* or NB311A genome (Fig. 1). As presented below, these relatively large plasmids harbor a number of functions that are uniquely beneficial to *N. hamburgensis* and clearly distinguish it from *N. winogradskyi* and NB311A.

Several “unique” (i.e., not found in the other *Nitrobacter* genome sequences) genomic islands, many of which appear to

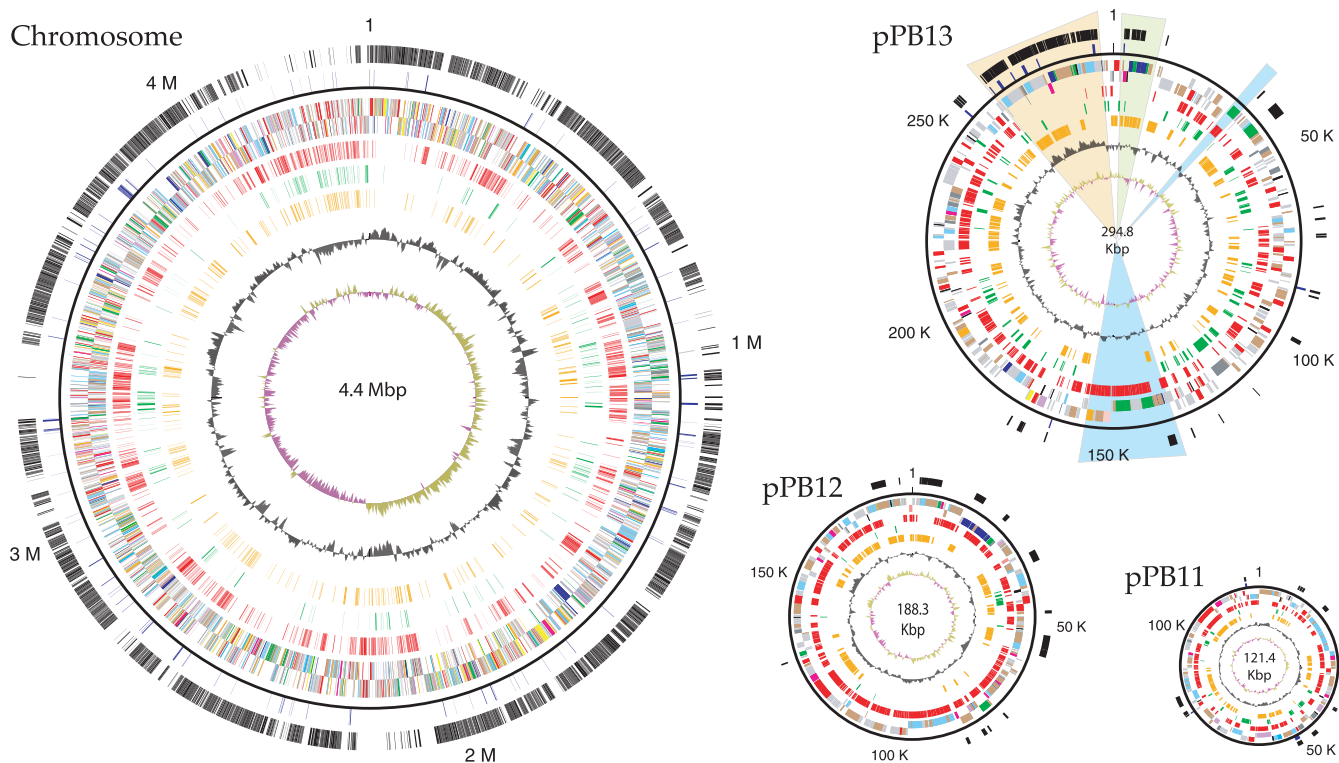


FIG. 1. Genome of *Nitrobacter hamburgensis* X14 (ATCC 25391). The outer circle depicts the locations of genes conserved in all three sequenced *Nitrobacter* genomes. Genes that are conserved in *Nitrobacter* but not *R. palustris* or *B. japonicum* (the subcore) are indicated in the second circle. The third and fourth circles depict predicted protein-encoding and structural RNA genes in *N. hamburgensis* on the plus and minus strands, respectively (green, energy metabolism; red, DNA replication; magenta, transcription; yellow, translation; orange, amino acid metabolism; dark blue, carbohydrate metabolism; pale red, nucleotide metabolism; black, coenzyme metabolism; cyan, lipid metabolism; light blue, cellular processes; brown, general function; gray, hypothetical and conserved hypothetical genes; pale green, structural RNAs). Genes “unique” to *N. hamburgensis* (not present in *N. winogradskyi* or NB311A) are depicted in circle five (red). The sixth and seventh circles indicate the locations of all annotated pseudogenes (green) and paralogs (orange), respectively. The eighth circle indicates GC bias, and the ninth circle indicates GC skew. The highlighted region of pPB13 depicts the locations of key gene clusters: autotrophic island (peach), RuBisCO and pentose phosphate pathway genes (green), and the cytochrome oxidases (*aa*₃ and *bd*-ubiquinol types) (blue).

have been acquired by conjugation or transduction, also help account for the genomic and physiological separation of *N. hamburgensis* and *N. winogradskyi*. For example, a 240-kb island (Nham_3756 to Nham_4008; Fig. 1; chromosome locus 4.13 to 4.37 Mbp), which contains a high concentration of pseudogenes and paralogs, appears to have originated from plasmid or phage sources, since it harbors putative plasmid replication initiator proteins (Nham_3835 and Nham_3863), a partitioning protein (Nham_3861), and phage integrase and phage-related proteins (Nham_3842 and Nham_4008). Similarly, a portion of a 4.2-kb phage element (rep28; see Table S1 in the supplemental material) is found replicated on the *N. hamburgensis* chromosome and contains a phage-related methylase, an uncharacterized phage protein, and two hypothetical proteins found only in *N. hamburgensis*. These and other similar elements lie within other large chromosomal regions (Nham_0784 to Nham_0835, Nham_0838 to Nham_0943, and Nham_1145 to Nham_1186) that are not present in the *N. winogradskyi* genome and appear to have integrated into *N. hamburgensis* (or to have been lost from the other *Nitrobacter* genomes) since delineation from a common ancestor. Based on genome sequence data in REBASE (47), *N. hamburgensis* and *N. winogradskyi* also encode an above-average quantity of restriction-modification (RM) systems (11 in *N. hamburgensis* [2.39 RM genes per Mbp] and 9 in *N. winogradskyi* [2.64 RM genes per Mbp]), the majority of which are type II RM systems. These RM systems should provide defense against genome contamination by phage or other foreign DNA. On the other hand, *N. hamburgensis* appears to have been a successful target of phages at some point in its evolutionary history. Recent evidence indicates that type II RM complexes may function as “selfish” mobile genetic elements and can promote homologous recombination in the host bacteria (as a defense mechanism), resulting in more genomic rearrangements and diversity (32, 33, 44). Clearly, unraveling the complex history and role of phages, RM systems, and other mobile genetic elements (transposons) in the diversification and evolution of these closely related *Nitrobacter* species will require further investigation.

Interspecies comparisons. A global comparative analysis of all genes was completed to assess both the common and unique elements of each of the sequenced *Nitrobacter* species (Fig. 2). A total of 2,179 “gene types” (excluding paralogs/gene duplications) were conserved in each *Nitrobacter* genome. The majority (86%) of the genes in the genome of *N. winogradskyi* are conserved in either *N. hamburgensis* or NB311A; therefore, most of the genome-inferred metabolic potential of *N. winogradskyi* described previously can be extrapolated to these other species (53). In contrast, *N. hamburgensis* and NB311A collectively carry approximately 2,801 gene types not found in *N. winogradskyi* (approximately one-third of each genome; 1,301 and 1,198 gene types, respectively). Surprisingly, although the *N. hamburgensis* genome is >900 kb larger than the NB311A draft sequence, our analysis indicated that NB311A harbors roughly the same number of gene types (3,881 versus 3,876) as *N. hamburgensis*.

The organization of unique genes by COG groups revealed similar gene distribution patterns for all three species, suggesting that many of these genes (based solely on COG groupings) appear to be functional analogs (S. R. Starkenburg, unpub-

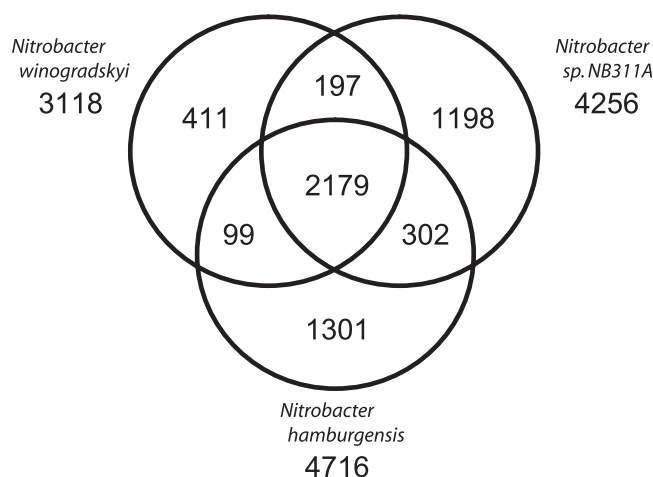


FIG. 2. Global gene conservation in *Nitrobacter*. Each circle represents the total number of gene types in each genome. Overlapping regions depict the number of genes types shared between the respective genomes. The numbers outside the circles indicate the total number of genes identified in each genome, including paralogs/gene duplications.

lished results). Nevertheless, manual analysis of genome-specific sequences revealed that each genome did contain unique genetic material (Table 2), which potentially confers specific functions relevant to the ecological niche of each bacterium. Many of the unique *N. hamburgensis* genes/functions are discussed below. With regard to *N. winogradskyi*, of the 411 genes not found in either of the other two *Nitrobacter* species, only 124 could be assigned a putative function, including an alkane-sulfonate monooxygenase, two nitrate/sulfonate/bicarbonate ABC transporters, and synthesis genes for the pyrroloquinoline quinone cofactor.

Several unique gene clusters were identified in NB311A (Table 2), including some that may be indicative of adaptation to a marine lifestyle. NB311A uniquely possesses genes that encode a chloride channel (NB311A_05795), an $\text{Na}^+/\text{Ca}^{2+}$ antiporter (NB311A_09276), and several cation-dependent ATPases. NB311A also harbors a four-gene cluster (NB311A_1874 to NB311A_1879), which may code for synthesis of ectoine-like osmoprotectants. The putative NB311A ectoine synthesis peptides have 47, 57, 51, and 50% identity to the ectoine synthesis proteins (EctABCD) in the moderate halophile *Chromohalobacter salexigens* DSM 3043, which has been shown to produce ectoines as osmo- and thermoprotectants (21, 22, 60). All three genomes were found to encode a putative Na^+/H^+ antiporter (*nhaA*), which is required for *Escherichia coli* to survive high-salt stress (2, 51). Consequently, we carried out a preliminary investigation of the salt tolerance of *N. hamburgensis* and *N. winogradskyi* by monitoring growth and nitrite consumption in cultures amended with 0, 100, 200, 400, and 600 mM NaCl (culture medium amended with 600 mM NaCl is equivalent to the salinity of seawater). *N. winogradskyi* grew normally in standard growth medium amended with 0 to 200 mM NaCl. In cultures amended with 400 mM NaCl, the growth rate decreased by 50%, yet the growth yield of *N. winogradskyi* was unaffected. Growth was not observed when cultures were amended with 600 mM NaCl, even though the cells continued to oxidize nitrite. *N. hamburgensis* was less salt resistant, as growth was inhibited at >200 mM NaCl, although the cells

TABLE 2. Unique genes and putative functional biases in the genus *Nitrobacter*

Organism	Category (functions)
<i>N. winogradskyi</i> Nb-255.....	Transport (NO ₃ ⁻ /sulfonate/CO ₃ ²⁻ [2], iron uptake systems, nickel/cobalt, PO ₄ ³⁻ porin, uncharacterized ABC transporter components), miscellaneous (histidine biosynthesis, multiple FecIR genes, pyrroloquinoline quinone biosynthesis)
<i>N. hamburgensis</i> X14.....	Transport (ammonia permease, K ⁺ transport, uncharacterized ABC transport components), carbon metabolism (formate dehydrogenase, carbon monoxide dehydrogenase-like, L-lactate dehydrogenase, malate dehydrogenase, pyruvate-formate lyase, homogentisate/phenylacetate degradation), energetics (cytochrome <i>c</i> oxidase, cytochrome <i>bd</i> ubiquinol oxidase, cytochrome <i>b</i> ₅₆₁ [4], cytochrome P460, flavodoxin reductase, sulfur oxidation genes [<i>soxXYZAB</i>]), nitric oxide reductase [<i>sNOR</i>]), miscellaneous (type II secretion, serine proteases, conjugal transfer, heavy metal resistance, arsenite oxidase)
<i>Nitrobacter</i> sp. strain Nb-311A.....	Transport (TonB systems, Ca ²⁺ /Na ²⁺ antiporter, Cl ⁻ channel, chromate, uncharacterized ABC transporter components, Mg/cobalt, sulfate permeases, Co/Zn/Cd efflux), replication (DNA replication/repair, DNA polymerases IV and III, <i>minCDE</i> septum formation), miscellaneous (polysaccharide synthesis and export, UspA stress genes, ectoine synthase, cation ATPases, pyoverdine synthesis, hydroxamate siderophore syn. [<i>IucC</i> family])

continued to oxidize nitrite in cultures containing up to 400 mM NaCl. Nitrite oxidation of *N. hamburgensis* was completely inhibited by 600 mM NaCl (S. R. Starkenburg, unpublished results). In light of these results, all members of the genus *Nitrobacter* appear to be quite halotolerant, although many of the aforementioned genes in NB311A presumably provide additional means to manage osmotic stress and may enable NB311A to thrive in marine coastal environments.

Plasmid analysis. Previous reports indicated that *N. hamburgensis* contains three plasmids, designated pPB11, pPB12, and pPB13 (24, 35), yet little was known about how these plasmids support the lifestyle of *N. hamburgensis*. Each of the plasmid genes was analyzed for functional content through clustering of orthologous groups of proteins (COG) (Fig. 3). The largest plasmid, pPB13, was found to be biased toward

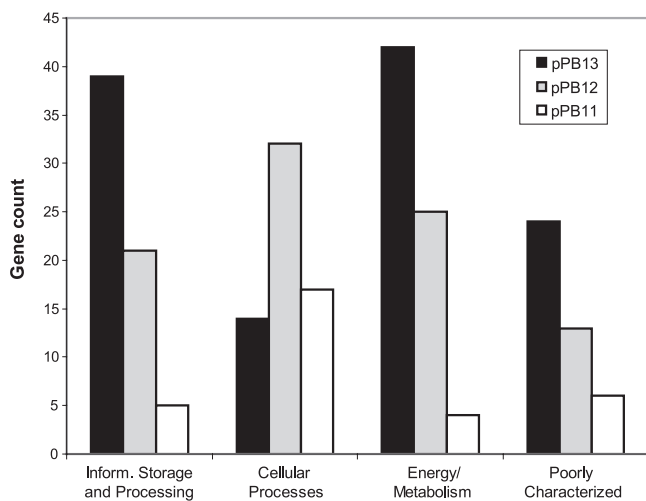


FIG. 3. Functional distribution of *N. hamburgensis* plasmid genes based on COG assignments. Genes in COG groups J, K, and L are included in "information storage and processing." Genes in COG groups D, V, T, M, N, U, and O are included in "cellular processes." Genes in COG groups G, E, F, H, I, P, and Q are included in "metabolism." Genes in COG groups R and S are included in "poorly characterized." Of the 494 plasmid genes, 242 could be assigned to a COG group. The remaining plasmid genes were excluded from the analysis.

carbon/energy metabolism (28 genes; see below) and information storage/processing, although most of the genes in the latter category encode transposases (30 genes). Conversely, the small plasmid pPB11 is dominated by conjugation/pilus formation genes; part of this region (~2.5 kb) appears to have been duplicated within pPB11, and a larger (~9.8-kb) portion has been duplicated in pPB13. pPB12 appears to be a functional hybrid of the other two plasmids, containing gene clusters for conjugation and energy and carbon metabolism, plus a suite of genes for heavy metal resistance, including those for heavy metal efflux (Nham_4358 and Nham_4359; Nham_4404 and Nham_4405; Nham_4529 to Nham_4535), mercury resistance (Nham_4416 to Nham_4421), and copper resistance (Nham_4364 and Nham_4365; Nham_4380; Nham_4382 to Nham_4385; Nham_4397 to Nham_4399; Nham_4422 to Nham_4424; Nham_4492). A putative arsenite oxidase gene and accessory genes were also located on pPB12 (Nham_4425 to Nham_4432), although it is unknown whether these genes are involved in arsenic detoxification or are linked to a respiratory chain. A few additional transport functions encoded on pPB12 include a CDS for a P-type ATPase Mg²⁺ importer (Nham_4377), a predicted TrkA K⁺ transporter (Nham_4433), and a putative arabinose transporter (Nham_4434). Notably, the only copy in the genome of an ATP-dependent glucokinase (Nham_4371) is located on pPB12.

A significant feature of pPB13 is the presence of a large, ~28-kb gene cluster which may be indispensable for autotrophic growth of this organism (Fig. 1). This "autotrophic island" encodes the large and small subunits of a type I ribulose-bisphosphate carboxylase (RuBisCO) (Nham_4332 to Nham_4333), the sole complement of genes necessary for carboxysome formation, and includes a tandem repeat of a four-gene cluster which contains a chain L-like subunit of complex I and a PII protein. Although most of the plasmid-borne genes are unique to *N. hamburgensis*, ~21 kb of this ~28-kb autotrophic island is conserved in the chromosomes of *N. winogradskyi* and NB311A (Fig. 1). This gene cluster was most likely acquired by the ancestor to all three *Nitrobacter* genomes via a lateral gene transfer event, because the GC content of this island is 4.6% and 3.4% higher than the GC averages of the plasmids and genome, respectively,

and the genes in the autotrophic island are most similar and syntenous to the RuBisCO and carboxysome gene clusters in the *Betaproteobacteria*, *Nitrosomonas eutropha*, and *Thiobacillus* spp.

Many examples of DNA exchange and duplication (beyond insertion sequence element transposition) between the plasmids and the chromosome have been identified (see Table S1 in the supplemental material). In addition to the autotrophic island described above, several other Calvin cycle enzymes are also located on pPB13. A 6.1-kb region (rep18) encodes a second, nonparalogous copy of a type I RuBisCO (see “C₁ carbon metabolism” below) and single copies of fructose-1,6-bisphosphatase, phosphoribulokinase, and ketose-bisphosphate aldolase. This second autotrophic island is >99% identical to a region on the chromosome (Nham_3749 to Nham_3754). Interestingly, this similarity does not extend to an upstream and divergently transcribed LysR-type regulator (Nham_4044 in pPB13, Nham_3755) in the chromosome, which may be responsible for the differential regulation of these paralogous gene clusters. In addition to the autotrophic islands, pPB13 carries two more regions nearly identical to chromosomal loci, including a 6.7-kb gene cluster (Nham_4077 to Nham_4081, rep12) which includes genes for an aconitase and a DNA-binding ferritin-like protein. This 6.7-kb gene cluster is >99% identical to the chromosomally located gene cluster (Nham_0912 to Nham_0916). pPB12 also carries two additional loci (Nham_4530 to Nham_4359; Nham_4404 and Nham_4405) that are similar to chromosomal locus Nham_1848 to Nham_1854 and encode products that are also involved in heavy metal resistance.

Regulation and signaling. To assess the genomic repertoire of signaling and regulatory capacity, the pertinent genes in *Nitrobacter* were compared with respective genes from the alphaproteobacterial relatives, *B. japonicum* and *R. palustris*, for which these data were available. The *N. hamburgensis* genome encodes a moderate abundance of signaling proteins, more than the other *Nitrobacter* strains but about one-half and one-third of the signaling capacities in *R. palustris* and *B. japonicum*, respectively. In contrast, *N. hamburgensis* and NB311A contain about half the number of *fecI*-like extracytoplasmic transcription factors (ECFs) (a subfamily of σ^{70}) as in *N. winogradskyi*. Many of the ECF genes in *N. winogradskyi* and NB311A are proximal to *fecR* and/or siderophore receptor genes, suggesting that most of these proteins function to positively regulate iron uptake. Surprisingly, the *N. hamburgensis* genome is completely devoid of *fecR* homologs and contains fewer siderophore receptor genes, and none of the ECF proteins are adjacent to iron-related proteins. Thus, *N. hamburgensis* appears to have evolved a different iron management strategy than either *N. winogradskyi* or NB311A and presumably relies solely on the global iron regulator FUR to control intracellular iron levels instead of responding to extracellular iron concentrations via FecIR.

In comparison to those of *B. japonicum* and *R. palustris*, the *Nitrobacter* genomes contain fewer genes encoding proteins with EAL and GGDEF domains, which likely function in the synthesis and hydrolysis of the intracellular signaling compound cyclic diguanylate (49). The *Nitrobacter* genomes contained a similar number of genes encoding PAS/PAC-domain proteins as *B. japonicum*, which often function as redox sensors (62). Because *Nitrobacter* can grow both aerobically and anaer-

obically (1, 17, 18), these sensors may be important for the functioning of nitrification aggregates at the oxic/anoxic interface by sensing a low redox potential in the environment to induce gene expression needed to switch to an anaerobic metabolism such as nitrate respiration.

Histidine protein kinases (HPKs) and response regulator proteins (RRs) constitute two-component regulatory systems that are often dedicated to the sensing and mitigation of environmental stress conditions. With a total of 46 HPKs and 45 RRs, *N. hamburgensis* has a respectable complement of potentially functional two-component proteins that are expressed from genes arranged in tandem (20 paired HPK and RR genes) as well as singletons (26 unpaired HPK genes and 24 unpaired RR genes). Some of the genes encoding chemotaxis components were grouped with HPKs and RRs with EAL, GGDEF, or GAF domains, suggesting an interconnection between chemotaxis and other signal transduction systems.

Sulfur Metabolism. All sequenced *Nitrobacter* species have the capacity to assimilate sulfur by reducing sulfate to sulfide, which is then incorporated into cysteine. The use of reduced sulfur as an energy source has not been reported for *Nitrobacter*, but *R. palustris* can grow photolithoautotrophically using thiosulfate as an electron donor (48, 58). Intriguingly, *N. hamburgensis* has a small operon containing several genes associated with dissimilatory sulfur oxidation, *soxXYZA_B* (Nham_3671 to Nham_3676). The genes in this cluster encode three of the four main protein complexes which catalyze the oxidation of reduced sulfur, i.e., SoxYZ, SoxAX, and SoxB (19, 20, 46, 57). The fourth protein complex, SoxCD, which completes the oxidation of thiosulfate by oxidizing sulfur to sulfate (20), is not present in *N. hamburgensis*. With respect to experimentally validated gene products, the arrangement and protein identity of the *N. hamburgensis soxXYZA_B* gene cluster are most similar to those for genes found in *Chlorobium*. Similarly, *Chlorobium* spp. do not contain a classical SoxCD complex but still have the ability to oxidize thiosulfate or sulfide anaerobically using an alternate sulfur oxidase/dehydrogenase (12). Indeed, two genes annotated as encoding subunits of a sulfite oxidase (Nham_1093 and Nham_1094), are found elsewhere in the *N. hamburgensis* genome. Unlike the other *sox*-like genes, these two genes are conserved in *N. winogradskyi* (but not NB311A) and have some sequence similarity with *soxCD* in sulfur oxidizers such as the obligate sulfur oxidizer *Thiomicrospira crunogena* XCL-2 (50). Whether any of these putative *N. hamburgensis sox* genes function in respiratory sulfur oxidation, detoxification, or assimilation remains unknown and awaits further experimentation.

Dissimilatory nitrogen metabolism. Nitrite-dependent lithotrophic growth in *Nitrobacter* is catalyzed by a reversible nitrite oxidoreductase (NXR). NXR is a heterodimer containing alpha (NxrA) and beta (NxrB) subunits and is evolutionarily related to the Nar-type dissimilatory nitrate reductase (30). Similar to *N. winogradskyi*, multiple copies of *nxA* ($n = 3$) and *nxB* ($n = 2$) are carried in the *N. hamburgensis* genome, but only one central gene cluster (Nham_3443 to Nham_3451) encodes the putative accessory proteins of NXR. In addition to *nxA* and *nxB*, several genes in this cluster are conserved in all three *Nitrobacter* genomes, including homologs to nitrate reductase accessory proteins NarJI (Nham_3446 and Nham_3447), a peptidyl prolyl *cis-trans*-isomerase (*nrxX*, Nham_3448), a cytochrome *c* (Nham_3450), and putative proteins involved in the

transport of nitrite and/or nitrate (*narK*, Nham_3444; TDT family of transporters, Nham_3443).

Some *Nitrobacter* species have been shown to grow anaerobically using nitrate as a terminal electron acceptor when coupled to the oxidation of simple organic compounds (1, 18), and the terminal product of denitrification in *Nitrobacter* is reported to be nitrous oxide (17, 18). As was the case for *N. winogradskyi*, additional homologs of nitrate reductase genes other than NXR were not identified in *N. hamburgensis* or NB311A, and likewise, a gene cluster encoding a *nirK*-type nitrite reductase is conserved in all three *Nitrobacter* genomes. Together these enzymes presumably function to reduce nitrate to nitric oxide under anaerobic conditions. Each genome also contains a putative flavin mononucleotide-dependent nitroreductase which, if functional, could be involved in detoxification or respiration. In contrast, a gene cluster (Nham_2710 to Nham_2712) encoding a nitric oxide reductase homologous to sNOR in the heme-copper oxidase superfamily was found only in *N. hamburgensis* and not the other two *Nitrobacter* genomes (8). Similarly, a cytochrome P460 (*cytL*, Nham_2497) whose translated product contains an infrequently used heme-coordination motif, CGxxCH (13), was found only in *N. hamburgensis*. Although the function of cytochrome P460 has not been resolved, related proteins within the larger cytochrome P460 family have been proposed to mediate the transfer of NO during denitrification and/or to protect bacteria from NO toxicity (13, 45). An NO detoxification mechanism via oxidation would be advantageous for *Nitrobacter*, because instead of forming nitrous oxide (via sNOR) or ammonia [via NAD(P)H-siroheme nitrite reductase, Nham_2963 to Nham_2965], cytochrome P460 may recycle NO back to nitrite. Understanding of the physiological functions of *nirK* and cytochrome P460 in nitrogen oxide metabolism of *Nitrobacter* awaits experimentation.

Assimilatory nitrogen metabolism. In terms of nitrogen assimilation, each NOB genome contains an assimilatory nitrite reductase (*nirBD*), which permits the production of ammonia from nitrite. Assimilatory nitrate reductases were not found in any *Nitrobacter* genome. In contrast to the case for *N. winogradskyi*, which lacks an ammonia permease or urea catabolic genes (53), the *N. hamburgensis* and NB311A genomes both contain ammonia permeases (Nham_0084) and two genes annotated as urea carboxylase (Nham_2041; COG1984) and allophanate hydrolase (Nham_2040; COG2049). The latter two enzymes putatively contribute to ATP-dependent urea amidolyase activity. All NOB genomes lack genes encoding a classical urease (ATP-independent urea hydrolase) or a urea transporter identified previously in several ammonia-oxidizing bacteria (34); however, *N. hamburgensis* contains five clusters of genes that encode branched-chain amino acid (urea/short-chain amide) ABC transport systems. Although it would be a costly solution given the ATP dependence of all the enzymes, *N. hamburgensis* may obtain urea from the environment or from salvaging protein-nitrogen via the urea cycle, thereby providing the means to regulate its internal pH or provide associated ammonia-oxidizing bacteria with ammonia and CO₂.

C₁ carbon metabolism. Previous DNA hybridization studies concluded that *N. hamburgensis* contained two copies of the large subunit of RuBisCO (*cbbL*), one encoded on pPB13 and

the other on the chromosome (24). Three copies of RuBisCO were identified in the *N. hamburgensis* genome. Two sequence-divergent copies of a type I RuBisCO are located on pPB13 (Nham_4049 and Nham_4050; Nham_4332 and Nham_4333). The third set of RuBisCO-encoding genes (Nham_3750 and Nham_3751) found on the chromosome is identical to the RuBisCO genes Nham_4049 and Nham_4050 on pPB13. Intriguingly, both sequence-divergent RuBisCO copies are preceded by PII-like regulatory proteins, and this arrangement is conserved in all three *Nitrobacter* genomes. PII proteins are ubiquitous in bacteria and are most commonly known for their role in controlling nitrogen assimilation (39). These particular PII homologs may be serving a regulatory role in carbon fixation, or alternatively, a coupled regulatory link between carbon and nitrogen can be envisioned. Although coordination of nitrogen and carbon metabolism is not uncommon, finely tuned control of nitrogen and carbon assimilation via PII-type regulators may be crucial for *Nitrobacter* to thrive on an energy-limited substrate such as nitrite.

Most strikingly, the *N. hamburgensis* genome contains four gene clusters, plus a lone CDS, encoding multiple homologs of molybdopterin-containing carbon monoxide dehydrogenase (Mo-CODH). In contrast, only one Mo-CODH homolog is encoded in *N. winogradskyi*. The largest of these clusters (Nham_2601 to Nham_2608) has high similarity and gene synteny to those identified in the *N. winogradskyi* (53), *Nitrobacter* sp. strain 311A, *Bradyrhizobium japonicum* USDA 110, and *Rhodospseudomonas palustris* CGA009 genomes (Table 3). This unusual Mo-CODH gene cluster lacks a CDS for *coxL/cutL*, the large subunit of Mo-CODH (53). However, these lone CDSs found in the same genomes are highly conserved and contain the AYRGAGR active site and other motifs of form II CoxL proteins (29). Although growth on, or utilization of, CO by *R. palustris* has not been reported, *B. japonicum* USDA 110 is capable of aerobic growth on CO as a sole carbon and energy source (40), albeit at a very low rate. *B. japonicum* USDA 110 can also oxidize CO at the expense of nitrate reduction, but without growth, under anaerobic conditions (28). The N-terminal sequence of CoxL purified from *B. japonicum* USDA 110 apparently matched the translated sequence of blr0336 (M. Lorite, unpublished results), indicating that this gene cluster is expressed for carboxydrotrophy. Whether any *Nitrobacter* sp. is capable of CO metabolism remains to be determined; however, *N. hamburgensis* has the largest and most diverse Mo-CODH-like gene inventory thus far among the *Nitrobacter* sp. genomes. In contrast to *N. winogradskyi*, *N. hamburgensis* contains several genes encoding cytochrome *b*₅₆₁, an important electron transfer component in aerobic carboxydrotrophic bacteria (42, 43). In fact, the *N. hamburgensis* genome contains more complete copies of these Mo-CODH-like genes than it does of NXR, which further suggests that these proteins may play a vital, yet unknown role in the lifestyle of *Nitrobacter*.

Unlike the genomes of *N. winogradskyi* and NB311A, the genome of *N. hamburgensis* contains four contiguous gene clusters, two in the forward (Nham_3124 to Nham_3128; Nham_3132 and Nham_3133) and two in the reverse (Nham_3129 to Nham_3131; Nham_3134 and Nham_3135) orientation, that resemble genes for formate dehydrogenase (FDH), the TorD chaperone, and enzymes for biosynthesis of the molybdopterin cofactor. Strains of *N. winogradskyi*

TABLE 3. CODH gene clusters in *Nitrobacter*, *B. japonicum*, and *R. palustris*

Subunit	<i>N. hamburgensis</i> gene cluster	<i>N. winogradskyi</i> Nb-255		<i>Nitrobacter</i> sp. strain Nb-311A		<i>B. japonicum</i> USDA 110		<i>R. palustris</i> CGA009	
		Nearest neighbor	% Protein sequence identity	Nearest neighbor	% Protein sequence identity	Nearest neighbor	% Protein sequence identity	Nearest neighbor	% Protein sequence identity
CoxG	Nham_2608	Nwin_2206	77	NB311A_18136	77	blI5666	84	RPA3804	81
CoxS/CutS	Nham_2607	Nwin_2205	93	NB311A_18141	91	blI5665	91	RPA3803	90
CoxM/CutM	Nham_2606	Nwin_2204	83	NB311A_18146	85	blI5664	84	RPA3802	84
ATPase	Nham_2605	Nwin_2203	87	NB311A_18151	89	blI5663	87	RPA3801	86
CoxE	Nham_2604	Nwin_2202	80	NB311A_18156	79	blI5662	76	RPA3800	79
CoxF	Nham_2603	Nwin_2201	85	NB311A_18161	86	blI5661	89	RPA3799	88
CoxF	Nham_2602	Nwin_2200	86	NB311A_18166	86	blI5660	79	RPA3798	80
MobA-like	Nham_2601	Nwin_2199	89	NB311A_18171	90	blI5659	77	RPA3797	76
CoxL/CutL	Nham_1307	Nwin_1079	86	NB311A_11547	87	blI5914	78	RPA3974	79
CoxS/CutS	Nham_1039	NP ^a		NB311A_01949	91	blr5209	79	NP	
CoxM/CutM	Nham_1040	NP		NB311A_01944	89	blr5210	71	NP	
CoxL/CutL	Nham_1041	NP		NB311A_01939	88	blr5211	67	NP	
CoxS/CutS	Nham_1453	NP		NP		blr0335	81	RPA4666	82
CoxL/CutL	Nham_1454	NP		NP		blr0336 blr0337	84	RPA4667	85
CoxM/CutM	Nham_1455	NP		NP			72	RPA4668	74
CoxL/CutL	Nham_1181 ^b	NP		NP		NP		NP	
CoxS/CutS	Nham_1182 ^c	NP		NP		NP		NP	
CoxL/CutL	Nham_1183 ^d	NP		NP		NP		NP	

^a NP, not present.

^b Nearest neighbor for Nham_1181, CutL from *Magnetospirillum magnetotacticum* MS-1 (Magn03009301) (45%).

^c Nearest neighbor for Nham_1182, ferredoxin from *Burkholderia ambifaria* (BambDRAFT_3191) (68%).

^d Nearest neighbor for Nham_1183, cytochrome *c* from *Mesorhizobium* sp. strain BNC1 (MesoDRAFT_4421) (46%).

have been shown to oxidize formate (41, 59), although the activity was thought to be catalyzed by NXR (11). Similar three-subunit metalloenzyme FDHs have been shown to catalyze the anaerobic oxidation of formate, which is produced from pyruvate, to CO₂ (27). Several *Nitrobacter* spp., including *N. hamburgensis*, are capable of both aerobic and anaerobic growth on pyruvate with O₂ or nitrate as the electron acceptor, respectively (5). Thus, it remains to be determined whether this putative FDH functions in aerobic or anaerobic environments.

Heterotrophy. Growth of *N. winogradskyi* and *N. hamburgensis* is enhanced in the presence of C₂, C₃, and C₅ carbon molecules (3, 4, 52), although growth on hexose sugars or aromatic compounds has not been reported for any *Nitrobacter* strain. The genome of *N. winogradskyi* appears to be devoid of active transporters for sugars, and a classical glycolysis pathway could not be constructed because a phosphofructokinase gene could not be identified (53). In contrast, the inability of *N. hamburgensis* or NB311A to metabolize hexose sugars is less obvious, given that a complete glycolysis pathway was identified in both *N. hamburgensis* and NB311A. Additional sleuthing for genes involved in C₆ metabolism revealed two plasmid-encoded copies of glucose-methanol-choline oxidoreductase genes (Nham_4194 and Nham_4244) and a unique gene cluster with homology to an ABC-type general sugar transporter (Nham_1206 to Nham_1209), which together provide the in silico potential to transport and initiate oxidation of hexoses.

Many other genes which may further extend the organic carbon substrate range of *N. hamburgensis* were identified. A gene cluster encoding the subunits of a putative glycolate oxidase and a cytochrome *c550* (*glcDEF*; Nham_3202 to Nham_3205) were identified in all *Nitrobacter* genomes. Genetic and functional

evidence indicates that a glyoxylate bypass of the tricarboxylic acid cycle operates in *Nitrobacter*, and thus, glyoxylate (the product of *glcDEF*) could serve as an additional carbon source. Two NAD-independent (flavin adenine dinucleotide/flavin mononucleotide-dependent) oxidoreductases (Nham_4010 and Nham_1112) were also located, which potentially encode putative D- or L-isomer-specific lactate dehydrogenases. If functional, these enzymes could potentially oxidize D- and/or L-lactate to pyruvate, providing both energy and carbon to the cell. Indeed, both D- and L-lactate dehydrogenase activities were observed in *R. palustris* (25). Limited metabolism of aromatic carbon compounds may also be possible given that a cluster of 19 genes that encode the pathways for homogentisate and phenylacetate metabolism (Nham_0920 to Nham_0938) were identified. As is the situation for most potential organic substrates, candidate genes for the transport and uptake of the aforementioned compounds were not readily identified in these bacteria.

Gene clusters encoding respiratory terminal oxidases and cytochromes are more abundant in *N. hamburgensis* than in the other two *Nitrobacter* species. Previous investigations have indicated that *N. hamburgensis* has a higher specific affinity for oxygen under suboxic conditions than *N. winogradskyi* (36, 37), and distinct respiratory chains and terminal oxidases have been suggested to function in *N. hamburgensis*, as different *b*- and *c*-type cytochromes are present during autotrophic growth and heterotrophic growth, respectively (6, 31). In addition to the *aa*₃-type cytochrome *c* oxidase discussed below, four *b*-type cytochrome genes plus a plasmid-carried cytochrome *bd*-ubiquinol oxidase gene were found exclusively in the *N. hamburgensis* genome. The product of these quinol oxidase genes is a likely source of the major *b*-type cytochrome previously isolated from heterotrophically grown *N. hamburgensis* (31). *N. hamburgensis* contains three gene clusters encoding three cop-

TABLE 4. *Nitrobacter* subcore genes^a

Category and putative function	Gene(s) in:		
	<i>N. hamburgensis</i>	<i>N. winogradskyi</i>	NB311A
Polysaccharide biosynthesis			
Lipopolysaccharide biosynthesis protein	Nham_1054	Nwi_0538	NB311A03749
Glycosyl transferase, family 2	Nham_1055	Nwi_0539	NB311A03744
Glycosyl transferase	Nham_3303	Nwi_0540	NB311A03739
Capsule polysaccharide biosynthesis protein	Nham_1195	Nwi_0641	NB311A16262
Acylneuraminase cytidyltransferase	Nham_1201	Nwi_0645	NB311A16252
Hypothetical protein	Nham_1200	Nwi_0646	NB311A16247
NUDIX hydrolase	Nham_1197	Nwi_0648	NB311A16237
Haloacid dehalogenase-like hydrolase	Nham_1198	Nwi_0649	NB311A16232
NUDIX hydrolase	Nham_1199	Nwi_0650	NB311A16227
Nitrite metabolism, transport			
Hypothetical protein	Nham_3451	Nwi_0772	NB311A09094
Cytochrome <i>c</i> , class I	Nham_3450	Nwi_0773	NB311A09084
<i>nrxA1</i> ; nitrite oxidoreductase, alpha subunit	Nham_3449	Nwi_0774	NB311A09079
<i>nrxX</i> ; nitrite oxidoreductase, subunit X	Nham_3448	Nwi_0775	NB311A09069
<i>nrxB1</i> ; nitrite oxidoreductase, beta subunit	Nham_3447	Nwi_0776	NB311A09069
<i>nrxD</i> ; nitrite oxidoreductase, delta subunit	Nham_3446	Nwi_0777	NB311A09054
<i>nrxG</i> ; nitrite oxidoreductase, gamma subunit	Nham_3445	Nwi_0778	NB311A09049
<i>narK</i> ; nitrite/nitrate major facilitator superfamily transporter	Nham_3444	Nwi_0779	NB311A09044
TDT family transport protein	Nham_3443	Nwi_0780	NB311A09039
Cytochrome <i>c</i> , class IC (adjacent to <i>nirK</i>)	Nham_3282	Nwi_2649	NB311A15177
Cytochrome <i>c</i> , class I	Nham_3283	Nwi_2650	NB311A15182
Cytochrome <i>c</i> biogenesis factor	Nham_3285	Nwi_2652	NB311A15192
<i>nsrR</i> ; transcriptional regulator, BadM/Rrf2 family	Nham_3286	Nwi_2653	NB311A15197
Nitrite reductase [NAD(P)H] large subunit, NirD	Nham_2964	Nwi_0720	NB311A01969
<i>nrxB</i> homolog	Nham_3289	Nwi_0965	NB311A10815
<i>nrxA</i> homolog(s)	Nham_0951, Nham_2961	Nwi_2068	NB311A17691
Regulatory genes			
PII-like regulatory protein	Nham_4043, Nham_4324, Nham_4330	Nwi_1989, Nwi_2931	NB311A00865
Transcriptional regulator, LuxR family	Nham_2943	Nwi_0957	NB311A10770
Transcription regulatory protein GAL11 domain	Nham_2946	Nwi_0960	NB311A10785
Transcription factor jumonji/aspartyl beta-hydroxylase	Nham_1520	Nwi_1272	NB311A10046
Miscellaneous			
Sulfite reductase (NADPH) hemoprotein, beta subunit	Nham_0683	Nwi_0591	NB311A03464
Major facilitator superfamily MFS_1	Nham_1829	Nwi_1437	NB311A17399
Polyphosphate glucokinase	Nham_2098	Nwi_1575	NB311A17319
Phosphoesterase, PA-phosphatase related	Nham_2161	Nwi_1638	NB311A18878
Isoprenylcysteine carboxyl methyltransferase	Nham_1746	Nwi_1821	NB311A06266
Luciferase-like protein	Nham_0958	Nwi_2071	NB311A16699
TonB-dependent receptor	Nham_0957	Nwi_2072	NB311A16694
TadE-like protein	Nham_3156	Nwi_2536	NB311A14567
Cupin 2, conserved barrel	Nham_3657	Nwi_0906	NB311A18960
Na ⁺ /H ⁺ antiporter NhaA	Nham_4598	Nwi_2853	NB311A16719
Putative serine/threonine protein phosphatase	Nham_3383	Nwi_3015	NB311A06978
Nucleoside phosphorylase	Nham_3696	Nwi_3067	NB311A02747
Additional hypothetical proteins (75)			
Phage genes (6)			

^a All of the genes with a functional annotation had at least one homolog (E value of $\leq 1e^{-5}$) in a genome other than *R. palustris* or *B. japonicum*. Of the 75 hypothetical proteins, 41 had no match to any peptide in the KEGG genome database (see Table S2 in the supplemental material).

ies of an *aa*₃-type cytochrome *c* oxidase. The two chromosomally encoded cytochrome *c* oxidase gene clusters (Nham_0255 to Nham_0261; Nham_3457 to Nham_3463) are 100% identical at the protein sequence level and are homologous to two gene clusters encoding the only cytochrome *c* oxidases in *N. winogradskyi* (53). The plasmid-encoded cytochrome *c* oxidase gene cluster (pPB13; Nham_4177 to Nham_4180) contained genes for subunits I to IV, and the CDSs are 42, 32, 27, and 0% identical at the protein sequence level to chromosomal homologs of subunits I, II, III, and IV, respectively. Curiously, a

molybdopterin oxidoreductase (Nham_4186 to Nham_4188) with sequence similarity to polysulfide reductases and *nrf*-type nitrite reductase is also encoded in the plasmid-borne cytochrome *c* oxidase gene cluster. Another separate gene cluster containing subunits I and II of cytochrome *c* oxidase plus an adjacent *senC* gene was identified (Nham_2710 to Nham_2712) and, as mentioned above, is homologous to sNOR-type nitric oxide reductases. The sNOR gene cluster is absent from the *N. winogradskyi* and NB311A genomes but is present in all of the ammonia-oxidizing and some sulfur-oxidizing bacterial genomes (55). This

gene cluster was up-regulated in a nitrite reductase-deficient mutant of *Nitrosomonas europaea*, indicating involvement of sNOR in the nitrosative stress response (10).

Nitrobacter subcore inventory. To gain further insight into NOB physiology, a final comparative analysis of the *Nitrobacter* genomes and all of the sequenced strains of *R. palustris* and *B. japonicum* was conducted. The facts that *R. palustris* and *B. japonicum* are metabolically versatile, do not use nitrite as an energy source, and are close evolutionary relatives to *Nitrobacter* provide a unique opportunity to explore the genetic foundation of being a nitrite oxidizer. Using the *Nitrobacter* core genome as the query database, all core genes with high sequence identity to a gene(s) in any strain of *R. palustris* or *B. japonicum* were removed. About 94% of the *Nitrobacter* core was conserved in either *R. palustris* or *B. japonicum*, leaving 116 gene types uniquely conserved in each *Nitrobacter* genome. Within the 116-gene *Nitrobacter* "subcore," 75 genes were identified as encoding hypothetical proteins or contained domains of unknown function (see Table S2 in the supplemental material). Forty-six peptides (all hypothetical) had no match in the KEGG genome database (E value cutoff of $\geq 1e-5$), and of the remaining 70 subcore peptides that had homologs in other prokaryotic genomes, the majority ($n = 61$) were most similar to peptides found within other *Proteobacteria*.

Among the functionally annotated subcore gene set ($n = 41$) (Table 4), two gene clusters appear to encode polysaccharide synthesis proteins (Nham_1195 to Nham_1201; Nham_1054 and Nham_1055), specifically glycosyl transferases, hydrolases, and sialic-acid based homopolysaccharide formation genes, some of which have little sequence similarity to any alphaproteobacterial peptides. Approximately half of the subcore genes with functional annotations appear to be associated with nitrite metabolism, transport, and regulation, including the gene cluster encoding the subunits of the NXR and the *c*-type cytochromes and a putative NsrR-like regulatory protein adjacent to *nirK*. In addition to the main NXR gene cluster which encodes the critical enzyme system for nitrite oxidation in the genus *Nitrobacter*, the scattered, lone copies of *nxA* and *nxB* were also included in the subcore inventory. How (or if) these additional *nxA* and *nxB* gene duplications function remains to be experimentally determined.

Strikingly, the *R. palustris* and/or *B. japonicum* genomes contain homologs of several genes in the *Nitrobacter* subcore inventory, yet these genes are orthologous to genes outside the *Alphaproteobacteria* lineage. For example, *R. palustris* and *B. japonicum* both contain *nirK*, yet the *Nitrobacter* *ncgABC-nirK* gene cluster is syntenous to and has higher sequence similarity with the *ncgABC-nirK* gene cluster in ammonia-oxidizing *Nitrosomonas* spp., suggesting that it was horizontally transferred between the niche-sharing *Nitrobacter* and *Nitrosomonas* bacteria (8). Similarly, *R. palustris* and *B. japonicum* contain PII protein-encoding genes, yet the *Nitrobacter* PIIs adjacent to RuBisCO are more similar to those in the *Betaproteobacteria*, *Nitrosomonas eutropha*, and the denitrifying *Thiobacillus denitrificans*. Furthermore, the *R. palustris* and *B. japonicum* genomes contain periplasmic nitrate reductase (NapABC) but do not encode NarGH-type nitrate reductase (evolutionarily related to NXR), and the closest protein homologs of the *Nitrobacter* NXR are the putative nitrite oxidoreductase in *Nitrococcus mobilis* (~68% protein sequence identity), a nitrite-oxidizing gammapro-

teobacterium, and NarGH in the deltaproteobacterium *Geobacter metallireducens* (~59% protein sequence identity). In sum, the subcore appears to carry a collection of genes which are not indicative of its evolutionary origins but instead reflect the ecological niche (nitrification and denitrification) of *Nitrobacter* achieved through assimilation, modification, and expression of genes acquired from more distant bacterial lineages.

In conclusion, the genome sequence of *N. hamburgensis*, compared with those of *N. winogradskyi* and NB311A, has helped verify and narrow the genetic basis of nitrite oxidation in this famous *Alphaproteobacteria* lineage. Simultaneously, many putative gene candidates were identified which may expand the metabolic capabilities within the genus *Nitrobacter* and account for the phenotypic variations that are known to exist between *N. winogradskyi* and *N. hamburgensis*. Although the genome of *N. hamburgensis* is the largest of the *Nitrobacter* genomes, it is less organized and more fragmented, given its seemingly high content of pseudogenes, paralogs, mobile genetic elements, and phage remnants. Nevertheless, *N. hamburgensis* appears to have maintained a greater level of metabolic flexibility, especially in terms of organic and inorganic carbon use. Extensive duplications of several gene clusters (e.g., NXR, terminal oxidases, CODH, RuBisCO, etc.) in *N. hamburgensis* imply an increase in metabolic capacity and/or the ability to differentially express paralogous gene clusters based on environmental stimuli. In contrast, the *N. winogradskyi* genome is smaller, contains fewer paralogs, and may be restricting the organism to chemolithoautotrophic metabolism. Future genetic comparisons between additional *Nitrobacter* strains and other lineages of nitrite oxidizers, such as *Nitrospira* and *Nitrococcus*, will further advance our understanding of lithotrophic metabolism and the role of NOB in nitrification.

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