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Genome Sequence of the Chemolithoautotrophic Nitrite-Oxidizing Bacterium *Nitrobacter winogradskyi* Nb-255

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The alphaproteobacterium *Nitrobacter winogradskyi* (ATCC 25391) is a gram-negative facultative chemolithoautotroph capable of extracting energy from the oxidation of nitrite to nitrate. Sequencing and analysis of its genome revealed a single circular chromosome of 3,402,093 bp encoding 3,143 predicted proteins. There were extensive similarities to genes in two alphaproteobacteria, *Bradyrhizobium japonicum* USDA110 (1,300 genes) and *Rhodopseudomonas palustris* CGA009 CG (815 genes). Genes encoding pathways for known modes of chemolithotrophic and chemoorganotrophic growth were identified. Genes encoding multiple enzymes involved in anapleurotic reactions centered on C_2 to C_4 metabolism, including a glyoxylate bypass, were annotated. The inability of *N. winogradskyi* to grow on C_6 molecules is consistent with the genome sequence, which lacks genes for complete Embden-Meyerhof and Entner-Doudoroff pathways, and active uptake of sugars. Two gene copies of the nitrite oxidoreductase, type I ribulose-1,5-bisphosphate carboxylase/oxygenase, cytochrome *c* oxidase, and gene homologs encoding an aerobic-type carbon monoxide dehydrogenase were present. Similarity of nitrite oxidoreductases to respiratory nitrate reductases was confirmed. Approximately 10% of the *N. winogradskyi* genome codes for genes involved in transport and secretion, including the presence of transporters for various organic-nitrogen molecules. The *N. winogradskyi* genome provides new insight into the phylogenetic identity and physiological capabilities of nitrite-oxidizing bacteria. The genome will serve as a model to study the cellular and molecular processes that control nitrite oxidation and its interaction with other nitrogen-cycling processes.

Nitrification, the microbiological process by which ammonia is converted to nitrate, is a major component of the global nitrogen cycle, plays a crucial role in transformation of fertilizer nitrogen in agricultural systems, and is a key component of nitrogen removal in wastewater treatment. Excess production of soluble nitrogen by nitrification results in the contamination of potable water and eutrophication of aquatic and terrestrial ecosystems, while the gaseous by-products of nitrification, nitric oxide and nitrous oxide, are two of the most potent greenhouse gases. As anthropogenic inputs of fixed nitrogen continue to expand to meet the demands of a growing global population, intimate knowledge of the nitrification process and the microorganisms that control this process will be necessary to address environmental nitrogen imbalances.

Nitrobacter winogradskyi Nb-255 and other nitrite-oxidizing bacteria participate in nitrification by converting nitrite, the end product of ammonia oxidation, into nitrate according to the reaction $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$. Nitrite functions as an electron donor for the reduction of NAD via reverse electron flow and the generation of ATP by oxidative phosphorylation (36).

As a facultative chemolithoautotroph, *N. winogradskyi* gains energy from nitrite oxidation, and fixes carbon dioxide as its source of carbon. Additionally, in the absence of nitrite, various *Nitrobacter* species can utilize organic compounds as sole carbon and energy sources. However, chemoorganotrophic growth is typically much slower than that occurring when nitrite is supplied as the energy source (16). All tricarboxylic acid cycle enzyme activities have been detected (84) and energy can be gained from the oxidation of pyruvate, acetate, formate, α-ketoglutarate, and glycerol (14, 79). In some strains of *Nitrobacter*, growth on a combination of nitrite and the aforementioned organic carbon sources (mixotrophy) can outpace either chemolitho- or chemoorganotrophic growth (16, 84).

The nitrite-oxidizing bacteria are currently classified into four genera. *Nitrobacter*, *Nitrospina*, and *Nitrococcus* are proteobacteria and members of the alpha-, delta-, and gammaproteobacteria (89), respectively. The fourth genus, *Nitrospira*, is a member of the class *Nitrospira* within the phylum *Nitrospirae*. Historically, members of the *Nitrobacter* genus have been used as the primary model organism for studying nitrite oxidation. Based on 16S rRNA gene sequence analysis, strains in the genus *Nitrobacter* are most similar to the metabolically versatile phototrophic bacterium *Rhodopseudomonas palustris* and the dinitrogen-fixing legume symbiont *Bradyrhizobium japonicum* (89).

The nitrite-oxidizing bacteria have been available in pure culture for over a century, but detailed studies at the molecular level are scarce. To date, research on *N. winogradskyi* has focused primarily on the description of its physiology, which in most instances is limited to the proteins involved in electron

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transport, nitrite oxidation, carbon fixation, and intermediary carbon metabolism (2, 15, 16, 101). Currently, there is considerable interest in the ecology of nitrification and the composition of ammonia-oxidizing bacteria and nitrite-oxidizing bacterial communities in various ecosystems (19, 39, 46, 51). Whole-genome sequences from nitrite-oxidizing bacteria will provide new insight into their phylogenetic identity, reveal potentially novel physiological capabilities, and serve as a model to study the cellular and molecular processes that control nitrite oxidation. Here we present the analysis of the first complete genome sequence from a nitrite-oxidizing bacterium, *N. winogradskyi* Nb-255.

MATERIALS AND METHODS

Organism source and culturing. *Nitrobacter winogradskyi* Nb-255 was obtained from the American Type Culture Collection as strain ATCC 25391. Batch cultures of *N. winogradskyi* were grown chemolithoautotrophically in *Nitrobacter* medium 480 (www.atcc.org/mediapdfs/480.pdf) with nitrite as the sole added electron donor. Cultures were verified to be free of heterotrophic contamination by plating 0.1-ml aliquots on 1/10 nutrient broth plates. Genomic DNA was isolated from *N. winogradskyi* batch cultures by using the Promega (Madison, WI) Wizard genomic DNA purification system according to the manufacturer's suggested protocol.

Genome library construction, sequencing, and assembly. Sequencing of DNA was done by using the whole-genome shotgun method as previously described (23, 34). Briefly, random 3- and 8-kb DNA fragments were isolated and cloned into pUC18 and pMCL200, respectively, for amplification in *Escherichia coli*. A larger fosmid library was constructed containing approximately 40-kb inserts of sheared genomic DNA cloned into the pCC1Fos cloning vector. Double-ended plasmid sequencing reactions were done at the Department of Energy Joint Genome Institute using ABI 3730xl DNA analyzers and MegaBACE 4500 genetic analyzers as described on the Joint Genome Institute website (http://www.jgi.doe.gov/).

Approximately 47,312 reads of sequence were assembled, producing an average of 7.4-fold coverage across the genome. Processing of sequence traces and base calling and assessment of data quality were performed with PHRED and PHRAP, respectively (32, 33). Assembled sequences were visualized with CONSED (40). The initial assembly consisted of 42 contigs (≥20 reads/contig). Gaps in the sequence were primarily closed by primer walking on gap-spanning library clones or PCR products generated from genomic DNA. True physical gaps were closed by combinatorial (multiplex) PCR. Sequence finishing and polishing added 272 reads and assessment of final assembly quality was completed as described previously (23).

Sequence analysis and annotation. Automated gene modeling was completed by combining results from Critica, Generation, and Glimmer modeling packages, and comparing the translations to GenBank's nonredundant database using the basic local alignment search tool for proteins (BLASTP). The protein set was also searched against the KEGG, InterPro, TIGRFams, PROSITE, and Clusters of Orthologous Groups of protein (COGs) databases to further assess function. Manual corrections to automated functional assignments (<2% of the genome) were completed on an individual gene-by-gene basis as needed. Comparative analyses of bacterial genomes and gene neighborhoods were completed using the Joint Genome Institute Integrated Microbial Genomes web-based interface (http://img.jgi.doe.gov/pub/main.cgi).

Nucleotide sequence accession number. The sequence and annotation of the complete *N. winogradskyi* Nb-255 genome are available at GenBank/EMBL/DDBJ under accession number CP000115.

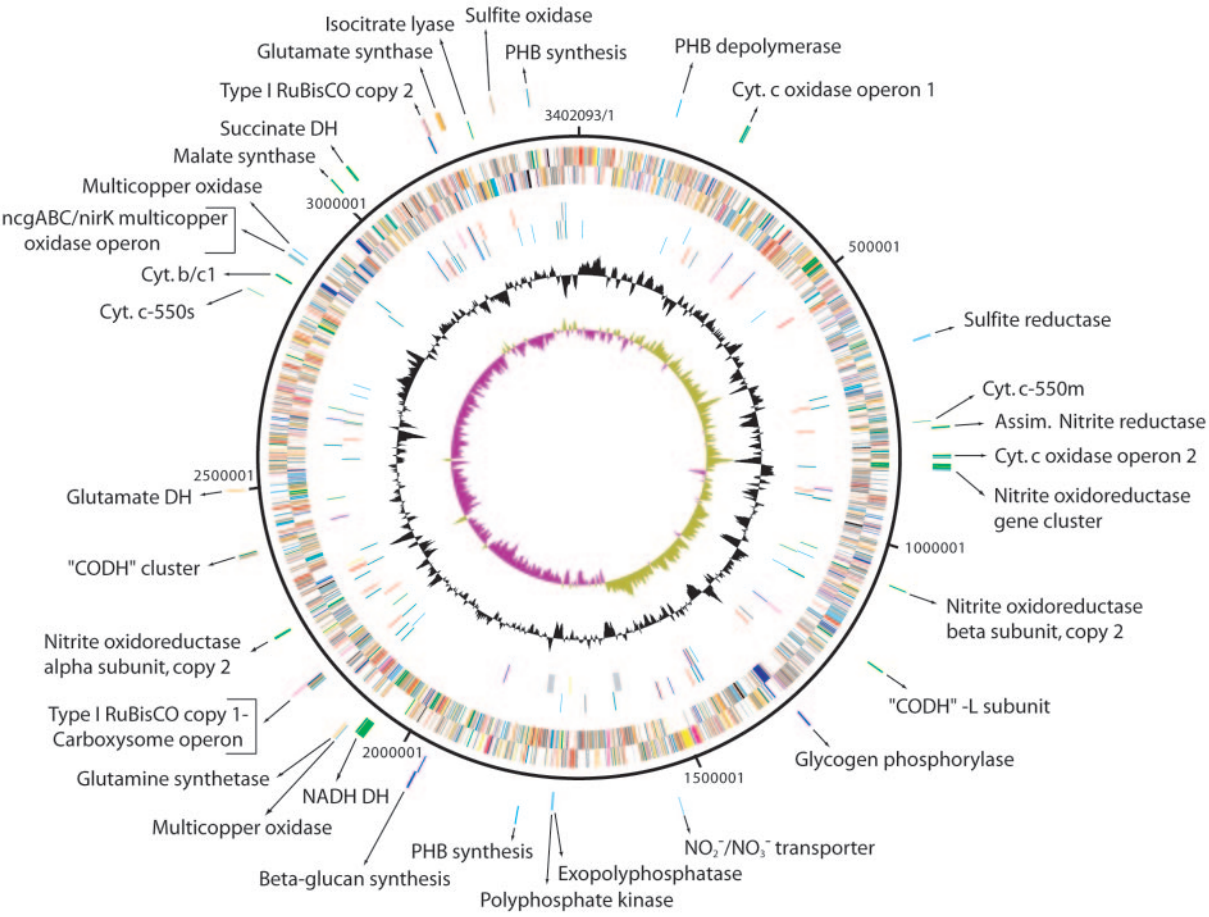
RESULTS

General characteristics. The genome of *N. winogradskyi* Nb-255 (ATCC 25391) is composed of a single circular chromosome of 3,402,093 bp encoding 3,143 characterized and putative proteins (Fig. 1). The single copies of the 16S and 23S rRNA genes had 98 and 95% identity to *Bradyrhizobium japonicum* USDA110 and *Rhodopseudomonas palustris* CGA009, respectively. Based on BLASTP searches, function was predicted for 2,145 genes (67% of the genome), and 281 genes were unique to *N. winogradskyi*. Based on BLAST analysis of KEGG assignments, the most extensive similarities were to genes in other alphaproteobacteria, namely *B. japonicum* (1,300 genes), followed by *R. palustris* (815 genes). Strikingly, the next most frequent BLAST hits (85 genes) were to the ammonia-oxidizing betaproteobacterium *Nitrosomonas europaea*. Of the 85 genes that were most similar to homologs in *N. europaea*, 28 were annotated as transposases (mostly IS*4* family), and 15 were hypotheticals. The remaining genes encoded a multicopper oxidase/cytochrome cluster (see "Dissimilatory nitrate reduction" section below), a glycolate oxidase, sulfite reductase (nwi0590-0591) flavoprotein subunits, and three siderophore receptors.

Complex repetitive sequences. The genome has been the recipient of numerous integration events and contains 276 coding sequences (9% of the total) for transposases, integrases, resolvases, and inactivated derivatives thereof, many of which are grouped into families (>98% nucleotide sequence identity) of complex repetitive elements. The genome contains 111 repeated, full or partial copies of transposase-encoding insertion sequence (IS) elements that represent eight different families (Table 1). Within these families, the IS element copies are nearly identical (>98% identity) and range from 2 to 23 copies per family. Two IS families, ISnw1 and ISnw2, are highly similar (80% identity) and represent an IS superfamily within *N. winogradskyi*. This IS superfamily resembles the *Caulobacter crescentus* ISCc3 and encodes both transposase subunits A and B. In addition, the genome carries two copies of a ~4.5-kb element that encodes a number of phage-related products (nwi1485 to nwi1479 and nwi1623 to nwi1628) and interestingly lies within two larger phage-like regions.

Analysis of homologous genes revealed that the *N. winogradskyi* genome may have recently undergone a number of gene duplications. For example, there are two identical copies of a cytochrome *c* oxidase gene cluster (nwi0223 to nwi0228 and nwi0761 to nwi0766). This 5.4-kb exact duplication contains identical copies of the cytochrome *c* oxidase subunit I, II, and III, heme O synthetase, CoxF, and the cytochrome *c* oxidase assembly protein CtaG. There are also two copies of the nitrite oxidoreductase α subunit (nwi0774 and nwi2068, 94% identity) and β subunit (nwi0776 and nwi0965, 97% identity). Some of these duplications may have already developed new functions, as reflected by the assimilation of these repeated cassettes into different genes. For example, two nearly identical (>99.8%) copies of a 1,593-bp region contribute differently to two genes. For gene nwi2076, the repetitive element constitutes almost the entire length of the 1,602-bp gene, while in nwi1732, this sequence comprises only the 3′ portion of the 2,595-bp gene. Both of these have been annotated as putative TonB-dependent siderophore receptors. Additionally, there are two copies of a putative TonB-dependent biopolymer transport system (nwi2038 to nwi2040 and nwi0705 to nwi0703); however, only the 3′ end of the ExbB proton channel component (nwi0703 and nwi2040) shows conservation (98% identity), while the 5′ ends are of different sizes and are unique.

Transcription, translation, and cell division. Genes for the α, β, β′, and ω components of the core bacterial RNA polymerase were identified and are most similar to the same components in *B. japonicum*. All essential ribosomal proteins are accounted for in the genome. Typical prokaryote translation factors if-1, if-2, and if-3 and elongation factors Tu and G were



Total bases	3,402,093	Protein coding genes	3143
DNA coding bases	2,888,358	With predicted function	2145
GC %	62.05 %	Without predicted function	998
tRNA	49	Pseudogenes	21
5S/16S/23S	1/1/1		

FIG. 1. Chromosome of *Nitrobacter winogradskyi* Nb-255 (ATCC 25391). The outer two circles indicate the locations of key energetic and metabolic features. The third and fourth circles depict predicted protein-encoding and structural RNA genes 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). The fifth and sixth circles depict the location of IS elements and phage regions (gray bars) on the plus and minus strands, respectively. The seventh circle indicates GC bias; and the eighth circle indicates GC skew.

all contiguous with genes coding for ribosomal proteins. There were 49 genes for amino acyl-tRNA biosynthesis. Genes encoding RNA processing functions include RNase PH (final 3′ trimming and modification of tRNA precursors), RNase D (a 3′ exonuclease acting on tRNA), and RNase E and RNase G (maturation of the 5′ end of 16S RNA).

Genes putatively encoding homologs for the tubulin-like FtsZ (nwi1058), and for cell division proteins that associate with Ftsz, FtsA (nwi1057), FtsQ (nwi1056), FtsW, (nwi1050), FtsI (nwi1045), and FtsK (nwi0083), were identified. Like other alphaproteobacteria, *N. winogradskyi* lacks homologs of MinCDE, SulA, ZipA, FtsL, and FtsN (104). *N. winogradskyi* possesses homologs of CtrA (nwi0525) and GcrA (nwi0512), which in *Caulobacter crescentus* form the core oscillator of the genetic circuit controlling cell cycle progression and asymmetric polar morphogenesis (44). The CtrA-GcrA interaction may also have a role in controlling *N. winogradskyi* cell division, which occurs by polar swelling, and results in asymmetric cells.

Regulation and signaling. *N. winogradskyi* has 322 genes (ca. 10% of the genome) devoted to regulation and signaling (Table 2). Genes for σ^{32}-, σ^{70}-, and σ^{54}-like transcription factors were identified. Fourteen specialized *fecI*-related σ^{24} homologs are present, most of which are proximal to *fecR* and siderophore receptor genes. A ferric uptake regulator (nwi0013) was identified in addition to a zinc uptake regulator (nwi0493), and an iron response regulator (nwi0035), the last

TABLE 1. Summary of complex repetitive sequences

Sequence group	Size (bp)	No. of copies	% Identity[a]
Gene, operon, and/or region			
Cytochrome *c* oxidase gene clusters (nwi0223–nwi0228; nwi0761–nwi0766)	5,432	2	100
Phage-related gene clusters (nwi1485–nwi1477; nwi1628–nwi1621)	4,406	2	98.9
Nitrite oxidoreductase α-subunit (nwi2068; nwi0774)	3,645	2	94
Nitrite oxidoreductase β-subunit (nwi0965; nwi0776)	1,542	2	97
TonB-dependent receptor (nwi2076; nwi1732)	1,562	2	99.8
TonB-dependent transport system (nwi2038–nwi2040; nwi0705–nwi0703)	2,117	2	98.3
IS elements			
ISnw*1* (two transposases)	1,511	23	>99.8
ISnw*2* (two transposases)	1,512	11	>98
ISnw*3* (two transposases)	1,050	18	>99.8
ISnw*4* (one transposase)	1,016	10[b]	>99.8
ISnw*5* (one transposase)	902	14	>99.3
ISnw*6* (two transposases)	855	19	>98.8
ISnw*7* (one transposase)	947	14[b]	>99.6
ISnw*8* (two transposases)	1,321	2	100

[a] Nucleotide sequence identity between repeats.
[b] Two of the copies contain only partial repeats of the IS element.

of which could be mediating iron-dependent control of heme synthesis (71). *N. winogradskyi* has 28 signal transduction histidine kinases of varied function, two of which are nitrogen related (NtrB) and seven of which are periplasmic sensor signal transduction related.

Genes encoding 13 CheY-like receiver domain proteins and four LuxR-like transcriptional regulators were located. One CheY protein containing a NarL receiver domain may be a nitrite/nitrate response regulator with a NarL receiver domain. A complete chemotaxis operon with a CheW-like response regulator receiver (nwi0520) is present and adjacent to flagellar protein synthesis genes (nwi0529), which may indicate coordination of motility and chemotaxis signal transduction proteins to facilitate transport of the cell to the location of substrates. The *N. winogradskyi* genome contains genes (nwi0626 to nwi0627) encoding a functional two-component regulatory system, LasRI (but lacks RhlRI), which plays key roles in connecting quorum sensing, motility, stationary-phase response and synthesis of virulence and stress tolerance factors in many bacteria (97). Genes for basal transcription factors (*nusG*, nwi1345 and nwi1549; *greA*, nwi0922 and nwi2450; *rhoA*, nwi0100; *nusA*, nwi0022; and *nusB*, nwi1722 and nwi0156) were also accounted for in the genome.

TABLE 2. Regulatory and signaling proteins

No. of proteins	Category[a]
136	Transcription/elongation/termination factors
17	Sigma factors (1-σ^{32}, σ^{70}, and σ^{54}; 14-ECF σ^{24})
1	Anti/anti-anti sigma factors
5	Termination/antitermination factors (e.g. Rho, NusA, NusB, NusG)
2	Elongation factors
109	Transcription factors (27 Fis, 8 LysR, 8 two-component transcriptional regulator winged-helix family, 8 XRE family)
94	Signal transduction proteins (3 chemotaxis, 94 nonchemotaxis signal transduction)
28	Signal transduction histidine kinases
14	Cyclic nucleotide signal transduction
4	PII-like nitrogen regulatory proteins
45	Miscellaneous (13 CheY, 12 putative FecR, 7 PAS/PAC domain)

[a] PAS, *p*eroid clock protein–*a*ryl hydrocarbon nuclear translocator–*s*ingle-minded proteins; PAC, PAS-associated C-terminal motif.

Multiple phosphotransferase system signaling genes were identified. The gene cluster at nwi0344 to nwi0348 encodes Hpr, EIIA, HPr kinase, and homologs of the ChvIG/ExoRS two-component sensory system (Fig. 2C). A phosphoenolpyruvate-utilizing EI enzyme (nwi0378) was also annotated along with a second EIIA2 (*ptsN*, nwi0179) gene immediately upstream of σ^{54} (*rpoN*). Gene homologs for sugar permeases (e.g., EIIB, EIIC, and EIID) were not identified. The absence of sugar permease genes combined with the presence of an Hpr kinase gene suggest that the phosphotransferase-type signaling molecules in *N. winogradskyi* are not involved in sugar transport (18). Alternatively, the phosphotransferase proteins may be stimulating RpoN expression (60, 90) and/or involved in the regulation of ChvIG/ExoRS, which has been shown to regulate acid-inducible genes in *Agrobacterium tumefaciens* (54), and succinoglycan exopolysaccharide production and flagellum biosynthesis in *Sinorhizobium meliloti* (56, 103).

Energy generation and electron transport. Reductant (NADH) gained from the metabolism of organic substrates is assumed to fuel oxidative phosphorylation via conventional respiratory machinery (15). Indeed, genes for complexes I to V of a classical electron transport system were identified.

In chemolithotrophic growth, nitrite is oxidized to nitrate via a molybdopterin-containing nitrite oxidoreductase (2, 15). Electrons are released from the cytochrome a_1c_1 component of the nitrite oxidoreductase and subsequently transferred to cytochrome *c* oxidase (Cox) through cytochrome *c*550 (101). Cytochrome *c*-550 and the *cox* operon have been studied in detail in *N. winogradskyi* strain agilis (ATCC 14123). DNA hybridization to *coxA*, *coxB*, and *coxC* probes revealed multiple copies of the cytochrome oxidase operon (11). Both soluble and membrane-bound class IA/B cytochrome *c*-550 proteins were purified and shown to be electron donors for Cox (67, 87, 102). Among the *c*-type cytochrome genes identified, orthologs for both cytochrome *c*-550 proteins (c-550s, nwi2582, and c-550m, nwi0712) were located. A paralog of *c*-550m (nwi0287) was also identified in addition to two class IC cytochromes (COG2863, nwi0159, and nwi0670). Further analysis is needed to assign functions to these or other *c*-type cytochromes in the electron transport system.

Congruent with previous investigations of *N. winogradskyi* strain agilis (11), two distinct operons encoding paralogs of six cytochrome *c* oxidase (complex IV) synthesis genes were identified in the genome (see "Complex repetitive sequences" above). Additional cytochrome oxidases were not evident in the genome although gene homologs of two subunits (*cyoBC*) of a cytochrome *o* oxidase are adjacent to the cytochrome *c* oxidase genes at the nwi0761 and nwi0766 loci. Electrons from nitrite oxidation are considered to enter the respiratory chain at the level of cytochrome a_1 (2, 15, 100, 101). Cytochrome a_1 requires a heme A cofactor that is biosynthesized from heme B

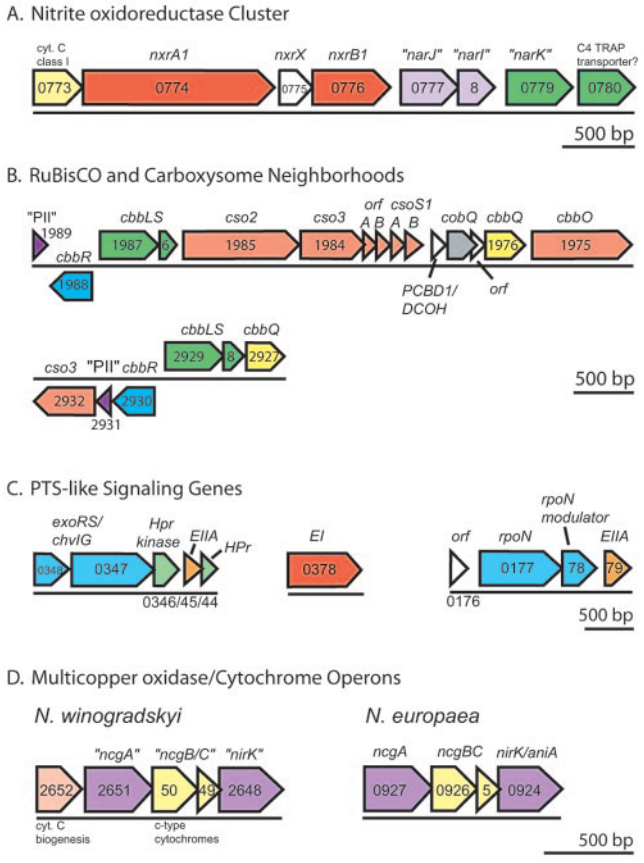


FIG. 2. Organization of *N. winogradskyi* gene clusters. Each arrow represents one gene. The *N. winogradskyi* locus numbers are indicated within the arrows and putative gene names are above each arrow. In panel D, a gene cluster from *N. europaea* (ATCC 19718) is shown for comparison.

via heme O as a stable intermediate (35, 65). This pathway requires the sequential activity of heme O synthase (CoxA/CtaB homologs) (35, 86) and heme A synthase (CtaA homologs) (86), respectively. Each cytochrome *c* oxidase gene cluster contains a CyoE/CtaB homolog (nwi0225 and nwi0763), although a single Cox15/CtaA (nwi1418) homolog is segregated from these cytochrome operons and is clustered with one of two predicted NarK homologs (nwi1419), which are implicated in transport of nitrate or nitrite.

In the past, the nitrite oxidoreductase enzyme has been abbreviated as NOR, an abbreviation that has also been used for nitric oxide reductase. To eliminate this confusion, we propose a new abbreviation for *n*itrite o*x*ido*r*eductase, NXR. Nitrite oxidation by NXR is reversible and NXR can catalyze the reduction of nitrate to nitrite; this transformation is believed to be part of the denitrification pathway carried out by *N. winogradskyi* (see below) (2, 15, 27). Formate may also be a substrate for NXR, and is oxidized to carbon dioxide (27).

The NXR enzyme complex is a heterodimer consisting of one α subunit (large subunit) and one β subunit (small subunit), encoded by *nxrA* and *nxrB*, respectively (82). The *N. winogradskyi* genome contains two copies each of *nxrA* and *nxrB*. One copy of *nxrA* is one gene upstream of *nxrB* (*nxrA1* at nwi0774 and *nxrB1* at nwi0776; Fig. 2A), while the other copies are segregated at distant points in the genome (*nxrA2* at nwi2068 and *nxrB2* at nwi0965). There are no other genes closely associated with the segregated copy of *nxrA2*; the segregated copy of *nxrB2* lies downstream of a gene annotated as encoding a hypothetical protein.

The NXR complex is membrane associated and has been localized to the cytoplasmic face of the cell membrane (81, 82). Signal peptides were not detected by SIGNALP (10) analysis of either NxrA or NxrB. However, NxrA and NxrA were predicted by TOPPRED (94) and TMPRED (43) analysis to form two or three transmembrane α-helices, suggesting that the α-subunit anchors the NXR complex to the cytoplasmic membrane.

Previous sequence analysis in *N. hamburgensis* identified an open reading frame (ORF), *norX*, located between *nxrA1* (*norA*) and *nxrB1* (*norB*) (50). A putative homolog of *norX*, *nxrX* (nwi0775), was identified in *N. winogradskyi* (Fig. 2A) and is predicted to encode a peptidyl-prolyl *cis-trans* isomerase (EC 5.2.1.8), which may aid in the folding of NXR. Inspection of the nucleotide sequences of *nxrX* and *norX* revealed a single base "deletion" in *nxrX*. The absence of a thymine between bases 863769 and 863770 caused a shift in the reading frame, altering the putative start codon and 5′ end of *nxrX*/nwi0775. The start codon of *nxrX* annotated in *N. winogradskyi* begins 22 bases upstream of the start site reported for *norX*. The correct start site and functionality of *nxrX*/*norX* remain to be validated.

The *nxrA1XB1* cluster appears to be organized in an operon with four additional genes that might provide accessory functions to NXR (Fig. 2A). Immediately upstream of *nxrA1*, nwi0773 is predicted to encode a *c*-type cytochrome that may be a part of the electron transport system coupled to oxidation and reduction of nitrite (2, 15, 100, 101). Immediately downstream of *nxrB1*, two genes are predicted to encode homologs of NarJ (nwi0777) and NarI (nwi0778), which are subunits of the dissimilatory nitrate reductase common to heterotrophic denitrifiers (105). NarJ, the nitrate reductase δ subunit, inserts the molybdenum cofactor in nitrate reductase A (13, 92), and could play a similar role in NxrA biosynthesis. NarI (nitrate reductase γ subunit) is a *b*-type cytochrome that serves as the electron acceptor from the quinone pool and electron donor to the molybdenum cofactor in the α-subunit.

The last two genes in the *nxr* cluster are predicted to encode a NarK-like nitrate/nitrite transporter (nwi0779) and a transporter for C_4 dicarboxylic acids/malic acid or tellurium (nwi0780). NarK is a member of the major facilitator superfamily of transporters that has been reported to function as a nitrate-proton symporter, a nitrate-nitrite antiporter, and a nitrite uniporter (26, 98). Active uptake of nitrite or efflux of nitrate would both be important to maintaining NXR activity, and the function(s) of NarK in *N. winogradskyi* remains to be determined. The relationship of the nwi0780 product to NXR activity is unknown.

Dissimilatory nitrate reduction. *N. winogradskyi* can grow anaerobically and gain energy by coupling oxidation of organic compounds to nitrate reduction (17, 37). As mentioned above, nitrite oxidation mediated by NXR is reversible, and the nitrate reductase activity that initiates denitrification is presumably provided by this enzyme. NxrA and NxrB are homologs of the NarGH polypeptides that comprise the large and small subunits of dissimilatory nitrate reductase A in heterotrophic

denitrifiers (50). Also as mentioned above, homologs of the NarJI subunits of nitrate reductase are also clustered with NxrAB. With the exception of *nxrA2* and *nxrB2*, *N. winogradskyi* possesses no other *narGHJI*-like genes. *N. winogradskyi* lacks a periplasmic nitrate reductase, and in this respect differs from its closest phylogenetic relative, *B. japonicum*.

In *Nitrobacter vulgaris*, nitrite reductase activity (reduction of nitrite to nitric oxide) was measured in a membrane-bound *N*,*N*-diethyldithiocarbamate-sensitive protein that copurified with NXR (1). The two major groups of enzymes that function in this capacity are the trimeric copper-containing oxidases (CuNIR) and the dimeric cd_1-type cytochromes (24, 73, 105). *N. winogradskyi* is not predicted to produce the latter of these, as it lacks genes for biosynthesis of the heme D_1 cofactor. However, *N. winogradskyi* possesses four predicted multicopper oxidases encoded by nwi1901, nwi2648, nwi2651, and nwi2661.

Two of the putative multicopper oxidase genes (*nirK*, nwi2648; and *ncgA*, nwi2651) are clustered in an apparent operon with genes for biogenesis of *c*-type cytochromes (nwi2652, *ncgB*; nwi2650, *ncgC*; and nwi2649). Orthologs of these genes and the operon structure are conserved in the ammonia-oxidizing bacteria *Nitrosomonas europaea* and *N. eutropha* (23) (Fig. 2D). In *N. europaea*, the results of mutational analyses indicate that the nwi2648 ortholog (NirK) and other genes in the cluster function in detoxification of and tolerance to nitrite and possibly nitric oxide (6, 8). These genes could have a similar role in *N. winogradskyi* (see "Environmental defense and stress" below). Of the two remaining multicopper oxidases, only nwi2661 lacks the motif for a type III copper site, the absence of which is characteristic of the copper-containing nitrite reductases (31). Which, if any, of these putative multicopper oxidase genes function in a dissimilatory nitrate reduction pathway remains to be determined.

While nitrous oxide is reported to be the terminal product of respiratory nitrate reduction by *Nitrobacter* spp. (37), the predominant form has not been independently confirmed. Nitrous oxide has been detected in nonspecified amounts (36, 37), yet the *N. winogradskyi* genome lacks predicted homologs of a nitric oxide reductase. If nitrous oxide is the primary end product of respiratory denitrification, the pathway by which it would be formed is uncertain. Presumably, nitrous oxide is produced from the reduction of nitric oxide, and two nitric oxide reductase (NOR) classes are recognized, cytochrome *c* dependent (cNOR) and quinol dependent (29, 42, 73). There were no BLAST hits in the *N. winogradskyi* genome for quinol-dependent homologs.

Two putative proteins had weak similarities (23 to 26% identity) with the NorC (nwi2313) and NorB (nwi2314) subunits of the cNOR complex. The cNOR catalytic subunit is NorB and the predicted product of nwi2314 is much larger than that of typical NorB homologs, 839 amino acids versus ca. 460 amino acids, respectively. The products of nwi2313 and nwi2314 are predicted to possess the COX1 and COX2 domains that are characteristic of the heme-copper cytochrome oxidase family of which cNOR is a member. However, cNOR is distinguished from other heme-copper cytochrome oxidases in that the NorB dinuclear center contains nonheme iron instead of copper, and four conserved glutamic acid residues in NorB have been implicated in coordinating the nonheme iron ligand (73). Because the product of nwi0562 aligns poorly with NorB homologs, it is difficult to ascertain if this distinguishing feature exists in the putative polypeptide.

Autotrophy. Carbon dioxide fixation in *N. winogradskyi* is mediated by a type I ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) via the Calvin-Benson-Bassham (CBB) cycle (15, 16). Genes for all of the enzymes of a functional CBB cycle are present. The typical sedoheptulose 1,7-bisphosphatase (EC 3.1.3.37) and the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) genes are absent and are functionally replaced in *N. winogradskyi* by homologs of fructose 1,6-bisphosphatase (EC 3.1.3.11, nwi2694) and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, nwi2735), respectively (23, 59, 76).

Two copies of *cbbL* and *cbbS*, encoding the large and small subunits, respectively, of type I RuBisCO were identified (Fig. 2B; nwi2928-2929 and nwi1987-1986). Genes encoding the structural components of carboxysomes are immediately downstream of *cbbS* (nwi1986). The two RuBisCO copies do not appear to be paralogs given that the sequence of the nwi2928-2929 locus is most similar to those of the RuBisCO genes of its close relatives in the alphaproteobacteria, *B. japonicum* and *R. palustris*, while the RuBisCO genes (nwi1987-1986) are most similar to those in the gammaproteobacteria *Thiobacillus* and the ammonia-oxidizing betaproteobacteria genera *Nitrosospira* and *Nitrosomonas*. Likewise, the organization of the nwi1975 to nwi1987 RuBisCO/carboxysome gene cluster is nearly identical to that in the gammaproteobacteria *Acidithiobacillus ferrooxidans* (22) and *Thiobacillus denitrificans*.

Both copies of *cbbL* are preceded by divergently transcribed *cbbR* LysR-type regulators (Fig. 2B) and putative signal transduction proteins containing PII-like domains (nwi1989 and nwi2931). While the presence of an adjacent *cbbL* regulator is common, the location of PII-like proteins near RuBisCO appears to be rare. Outside of the *Nitrobacter* lineage, only one other PII homolog (tbd2652 from *T. denitrificans* sp. strain ATCC 25259) of nwi1989 or nwi2931 is adjacent to genes encoding RuBisCO or carboxysome peptides. Although the function of these PII-like proteins is not known, their presence next to both RuBisCO gene clusters potentially indicates coordinated regulatory control of nitrogen assimilation in response to availability of carbon.

Carbon monoxide dehydrogenase homologs. Genes putatively encoding an aerobic-type carbon monoxide dehydrogenase (CODH) were identified. The predicted CODH complex is comprised of large, medium, and small subunits encoded by *coxL*/*cutL*-like (nwi1079), *coxM*/*cutM*-like (nwi2204), and *coxS*/*cutS*-like (nwi2205) genes, respectively. The last two genes are adjacent on the reverse strand, while the *coxL*/*cutL*-like gene (nwi1079) is located some distance away on the forward strand. This distribution differs from that of most other *cox*/*cut*-like genes, where genes encoding all three subunits are typically clustered (38, 49, 74).

Immediately downstream of nwi2204 is a cluster of five ORFs that could be involved in the biosynthesis of the molybdopterin cofactor required by CoxL/CutL. The motifs of these gene products identified by Pfam analysis include an AAA+ family ATPase (nwi2203), a VWA (von Willebrand factor type A domain)-containing CoxE-like protein (nwi2202), a protein of the XdhC and CoxF family (nwi2201 and nwi2200), and a probable molybdopterin-binding protein (nwi2199). Immedi-

ately upstream of *coxS*/*cutS*-like nwi2205, the predicted product of nwi2206 appears to be a CoxG-like protein; the function of CoxG is unknown, but CoxG-like genes are conserved in carbon monoxide-oxidizing bacteria (38, 74).

Heterotrophy. Metabolic pathways which support known modes of heterotrophic growth in *N. winogradskyi* were constructed from putative gene annotations. Enzymatic activities measured from auto-, mixo-, and heterotrophically grown cells of *N. winogradskyi* strain agilis demonstrated a complete tricarboxylic acid cycle (83). Growth of *N. winogradskyi* on hexose sugars such as glucose and fructose has not been reported. Consistent with these findings, a gene encoding a putative phosphofructokinase was not identified in the genome, which would sever classical glycolysis. The Enter-Doudoroff pathway also could not be reconstructed. This was due to the absence of a 2-keto-3-deoxy-6-phosphogluconic acid aldolase gene, even though an adequate homolog for a phosphogluconate dehydratase (nwi0213) was identified.

Genes encoding enzymes necessary for pyruvate, acetate, and glycerol metabolism were identified. For pyruvate metabolism, genes were identified for encoding pyruvate dehydrogenase (nwi1817-1818), phosphoenolpyruvate carboxylase (nwi2278), and malic enzyme (nwi1587), confirming previous investigations with *N. winogradskyi* strain agilis (84). Pyruvate phosphate dikinase (nwi2709) and phosphoenolpyruvate carboxykinase genes (nwi0350) were also identified, allowing *N. winogradskyi* greater metabolic flexibility around pyruvate.

Similarly, heterotrophic growth by *N. winogradskyi* on acetate was demonstrated (14, 28, 79). Genes for catabolic enzymes that would funnel acetate into the tricarboxylic acid cycle via conversion to acetyl-coenzyme A through either acetyl-phosphate or acetyl-adenylate intermediates were located. The presence of genes for key glyoxylate cycle enzymes, isocitrate lyase and malate synthase, permits the regeneration of oxaloacetate and prevents a C_4 deficiency that would occur during growth on acetate or other C_2 or C_3 compounds. Finally, with regard to glycerol, homologs of glycerol kinase (nwi0281), glycerol-3-phosphate dehydrogenase genes (nwi0469 and nwi2990), and triosephosphate isomerase (nwi1835) were found, which would allow the conversion of glycerol to the glycolysis intermediate glyceraldehyde-3-phosphate.

Although growth of *Nitrobacter* on glycerol has been demonstrated (37), an inner membrane glycerol porin was not identified. An aquaporin, *aqpZ* (nwi1000) (in the same major intrinsic protein family and COG functional group as glycerol porin channels), was located, but analysis of conserved residues in *aqpZ* indicate this gene encodes an aquaporin (85), which is generally selective for water.

Vitamins and coenzymes. Genes encoding complete pathways for the de novo synthesis of heme, NAD, pyrroloquinoline quinone, biotin, pantothenate, folate, acyl carrier protein, and coenzyme A were identified. Classical pathways could not be confirmed for cobalamin (B_{12}), thiamine, or pyridoxal (B_6). Surprisingly, a complete classical pathway for the biosynthesis of menaquinone or ubiquinone could not be reconstructed from annotated genes, as homologs encoding a chorismate lyase (*ubiC*) or 3-octaprenyl-4-hydroxybenzoate decarboxylase (*ubiD* or *ubiX*) were lacking from the *N. winogradskyi* genome. Prior investigators have indicated that *N. winogradskyi* strain agilis possessed ubiquinone (2), which, if correct, would suggest that biosynthesis of this molecule may occur via a nonclassical pathway.

Lipid and lipopolysaccharide biosynthesis. Several studies have shown that the fatty acid composition of phospholipids in *N. winogradskyi* is almost exclusively vaccenic acid (18:1,11c) (4, 55). Two FabA-encoding genes (nwi0034 and nwi1578) were identified that carry out the desaturation of fatty acids and the subsequent conversion of *trans* to *cis* double-bond configurations. Genes were identified that are involved in assembly of a complete lipopolysaccharide, including lipid A, core oligosaccharide, and an O-antigen structure. In addition, two paralogous genes (nwi2396 and nwi0647) were identified that code for synthesis of *N*-acetylneuraminic acid, and/or formation of the capsular homopolysaccharide sialic acid.

Another interesting feature of polysaccharide biosynthesis was the identification of genes associated with the regulation (*ndvD*, nwi1788) (25), synthesis (*ndvC*, nwi1787), glycosyl transfer (*ndvB*, nwi1789), and transport (*ndvA*, nwi2681) of membrane-associated cyclic β-glucans that accumulate in the periplasmic space. This class of compounds has received some attention in *Rhizobium* and *Bradyrhizobium* spp., where they are synthesized under low osmotic strength and are thought to play an unspecified role in nodulation.

Amino acid biosynthesis. Genes for the biosynthesis of all 20 amino acids were identified. The *N. winogradskyi* genome lacks both an asparagine synthetase and an asparaginyl-tRNA synthetase. Thus, the sole pathway to synthesize asparagine and charge a tRNA appears to be through transamidation of an aspartyl-tRNA (63) via a class II aspartyl-tRNA synthetase (nwi1588, EC 6.1.1.12) and a glutamine-dependent Asp-$tRNA^{Asn}$ amidotransferase encoded by *gatCAB* (nwi2003, nwi2001, and nwi1997).

Storage compounds. Many cytoplasmic inclusions have been reported in *Nitrobacter* cells, including poly-β-hydroxybutyrate (PHB), polyphosphates, and glycogen (14, 96). *N. winogradskyi* Nb-255 contains the genes necessary to synthesize and metabolize PHB and polyphosphate, but not glycogen. Homologs of genes encoding the necessary enzymes for PHB synthesis, *phbA*, *phbB*, and *phbC* (nwi3060, nwi3061, and nwi1650, respectively) and PHB breakdown, PHB depolymerase (nwi0130), are present. Polyphosphate chains are produced via an ATP-dependent polyphosphate kinase (nwi1594) and cleaved with an adjacent exopolyphosphatase (nwi1593). A complete glycogen synthesis pathway could not be reconstructed from the annotated genes. Neither glycogen synthase (EC 2.7.7.27) nor ADP glucose pyrophosphorylase (EC 2.4.1.21) could be identified; however, a gene encoding a glycogen breakdown enzyme, glycogen phosphorylase (EC 2.4.1.1, nwi2414), is present.

Mineral nitrogen assimilation into amino acids. Incorporation of ammonium, hydroxylamine, nitrate, and nitrite into cell nitrogen has been demonstrated in some *Nitrobacter* strains (53). Homologs for a hydroxylamine reductase and an assimilatory nitrate reductase (which function to assimilate hydroxylamine and nitrate, respectively) were not identified in the genome. Nitrite assimilation is likely to be mediated by an NAD(P)H-dependent nitrite reductase encoded by *nirB* (nwi0719) and *nirD* (nwi0720), which is most similar to *nirBD* in *Mesorhizobium loti*. The NirBD complex is a cytoplasmic, siroheme-containing enzyme that utilizes NAD(P)H or ferredoxin as the

TABLE 3. Summary of transporters

Transporter family[a]	No.	Function(s)
ABC type I/II	18/1[b]	Heme, alkanesulfonate, Fe, molybdate, Mn/Zn, Mg/Co, polar/branched amino acids, polyamines, sulfonate, sulfate, nitrate, peptides, glycine betaine/proline, type II unknown
MFS	8	Cyanate permease, metals, multidrug resistance, general sugar, nitrite/nitrate
RND	7	Heavy metal efflux (Ag, Co, Zn, Cd), unknown
TRAP	1	C_4 dicarboxylate (malate, succinate)
CPA-1	1	Flux of Na^+/H^+ ions
SMR	3	Unknown multidrug resistance
Antiporter/symporter	3	$Na^+/H^+/K^+$
Miscellaneous	8	Arsenate efflux, sulfate permease, nitrite/formate, Mg and Mn efflux, unknown porins

[a] ABC, ATP binding cassette; MFS, major facilitator superfamily; RND, resistance-nodulation-cell division; TRAP, tripartate ATP-dependent periplasmic; CPA, cation:proton antiporter; SMR, small multidrug resistance.
[b] 18 type I, 1 type II.

electron donor (77). NirBD-mediated, nitrite-dependent ammonium formation could serve any of three different functions: nitrogen assimilation, nitrite detoxification, or NAD(P) regeneration (66, 77). Denitrifying cultures of *N. winogradskyi* have been reported to accumulate ammonium (37).

Because *N. winogradskyi* possesses genes for glutamine synthetase (GS, nwi1904), glutamate synthase (GOGAT, nwi2953-2954), and an assimilatory glutamate dehydrogenase (nwi2286), it has the potential to switch between GS-GOGAT and glutamate dehydrogenase depending upon the nitrogen source. For example, *N. winogradskyi* growing in pure culture on nitrite as a sole nitrogen source would likely utilize the GS-GOGAT system for nitrogen assimilation. However, in many environments, *N. winogradskyi* and other nitrite-oxidizing bacteria will be exposed to higher levels of ammonium than of nitrite, which may trigger a shift to utilization of the glutamate dehydrogenase system.

Several other genes associated with regulation of nitrogen metabolism (including GS expression) were identified in the *N. winogradskyi* genome, including *ntrB*/*C* and *ntrX*/*Y*. Genes putatively encoding GlnB, GlnK (PII), and GlnD (uridyltransferase) were identified, including one copy of *glnB* adjacent to *glnA*. Finally, *glnE* (nwi1199), encoding the GS adenylating enzyme, was identified. Further study is required to determine how *N. winogradskyi* regulates nitrogen assimilation in response to the inorganic nitrogen status of its environment.

N winogradskyi possesses all of the enzymes of the urea cycle and theoretically should be able to produce urea. However, there was no evidence of a urease gene or a complete urea carboxylase. Thus, if *N. winogradskyi* salvages nitrogen from turnover of proteins, urea might be excreted and, if so, may be available for uptake by nearby ureolytic ammonia-oxidizing bacteria.

Transport and secretion. Approximately 10% of the *N. winogradskyi* genome encodes genes for transport and secretion. Most transport systems are of the ATP binding cassette (ABC) type I, major facilitator superfamily, and resistance-nodulation-cell division families (Table 3). Single ABC type II, tripartite ATP-dependent periplasmic, and cation:proton antiporter 1-type transporters were also identified in addition to three small multidrug resistance type and at least three antiport/symport systems for sodium, protons and potassium. The genome lacks genes for active transporters of sugars, although an OprB-like outer membrane porin (nwi0329) and a putative major facilitator superfamily family 1 transporter (nwi3027) were annotated, the combination of which could enable carbohydrate uptake (68, 99). There were no other genes encoding import systems for monosaccharides, further supporting this organism's inability to utilize these molecules for growth.

Considering the limited heterotrophic ability of this bacterium, an interesting feature of this genome was the presence of multiple transporters for various organic nitrogen molecules. ABC type I transport systems for import of polar amino acids (His, Glu, Gln, and Arg) plus two complete systems for import of branched amino acids were identified. A *potGHIF* homolog for import of polyamines (putrescine and spermidine) and genes encoding all of the subunits of a peptide import system (*oppABCDF*) are also present. Furthermore, homologs of import systems for proline/glycine betaine and dicarboxylate molecules (glutamate, aspartate, succinate, and malate) were also found.

Approximately 70 genes in the *N. winogradskyi* genome are dedicated to iron acquisition. Considering that NXR contains multiple Fe-S centers, and that 10 to 30% of the total protein in *N. winogradskyi* cells is NXR (15, 88), acquisition of iron is crucial for survival. FeoAB-encoding genes (nwi2975-2976) were identified, enabling transport of ferrous iron under acidic or anaerobic conditions (3). *N. winogradskyi* possesses 26 putative iron siderophore receptor genes to acquire ferric iron during aerobic growth at neutral pH. These outer membrane receptors are supported by four TonB-ExbD-ExbB gene sets and two ABC transport systems, which would facilitate transport of siderophores into the cytoplasm (3, 20, 64). Similarly to *R. palustris*, a cluster of three genes for synthesis of a "rhizobactin-like" hydroxymate siderophore are present. Two additional genes, predicted by general function, are members of a COG that have been associated with an arylsulfate sulfotransferase involved in catechol siderophore biosynthesis. The genome of *N. winogradskyi* also encodes an HmuTUV-like ABC transport system for hemin uptake.

Genes encoding multiple transporters were also identified for uptake of sulfur, inorganic nitrogen and phosphorus. Two NarK homologs (nwi0779 and nwi1419) were identified which could function to transport nitrite or nitrate. Three ABC type I transporters for sulfonate and/or nitrate, and one for sulfate/thiosulfate are also present. Additionally, an alkanesulfonate monooxygenase homolog (*ssuD*, nwi0682) was identified, which potentially functions to desulfonate short-chain alkanes as a source of sulfur. The *ssuD* gene is adjoined by complementary ABC transport components (*ssuA*, *ssuB*, and *ssuC*).

With respect to phosphorus, *N. winogradskyi* seems to have at least one high-affinity and one low-affinity transport system.

Genes encoding the low-affinity PitA (nwi2031) system were identified. These genes share the same organization as the Pit system characterized in *S. meliloti* (5, 93), which is thought to be constitutively expressed when phosphate is in excess. Genes encoding the well-characterized ABC-type high-affinity system PstSCAB/PhoU (nwi0505 to nwi0512) were also annotated. The *pst* genes, which are generally expressed under phosphate starvation (72, 95), are also flanked by the phosphate two-component global regulator system *phoBR*. A gene encoding an Opr-type phosphate porin (nwi2169) was identified, which may form a trimeric porin under phosphate-limited conditions (41, 69).

Genes encoding a complete Sec system for transport of proteins to the membrane or periplasm were identified. A Tat system (nwi1777 to nwi1779) for export of folded proteins across the inner membrane was also annotated. Based on analysis by TatP (9) and PrediSi (http://www.predisi.de), approximately 580 proteins are potentially secreted by *N. winogradskyi*, 240 of these by the Tat pathway. As in many other gram-negative organisms, genes encoding components of a type II protein secretion/type IV pilus assembly are conserved in this organism. The operonic organization of the pilus components is nearly identical to that of the pili operon previously described in *C. crescentus*, a stalk-forming alphaproteobacterium (78). A suite of Trb-like type IV secretion/conjugal transfer genes were identified as well.

Environmental stress and defense. *N. winogradskyi*, like nearly all other aerobic organisms, is expected to contain enzymes that convert active oxygen compounds such as superoxide and hydroperoxides into innocuous products (47, 52, 75). The genome of *N. winogradskyi* contains genes that encode a heme-containing catalase-peroxidase (HPI, *katG*, nwi0030), an iron-containing (Fe-SOD, *sodB*, nwi0913), and a copper-zinc-containing (Cu/Zn-SOD, *sodC*, nwi2796) superoxide dismutase. In addition, the genome contains several genes that code for a diverse complement of thioredoxin-dependent peroxide reductases, including alkyl hydroperoxide reductase (*ahpC*, nwi1738 and nwi0891), thioredoxin reductase (*ahpF*, COG0492 and nwi2453), other peroxiredoxins (COG0678 and nwi2686), glutathione peroxidase (COG0386 and nwi2639), glutathione reductase (COG1249 and nwi1223) as well as antioxidant proteins with peroxidase activity (EC,1.11.1.7; nwi0432 and nwi0492).

Despite the fact that *N. winogradskyi* lacks genes encoding monofunctional heme-containing (HPII, *katA*) and non-heme-containing (Mn-catalase) catalases as well as peroxide-scavenging cytochrome *c* peroxidase, its oxidative stress tolerance inventory is more complex than previously reported for *N. europaea* (23). In contrast, *N. winogradskyi* seems less well prepared than *N. europaea* to cope with reactive nitrogen stress. Whereas the genome of *N. winogradskyi* contains genes encoding yet uncharacterized multicopper oxidases, which have been implicated in NO reduction (45), it lacks a gene for cytochrome P460, which has a hydroxylamine detoxification role (12).

Also as noted above, the *nirK* ortholog occurs in an apparent operon in *N. winogradskyi*, the structure of which is conserved in *N. europaea* and *N. eutropha*. In all three of these organisms, the *nirK* cluster is preceded by a gene predicted to encode an Rrf2 family regulatory protein. In *N. europaea*, the Rrf2 homolog (NsrR) acts as a transcriptional repressor of the downstream operon, and NsrR repression is lifted by exposure to nitrite (7), and a nitrite-sensing role could also be imagined for the NsrR-like homolog (nwi2653) in *N. winogradskyi*. However, in *N. europaea* and *N. eutropha*, this regulatory gene is oriented divergently to the *nirK* operon (7, 91), while in *N. winogradskyi* it is oriented in the same direction as the operon. It's unknown how this difference in orientation may affect the regulatory activity of the Rrf2-like homolog in *N. winogradskyi*.

A variety of temperature-, osmotic strength-, and chemical stress-related genes were identified. Five gene paralogs encoding the major cold shock regulatory protein (CspA), more than one gene for the cold-induced β subunit of DNA gyrase, and a sizable complement of genes that encode a variety of heat shock proteins, including the chaperonins (GroES, GroEL, DnaJ, and DnaK), was observed. *N. winogradskyi* also seems to be equipped for protection from organic solvents (OstA, nwi1679), arsenic (ArsBC, nwi3124 to nwi3125) and cyanate (CynS, nwi1302; and CynX, nwi1437) toxicity, and osmotic stresses (glycine betaine system) and can regulate its pH via a pH-adaptive potassium efflux system (PhaA-F, nwi2654 to nwi2658).

Effective stress response also involves regulated degradative capacity and *N. winogradskyi* contains several genes encoding ATP-dependent proteases, including Lon and Clp. Genes for DNA repair systems such as RecA, RecB, RecG, and RecF were also located. DNA-specific exonuclease RecJ and exonuclease ABC complex ORFs are present at different locations in the genome (e.g., *uvrC*, *uvrB*, *uvrA*, and *uvrD*). Genes encoding MutS and MutL, key components for initiation of methyl-directed DNA mismatch repair, were accounted for, although a MutH-encoding gene was not identified.

Motility. *Nitrobacter winogradskyi* is motile and can form biofilms, and hence its genome should contain structural and regulatory genes necessary for flagellar synthesis and function in response to environmental cues and challenges. The complement of operons needed for chemotaxis and flagellum biosynthesis is complete compared with information available for other bacteria (21, 30). However, the organization of these genes and the operon locations in the genome are remarkably different. Of the five known classes of methyl-accepting chemotaxis proteins (21, 58, 80), the *N. winogradskyi* genome encodes three of them: Tsr (nwi2996), which directly senses serine, alanine, glycine, and aminoisobutyrate; Tar (nwi0530), which senses aspartate and glutamate directly and is responsive to cobalt and nickel; and a protein in the CheD/PilJ/McpH protein family (nwi0072). Genes for a ribose/glucose/galactose sensor (Trg-like), a binding protein-dependent dipeptide sensor (Tap), and the redox sensor (Aer) were not found. *N. winogradskyi* appears to have a methyl-accepting chemotaxis protein complement similar to that found in *N. europaea*, and its chemotactic activity is likely regulated through the Che protein phosphorelay (CheWAY and CheBR) (23). It needs to be experimentally verified whether the *N. winogradskyi* methyl-accepting chemotaxis proteins respond to the same signals as found in gammaproteobacteria such as *E. coli* and *Pseudomonas aeruginosa*.

Surprisingly, the *N. winogradskyi* genome lacks the genes encoding the flagellar master operon *flhDC*, which is required for the transcriptional initiation of flagellation and chemotaxis both through direct activation and derepression of operons

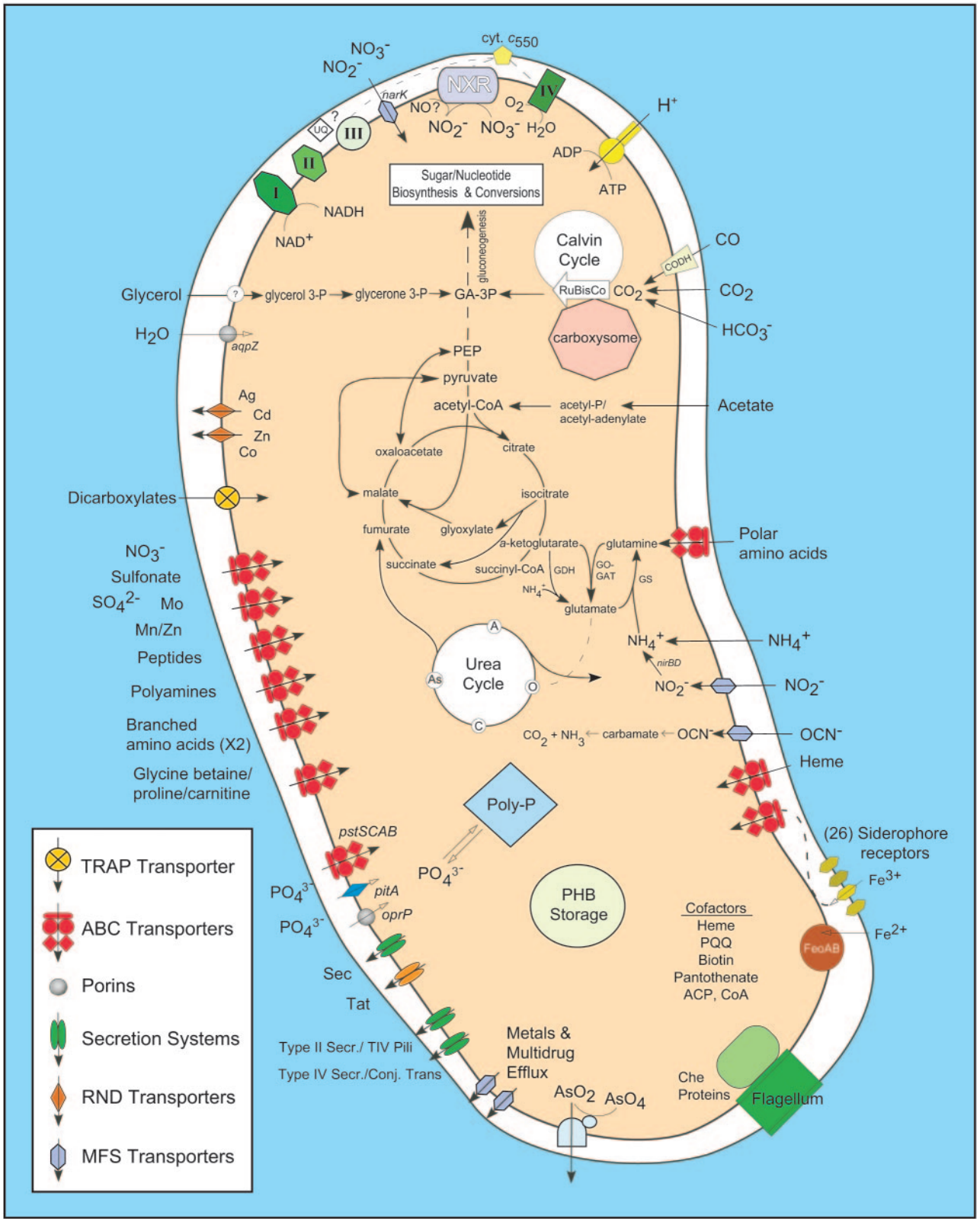


FIG. 3. *N. winogradskyi* cell diagram. Cellular processes depicted are based on putative gene annotations. Roman numerals refer to electron transport components of enzyme complex I (NADH-ubiquinone reductase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase).

and indirectly through control of the FliA protein, an alternative sigma factor (σ^{28}). A *fliA* homolog, anti-sigma *flgM*/*fliT* (58), and a *cheZ* phosphatase were also not observed. Hence, it appears that flagellation and motility may be differently regulated in *N. winogradskyi*.

DISCUSSION

Analysis of the *N. winogradskyi* genome both verifies and extends our understanding of its facultative lithoautotrophic lifestyle (summarized in Fig. 3), in which nitrite and carbon dioxide are utilized as sole energy and carbon sources, respectively. While *N. winogradskyi* is not obligately tied to nitrite oxidation to gain growth-supporting energy, it appears to have a preference for nitrite as an electron donor source despite the highly positive redox potential of the nitrite-nitrate couple (E'^{o}, 430 mV). The inability of *N. winogradskyi* to grow on C_6 molecules is consistent with its genome sequence, which lacks genes for complete Embden-Myerhof and Entner-Doudoroff pathways and does not code for the production of transporters for active uptake of sugars. Genes encoding multiple enzymes involved in anapleurotic reactions, including a glyoxylate bypass, were identified, which is consistent with reports of growth by *N. winogradskyi* on C_2 and C_3 molecules (28, 79). However, the *N. winogradskyi* genome sequence does explain why growth on dicarboxylates such as malate or succinate has not been observed, as it is predicted to encode a dicarboxylate transporter and a complete tricarboxylic acid cycle.

While the genome sequence indicates *N. winogradskyi* possesses CODH homologs, it is unknown if these genes produce a functional enzyme or couple CODH activity to carbon monoxide-dependent growth. It is unknown if segregation of the putative *coxL*/*cutL* from *cox*/*cutSM* affects expression and assembly of the subunits. Also, *N. winogradskyi* lacks the capacity to produce cytochrome b_{561}, a key electron transfer chain component typically associated with carbon monoxide-dependent growth of aerobic carboxidotrophic bacteria (61, 62). Utilization of carbon monoxide as a sole carbon and energy source to support growth has been demonstrated for the closest relative to *N. winogradskyi*, *B. japonicum* USDA110 (57). However, the *B. japonicum* USDA110 genome possesses multiple copies of putative CODH-encoding genes, including three *cox*/*cutSML* clusters, and at least two ORFs putatively encoding cytochrome b_{561}. Because of these differences, the potential of *N. winogradskyi* to exhibit CODH activity or carbon monoxide-dependent growth cannot be extrapolated from that demonstrated for *B. japonicum* USDA110, and awaits experimental analysis.

Given the challenges faced by *N. winogradskyi* and other nitrite-oxidizing bacteria growing on an energy-limited substrate, it will be fascinating to explore how this organism partitions the flux of reductant and ATP into the synthesis of various polymers (polyphosphate, PHB, beta-glucans, exopolysaccharides) that are nonessential under some conditions and essential under others. *Nitrobacter* accumulates PHB when grown heterotrophically with nitrate under low-oxygen conditions (37). PHB storage has been studied widely in bacteria, including members of the genus *Rhizobium*, where it can accumulate to high levels under free-living and symbiotic conditions (48).

Interestingly, dicarboxylate carbon sources such as malate and succinate result in the accumulation of PHB under some conditions and not others. Recently, Poole and Allaway (70) speculated that the availability of ammonium might be critical in promoting carbon assimilation into protein and preventing the accumulation of carbon in PHB. As mentioned above, *N. winogradskyi* possesses genes that code for many enzymes associated with the metabolism of acetate and pyruvate, which might influence the flow of carbon into the tricarboxylic acid cycle, lipids, and PHB. It will be interesting to examine how ammonium versus nitrite availability influences the flow of intracellular partitioning of carbon by *N. winogradskyi*.

Another interesting feature of the Nb-255 genome was the identification of multiple putative transporters capable of importing amino acids, peptides, and cyanate. These annotations suggest that access to organic nitrogen sources may be important, particularly during heterotrophic growth. Under lithotrophic growth conditions, nitrite serves as the sole source of nitrogen for biosynthesis and energy, as the genome encodes an assimilatory nitrite reductase (NirBD) and NarK-like transporters. The repression of NXR during heterotrophic growth (83) requires *N. winogradskyi* to utilize alternative sources of nitrogen for biosynthesis. Indeed, heterotrophic growth of *N. winogradskyi* generally occurs in cultures containing complex organic nitrogen sources such as yeast extract, peptone, and Casamino Acids (14, 28, 79, 84). Although *N. winogradskyi* can assimilate ammonium and nitrate (53), it may rely on organic nitrogen sources for biosynthesis in environments low in nitrate or nitrite, and may also have adapted to low ammonium, which is readily transformed to nitrite by ammonia-oxidizing bacteria. Clearly, a closer examination of the uptake and metabolism of exogenously supplied amino acids and inorganic nitrogen sources is warranted.

N. winogradskyi is the third member of the *Bradyrhizobiaceae* for which the genome has been fully sequenced. The *N. winogradskyi* genome (3.4 Mb) is smaller than that of the other two members, *R. palustris* (5.4 Mb) and *B. japonicum* (9.1 Mb), yet over two-thirds of the predicted proteins in the *N. winogradskyi* genome have the highest degree of sequence identity to homologs in *B. japonicum* and *R. palustris*. *R. palustris* is arguably the most metabolically versatile bacterium known, while *N. winogradskyi* may be moving towards an obligate dependence on nitrite oxidation. Many deletions of metabolic capabilities possessed by an ancestor common to *N. winogradskyi*, *B. japonicum*, and *R. palustris* are evident throughout the *N. winogradskyi* genome. For example, genes encoding remnants of nitrogen fixation, thiosulfate/sulfite oxidation, and nodule development pathways (which are functional in *B. japonicum* and *R. palustris*) were identified in *N. winogradskyi*. Whole-genome comparisons between these family members will undoubtedly aid in understanding niche selection, gene duplication events, genome reduction strategies, and horizontal transfer of DNA from other lineages.

The genome sequence of *N. winogradskyi* facilitates genomic comparisons with related species and may also advance our understanding of the interactions of nitrite-oxidizing bacteria with other microbes with which they closely associate in the environment, particularly ammonia-oxidizing bacteria. Nitrite rarely accumulates in soils, wastewater, or activated sludge, indicating that nitrite and ammonia oxidization are coupled in nature, and the nitrite-oxidizing bacteria are frequently observed to be physically clustered with ammonia-oxidizing bacteria. Outside of those of the *Bradyrhizobiaceae*, the proteins

annotated in *N. winogradskyi* were most frequently similar to orthologs (reciprocal best BLASTP hits) in *N. europaea*, suggesting exchange of genetic material. The functions of many of these genes are currently unknown, but their co-occurrence in distinct evolutionary lineages of nitrifying bacteria may indicate their global importance for nitrification. It also remains to be seen how these genes and other processes (e.g., the *ncgA*/*B*/*C*/*nirK* gene cluster, N flux, exopolysaccharide synthesis, and quorum sensing) function in a nitrifying community. Information gained from this and other nitrifier genomes will help clarify the potential interactions and coordination of nitrite and ammonia oxidation.

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