AN ABSTRACT OF THE DISSERTATION OF

Shawn R. Starkenburg for the degree of Doctor of Philosophy in Microbiology presented on December 18, 2007

Title: An Investigation of Carbon and Nitrogen Metabolism through a Genomic Analysis of the Genus *Nitrobacter*

Abstract approved:

_____

Peter J. Bottomley

The chemolithoautotrophic nitrite oxidizing bacteria (NOB) participate in the biogeochemical cycling of nitrogen by catalyzing and conserving energy from the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) via a nitrite oxidoreductase (NXR). The main objective of this work was to comparatively annotate and analyze the genome sequences of *Nitrobacter winogradskyi* NB255 and *Nitrobacter hamburgensis* X14 and use this information to extend our understanding of nitrogen and carbon metabolism in NOB. Through the analysis of the *N. winogradskyi* genome, genes encoding pathways for known modes of lithotrophic and heterotrophic growth were identified, including multiple enzymes involved in anapleurotic reactions centered on C2 to C4 metabolism. *N. winogradskyi* lacked genes encoding a complete glycolysis pathway and for the active transport of sugars. The *N. hamburgensis* genome harbored many genes not found in *N. winogradskyi*, including a complete glycolysis pathway, unique electron transport components, and putative pathways for the catabolism of aromatic, organic and one-carbon compounds. FAD-dependent oxidases were identified in the genome of *N. hamburgensis* which suggested that lactate could be metabolized, providing reductant and carbon to the cell. Indeed, D-lactate enhanced the growth rate and yield of *N. hamburgensis* in the presence of NO_2^- and served as a sole energy and carbon source in the absence of NO_2^-. Although lactate consumption occurred constitutively in

lithoautotrophically grown cells, evidence was obtained for physiological adaptation to lactate. D-lactate grown cells consumed and assimilated lactate at a faster rate than NO_2^- grown cells, and D-lactate-dependent O_2 uptake was significantly greater in cells grown heterotrophically or mixotrophically compared to cells grown lithoautotrophically. However, D-lactate could not substitute for CO_2 as the sole carbon source (lithoheterotrophy) during growth in the presence of NO_2^-. Through a comparative analysis of the *Nitrobacter* 'core' genome, many genes involved in NO_2^- metabolism were identified, including a dissimilatory nitrite reductase (NirK). The putative *nirK* in *N. winogradskyi* was maximally transcribed under low oxygen in the presence of NO_2^- and transcription was not detected under anaerobic conditions. Although production of NO under aerobic conditions was not detected, NO was consumed in a cyanide-sensitive process and reversibly inhibited NO_2^--dependent O_2 uptake.

An Investigation of Carbon and Nitrogen Metabolism through a Genomic Analysis of the Genus *Nitrobacter*

by

Shawn R. Starkenburg

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented December 18, 2007
Commencement June 2008

Doctor of Philosophy dissertation of Shawn R. Starkenburg presented on December 18, 2007

APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____

Shawn R. Starkenburg, Author

ACKNOWLEDGEMENTS

This work was completed through the support of many great people. I am grateful to my undergraduate professors, Sara Tolsma, Tim Lubben, and Fred Van Dyke for their personal and professional investment in me as a young student of science. Without their support and guidance, I would not have chosen a career in research. Many thanks also go to John Swart and Nick Cianciotto, who gave me the opportunity to gain professional experience in applied and academic research labs and lifted the veil of uncertainty with regard to my ability to pursue an advanced degree.

I am also grateful for the mentoring I have received from my co-advisors, Peter Bottomley and Dan Arp. Despite his taxing schedule, Dan always maintained an open door policy when I needed to chat about results or ask for professional advice. Thanks, Dan, for providing a good model of how to ethically approach research, for giving me the opportunity to complete an internship, and to learn how to manage a large research project. Many thanks also go to Peter Bottomley, whom, from day one, has been unwavering in his to commitment to mentor me through this dissertation. Thanks for approaching my scientific development with a sincere, pragmatic approach and for supporting my time away from professional endeavors to invest in my daughter, Naomi.

I would also like to thank my committee members, Lewis Semprini, Joyce Loper, and Larry Curtis for their time and interest in my work. Thanks, Patrick, for the technical assistance, professional development, and for your kindness during my internship. Thanks to all the members of the Arp, Bottomley, and Myrold lab groups for your camaraderie, kindness, and laughs. Special thanks to Norman Hommes, for being a good friend, an exercise partner, for your guidance and technical assistance in the lab, and for being a constant sounding board for ideas and problem solving over the course my graduate school career. Thanks, Brad, for the frizbee and football in the hall during the power outages, and thanks, Luis, for always helping with technical glitches. Special thanks also go to Anne Taylor- graduate school was more enjoyable because of your tutelage, the lunchtime talks, and your generous gifts of chocolate (and aspirin).

Finally, I would like to thank my family for their support. Thanks, Diane, for your patience and encouragement through many times of doubt and uncertainty. I would not have completed this degree without your innumerable personal and professional sacrifices. This dissertation is as much yours as it is mine. I would also like to thank my daughter, Naomi, for the reality checks, for putting up with Saturday trips to the lab, and for helping me remember which facets of life truly matter.

CONTRIBUTION OF AUTHORS

Patrick S.G. Chain managed the assembly of the genomes of *N. winogradskyi* and *N. hamburgensis*, and contributed to sequence analysis and manuscript preparation of the 'Complex Repetitive Elements' subsection in Chapter 2 and 3. Luis A. Sayavedra-Soto contributed to the genomic analysis of 'Transcription, Translation, and Regulation' and manuscript preparation in Chapter 2 and verification of annotations regarding 'Regulation' in Chapter 3. Loren Hauser and Miriam Land assisted in the automated annotation and construction of the online interface of the *N. winogradskyi* genome in Chapter 2. Frank Larimer contributed to construction of the online genome interface and data analysis in Chapter 2. In Chapter 3, Frank Larimer assisted technically with cross-genomic comparisons by constructing the bioinformatics tools to facilitate such comparisons in Chapter 3. Stephanie A. Malfatti contributed technically to genome assembly in Chapter 2. Martin G. Klotz contributed to manuscript preparation of Chapters 2 and 3 and to genomic analysis of 'Cell Signaling' and 'Enviromental Stress and Defense' in Chapter 2 and 'Regulation and Signaling' and 'Assimilatory Nitrogen Metabolism' in Chapter 3. Mira Gentry contributed to genomic 'Regulation and Signaling' analysis in Chapter 3. Lisa Stein contributed to genomic analysis of 'C-1 Metabolism', 'Dissimilatory Nitrogen Metabolism' and manuscript preparation of Chapter 3. William J. Hickey contributed extensively to the genomic analysis and annotation and manuscript preparation of Chapter 2. Bess Ward contributed to genomic analysis of 'Sulfur Metabolism' and manuscript preparation in Chapter 3. Daniel J. Arp contributed to manuscript preparation of Chapters 2 and 3 and to data interpretation, study design and manuscript preparation of Chapters 4 and 5.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

An Investigation of Carbon and Nitrogen Metabolism through a Genomic Analysis of the Genus *Nitrobacter*

Chapter 1

General Introduction

Nitrogen (N) is required for life and is abundant in the form of dinitrogen gas (N_2) in the earth's atmosphere. Nonetheless, N_2 is not directly useable by most organisms and first needs to be transformed into a biologically available form. In the absence of human activity, transformation of N_2 into bioavailable molecules is mediated primarily by microorganisms. These microorganisms 'fix' nitrogen by converting N_2 into ammonia (NH_3) which is subsequently oxidized to nitrate (NO_3^-) through a process called nitrification.

Nitrification is a two step, aerobic process by which NH_3 is converted to NO_3^- via nitrite (NO_2^-) by microorganisms. The first step in nitrification, the conversion of ammonia to NO_2^-, is mediated by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA)(21, 91). The second step of nitrification is controlled by 'nitrite oxidizing bacteria' (NOB) which convert NO_2^- into NO_3^-. To date, archaea that can oxidize NO_2^- for energy have not been discovered. As chemolithoautotrophs, both ammonia oxidizing microorganisms (AOM) and NOB conserve energy from the oxidation of ammonia and NO_2^-, respectively, and fix carbon dioxide through the Calvin cycle as a source of carbon for biosynthesis (21, 23). Although ammonia oxidation and NO_2^- oxidation can occur independently, NO_2^- does not typically accumulate in soil or marine environments (104, 137) and NOB are often physically clustered with AOB (65, 139) indicating a tight coupling between ammonia oxidation and NO_2^- oxidations. Nitrifying microorganisms are ubiquitous in natural environments, found in fresh and brackish water, aerobic and anaerobic soils, on natural building stones (127) and have recently been identified in a subsurface radioactive thermal spring (180). Along with

AOM, NOB also play a significant role in sewage treatment systems, mediating the removal of excess N from wastewater.

Anthropogenic inputs of ammonia and NO_3^- into the environment now outweigh the natural inputs of bioavailable N (60, 61). Excess production of soluble N by nitrification can result in the contamination and eutrophication of aquatic and terrestrial ecosystems, while the gaseous products of nitrification, nitric oxide (NO) and nitrous oxide (N_2O), are two of the most potent greenhouse gases. As anthropogenic inputs of fixed N continue to increase to meet the demands of a growing global population, intimate knowledge of nitrification, and the microorganisms that control it, will be necessary to mitigate the aforementioned harmful effects, promote efficient use of fertilizer N in agricultural systems, and expedite removal of N from wastewater.

NOB Diversity

NOB are currently classified into four lineages, *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira*, which are members of the Alpha-, Delta- and Gammaproteobacteria (167) and the phylum *Nitrospireae*, respectively. Historically, *Nitrobacter* has been used as the primary model organism for studying NO_2^- oxidation. *Nitrobacter is* a member of the Family *Bradyrhizobiacaea* and is closely related (97-98% identity of 16s rRNA) to *Bradyrhizobium* and *Rhodopseudomonas*. *Rhodopseudomonas palustris* can grow either chemotrophically or phototrophically and is arguably one of the most metabolically versatile bacterium known (96) and *Bradyrhizobium japonicum* is a N_2-fixing legume symbiont and harbors one of the largest prokaryote genomes. However, *Nitrobacter* is unique in that neither of these close relatives have the ability to use NO_2^- as an energy source. Relative to the other NOB lineages, *Nitrobacter* is easy to culture and is typically the most common NOB isolated which established the dogma that *Nitrobacter* played a dominant role in NO_2^- oxidation. Over the last decade, the paradigm has shifted. The first reported attempt to identify *Nitrobacte*r in situ in wastewater treatment plants using 16s rRNA probes was unsuccessful (177). Further analysis using non-culture based approaches indicated that *Nitrospira*, not *Nitrobacter*, was the numerically dominant lineage in many wastewater

treatment systems (40, 41, 83, 139) and later was shown to be prevalent in other aquatic and soil environments (7, 56, 78). Despite the numerical dominance of *Nitrospira* in many ecosystems, *Nitrobacter* has subsequently been detected and isolated in wastewater (8, 114, 145) and recent investigations have indicated that *Nitrobacter* may have a selective advantage over *Nitrospira* under high NO_2^- loads (7, 40), high oxygen tension (108) and salt stress (118). As the majority of this work has been completed with enrichment cultures derived from wastewater, the environmental factor(s) which give one NOB lineage a selective advantage over another in other environments has not been fully explored.

Nitrite Oxidoreductase

Energy conservation via NO_2^- oxidation is mediated by the enzyme nitrite oxidoreductase (NXR) according to the following reaction:

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-. \qquad [1]$$

The source of oxygen in reaction [1] is water (8) and electrons gained from this reaction drive the reduction of NAD^+ via reverse electron flow and the generation of ATP by oxidative phosphorylation (54). The energy yield from NO_2^- oxidation is low compared to the energy conserved from reduced organic carbon sources as reflected by the fact that 85-115 moles of NO_2^- must be oxidized to fix 1mole of CO_2 (21). The cytoplasmic membrane contains many electron dense invaginations and as Spieck, et al (150) observed, these membranes are densely packed with NXR, presumably to maximize the number of NO_2^- oxidation sites. Indeed, under NO_2^- oxidizing conditions, 10-30% of the total cellular protein is estimated to be NXR (21, 164).

NXR is composed of an alpha and a beta heterodimer coded for by *nxrA* and *nxrB*, respectively (150). In the operon *nxrA* and *nxrB* are separated by *nxrX*, a putative peptidyl- prolyl-cis-trans isomerase which may aid in folding of NXR. NxrA encodes the site of NO_2^- oxidation activity and is dependent upon molybdopterin as a cofactor. NxrB contains many conserved cysteine residues which stabilize the Fe-S centers

necessary for transporting electrons to terminal oxidases for ATP generation and the synthesis of NADH (156).

Organic Carbon Metabolism

Despite the selective advantage of being able to oxidize NO_2^- to support growth, the energy conserved from NO_2^- is very low (21). Thus, the ability of NOB to use organic carbon (C) as a carbon or energy source would be beneficial to reduce the need for high amounts of NO_2^- and, efficient use of reduced carbon may give one NOB clade a competitive advantage over another in situations where NO_2^- is limiting. Although NOB of two genera (*Nitrospina* and *Nitrococcus*) are still considered obligately chemolithoautotrophic, most *Nitrobacter* species are capable of mixotrophic growth on both NO_2^- and organic energy sources (12, 65). Under aerobic conditions (but not anaerobic conditions), *Nitrospira*-like bacteria incorporated pyruvate into cell material suggesting that these bacteria can grow mixotrophically as well (40). Activities of all TCA cycle enzymes have been identified in *Nitrobacter,* although succinate dehydrogenase activity was very low (154). *Nitrobacter* is unique among NOBs in that some species can grow heterotrophically in the absence of NO_2^- using a narrow range of simple organic carbon (C) compounds such as pyruvate, acetate, and glycerol in the absence of NO_2^- (20, 147). Formate and α-ketoglutarate are also metabolized by *Nitrobacter* although the ability of these carbon sources to support growth has not been confirmed.

The effective use and metabolism of organic carbon varies among the *Nitrobacter* lineage. For example, *N. winogradskyi* can use NO_2^- and organic material concurrently while other *Nitrobacter* species exhibit diphasic growth, first using NO_2^- then organic material (23). *N. winogradskyi* can grow slowly on acetate relative to growth on NO_2^-, while *N. hamburgensis* X14 appears to grow best in medium containing both NO_2^- and organic carbon (23, 24, 155). Despite the different responses to organic carbon with the genus *Nitrobacter,* the physiologic and genetic basis for these phenotypes has not been determined. Futhermore, mixotrophic and heterotrophic studies of *Nitrobacter* were commonly completed in poorly defined complex medium

containing peptone, casamino acids, and yeast extract (24, 70, 87, 159), although it is not known how or if these media components benefit *Nitrobacter*.

Denitrification in *Nitrobacter*

Some species of *Nitrobacter* can also grow anaerobically and gain energy by coupling the oxidation of pyruvate or glycerol to NO_3^- reduction (25, 55). The terminal products of denitrification in *N. winogradskyi* are reported to be NO and N_2O (1, 54, 55). The reaction in equation [1] can be catalyzed in reverse by NXR under anaerobic conditions (55, 159) as NxrAB is evolutionarily related to the NarGH polypeptides that comprise the large and small subunits of dissimilatory nitrate reductases in heterotrophic denitrifiers (87). Although the mechanisms for NO and N_2O formation are not known, in *Nitrobacter vulgaris*, dissimilatory nitrite reductase activity (reduction of NO_2^- to NO) was measured from a protein that co-purified with NXR under anaerobic conditions (1). The two major groups of enzymes that function in this capacity are the trimeric copper-containing oxidases (NIR) and the dimeric cd_1-type cytochromes (34, 131, 196). The purified NO_2^- reductase was enriched in copper and was sensitive to *N*,*N*-diethyldithiocarbamate (DDC) suggesting that this enzyme was a copper-containing nirK-type nitrite reductase (1).

Although the aforementioned NO_2^- reductase activity was higher under anaerobic conditions, intriguingly, enzymatic activity was also detected in air saturated medium (1) suggesting that the reduction of NO_2^- to NO also functions under aerobic conditions. Additionally, Freitag, et al (54) observed that when NO was added to either anaerobic or aerobic cell suspensions, NADH was synthesized. These observations led to the hypothesis that NO may play a role in the NO_2^- oxidizing system of *Nitrobacter*. Electrons from NO_2^- oxidation via NXR enter the respiratory chain at the level of complex III and thus a high energetic hurdle must be overcome to synthesize NADH via reverse flow of electrons from NO_2^-. From an energetic perspective, NO should be a more favorable electron donor than NO_2^- given the less positive redox potential (E_o'= +387 mV) of the NO_2^-/NO couple relative to NO_3^-/NO_2^- (E_o'= +420 mV).

Further investigations into the metabolism of NO by this or any other NOB has not been completed, yet, many recent reports have emphasized a greater significance for the function of NirK and NO production during the *aerobic* oxidation of ammonia in *Nitrosomonas* (9-11, 31, 32, 138). Release of NO during aerobic ammonia oxidation was historically interpreted to be caused by auto-oxidation of hydroxylamine (an intermediate in the oxidation of ammonia to NO_2^-). Recently, it was discovered that ammonia oxidation by *Nitrosomonas eutropha* was inhibited when NO was stripped from aerobic cultures and the ensuing recovery of ammonia oxidation occurred much faster when NO was added back the cultures (192). Nirk was also found to be active and expressed under aerobic conditions, and in a *nirK*-deficient strain of *N. europaea,* the growth rate and cell yield during ammonia oxidation were reduced compared to the wild-type (32, 138). Additionally, expression of NirK in *N. europaea* was controlled by the concentration of NO_2^- and pH, but unlike an archetypal nitrite reductase that is expressed during anaerobic denitrification, oxygen limitation did not induce its expression (10). In summary, these data indicate that in AOB, NirK and NO appear to be required for optimum energy yield and growth on ammonia under aerobic conditions but it remains to be determined how, or if, NO functions in the NO_2^- oxidizing system of *Nitrobacter*.

NOB Genomics

Despite the fact that NO_2^- oxidizers have been available in culture for over a century, 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 enzymes involved in energy generation, NO_2^- oxidation, carbon fixation and intermediary C metabolism (2, 21, 23, 186). Prior to the genomics era, only a handful of genes were sequenced from any *Nitrobacter* strain (70, 87, 162). For example, in the early 1990's, *nxrB* and *nxrX* were sequenced and *nxrB* was shown to be similar to dissimilatory nitrate reductase subunit, *narH*, but only a partial sequencing of *nxrA* was completed (87). As mentioned above, *Nitrobacter* is closely related to *Rhodopseudomonas*, arguably, the most metabolically versatile bacterium

known (96), yet, the genetic and molecular basis of what evolutionarily separates the 'metabolically limited' *Nitrobacter* from its physiologically versatile relatives has remained a mystery.

Over the last three years, multiple genomes from NOB and AOM have been sequenced. With respect to *Nitrobacter*, the first genome of a representative strain of the classical species, *N. winogradskyi* (NB255) was sequenced in 2005 (151). Shortly thereafter, the genome sequences of the soil isolate, *N. hamburgensis* and a marine variant of *N. winogradskyi*, NB311A (the latter of which has never been studied) were also released. The availability of genome sequences of these three species has provided an opportunity to gain new insight into the genetic basis of NO_2^- oxidation, the phylogenetic identity of NOB, and will clarify and extend the physiological capabilities of these unique organisms.

Thesis Objectives

The main objective of this work was to annotate and analyze the genome sequences of *N. winogradskyi* and *N. hamburgensis* and use this analysis to inform and extend our understanding of nitrogen and carbon metabolism in NOB.

Chapter 2 describes the analysis of the genome sequence of *N. winogradskyi*. This was the first complete genome sequence from a NOB and thus a comprehensive analysis of several facets of cell physiology were analyzed including energy conservation and electron transport, dissimilatory and assimilatory nitrogen metabolism, utilization of organic and inorganic carbon sources, lipid and lipopolysacchride formation, and the putative mechanisms of environmental stress, and transport of nutrients.

Chapter 3 describes the analysis of the genome sequence of *N. hamburgensis* through a comparison of this genome to *N. winogradskyi* and the marine isolate, *Nitrobacter sp. NB311A*. Because *N. hamburgensis* and *N. winogradskyi* have different growth phenotypes, I assessed the genomic basis for the aforementioned variations in growth and physiology and determined the unique genetic and structural characteristics of each *Nitrobacter* genome. Secondly, construction of a *Nitrobacter* composite or

'core' genome (representing the common genetic elements between the three *Nitrobacter* species) was completed to gain further insight into the common genetic basis of NO_2^- oxidation. Using the core genome, a comparative analysis was also conducted with the *Nitrobacter* genomes and all of the sequenced strains of *R. palustris* and *B. japonicum* to determine the unique genetic features of *Nitrobacter* not found in its metabolically versatile, non- NO_2^- oxidizing, relatives.

Chapter 4 describes an investigation of lactate metabolism by *N. hamburgensis*. This investigation was prompted by the fact that three FAD-dependent oxidases were identified in the genome that could potentially oxidize lactate to pyruvate as a source of carbon and energy. The growth response of *N. hamburgensis* to D- and L- lactate was measured in the presence and absence of NO_2^- to determine if lactate could support heterotrophic or mixotrophic growth of this bacterium. The physiological adaptation to lactate was assessed by measuring the rate of consumption, its support of respiration, and the distribution of lactate carbon in heterotrophic, mixotrophic, and autotrophic cells. The effect of NO_2^- on lactate metabolism was also determined.

Chapter 5 describes an investigation into the function of a putative nitrite reductase and the effect of its product, NO, on viable cells of *N. winogradskyi*. Sequencing of the *N. winogradskyi* genome revealed that the chromosome contained an operon encoding a gene (Nwin_2648) that encoded a putative *nirK* type nitrite reductase (151). Surprisingly, although NirK is encoded in the genome of *N. winogradskyi's* close Alphaproteobacterial relative, *Rhodopseudomonas palustris,* the *N. winogradskyi nirK* was most similar to a homolog in the ammonia oxidizing Betaproteobacterium, *Nitrosomonas* (31), providing cirmcumstantial evidence that this enzyme may be functioning during aerobic NO_2^- oxidation. I measured the transcription of *nirk* under aerobic and anaerobic conditions and deterimined if oxygen, pH, and/or NO_2^- controlled its expression. The production of NO was assessed under full aerobic conditions. Due to the reactive nature of NO, the effects of NO on respiration were studied, and the mechanisms and end product(s) of NO consumption by *N. winogradskyi* were investigated.

Chapter 2

The genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255

Shawn R. Starkenburg, Patrick Chain, Luis A. Sayavedra-Soto, Loren Hauser, Miriam L. Land, Frank Larimer, Stephanie A. Malfatti, Martin G. Klotz, Peter J. Bottomley, Daniel J. Arp, and William J. Hickey

Published in:
Applied and Environmental Microbiology
American Society for Microbiology,
2006, Vol. 72, No. 3, p. 2050-2063

ABSTRACT

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 3402093 bp encoding 3143 predicted proteins. There were extensive similarities to genes in two Alphaproteobacteria, *Bradyrhizobium japonicum* USDA110 (1300 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 C2 to C4 metabolism, including a glyoxylate bypass, were annotated. The inability of *N. winogradskyi* to grow on C6 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.

INTRODUCTION

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 byproducts 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 (NOB), participate in nitrification by converting nitrite, the end product of ammonia oxidation, into nitrate according to the following 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 (54).

As a facultative chemolithoautotroph, *N. winogradskyi* derives 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, chemoorganotroph growth is typically much slower than that occurring when nitrite is supplied as the energy source (23). All tricarboxylic acid (TCA) cycle enzyme activities have been detected (155) and energy can be gained from the oxidation of pyruvate, acetate, formate, α-ketoglutarate, and glycerol (20, 83, 147). In some strains of *Nitrobacter,* growth on a combination of nitrite and the aforementioned organic carbon sources (mixotrophy) can outpace either chemolitho- or chemoorgano-trophic growth (23, 155).

The NOB are currently classified into four genera. *Nitrobacter*, *Nitrospina*, *Nitrococcus* are Proteobacteria and members of the Alpha-, Delta-, and Gamma-proteobacteria (167) classes, 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 bacteria, *Rhodopseudomonas palustris*, and the dinitrogen-fixing legume symbiont, *Bradyrhizobium japonicum* (167).

The NOB 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 transport, nitrite oxidation, carbon fixation and intermediary carbon metabolism (2, 21, 23, 186). Currently, there is considerable interest in the ecology of nitrification and the composition of ammonia-oxidizing bacteria (AOB) and NOB communities in various ecosystems (27, 65, 76, 91). Whole genome sequences from NOB will provide new insight into their phylogenetic identity, reveal potentially novel physiological capabilities, and will serve as a model to study the cellular and molecular processes that control nitrite oxidation. In this study, an analysis of the first complete genome sequence from a nitrite oxidizing bacterium, *N. winogradskyi* Nb-255, is presented.

MATERIALS AND METHODS

Organism source and culturing. *Nitrobacter winogradskyi* Nb-255 was obtained from the American Type Culture Collection (ATCC) as strain ATTC 25391. Batch cultures of *N. winogradskyi* were grown chemoautolithotrophically 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 (33, 52). 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 DOE Joint Genome Institute using ABI 3730xl DNA Analyzers and MegaBACE 4500 Genetic Analyzers as described on the JGI 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, base calling and assessment of data quality were performed with PHRED and PHRAP, respectively (50, 51). Assembled sequences were visualized with CONSED (66). The initial assembly consisted of forty-two contigs ($\geq$ 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 (33).

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 basic local

alignment search tool for proteins (BLASTP). The protein set was also searched against 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 analysis of bacterial genomes and gene neighborhoods were completed using the JGI Integrated Microbial Genomes (IMG) 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 is available at GenBank/EMBL/DDBJ 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 3143 characterized and putative proteins (Fig. 2.1). The single copies of 16S- and 23S-rRNA genes had 98 % and 95 % identity to *Bradyrhizobium japonicum* USDA110 and *Rhodopseudomonas palustris* CGA009, respectively. Based on a BLASTP searches, 281 open reading frames (ORFs) appear unique to *N. winogradskyi* and of the remainder 2837 (66 %) could be assigned a putative function. Based on BLAST analysis of KEGG assignments, the most extensive similarities were to genes in other Alphaproteobacteria, namely *B. japonicum* (1300 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 IS4 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 IS elements that represent eight different families (Table 2.1). 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-nwi1479, nwi1623-nwi1628) and interestingly, lies within two, larger phage-like regions.

Analysis of homologous genes revealed that *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 (nwi0223-0228 and nwi0761-0766). 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 2595 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 (nwi2038-40 and nwi0705-0703), however, only the 3' end of the ExbB proton channel component (nwi0703, nwi2040) shows conservation (98% identity), while the 5' ends are of different size 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, if-3 and elongation factors Tu and G were all contiguous with genes coding for ribosomal proteins. There were 49 genes for amino acyl-tRNA biosynthesis. Genes encoding RNA processing functions include Ribonuclease PH (final 3'-trimming and modification of tRNA precursors), ribonuclease D (a 3'-exonuclease acting on tRNA), and RNase E and RNase G (maturation of the 5' end of 16S RNA).

Genes were identified putatively encoding homologs for the tublin-like FtsZ (nwi1058), and for cell division proteins that associate with Ftsz; FtsA (nwi1057), FtsQ (nwi1056), FtsW, (nwi1050), FtsI (nwi1045) and FtsK (nwi0083). Like other Alphaproteobacteria, *N. winogradskyi* lacks homologs of MinCDE, SulA, ZipA, FtsL and FtsN (191). *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 (73). 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 2.2). 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. The global iron regulator (FUR, nwi0013) was identified in addition to a zinc uptake regulator (ZUR, nwi0493), and an iron response regulator (IRR, nwi0035), the latter of which could be mediating iron-dependent control of heme synthesis (128). *N. winogradskyi* has 28 signal transduction histidine kinases of varied function, two of which are nitrogen related (NtrB) and seven 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 (nwi0626-0627) 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 (181). Genes for basal transcription factors (*nusG*: nwi1345, 1549, *greA*: nwi0922, 2450, *rhoA*: nwi0100, *nusA*: nwi0022, *nusB*: nwi1722, 0156) were also accounted for in the genome.

Multiple phosphotransferase system (PTS) signaling genes were identified. The gene cluster at nwi0344-nwi0348 encodes HPr, EIIA, HPr kinase, and homologs of the ChvIG/ExoRS two component sensory system (Fig. 2C). A phosphoenolpyruvate (PEP) utilizing EI enzyme (nwi0378) was also annotated along with a second EIIA 2 (*ptsN*: nwi0179) gene immediately upstream of σ^{54} (*rpoN*). Gene homologs for sugar permeases (*e.g.*, EIIB, EIIC, EIID) were not identified. The absence of sugar permease genes combined with the presence of an Hpr kinase gene suggest that the PTS-type signaling molecules in *N. winogradskyi* are not involved in sugar transport (26). Alternatively, the phosphotransferase proteins may be stimulating RpoN expression (110, 168) and/or involved in the regulation of ChvIG/ExoRS, which has been shown to regulate acid inducible genes in *Agrobacterium tumefaciens* (98), and succinoglycan EPS production and flagellum biosynthesis in *Sinorhizobium meliloti* (100, 190).

Energy generation and electron transport. Reductant (NADH) gained from the metabolism of organic substrates is assumed to fuel oxidative phosphorylation via conventional respiratory machinery (21, 23) . Indeed, genes for Complexes I-V of a classical electron transport system (ETS) were identified. In chemolithotrophic growth, nitrite is oxidized to nitrate *via* a molybdopterin-containing nitrite oxidoreductase (2, 21). 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 (186). 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 (15). Both soluble and membrane-bound class IA/B cytochrome *c*-550 proteins were purified, and shown to be electron donors for Cox (121, 162, 189). Among the c-type cytochrome genes identified, orthologs for both cytochrome *c*-550 proteins (c-550s: nwi2582, 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 function to these or other *c*-type cytochromes in the ETS.

Congruent with previous investigations of *N. winogradskyi* strain agilis (15), 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- nwi0766 loci. Electrons from nitrite oxidation are considered to enter the respiratory chain at the level of cytochrome a_1 (2, 21, 185, 186). Cytochrome a_1 requires a heme A cofactor that is biosynthesized from heme B via heme O as a stable intermediate (53, 116). This pathway requires the sequential activity of heme O synthase (CoxA/CtaB homologs, (53, 160)) and heme A synthase (CtaA homologs, (160)), respectively. Each cytochrome *c*-oxidase gene cluster contains a CyoE/CtaB homolog (nwi0225, 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 referred to as NOR, a designation that has also been applied to nitric oxide reductase. To eliminate this confusion, we propose a new designation for nitrite oxidoreductase: 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, 21, 38)). Formate may also be a substrate for NXR, and is oxidized to carbon dioxide (38).

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 (150). The *N. winogradskyi* genome contains two copies each of *nxrA* and *nxrB*. One copy of *nxrA* is one gene upstream of *nxrB* (*nxrA1*: nwi0774; *nxrB1*: nwi0776; Fig. 2A), while the other copies are segregated at distant points in the genome (*nxrA2*: nwi2068; *nxrB2*: 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 (149, 150). Signal peptides were not detected by SIGNALP (14) analysis of either NxrA or NxrB. However, NxrA and NxrA were predicted by TOPPRED (176) and TMPRED (72) 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 ORF, *norX*, located between *nxrA1* (*norA*) and *nxrB1* (*norB*) (87). A putative homolog of *norX, nxrX* (nwi0775), was identified in *N. winogradskyi* (Fig. 2.2A) 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 863769-70 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* remains 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. 2.2A). Immediately upstream of *nxrA1*, nwi0773 is predicted to encode a *c*-type cytochrome that may be a part of the ETS coupled to oxidation and reduction of nitrite (2, 21, 185, 186).

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 (196). NarJ, the nitrate reductase δ-subunit, inserts the molybdenum cofactor in nitrate reductase A (19, 174), 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 (MFS) of transporters that has been reported to function as a nitrate-proton symporter, a nitrate-nitrite antiporter, and a nitrite uniporter (37, 182). 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* remain 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 (25, 54, 55). 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 (87). 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 co-purified 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 (34, 131, 196). *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; *ncgA*, nwi2651) are clustered in an apparent operon with genes for biogenesis of *c*-type cytochromes (nwi2652, *ncgB*: nwi2650, *ncgC*: nwi2649). Orthologs of these genes and the operon structure are conserved in the ammonia oxidizing bacteria, *Nitrosomonas europaea* and *N. eutropha* ((33), Fig. 2.2D). In *N. europaea*, results of mutational analyses indicate that the nwi2648 ortholog (NirK) and other genes in the cluster function in detoxification/tolerance of nitrite and possibly nitric oxide (9, 11). These genes could have a similar role in *N. winogradskyi* (see "Environmental Defense and Stress" below). Of the two remaining multicopper oxidases, only that predicted for nwi2661 lacks the motif for a Type III copper site, the absence of which is characteristic of the copper-containing nitrite reductases (46). 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 (55)* , the predominant form has not been independently confirmed. Nitrous oxide has been detected in non-specified amounts (54, 55), 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; the cytochrome *c*-dependent (cNOR) and quinol (qNOR)-dependent (42, 71, 131). There were no BLAST hits in the *N. winogradskyi* genome for qNOR homologs. Two putative proteins had weak similarities (23-26% 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 vs. ca. 460 amino acids, respectively. 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 non-heme iron instead of copper, and four conserved glutamic acid residues in NorB have been implicated in coordinating the non-heme iron ligand (131). 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 (21, 23). Genes for all of the enzymes of a functional CBB cycle are present. The typical sedoheptulose 1,7-bis-phosphatase (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-bis-phosphatase (EC 3.1.3.11, nwi2694) and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, nwi2735), respectively (33, 109, 143).

Two copies of *cbbL* and *cbbS*, encoding the large and small subunits, respectively of Type I RuBisCO, were identified (Fig. 2.2B; nwi2928-2929; 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 sequence similarity of the nwi2928-2929 locus is most similar to 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 nwi1975-1987 RubisCO/carboxysome gene cluster is nearly identical to that in the Gammaproteobacteria, *Acidithiobacillus ferrooxidans* (30) and *Thiobacillus denitrificans.*

Both copies of *cbbL* are preceded by divergently transcribed *cbbR* LysR-type regulators (Fig. 2.2B) and putative signal transduction proteins containing PII-like domains (nwi1989, 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*. ATCC 25259) of nwi1989 or nwi2931is 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 latter 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 (59, 84, 135).

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). Immediately 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 (59, 135).

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 hetero-trophically grown cells of *N. winogradskyi* strain agilis, demonstrated a complete TCA cycle (154). Growth by *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 its genome, which would sever classical

glycolysis. The Enter-Doudoroff pathway could not be reconstructed either due to absence of a 2-keto-3-deoxy-6-phosphogluconic (KDPG) 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), PEP carboxylase (nwi2278), and malic enzyme (nwi1587), confirming previous investigations with *N. winogradskyi* strain agilis (155). Pyruvate phosphate dikinase (nwi2709) and PEP carboxykinase genes (nwi0350) were also identified, allowing *N. winogradskyi* greater metabolic flexibility around pyruvate. Similarly, heterotrophic growth by *N. winogradskyi* on acetate was demonstrated (20, 43, 147), and genes for catabolic enzymes that would funnel acetate into the TCA cycle *via* conversion to acetyl-CoA 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 C4 deficiency that would occur during growth on acetate or other C2 or C3 compounds. Homologs of glycerol kinase (nwi0281), glycerol-3-phosphate dehydrogenase genes (EC 1.1.1.94, nwi0469; EC 1.1.3.21, nwi2990), and triosphosphate isomerase (EC 5.3.1.1, nwi1835) were found, which would allow the conversion of glycerol to the glycolysis interemediate, glyceraldehyde-3-phosphate. Although growth of *Nitrobacter* on glycerol has been demonstrated (55), 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 (158), 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 (ACP) and CoA were identified. Classical pathways could not be confirmed for cobalamin (B12), thiamine, or pyridoxal (B6). 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* (*agilis*) possessed ubiquinone (2), which, if correct, would suggest that biosynthesis of this molecule may occur *via* a non-classical pathway.

Lipid and lipopolysaccaride (LPS) biosynthesis. Several studies have shown that the fatty acid composition of phospholipids in *N. winogradskyi* is almost exclusively vaccenic acid (18:1,11c) (5, 99). Two FabA encoding genes (nwi0034, nwi1578) were identified that carries 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 LPS including lipid A, core oligosaccharide, and an O-antigen structure. In addition, two paralogous genes (nwi2396, nwi0647) were identified that code for synthesis of N-acetyl neuraminic 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) (35), 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 conditions, 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 t-RNA appears to be through transamidation of an aspartyl-tRNA(113) *via* a class II aspartyl-tRNA synthetase (nwi1588, EC 6.1.1.12) and a glutamine-dependent Asp-tRNA^{Asn} amidotransferase encoded by *gatCAB* (nwi2003,2001,1997).

Storage compounds. Many cytoplasmic inclusions have been reported in *Nitrobacter* cells, including poly-β-hydroxybutyrate (PHB), polyphosphates, and glycogen (20, 179). *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, nwi1650) 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 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 (95). 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 electron donor (144). NirBD-mediated, nitrite-dependent ammonium formation could serve three different functions: nitrogen assimilation, nitrite detoxification, or NAD(P) regeneration (117, 144). Denitrifying cultures of *N. winogradskyi* have been reported to accumulate ammonium (55).

Because *N. winogradskyi* posseses genes for glutamine synthetase (GS: nwi1904), glutamate synthase (GOGAT: nwi2953-2954) and an assimilatory glutamate dehydrogenase (GDH: nwi2286), it has the potential to switch between GS-GOGAT and GDH 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 NOB will be exposed to higher levels of ammonium than of nitrite, which may trigger a shift to utilization of the GDH 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 (uridyl transferase) 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 (MFS), and Resistance-Nodulation-Cell Division (RND) families (Table 2.3). Single ABC type II, TRAP, and CPA-1 type transporters were also identified in addition to three multidrug SMR 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 MFS family 1 transporter (nwi3027) were annotated, the combination of which could enable carbohydrate uptake (123, 184). 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. The ABC type I transport systems for import of polar amino acids (His/Glu/Gln/Arg) plus two complete systems for import of branched amino acids were identified. A *potGHIF* homolog for import of polyamines (putrescine/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/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 10-30% of the total protein in *N. winogradskyi* cells is NXR (21, 163), 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* posseses 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, 28, 115). Similar 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, 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, 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, which share the same organization as the Pit system characterized in *S. meliloti* (6, 175), 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-nwi0512), were also annotated. The *pst* genes, which are generally expressed under phosphate starvation (129, 178), are also flanked by the phosphate two-component global regulator system, *phoB/R*. A gene

encoding an Opr-type phosphate porin (nwi2169) was identified, which may form a trimeric porin under phosphate limited conditions (69, 124).

Genes encoding a complete Sec system for transport of proteins to the membrane or periplasm were identified. A Tat system (nwi1777-1779), for export of folded proteins across the inner membrane was also annotated. Based on analysis by TatP (13) and PrediSi (http://www.predisi.de), approximately 580 proteins are potentially secreted by *N. winogradskyi*, 240 of these by the Tat pathway. Similar to 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 pili components is nearly identical to the pili operon previously described in *C. crescentus*, a stalk-forming, Alphaproteobacterium (146). 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 (79, 93, 141). 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, nwi0891), thioredoxin reductase (*ahpF*, COG 0492: nwi2453), other peroxiredoxins (COG0678: nwi2686), glutathione peroxidase (COG 0386; nwi2639), glutathione reductase (COG1249: nwi1223) as well as antioxidant proteins with peroxidase activity (EC:1.11.1.7; nwi0432, 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* (33). 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 multi-copper oxidases, which have been implicated in NO-reduction (74), it lacks a gene for cytochrome P460, which has a hydroxylamine-detoxification role (16).

Also as noted above, the *nirK/aniA* 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 (10), 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 (10, 169), 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-, 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, DnaK) were observed. *N. winogradskyi* also seems to be equipped for protection from organic solvents (OstA: nwi1679), arsenic (ArsBC: nwi3124-3125) and cyanate (CynS: nwi1302; CynX: nwi1437) toxicity, osmotic stresses (Glycine betaine system), and to regulate its pH *via* a pH-adaptive potassium efflux system (PhaA-F: nwi2654-2658). Effective stress response also involves regulated degradative capacity and the *N. winogradskyi* contains several genes encoding ATP-dependent proteases including Lon and Clp. Genes for DNA repair systems such as RecA, RecB, RecG, RecF, were also located. DNA-specific exonuclease RecJ and exinuclease 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, hence its genome should contain structural and regulatory genes necessary for flagella synthesis and function in response to environmental cues and challenges. The complement of operons needed for chemotaxis and flagellum biosynthesis is complete when compared with available information from other bacteria (29, 44). 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 (MCPs) (29, 105, 148), the *N. winogradskyi* genome encodes three of them: 1.) Tsr (nwi2996), which directly senses serine, alanine, glycine, aminoisobutyrate, 2.) Tar (nwi0530), which senses aspartate, glutamate directly and is responsive to cobalt and nickel, and 3.) 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 MCP complement similar to what was found in *N. europaea*, and its chemotactic activity is likely regulated through the Che protein phospho-relay (CheWAY, CheBR) (33). It needs to be experimentally verified whether the *N. winogradskyi* MCPs respond to the same signals as found in Gammoaproteobacteria such as *E. coli* and *P. 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/derepression of operons and indirectly through control of the FliA protein, an alternative sigma factor (sigma-28). A *fliA* homolog, anti-sigma *flgM*/*fliT* (105), 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. 2.3), 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 C6 molecules is consistent with its genome sequence, which lacks genes for complete Embden Myerhof and Enter Doudoroff pathways, and does not encode 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 C2 and C3 molecules (43, 147). However, the *N. winogradskyi* genome sequence does not 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 TCA 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. Its unknown if segregation of the putative *coxL/cutL* from *cox/cutSM* may affect 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 (111, 112). 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* USDA 110 (103). However, the *B. japonicum* USDA 110 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* USDA 110, and awaits experimental analysis.

Given the challenges faced by *N. winogradskyi* and other NOB 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 (55) . PHB storage has been studied widely in bacteria, including the genus *Rhizobium,* where it can accumulate to high levels under free living and symbiotic conditions (81). 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 (125) 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 TCA 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 (154) 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 (20, 43, 147, 155). Although *N. winogradskyi* can assimilate ammonium and nitrate (95), 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 a genome has been fully sequenced. The *N. winogradskyi* genome (3.4Mb) is smaller than the other two members, *R. palustris* (5.4Mb) and *B. japonicum* (9.1Mb) 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 (that 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 NOB with other microbes with which they closely associates in the environment, particularly ammonia oxididizing bacteria. Nitrite rarely accumulates in soils, wastewater or activated sludge indicating that nitrite- and ammonia-oxidization are coupled in nature, and the NOB are frequently observed to be physically clustered with ammonia oxidizing bacteria. Outside of the *Bradyrhizobiaceae*, proteins annotated in *N. winogradskyi* were most frequently similar to orthologs (reciprocal best BLASTP hits) in *N. europaea*, suggesting exchange of genetic material. The function of many of these genes is 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, EPS synthesis, 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.

ACKNOWLEDGEMENTS

Sequencing was funded by the U.S. Department of Energy's Office of Biological and Environmental Research and carried out at the Joint Genome Institute. Additional funding was provided to S. R. Starkenburg by the Subsurface Biosphere Integrated Graduate Education and Research Traineeship (IGERT) grant 0114427-DGE at Oregon State University from the National Science Foundation's Division of Graduate Education. Finishing was completed primarily at the Lawrence Livermore National Laboratory. Computational annotation was carried out at the Oak Ridge National Laboratory. The authors thank E.O. Burton and M.C. Pelliterri for the preparation of *N. winogradskyi* genomic DNA. Special thanks to G.M. King, F.R. Tabita, and D.R. Söll for helpful comments and assistance.

TABLE 2.1. Summary of Complex Repetitive Sequences

Sequence Group	Size (bp)	Copy Number	% Identity
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-dependepent transport system (nwi2038-nwi2040; nwi0705-nwi0703)	2,117	2	98.3
IS elements:			
ISnw1 (two transposases)	1,511	23	>99.8
ISnw2 (two transposases)	1,512	11	>98
ISnw3 (two transposases)	1,050	18	>99.8
ISnw4 (one transposase)	1,016	10[a]	>99.8
ISnw5 (one transposase)	902	14	>99.3
ISnw6 (two transposases)	855	19	>98.8
ISnw7 (one transposase)	947	14[a]	>99.6
ISnw8 (two transposases)	1,321	2	100

[a] Two of indicated copies only contain partial repeats of the IS element.

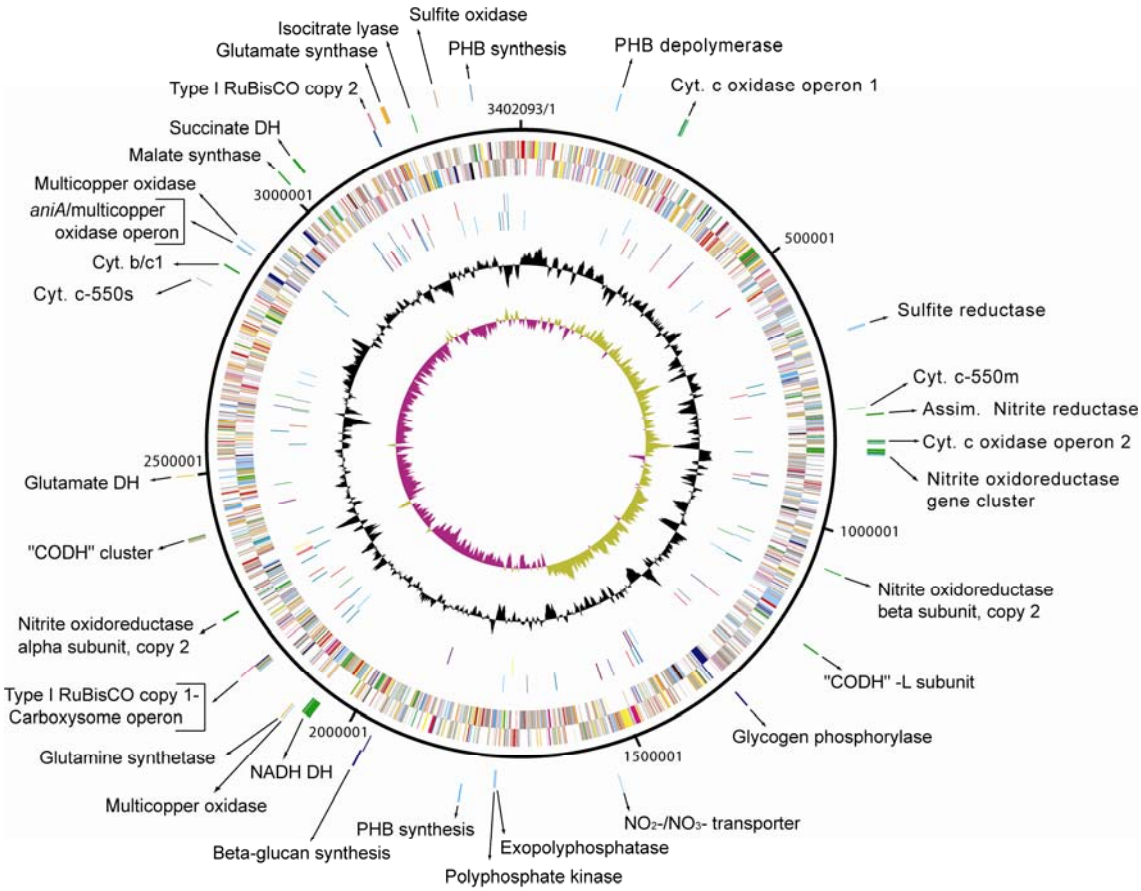
TABLE 2.2. Regulatory and Signaling Proteins

Number	Category
136	Transcription/Elongation/Termination Factors
17	Sigma Factors (1-σ_{32}, $_{70}$ and $_{54}$; 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 NON-chemotaxis signal transduction)
28	Signal Transduction Histidine Kinases (STHK)
14	Cyclic Nucleotide Signal Transduction
4	PII-like Nitrogen Regulatory Proteins
45	Miscellaneous (13 CheY, 12 putative FecR, 7 PAS/PAC domain)

TABLE 3.3. Summary of Transporters

Transporter Family[a]	Count	Function(s)
ABC Type I/II	18/1	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	C4 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]Transporter family abbreviations are as follows: 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



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		

Figure 2.1. The chromosome of *Nitrobacter winogradskyi* Nb-255 (ATCC 25391). The outer two circles indicate the location 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; the eighth circle, GC skew.

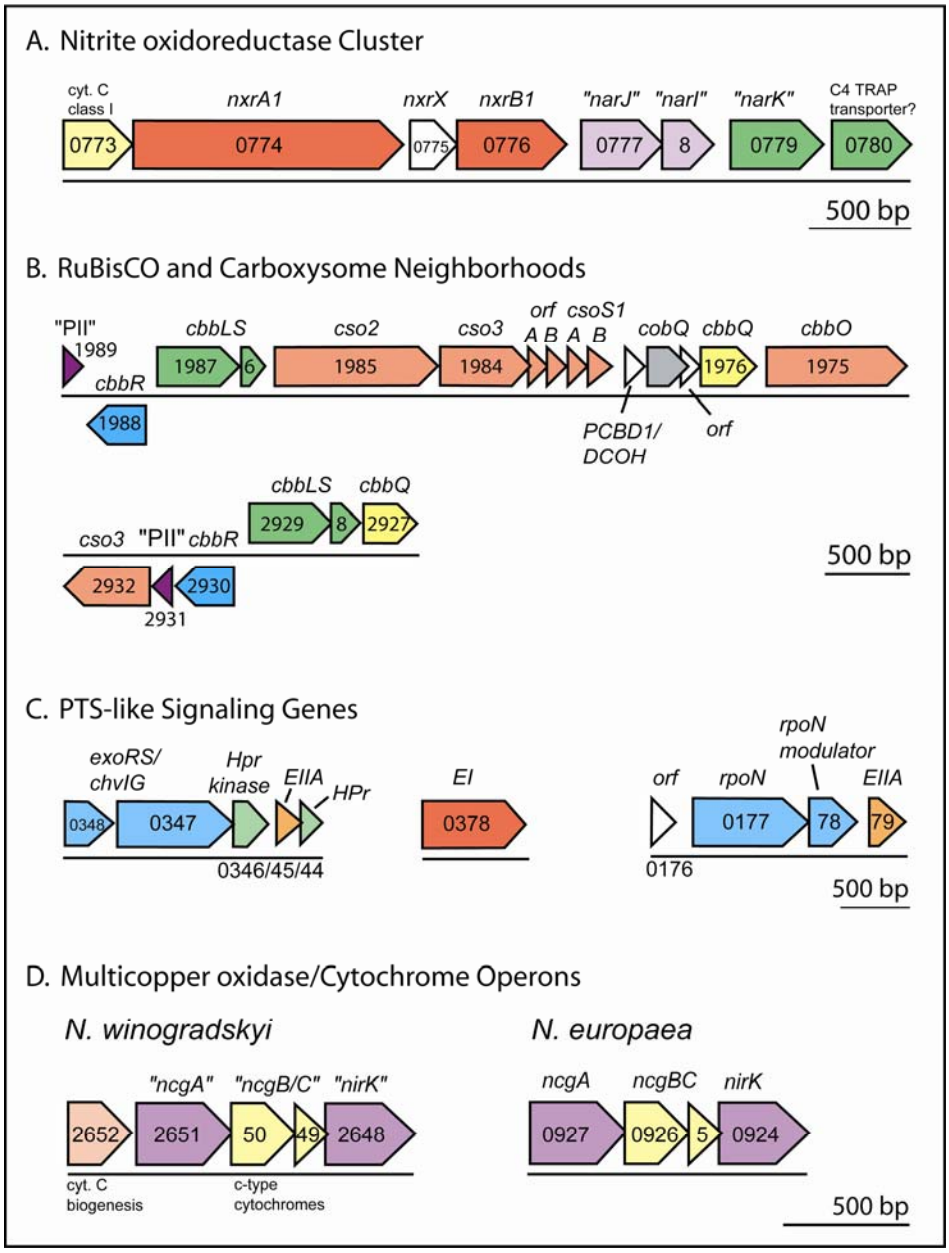


Figure 2.2. Organization of *N. winogradskyi* gene clusters. Each arrow represents one gene. The *N. winogradskyi* loci numbers are indicated within the arrows and putative gene names are above each arrow. In panel D., a gene cluster from *N. europaea* (ATCC 19718) is shown for comparison.

Figure 2.3 Legend. *N. winogradskyi* Cell Diagram. Cellular processes depicted are based on putative gene annotations. Roman numerals refer to electron transport components enzyme complex I (NADH-ubiquinone reductase), complex II (succinate dehydrogenase), complex II (ubiquinol-cytochrome *c* reductase), and complex (cytochrome *c* oxidase).

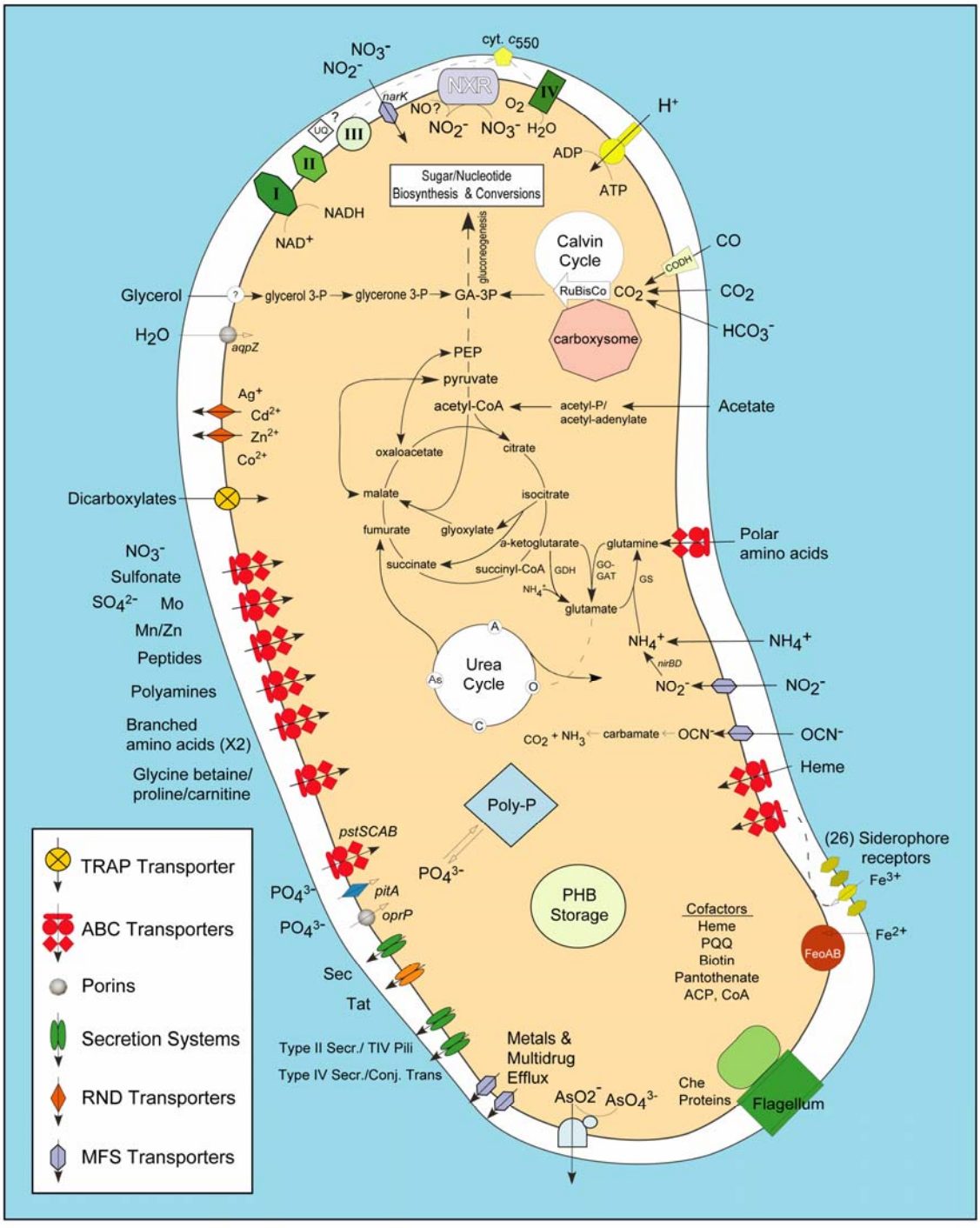


Figure 2.3. *N. winogradskyi* Cell Diagram

Chapter 3

The complete genome sequence of *Nitrobacter hamburgensis* X14 and a comparative genomic analysis of species within the Genus *Nitrobacter*.

Shawn R. Starkenburg, Frank W. Larimer, Lisa Y. Stein, Martin G. Klotz, Patrick S. G. Chain, Luis A. Sayavedra-Soto, Mira E. Gentry, Daniel J. Arp, Bess Ward, and Peter J. Bottomley

Submitted for Publication in:
Applied and Environmental Microbiology
American Society for Microbiology,
October 2007

ABSTRACT

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.* NB311A, respectively. Most of the plasmid-born 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.* NB311A. The *N. hamburgensis* chromosome also harbors many unique genes including heme-copper oxidases, cytochromes 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 (122 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. The subcore genes have diverged significantly from, or have origins outside, the Alphaproteobacteria lineage and may indicate some of the unique genetic requirements for nitrite oxidation in *Nitrobacter*.

INTRODUCTION

Nitrification is a two step process by which ammonia is converted to nitrate (NO_3^-) via nitrite (NO_2^-). 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 (N_2O), rank as two of the most potent greenhouse gases (77, 152, 183). *Nitrobacter hamburgensis* X14 and other nitrite oxidizing bacteria (NOB) participate in the process of nitrification by converting NO_2^-, the end product of ammonia oxidation, into NO_3^- according to the following reaction; $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$. NO_2^- 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 (54).

As facultative chemolithoautotrophs, members of the genus *Nitrobacter* oxidize NO_2^- and fix carbon dioxide (CO_2) 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 NO_2^- (20, 147). The effectiveness of organotrophy varies among members of *Nitrobacter g*enus. For example, *N. winogradskyi* can grow on acetate although optimal growth is achieved on NO_2^-, while *N. hamburgensis* X14 appears to grow best in media containing both NO_2^- and organic carbon (23, 24, 155). The genetic and full enzymatic basis for these differing phenotypes has not been explored.

Nitrobacter is a member of the Family *Bradyrhizobiacaea* and is closely related (97-98% identity of 16s rRNA) to the *Bradyrhizobium* and *Rhodopseudomonas* genera. *Rhodopseudomonas palustris* can grow either chemotrophically or phototropically and is arguably one of the most metabolically versatile bacterium known (96). The plant symbiont *Bradyrhizobium japonicum* has one of the largest prokaryote genomes, however, neither of these close *Nitrobacter* relatives has the ability to use NO_2^- as an energy source. Availability of the genome sequences of 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 led to a better understanding of the physiological requirements for NO_2^- oxidation.

In this study, an analysis of the genome sequence from *N. hamburgensis* X14 and a comparative analysis of the genomes within the genus *Nitrobacter*, is presented. Included in the *Nitrobacter* comparative analysis are the genomes of *Nitrobacter winogradskyi* NB255 and *Nitrobacter sp*. NB311A (NB311A). Curiously, NB311A was isolated near the west coast of central Africa, grows in seawater (B. Ward, unpublished results), yet its 16s rRNA gene sequence is 100% identical to the *soil* isolate, *N. winogradskyi*. *N. winogradskyi* was previously sequenced and analyzed (151) and a draft sequence of *Nitrobacter sp*. NB311A (NB311A) was recently made available. Additionally, five complete genomes of *Rhodopseudomonas palustris* and three from *Bradyrhizobium japonicum*, which are also available in public databases, were included in the analysis. Placed in the context of these *Bradyrhizobiaceae* members, a comparative analysis of *N. hamburgensis* with the other two *Nitrobacter* genomes provides the genetic framework for determining the metabolic variations and similarities that exist within *Nitrobacter*, and exploring the genomic basis of lithoautotrophy versus trophic flexibility among closely related bacteria.

MATERIALS AND METHODS

Construction, sequencing, and assembly. Genomic DNA from *N. hamburgensis* X14 was isolated, purified, and sheared into 3 kb, 8 kb and 40 kb fragments and ligated into 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 (33, 52) (see also JGI website http://www.jgi.doe.gov/).

Processing of sequence traces, base calling and assessment of data quality were performed with PHRED and PHRAP, respectively (50, 51). After quality control of the 66,403 total initial reads of 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 (66), and linked into 18 larger scaffolds using paired-end sequence information. Gaps between linked contigs were closed by either walking on gap-spanning clones or with PCR products generated from genomic DNA while physical (or un-captured) 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 (33).

Genome analysis and annotation. Automated gene modeling for *N. hamburgensis* was completed by combining results from Critica, Generation, and Glimmer modeling packages, and comparing the translations to GenBank's nonredundant (NR) database using basic local alignment search tool for proteins (BLASTP). The protein set was also searched against KEGG, InterPro, TIGRFams, PROSITE, EcoCYC, MetaCYC, and Clusters of Orthologous Groups of protein (COGs) databases to further assess function.

NB311A was isolated by Stan Watson in 1968 from surface waters in the tropical Eastern Atlantic Ocean of 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.venterinstitute.org/moore/) and

re-annotated using the same criteria as *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 11704 sequences of the CDS translations from *Nitrobacter hamburgensis* X14 (CP000319), *Nitrobacter winogradskyi* NB255 (CP000114), and *Nitrobacter sp.* NB311A (AAMY01000000) was assembled. An all-vs.-all analysis was performed using BLASTP (BLASTALL 2.2.13) using seg, with an *E*-value threshold of $1x10^{-5}$. 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 dataset displayed an average *E*-value = 1×10^{-113}, and an average percent identity = 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 species. The core sequences were extracted into a separate dataset and was subjected to BLASTP searches ($1e^{-10}$ and $1e^{-20}$ cutoffs) against a database consisting of *Bradyrhizobium japonicum* (BA000040, CP000494, CU234118) and *Rhodopseudomonas palustris* (BX571963, CP000250, CP000283, CP000301, CP000463) sequences (46111 peptides). Core sequences without a match ($\geq 1e^{-10}$) were designated the *Nitrobacter*-specific subcore. Manual searches for conserved gene clusters added seven peptides to the subcore using the following criteria; 1) the peptides were present in the $1e^{-20}$ subcore list, 2) were adjacent to peptide(s) in the $1e^{-10}$ database, and 3) the peptide identity was <50% similar to .peptides in the *R. palustris/B. japonicum* database.

Nucleotide sequence accession number. The sequence and annotation of the complete *N. hamburgensis* X14 chromosome is available at GenBank/EMBL/DDBJ

using accession number CP000319. The *N. hamburgensis* plasmids, pPB13, pPB12, pPB11, are available as plasmid 1, plasmid 2, and plasmid 3, using accession numbers CP000320, CP000321, 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, G+C=61.7%), pPB12 (188,318 bp, G+C=61.2%), and pPB13 (294,829 bp, G+C=60.4%). Based on BLAST analysis, *N. hamburgensis* shares more total CDSs with NB311A (1434) 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 NB311A than *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 the other two *Nitrobacter* species, containing ~1.6 and 0.9 Mbp more genetic material than *N. winogradskyi* and NB311A, respectively (Table 3.1). Several features of the *N. hamburgensis* genome account for its relatively large size. First, a disproportionately large number of pseudogenes and paralogs were identified, which account for 20% of the genome. 349 CDSs (~ 8% of the sequence space) were identified as pseudogenes in *N. hamburgensis* during automated annotation compared to 21 pseudogenes in *N. winogradskyi*. *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.* Secondly, the majority of the genes encoded on the *N. hamburgensis* plasmids do not have orthologs in the *N. winogradskyi* or NB311A genomes (Fig. 3.1). 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” (genes or genomic islands not found in the other *Nitrobacter* genome sequences) genomic islands, many of which appear to have been acquired by

conjugation or transduction, also help account for the genomic variation between *N. hamburgensis* and *N. winogradsky*. For example, a 240 kb island (Nham_3756-4008) appears to have originated from plasmid or phage sources, since it harbors putative plasmid replication initiator proteins (Nham_3835, Nham_3863), a partitioning protein (Nham_3861), as well as phage integrase and phage-related proteins (Nham_3842, Nham_4008). Similarly, a portion of a 4.2 kb phage element (rep28, Supplemental Table 3.5) is found replicated on the *N. hamburgensis* chromosome and contains a phage-related methylase, an uncharacterized phage protein, and two hypothetical proteins only found in *N. hamburgensis*. These and other similar elements lie within large chromosomal regions (Nham_0784-0835, Nham_0837-0943, Nham_1147-1186) that are not present in the *N. winogradskyi* genome and have clearly integrated into the *N. hamburgensis* genome since delineation from a common ancestor. Based on genome sequence data in REBASE (132), *N. hamburgensis* and *N. winogradskyi* also encode an above average quantity of restriction modification systems (RM) (*N. hamburgensis*: 11, 2.39 RM genes per Mbp; *N. winogradskyi*: 9, 2.64 RM genes per Mbp); the majority of which are Type-II RM systems. Classically speaking, these RM systems should provide defense against genome contamination by phage or other foreign DNA. On the other hand, *N. hamburgensis* seems to have been a successful target of phages at some point in its evolutionary history. Recent evidence indicates that Type II-RM complexes function as "selfish" mobile genetic elements and can promote homologous recombination in the host bacteria (as a defense mechanism), resulting in more genomic re-arrangements and diversity (89, 90, 119). 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.

Inter-Species 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. 3.2). The *Nitrobacter* composite or "core genome" consists of 2179 conserved gene types (excluding paralogs/gene duplications). The majority (86%) of the CDSs 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 (151). In contrast, *N. hamburgensis* and NB311A collectively encode approximately 2801 genes not found in *N. winogradskyi* (approximately one-third of each genome; 1301 and 1198 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 (3881 vs. 3876) as *N. hamburgensis*.

Organization of unique genes by COG groups revealed a similar gene distribution pattern for all three species suggesting that many of these genes (based solely on COG groupings) appear to be functional analogs (S.R. Starkenburg, unpublished results). Nevertheless, manual analysis of genome-specific sequences revealed that each genome did contain unique genetic material (Table 3.2), 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 monoxygenase, two NO_3^-/sulfonate/bicarbonate ABC transporters, and synthesis genes for the pyrroloquinoline quinone (PQQ) cofactor.

Several unique gene clusters were identified in NB311A (Table 3.2), including some that may be indicative of adaptation to a marine lifestyle. NB311A uniquely possesses genes that encode a chloride channel (NB311A_05795), a Na^+/Ca^{2+} antiporter (NB311A_09276), and several cation-dependent ATPases. NB311A also harbors a four gene cluster (NB311A_1874-1879) which may code for synthesis of ectoine-like osmoprotectants. The putative NB311A ectoine synthesis peptides have 47, 57, 51, 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 thermo-protectants (62, 63, 173). All three genomes were found to encode a putative Na^+/H^+ antiporter (*nhaA*), which is required for *E. coli* to survive high salt stress (4, 142). Consequently, a preliminary investigation of salt tolerance of *N.*

hamburgensis and *N. winogradskyi* was completed.. *N. winogradskyi* grew well in standard growth medium amended with 450 mM NaCl. Growth was not observed with 650 mM NaCl (the approximately salinity of seawater), even though the cells continued to oxidize nitrite. *N. hamburgensis* was less salt resistant as growth was inhibited at >250 mM NaCl and NO_2^--oxidation was inhibited at ≥450 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(70, 94), 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 3.3). The largest plasmid, pPB13, was found to be biased towards 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 which (~2.5 kb) appears to have been duplicated within pPB11 and a larger (~9.8 kb) portion of this region has been duplicated in pPB13. pPB12 appears to be a functional hybrid of the other two plasmids, containing gene clusters for conjugation, energy and carbon metabolism, plus a suite of genes for heavy metal resistance including those for heavy metal efflux (Nham_4358-59/4404-05/4529-35), mercury (Nham_4416-21), and copper resistance (Nham_4364-65/4380/4382-85/4397-99/4422-24/4492). A putative arsenite oxidase and accessory genes were also located on pPB12 (Nham_4425-32) although it is unknown if 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 Mg^{2+} 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 (Figure 3.1). This "autotrophic island" encodes the large and small subunits of a type I ribulose-bisphosphate carboxylase (RuBisCO; Nham_4049-50), 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 are conserved in the chromosomes of *N. winogradskyi* and NB311A (Figure 3.1). This gene cluster was most likely acquired by the ancestor to all three *Nitrobacter* genomes via lateral gene transfer because 1) the GC content of this island is 4.6% and 3.4% higher than the GC averages of the plasmids and genome, respectively, and 2) the sequence identity and the organization of genes in the cluster is most similar to homologs found outside the Alphaproteobacteria.

Many examples of DNA exchange and duplication (beyond ISE transposition) have been identified between the plasmids and the chromosome (Supplemental Table 3.5). 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, non-paralogous copy of a Type I RuBisCO (see "C1 Metabolism" section 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-Nham_3754). Interestingly, this similarity does not extend to an upstream and divergently transcribed LysR-type regulator (Nham4044 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 (Nham4077-Nham4081, rep12) which includes an aconitase and a DNA-binding ferritin-like protein). This 6.7 kb gene cluster is > 99% identical to the chromosomally located gene cluster (Nham0912-Nham0916). pPB12 also carries two additional loci (Nham_4530-4359, Nham_4404-4405) that are similar to chromosomal

locus Nham_1848-54 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 half and one third of the signaling capacity in *R. palustris* and *B. japonicum*, respectively. In contrast, *N. hamburgensis* and NB311A contain about half the number of *fecI*-like extracytoplasmic transcription factors (ECF, a subfamily of σ^{70}) when compared to *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 void of *fecR* homologs, 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 *intra*-cellular iron levels instead of responding to *extra*-cellular iron concentrations via FecIR.

In comparison to *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 (134). The *Nitrobacter* genomes contained a similar number of genes encoding PAS/PAC-domain proteins as *B. japonicum*, which often function as redox sensors (193). Because *Nitrobacter* can grow both aerobically and anaerobically (1, 54, 55), 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 NO_3^- respiration.

Histidine protein kinases (HPK) and response regulator proteins (RR) 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) as well as singletons (26 unpaired HPK genes, 24 unpaired RR). Some of the genes encoding chemotaxis components were grouped with HPK 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. Use of reduced sulfur as an energy source has not been reported in *Nitrobacter*, but *R. palustris* can grow photoautotrophically using thiosulfate as an energy source (133, 171). Intriguingly, *N. hamburgensis* has a small operon containing several genes associated with dissimilatory sulfur oxidation, *soxXYZA_B* (Nham_3671-3676). The genes in this cluster encode three of the four main protein complexes which catalyze the oxidation of reduced sulfur; SoxYZ, SoxAX, and SoxB (57, 58, 130, 157). The fourth protein complex SoxCD, which completes the oxidation of thiosulfate by oxidizing sulfur to sulfate (58), 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 is most similar to 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/dehydrogense (47). Indeed, two genes annotated as encoding subunits of a sulfite-oxidase (Nham_1093-94), 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 (140). 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. NO_2^- dependent lithotrophic growth in *Nitrobacter* is catalyzed by a reversible NO_2^- oxidoreductase (NXR). NXR is a

heterodimer containing an alpha (NxrA) and beta subunit (NxrB) and is evolutionarily related to the Nar-type dissimilatory nitrate reductases (87). Similar to *N. winogradskyi*, multiple copies of *nxrA* (n=3) and *nxrB* (n=2) are encoded in the *N. hamburgensis* genome but only one central gene cluster (Nham_3443-3451) encodes the putative accessory proteins of NXR. In addition to *nxrA* and *nxrB*, several genes in this cluster are conserved in all three *Nitrobacter* genomes including homologs to nitrate reductase accessory proteins NarJI (Nham_3446-7), a peptidyl prolyl cis-trans isomersase (*nxrX*, Nham_3448), a cytochrome *c* (Nham_3450), and putative proteins involved in the transport of NO_2^- and/or NO_3^- (*narK*, Nham_3444; TDT-family of transporters, Nham_3443).

Some *Nitrobacter* species have been shown to grow anaerobically using NO_3^- as a terminal electron acceptor when coupled to the oxidation of simple organic compounds (1, 55) and the terminal product of denitrification in *Nitrobacter* is reported to be N_2O (54, 55). As was the case in *N. winogradskyi*, additional homologs of nitrate reductases 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 NO_3^- to NO under anaerobic conditions. Each genome also contains a putative FMN-dependent nitroreductase which, if functional, could be involved in detoxification or respiration. In contrast, a gene cluster (Nham_2710-2710) encoding a nitric oxide reductase homologous to sNOR in the heme-copper oxidase superfamily was found only in *N. hamburgensis*, but not the other two *Nitrobacter* genomes (31). Similarly, a cytochrome P460 (*cytL*, Nham_2497) was only found in *N. hamburgensis* whose translated product contains an infrequently used heme-coordination motif, CGxxCH. This motif is found only in one of the two encoded cytochromes P460 (BAC50449) in the *B. japonicum* genome and in a cytochrome P460 protein from *Acidobacterium* (48). Cytochrome P460 has been shown to oxidize both hydroxylamine and NO in *Nitrosomonas europaea* (49, 74, 122). A NO detoxification mechanism via oxidation would be advantageous for *Nitrobacter*, because instead of forming N_2O (via sNOR) or ammonia (via NAD(P)H- siroheme nitrite reductase, Nham_2963-65), cytochrome

P460 could recycle NO back to NO_2^-. The physiological functions of *nirK* and cytochrome P460 in nitrogen oxide metabolism of *Nitrobacter* await 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 NO_2^-. Assimilatory nitrate reductases were not found in any *Nitrobacter* genome. In contrast to *N. winogradskyi*, which lacks an ammonia permease or urea catabolic genes (151), 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 AOB (92); 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 provide the means to regulate its internal pH or provide associated AOB with ammonia and CO_2.

C-1 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 (70). 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_4049-50; Nham_4332-4333). The third set of RuBisCO-encoding genes (Nham_3750-51) found on the chromosome is identical to the RuBisCO genes Nham_4049-50 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 (97). These particular PII homologs may be serving a regulatory role in carbon fixation, or alternately, 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 NO_2^-.

Most strikingly, the *N. hamburgensis* genome contains 4 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_2601-08) has high similarity and gene synteny to those identified in the *N. winogradskyi* (151), *Nitrobacter* sp. 311A, *Bradyrhizobium japonicum* USDA 110, and *Rhodopseudomonas palustris* CGA009 genomes (Table 3.3). This unusual Mo-CODH gene cluster lacks a CDS for *coxL*/*cutL*, the large subunit of Mo-CODH (151). 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 (86). 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 (103) albeit at a very slow rate. *B. japonicum* USDA 110 can also oxidize CO at the expense of NO_3^- reduction, but without growth, under anaerobic conditions (85). 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 carboxydotrophy. Whether any *Nitrobacter* spp. 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 from the *Nitrobacter* spp. genomes. In contrast to *N. winogradskyi*, *N. hamburgensi*s contains several genes encoding cytochrome b_{561}, an important electron transfer component in aerobic carboxidotrophic bacteria (111, 112). 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-

28/3132-33) and two in the reverse (Nham_3129-31/3134-35) orientation, that resemble formate dehydrogenase (FDH), the TorD chaperone, and enzymes for biosynthesis of the molybdopterin co-factor. Strains of *N. winogradskyi* have been shown to oxidize formate (106, 172), although the activity was thought to be catalyzed by NXR (38). Similar three-subunit metalloenzyme FDH have been shown to catalyze the anaerobic oxidation of formate, which is produced from pyruvate, to CO_2 (82). Several *Nitrobacter* spp. including *N. hamburgensis* are capable of both aerobic and anaerobic growth on pyruvate with O_2 or NO_3^- as the electron acceptor, respectively (22). 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 (20, 21, 147) although growth on hexose sugars or aromatic compounds has not been reported in 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 (151). 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 oxidoreductases (Nham_4194, Nham_4244), and a unique gene cluster with homology to an ABC-type general sugar transporter (Nham1206-9), which together provide the *in silico* potential to transport and initiate oxidation of hexoses.

Many other genes were identified which may further extend the organic carbon substrate range of *N. hamburgensis.* A gene cluster encoding the subunits of a putative glycolate oxidase and a cytochrome *c*550 (*glcDEF*; Nham_3202-5) were identified in all *Nitrobacter* genomes. Genetic and functional evidence indicate that a glyoxylate bypass of the TCA cycle operates in *Nitrobacter* and thus glyoxylate (the product of *glcDEF*) could serve as an additional carbon source. Three NAD-independent (FAD/FMN dependent) oxidoreductases (Nham_4010, Nham_3204, 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 reported in *R. palustris* (75). Limited metabolism of aromatic carbon compounds may also be possible given that a cluster of 19 genes was identified that encode the pathways for homogentisate and phenylacetate metabolism (Nham_0920-0938). 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 the other two *Nitrobacter* species. In *N. hamburgensis*, distinct respiratory chains and terminal oxidases have been suggested to function because different b- and *c*-type cytochromes are present during autotrophic growth and heterotrophic growth, respectively (23, 88). In addition to the aa_3-type cytochrome *c* oxidase discussed below, four b-type cytochrome genes, plus a plasmid encoded cytochrome bd-ubiquinol oxidase 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* (88). *N. hamburgensis* contains three four-gene clusters encoding aa_3-type cytochrome *c* oxidases (one of which is plasmid-borne) and another cluster (Nham_3452-54) encoding three subunits. The two cytochrome *c* oxidase gene clusters found on the chromosome (Nham_0255-61/3457-63) were 100% identical at the protein sequence level, and homologous to two gene clusters described previously as the only ones encoding cytochrome *c* oxidases in *N. winogradskyi* (151). The plasmid-encoded cytochrome *c* oxidase gene cluster (pPB13; Nham_4177-80) contained genes for subunits I-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_4186-88) 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_2710-12), 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 (153). This gene cluster was up-regulated in a nitrite reductase deficient mutant of *N. europaea*, indicating involvement of sNOR in nitrosative stress response (36).

Nitrobacter Subcore Inventory. To gain further insight into NOB physiology, a final comparative analysis was conducted between the *Nitrobacter* genomes and all of the sequenced strains of *R. palustris* and *B. japonicum*. The fact that *R. palustris* and *B. japonicum* are metabolically versatile, do not use NO_2^- as an energy source, and are close evolutionary relatives to *Nitrobacter*, provides a unique opportunity to explore the genetic foundation of being a NO_2^- oxidizer. Using the *Nitrobacter* core genome as the query database, all core genes with high homology to 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 122 gene types uniquely conserved in each *Nitrobacter* genome. Within the 122 gene *Nitrobacter*-"subcore", 79 genes encoded hypothetical proteins, 6 were phage or transposase-related, and 37 were given functional assignments.

Among the functionally annotated genes (Table 3.4), two gene clusters appear to encode polysaccharide synthesis proteins (Nwin_548-552, Nwi_0645-650), specifically glycosyl transferases, hydrolases, and sialic-acid based homopolysacchride formation genes, some of which have little homology to any prokaryotic peptides. Approximately half of the subcore genes appear to be associated with NO_2^- metabolism, transport, and regulation including the gene cluster encoding the subunits of the NXR and the cytochromes and putative regulatory protein adjacent to *nirK*. In addition to the main NXR gene cluster which encodes the critical enzyme system for NO_2^- oxidation in the genus *Nitrobacter*, the scattered, lone copies of *nxrA* and *nxrB* were also included in the subcore inventory. How (or if) these additional *nxrA* and *nxrB* gene duplications function remain to be experimentally determined.

Strikingly, the *R. palustris* and/or *B. japonicum* genomes do contain homologs of several genes in the *Nitrobacter* subcore inventory, yet these genes are more 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 (31). 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 *Nitrosomonas eutropha* and the denitrifying *Thiobacillus denitrificans*, another Betaproteobacteria. Furthermore, *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 identity), a NO_2^--oxidizing Gammaproteobacterium, and a NarGH in the Deltaproteobacterium, *Geobacter metallireducens* (~59% protein identity). In sum, the subcore appears to encode a collection of genes which are not indicative of its evolutionary origins, but instead reflects the ecological niche (nitrification, denitrification) of *Nitrobacter* achieved through assimilation, modification, and expression of genes acquired from more distant bacterial lineages.

CONCLUSION

The genome sequence of *N. hamburgensis,* when compared with *N. winogradskyi* and NB311A, has helped verify and narrow the genetic basis of NO_2^- oxidation in this historical 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, *N. winogradskyi* appears to be undergoing a reductionistic strategy, is more organized, and may be restricting itself to chemolithoautotrophic metabolism. Future genetic comparisons between additional *Nitrobacter* strains and other lineages of NO_2^- oxidizers, such as *Nitrospira* and *Nitrococcus*, will further advance our understanding of lithotrophic metabolism and the role of NOB in nitrification.

ACKNOWLEDGEMENTS

Sequencing was funded by the U.S. Department of Energy's Office of Biological and Environmental Research and carried out at the Joint Genome Institute. Additional funding was provided to S. R. Starkenburg by the Subsurface Biosphere Integrative Graduate Education and Research Traineeship (IGERT) grant 0114427-DGE at Oregon State University from the National Science Foundation's Division of Graduate Education. M.G. Klotz and M.E. Gentry were supported, in part, by incentive funds provided by the University of Louisville-EVPR office and the National Science Foundation (EF-0412129). Genome finishing was completed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory, University of California under contract W-7405-Eng-48. Computational annotation was carried out at Oak Ridge National Laboratory. Special thanks to John Waterbury, the Moore Foundation, and The Venter Institute for submitting, funding, and sequencing the draft genome of NB311A.

Figure 3.1. Legend. The genome of *Nitrobacter hamburgensis* X14 (ATCC 25391). The outer circle depicts the location 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 2nd circle. The third and fourth circles depict predicted protein-encoding and structural-RNA genes in *N. hamburgensis* on the plus and minus strand, 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* are depicted in circle five (red). The 6th and 7th circles indicate the location of all annotated pseudogenes (green) and paralogs (orange), respectively. The 8th circle indicates GC bias; the 9th circle, GC skew. The highlighted region of pPB13 depicts the location of key gene clusters; autotrophic island (peach), RuBisCO and pentose phosphate pathway genes (green), and the cytochrome oxidases (aa_3, bd-ubiquinol types) (blue).

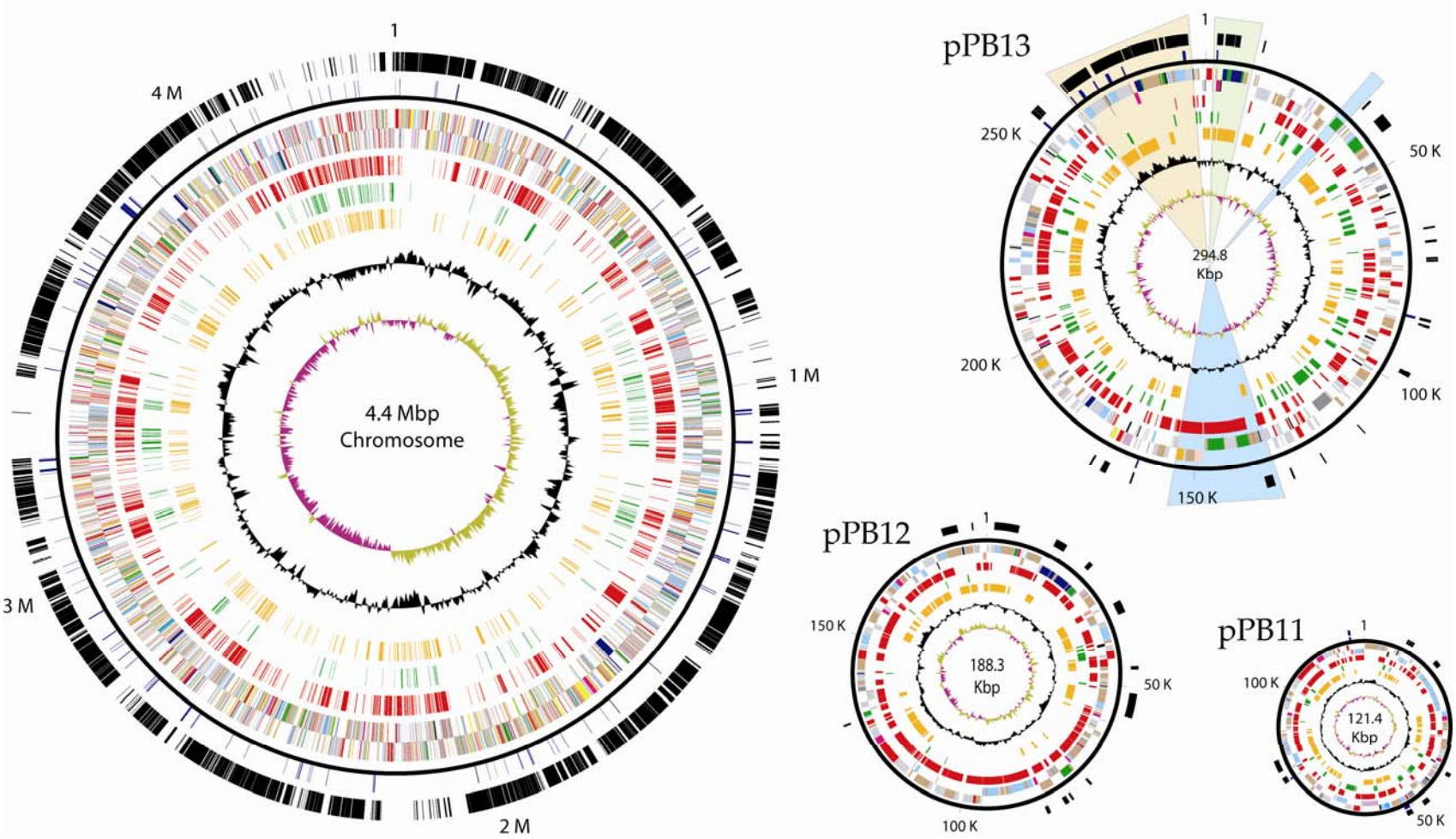


Figure 3.1. The genome of *Nitrobacter hamburgensis* X14

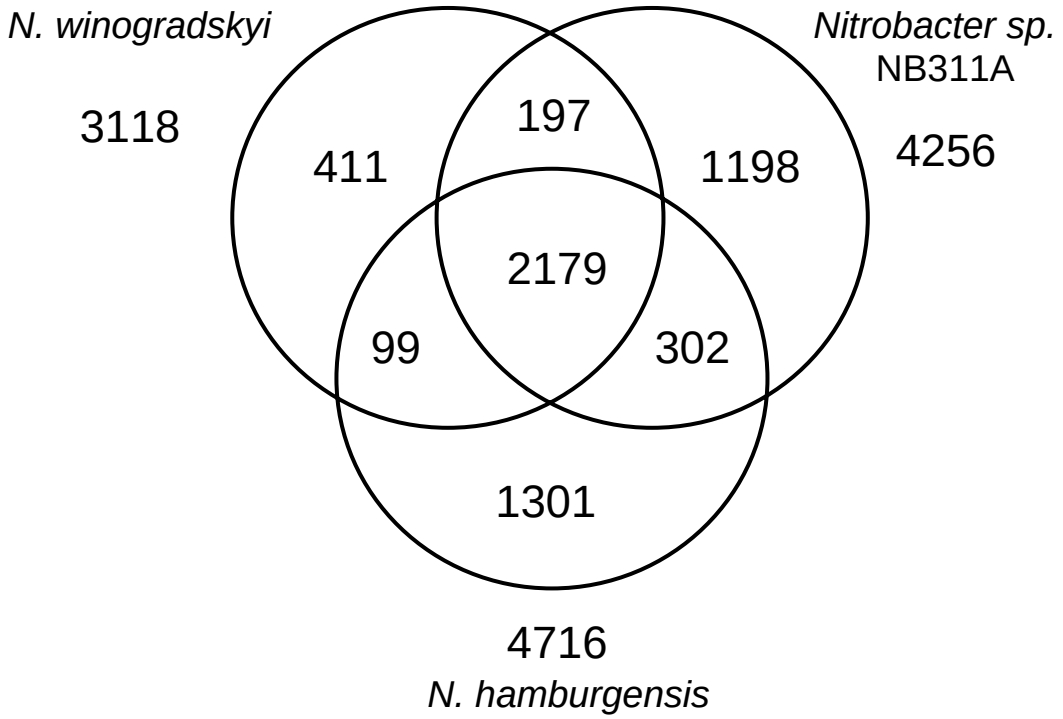


Figure 3.2. Global Gene Conservation in *Nitrobacter*. Each circle represents the total number of gene types in each genome. Overlapping regions depict the number of gene types shared between the respective genomes. The numbers outside the circles indicate the total number of genes identified in each genome, including paralogs/gene duplications.

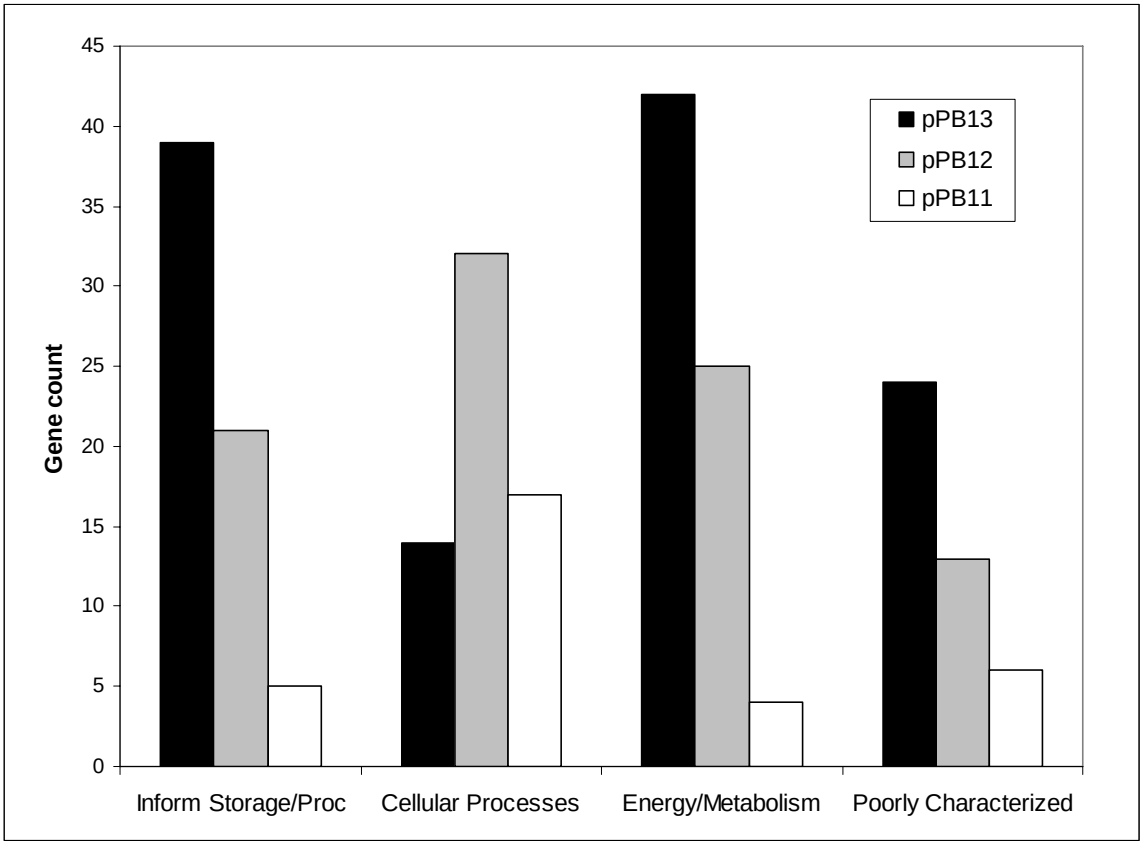


Figure 3.3. Functional Distribution of *N. hamburgensis* Plasmid Genes based on COG assignments. Genes in COG groups J, K, L are included in Information Storage and Processing. Genes in COG groups D,V,T,M,N,U,O are included in Cellular Processes. Genes in COG groups C,G,E,F,H,I, P, and Q are included 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.

TABLE 3.1. General Genome Characteristics

	N. hamburgensis **X14**	***N. winogradskyi*** **NB255**	***Nitrobacter sp.*** **NB311A Draft**
Origin	Soil	Soil	Marine
16s rRNA gene identity*	98%	100%	100%
Chromosome bases	4406967	3402093	~4105362
GC %	61.61%	62.05%	62%
Total genes	4716	3118	4256
Genes without predicted function	1848	993	1461
Pseudogenes	348	21	N.D.[+]
Paralogs	634	283	478
Paralog groups	251	74	143
Plasmids	3	0	N.D.
pPB13	294829 bp		
pPB12	188318 bp		
pPB11	121408 bp		

*16s rRNA identity relative to *N. winogradskyi* Nb-255
[+]Not Determined

Table 3.2. Unique Genes and Putative Functional Biases in the Genus *Nitrobacter*.

N. winogradskyi	***N. hamburgensis***	***Nitrobacter Nb311A***
Transport	Transport	Transport
(2) NO_3^-/Sulfonate/CO_3^{2-}	Ammonia permease	TonB systems
Iron Uptake systems	K^+ Transport	Ca^{2+}/Na^{2+} antiporter
Nickel/Cobalt	Uncharacterized ABC transport components	Cl^- channel
PO_4^{3-} porin	Carbon Metabolism	Chromate
Uncharacterized ABC transporter components	Formate Dehydrogenase	Uncharacterized ABC transporter components Mg/Cobalt
	Carbon Monoxide DH-like	
Miscellaneous	L-Lactate DH	Sulfate permeases
Histidine biosynthesis	Malate DH, pyruvate-formate lyase	Co/Zn/Cd efflux
Multiple FecIR genes	Homogentisate/Phenylacetate degradation	Replication
PQQ biosynthesis	Energetics	DNA Replication/Repair
	Cyt c oxidase	DNA Polymerase IV, III
	Cyt bd ubiquinol oxidase	*minCDE* septum formation
	(4) Cytochome b_{561}	Miscellaneous
	Cytochrome P_{460}	Polysaccharide synthesis, export
	Flavoredoxin reductase	UspA Stress genes
	Sulfur oxidation genes (*soxXYZAB*)	Ectoine synthase
	Nitric oxide reductase (*sNOR*)	Cation ATPases
	Miscellaneous	Pyoverdine synthesis
	Type II Secretion	Hydroxymate siderophore syn. (IucC Family)
	Serine Proteases	
	Conjugal Transfer	
	Heavy Metal Resistance	
	Arsenite oxidase	

Table 3.3. CODH Gene Clusters in ***Nitrobacter, B. japonicum, and R. palustris***

Subunit	Nham Gene Clusters	% Protein Sequence Identity of Nearest Neighbors							
		N. winogradskyi		*Nitrobacter* sp. 311A		*Bradyrhizobium japonicum* 110		*Rhodopsedomonas palustris*	
CoxG	Nham_2608	Nwin_2206	77%	NB311A_18136	77%	bll5666	84%	RPA3804	81%
CoxS/CutS	Nham_2607	Nwin_2205	93%	NB311A_18141	91%	bll5665	91%	RPA3803	90%
CoxM/CutM	Nham_2606	Nwin_2204	83%	NB311A_18146	85%	bll5664	84%	RPA3802	84%
ATPase	Nham_2605	Nwin_2203	87%	NB311A_18151	89%	bll5663	87%	RPA3801	86%
CoxE	Nham_2604	Nwin_2202	80%	NB311A_18156	79%	bll5662	76%	RPA3800	79%
CoxF	Nham_2603	Nwin_2201	85%	NB311A_18161	86%	bll5661	89%	RPA3799	88%
CoxF	Nham_2602	Nwin_2200	86%	NB311A_18166	86%	bll5660	79%	RPA3798	80%
MobA-like	Nham_2601	Nwin_2199	89%	NB311A_18171	90%	bll5659	77%	RPA3797	76%
CoxL/CutL	Nham_1307	Nwin_1079	86%	NB311A_11547	87%	bll5914	78%	RPA3974	79%
CoxS/CutS	Nham_1039	N.P.		NB311A_01949	91%	blr5209	79%	N.P.	
CoxM/CutM	Nham_1040	N.P.		NB311A_01944	89%	blr5210	71%	N.P.	
CoxL/CutL	Nham_1041	N.P.		NB311A_01939	88%	blr5211	67%	N.P.	
CoxS/CutS	Nham_1453	N.P.		N.P.		blr0335	81%	RPA4666	82%
CoxL/CutL	Nham_1454	N.P.		N.P.		blr0336	84%	RPA4667	85%
CoxM/CutM	Nham_1455	N.P.		N.P.		blr0337	72%	RPA4668	74%
CoxL/CutL	Nham_1181[a]	N.P.		N.P.		N.P.		N.P.	
CoxS/CutS	Nham_1182	N.P.		N.P.		N.P.		N.P.	
CoxL/CutL	Nham_1183	N.P.		N.P.		N.P.		N.P.	

[a]Nearest neighbors for Nham_1181: CutL from *Magnetospirillum magnetotacticum* MS-1 (Magn03009301), 45%; Nham_1182: ferredoxin from *Burkholderia ambifaria* (BambDRAFT_3191), 68%; Nham_1183: cytochrome *c* from *Mesorhizobium* sp. BNC1 (MesoDRAFT_4421), 46%.

Table 3.4. *Nitrobacter* Subcore Genes**.**

Putative Function	Gene ID(s)		
Polysaccharide biosynthesis	***N. hamburgensis***	***N. winogradskyi***	**NB311A**
lipopolysaccharide biosynthesis protein	Nham_1054	Nwi_0538	NB311A03749
glycosyl transferase, family 2	Nham_1055	Nwi_0539	NB311A03744
hypothetical protein	Nham_3303	Nwi_0540	NB311A03739
capsule polysaccharide biosynthesis protein	Nham_1195	Nwi_0641	NB311A16262
acylneuraminate cytidylyltransferase	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 c, class I	Nham_3450	Nwi_0773	NB311A09084
nxrA1; nitrite oxidoreductase alpha subunit	Nham_3449	Nwi_0774	NB311A09079
nxrX; nitrite oxidoreductase, subunit X	Nham_3448	Nwi_0775	NB311A09069
nxrB1; nitrite oxidoreductase beta subunit	Nham_3447	Nwi_0776	NB311A09069
nxrD; nitrite oxidoreductase, delta subunit	Nham_3446	Nwi_0777	NB311A09054
nxrG; nitrite oxidoreductase, gamma subunit	Nham_3445	Nwi_0778	NB311A09049
narK; nitrite/nitrate major facilitator superfamily transporter	Nham_3444	Nwi_0779	NB311A09044
TDT-family transport protein	Nham_3443	Nwi_0780	NB311A09039
cytochrome c, class IC, (adjacent to *nirK)*	Nham_3282	Nwi_2649	NB311A15177
cytochrome c, class I	Nham_3283	Nwi_2650	NB311A15182
Cytochrome c biogenesis factor	Nham_3285	Nwi_2652	NB311A15192
nsrR; transcriptional regulator, BadM/Rrf2 family	Nham_3286	Nwi_2653	NB311A15197
Nitrite reductase (NAD(P)H) large subunit, NirD	Nham_2964	Nwi_0720	NB311A01969
nxrB homolog	Nham_3289	Nwi_0965	NB311A10815
nxrA homolog(s)	Nham_0951, 2961	Nwi_2068	NB311A17691
Regulatory Genes			
PII-like Regulatory Protein	Nham_4043, 4324, 4330	Nwi_1989, 2931	NB311A00865
two component 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, betasubunit	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 (76)			
Phage genes (6)			

Table 3.5. Summary of complex repetitive sequences in the *Nitrobacter hamburgensis* X14 genome

Chromosome (NC_007964)				
Sequence Group (Putative Function)	**Size (bp)**	**Copy No.***	**% Identity#**	**Representative Gene(s)**
Gene, operon and/or region:				
rep12 (Hypothetical protein; Ferritin and Dps; Aconitate hydratase 1; Glyoxalase/bleomycin resistance protein/dioxygenase; Twin-arginine translocation pathway signal)	6,737	1	99.9	Nham_0912-0916
rep18 (Inositol phophatase/fructose-1,6-bisphosphatase; phosphoribulokinase/uridine kinase; Fructose-bisphosphate aldolase, class II, Calvin cycle subtype; ribulose bisphosphate carboxlase, large chain; ribulose bisphospate carboxylase, small chain; AAA ATPase,central region)	6,084	1	99.5	Nham_3749-3754
rep4 (Outer membrane autotransporter barrel)	1,262	2	99.9	Nham_1341
rep7 (Cytochrome-c oxidase)	5903	2	99.5	Nham_0255-0261
rep23 (Hypothetcial; Hypothetical)	2,022	2	99.5	Nham_2406-2407
rep24 (peptidase U35, phage prohead HK97; phage major capsid protein, HK97; Hypothetical; HNH nuclease/ phage PHI-105 holin-like protein)	2,614	2	98	Nham_2023-2026
rep28 (DNA methylase N-4/N-6; Hypothetical; Phage uncharacterized protein-like)	4,207	3	99	Nham_0799-0803
IS elements:				
rep1 (transposase orfA IS5 family element; transposase IS4)	814	5	95.6	Nham_1594-1595
rep2 (transposase, IS4) - 1 gene marked as pseudogene	1,312	24	99.9	Nham_0013
rep3 (transposase IS116/IS110/IS902)	1,470	3	99.7	Nham_2272
rep5 (tISRso5, ISRS05-transposase protein) - **~64% similar to rep6**	1,132	9	96.8	Nham_0809
rep6 (putaitve transposase) - **~64% similar to rep5**	1,093	1.5	99	Nham_0809
rep8 (transposase IS116/IS110/IS902)	1,479	3	96.8	Nham_1378
rep9 (Integrase, catalytic region; IstB-like ATP-binding protein)	2,450	8	98.4	Nham_0318-0319
rep10 (transposase IS116/IS110/IS902)	1,379	14	99.5	Nham_0109
rep13 (transposase, IS4)	1,634	2	100	Nham_1626
rep20 (transposase, IS4)	1,077	0.5	90.1	Nham_3889
rep21 (transposase, mutator type)	1,288	5	99.5	Nham_0110
rep22 (Integrase, catalytic region)	2,035	7	90.5	Nham_3078-3080

Table 3.5. Summary of complex repetitive sequences in the *Nitrobacter hamburgensis* X14 genome (Continued)

rep26 (ISSpo6, transposase orf A; ISSpo6, transpoase orfB) -	1,048	5	98.6	Nham_1154-1156
rep27 (transposase, IS4)	857	2.5	100	Nham_2931
rep29 (transposase, mutator type)	1431	2	98.9	Nham_2785

pPB13 (NC_007615)

Sequence Group	Size (bp)	Copy No.	% Identity	Representative Gene(s)
Gene, operon and/or region:				
rep12 (Hypothetical protein; Ferritin and Dps; Aconitate hydratase 1; Glyoxalase/bleomycin resistance protein/dioxygenase; Twin-arginine translocation pathway signal)	6,737	1	100	Nham_4077-4081
rep15 (traB; Hyp.; Pseudo; Psuedo; Hyp.; Pseudo; Hyp.; Hyp.; Heat shock protein Hsp20; Heat shock protein Hsp20; Hyp.; pilT protein-like; Hyp.; Hyp.; transposase IS116/IS110/IS902)	8,563	1	93	Nham_4305-4316
rep17 (NADH/Ubiquinone/plastoquinone (complex I); Hypothetical; Hypothetical; Nitrogen regulatory protein P-II (GlnB, GlnK))	6,232	2.25	96.4	Nham_4321-4324
rep18 (Inositol phophatase/fructose-1,6-bisphosphatase; phosphoribulokinase/uridine kinase; Fructose-bisphosphate aldolase, class II, Calvin cycle subtype; ribulose bisphosphate carboxlase, large chain; ribulose bisphospate carboxylase, small chain; AAA ATPase,central region)	6,084	1	100	Nham_4046-4051
rep19 (transcriptional regulator, LysR family)	757	2	99.9	Nham_4044
IS elements:				
rep1 (transposase orfA IS5 family element; transposase IS4)	814	1	84.1	Nham_4216
rep5 (tISRso5, ISRS05-transposase protein)	1,132	2	100	Nham_4085
rep6 (putative transposase)	1,093	3	99.9	Nham_4122
rep8 (transposase ID116/IS110/IS902)	1,479	4	99.7	Nham_4132
rep14 (putative transposase)	1,109	2.5	97.5	Nham_4130
rep20 (transposase, IS4)	1,077	4	99.5	Nham_4041
rep21 (transpoase, mutator type)	1,288	0.5	81.9	Nham_4167
rep22 (Integrase, catalytic region)	2,035	0.5	82.7	Nham_4138
rep26 (ISSpo6, transposase orf A; ISSpo6, transpoase orfB)	1,048	0.5	98.8	Nham_4150
rep29 (transposase, mutator type)	1431	1	100	Nham_4058

Table 3.5. Summary of complex repetitive sequences in the *Nitrobacter hamburgensis* X14 genome (Continued)

pPB12 (NC_007960)				
Sequence Group	**Size (bp)**	**Copy No.**	**% Identity**	**Representative Gene(s)**
Gene, operon and/or region:				
rep16 (nucleoside phosphorylase; and methylase)	1,511	2	99.8	Nham_4355
IS elements:				
None				

pPB11 (NC_007961)				
Sequence Group	**Size (bp)**	**Copy No.**	**% Identity**	**Representative Gene(s)**
Gene, operon and/or region:				
rep15 (traB; Hyp.; Pseudo; Psuedo; Hyp.; Pseudo; Hyp.; Hyp.; Heat shock protein Hsp20; Heat shock protein Hsp20; Hyp.; pilT protein-like; Hyp.; Hyp.; transposase IS116/IS110/IS902)	8,563	1	100,	Nham_4542-4556
rep16 (nucleoside phosphorylase; and methylase)	855	1	86.6	Nham_4627
IS elements:				
rep5 (tISRso5, ISRS05-transposase protein)	1,132	0.5	88.8	Nham_4619
rep6 (putaitve transposase - but annotated as Feruloyl esterase)	1,093	1	99.7	Nham_4600
rep13 (transposase, IS4)	1,634	1	100	Nham_4589
rep14 (putative transposase)	1,109	2.5	99.8	Nham_4599
rep20 (transposase, IS4)	1,077	0.5	96.5	Nham_4595

*Partial copies of a given repeat were counted if the identity was >80%

[#]represents the average % identity of all copies of given repeat

Chapter 4

Mixotrophic and Heterotrophic Growth on D-lactate and the Effect of Nitrite on Carbon Assimilation in *Nitrobacter hamburgensis*

Shawn R. Starkenburg, Daniel J. Arp, and Peter J. Bottomley

Prepared for Publication in:

Microbiology

Society for General Microbiology

ABSTRACT

Nitrobacter hamburgensis X14 is a facultative lithoautotroph that conserves energy from the oxidation of nitrite (NO_2^-) and fixes carbon dioxide (CO_2) as its sole source of carbon. The recent sequencing of the *N. hamburgensis* X14 genome catalyzed a re-examination of its mixotrophic and heterotrophic potential as three FAD-dependent oxidases were identified that may function to oxidize lactate, providing energy and carbon to the cell. The response of *N. hamburgensis* to D- and L-lactate in the presence (mixotrophy) and absence (heterotrophy) of NO_2^- was examined. Although L-lactate did not support heterotrophic growth or stimulate mixotrophic growth, D-lactate enhanced the growth rate and yield of *N. hamburgensis* in the presence of NO_2^- and served as a sole energy and carbon source in the absence of NO_2^-. Lithoautotrophically grown cells readily consumed D-lactate suggesting that the lactate metabolic pathway is constitutively expressed. In mixotrophically grown cells, D-lactate did not affect the rate of NO_2^- consumption compared to cells grown lithoautotrophically although NO_2^- slightly decreased the rate of lactate consumption. Nevertheless, a physiological adaptation to lactate occurred as D-lactate grown cells consumed and assimilated lactate at a faster rate than NO_2^- grown cells and the D-lactate-dependent O_2 uptake rate was significantly greater in cells grown heterotrophically or mixotrophically than in cells grown lithoautotrophically. Despite the fact that D-lactate stimulated growth in the presence of NO_2^-, D-lactate could not substitute for CO_2 as the sole carbon source (lithoheterotrophy).

INTRODUCTION

Nitrification is a two step, biologically mediated process through which ammonia is converted into nitrate (NO_3^-). Nitrite oxidizing bacteria (NOB) participate in the second step of nitrification by converting nitrite (NO_2^-) into NO_3^-. NOB use NO_2^- as their primary energy source to generate reductant (NADH and $FADH_2$), assimilate carbon dioxide (CO_2), and drive oxidative phophorylation. NO_2^- is a relatively poor energy substrate due to the positive midpoint potential of the NO_3^-/NO_2^- couple ($E_o' = +$ 420 mV). Since it is estimated that 85-115 moles of NO_2^- are required to fix one mole of CO_2 (21, 23), it is not surprising that some NOB can use organic carbon (C) as a carbon and energy source to reduce the burden of relying solely on NO_2^- to meet the energy demands of biosynthesis.

Although several phylogenetically distinct genera carry out NO_2^- oxidation, most of what is known about organic carbon metabolism of NOB has been derived from studies on members of the genus *Nitrobacte*r. *Nitrobacter* was originally thought to be obligately lithoautotrophic, relying solely on NO_2^- as an energy source and fixing CO_2 as its source of carbon until several studies, predominantly using strains of *Nitrobacter winogradskyi,* concluded that organic carbon was incorporated into cell material and simple organic substrates such as acetate, pyruvate, and glycerol, could support heterotrophic growth (20, 43, 147, 154, 155). Nevertheless, the preference of *N. winogradskyi* to grow lithoautotrophically is supported by the fact that heterotrophic growth rates are much slower than lithoautotrophy and, when CO_2 was stripped from cultures containing organic carbon and NO_2^-, these organic carbon sources could not substitute for CO_2 as a the sole carbon source (43, 80). With regard to mixotrophy, a combination of acetate and NO_2^- in cultures of *N. winogradskyi* could produce higher cell and protein yields yet, compared to lithoautotrophically grown cells, the growth rate was not stimulated (147).

Nitrobacter hamburgensis, a more recent isolate (c.1983) of the *Nitrobacter* genus, was described as having a greater heterotrophic potential than *N. winogradskyi* (24) as early descriptions reported that mixotrophic and heterotrophic growth rates were faster than lithoautotrophy (23, 24). Although many investigations using *N.*

hamburgensis have been conducted with cells grown mixotrophically, it is not well understood how organic carbon is utilized in this bacterium. Cell membranes from mixotrophic and heterotrophic cultures have been shown to contain different b-type cytochromes (88) indicating a physiological adaptation to growth in the presence of organic carbon. Nevertheless, detailed studies on how organic carbon is processed or influenced by NO_2^- in *N. hamburgensis* have not been completed and it is not known if organic carbon can be used as the sole carbon source (lithoheterotrophy) or if organic carbon positively or negatively influences the rate of NO_2^- oxidation.

The recent availability of the *N. hamburgensis X14* genome prompted us to re-examine mixotrophy and heterotrophy in this bacterium. Multiple genes that putatively encode lactate dehydrogenases (LDH) were identified in the genome. In contrast to the well-studied NAD-dependent LDHs, these genes encode homologs of FAD-dependent LDH which oxidize lactate to pyruvate and potentially could provide energy and/or carbon to the cell (64). A putative L-isomer specific LDH (EC 1.1.2.4, Nham_1112) was identified and two gene candidates, Nham_4010, and Nham_3204, contain protein domains of FAD-linked oxidases (EC 1.1.2.3), which also could possess LDH activity. In this study, the fate of lactate-C in mixotrophic and heterotrophic cultures of *N. hamburgensis* was examined. Lactate was able to enhance growth in the presence of NO_2^- and was also able to serve as a sole carbon and energy source in the absence of NO_2^-. Nevertheless, growth was inhibited and lactate assimilation was suppressed by NO_2^- when CO_2 was limited.

MATERIALS AND METHODS

Culture Conditions. Stock cultures of *Nitrobacter hamburgensis* X14 were grown in chemically defined medium. The base medium contained 750 µM $MgSO_4$, 30 µM $FeSO_4$-EDTA, 200 µM $CaCl_2$, and 1 µM $CuSO_4$. One ml of a trace metals stock solution (400 µM Na_2MoO_4, 1 mM $MnCl_2$, 350 µM $ZnSO_4$, 8.4 µM $CoCl_2$) was added per 2 L of base medium. After autoclaving, the base medium was amended with 20 ml/L of a phosphate buffer stock solution (480 mM KH_2PO_4, 42 mM NaH_2PO_4, pH 7.8). $NaNO_2$ (30-45mM) was added to the phosphate-buffered base medium as the

energy and nitrogen source for lithoautotrophic growth. Heterotrophic growth medium contained filter-sterilized sodium D-lactate (500 µM) (Sigma) and 2 mM $(NH_4)_2SO_4$ as a nitrogen source. Mixotrophic growth medium contained 30-45 mM $NaNO_2$, 2mM $(NH_4)_2SO_4$, and 500 µM sodium-D-lactate. Growth was monitored by determining the optical density at 600 nm. NO_2^- was measured spectrophotometrically or colorimetrically as previously described (68). Whole-cell protein content was assessed using the Bradford method modified by Nelson (120). Stock cultures were routinely checked for contamination by plating 100 µl culture samples on Luria broth agar plates or inoculating a 1/10 nutrient broth containing glucose (1 g/L). Culture purity was additionally verified by visualization of cells at 25,000X magnification with a transmission electron microscope.

Lactate consumption assay. Lithotrophically, mixotrophically, and heterotrophically grown *N. hamburgensis* cells were harvested by centrifugation from late log phase cultures, washed twice and re-suspended in sterile phosphate buffered base medium (pH 7.5, OD_{600} 0.3-1.0). Five ml aliquots of cell suspensions were added to 38 ml culture bottles and sealed with Teflon-faced gray butyl rubber stoppers (Supelco, Bellfonte, Pa.) and fastened with aluminum crimp seals. CO_2 in the headspace was measured by thermal conductivity gas chromatography (Model GC-8A, Shimadzu Corp,) using a 3 ft PORAPAK T column (Waters Associates, Inc., Columbia, Md.) running at 150º C with the detector set to 220º C. The concentration of D-lactate was measured with a D-lactate assay kit (Megazyme International; Wicklow, Ireland) according to the manufacturers instructions.

^{14}C-lactate incorporation. Resting cells of lithotrophically, mixotrophically, and heterotrophically grown *N. hamburgensis* cells were harvested by centrifugation from late log phase cultures and resuspended in sealed 38ml bottles containg 5ml of phosphate buffered base medium containing $(NH_4)_2SO_4$,1mM D-lactate, and 1.4 µCi of DL-[1-^{14}C]-lactate (Sigma-Aldrich; St. Louis, MO). *N. hamburgensis* was inoculated into these sealed bottles to an optical density of 0.4-0.6 at 600 nm. Each culture bottle contained a 1.5 ml tube with a piece of Whatman filter paper. 100 µl of 18 M KOH was added directly to the filter paper at the beginning of the experiment to trap evolved CO_2.

At the end of the experiment (T = 4 hours), the bottles were opened, and the KOH soaked filter paper was removed and added to a vial with scintillation fluid to measure $^{14}CO_2$. Cellular incorporation of radioactive ^{14}C from D-lactate was measured in cells that were harvested by centrifugation, washed twice, and resuspended in 1ml phosphate buffered base medium. A 200 µl aliquot of the resuspended cells was added to a vial with 3.5 ml scintillation fluid to measure the cellular ^{14}C incorporation. A one milliliter aliquot of the supernatant was added to a new sealed vial containing a KOH trap and was acidified with 20 µl of 12.1 M hydrochloric acid to evolve the residual $^{14}CO_2$ dissolved in the incubation medium.

RESULTS

Growth response of *N. hamburgensis* to D- or L-lactate. Experiments were conducted to determine the growth responses of *N. hamburgensis* to the presence of lactate. As both putative D- and L- specific LDH encoding genes were identified in the genome, *N. hamburgensis* was cultured in the presence of either D- or L-lactate with and without NO_2^- to assess its mixotrophic and heterotrophic potential. When *N. hamburgensis* was grown in medium supplemented with lactate and NO_2^-, the growth rate and final cell yield increased 50% and 60%, respectively, compared to cultures growing lithoautotrophically on medium containing NO_2^- and CO_2 (Figure 4.1A). Concomitantly, an increase in the total protein content was also observed in *N. hamburgensis* cells grown mixotrophically with D-lactate and NO_2^- (Figure 4.1C). Because D-lactate did not affect the gross rate of NO_2^- consumption (Figure 4.1B), the increased growth rate and cell yield indicated that less NO_2^- was consumed per cell under mixotrophic conditions. Growth was also observed in *N. hamburgensis* cultures incubated with D-lactate as the only source of energy (heterotrophy) (Figure 4.1A and 1C). Despite the fact that equal quantities of D-lactate were added to both mixotrophic and heterotrophic cultures, the cell yield and protein content of heterotrophically grown cells was reduced by 50% and 70%, respectively, compared to the growth stimulation observed when D-lactate was added to mixotrophic cultures containing D-lactate and NO_2^-.

In contrast to the growth response to D-lactate, L-lactate had no effect on growth rate or cell yield compared to control cultures growing on NO_2^- and CO_2. Furthermore, growth was not observed when L-lactate was added in the absence of NO_2^-. Similarly, the optical densities of cultures amended with both L- and D-lactate were not significantly different from cultures containing only D-lactate (data not shown) suggesting that L-lactate did not inhibit the growth of *N. hamburgensis* on D-lactate.

Because the majority of lactate-dependent growth stimulation occurred after most of the NO_2^- had been depleted, the effect of NO_2^- on lactate consumption was measured in resting cells (Table 4.1) harvested from both lithotrophic and mixotrophic cultures. Cells harvested from autotrophic cultures that were not exposed to lactate readily consumed lactate at 764 μg D-lactate-C/mg protein/h. The rate of lactate consumed by autotrophically grown cells was unaffected by the addition of NO_2^-. Mixotrophically grown cells consumed lactate at a faster rate (1181 μg D-lactate-C/mg protein/h) than autotrophically grown cells. In the presence of NO_2^-, the rate of lactate consumed by mixotrophically grown cells decreased by 20%.

Effect of CO_2 limitation. To determine if *N. hamburgensis* could use D-lactate as its sole carbon source in the presence of 35 mM NO_2^- (lithoheterotrophy), growth and NO_2^- consumption were monitored in a closed system that prevented the influx of atmospheric CO_2 (Table 4.2). In the closed system in the absence of an added carbon source, growth did not occur although approximately 10 mM NO_2^- was consumed over the duration of the experiment (4 days). When 500 μM D-lactate was provided as the sole source of carbon, measurable growth was not observed and NO_2^- consumption was similar to the control cultures without an added carbon source. When a limiting amount of sodium carbonate (187 μM) was added in the presence or absence of D-lactate, similar amounts of growth and NO_2^- consumption were measured (Table 4.2). The amount of NO_2^- consumed increased in response to increased concentrations of CO_2 and when sodium carbonate was provided in excess (750 μM), the optical density increased two fold over cultures containing 187 μM sodium carbonate and nearly all of the NO_2^- was consumed. Growth and NO_2^- consumption were not affected when a higher concentration (1500μM) of sodium carbonate was added, or if parallel culture

bottles were exposed to atmospheric CO_2 (data not shown). In contrast to the mild stimulation observed when D-lactate was added to CO_2-limited cultures, the growth yield of cultures containing both D-lactate and 750 μM sodium carbonate increased significantly (45%) compared to cultures containing 750 μM sodium carbonate alone.

Although NO_2^- only slightly affected lactate consumption during CO_2 replete conditions, it was still possible that NO_2^- affected lactate consumption when CO_2 was limiting. Growth, and NO_2^-, CO_2, and lactate consumption were measured in cultures amended with 0, 7.5, 15, 30 mM NO_2^- containing atmospheric levels of CO_2 and 500 μM D-lactate (Figure 4.2). In cultures amended with NO_2^-, the ambient CO_2 was quickly consumed and remained undetectable until after the NO_2^- was completely consumed. Although lactate was initially consumed at a low rate in all cultures, the rate of lactate consumption increased in cultures without NO_2^- and as the NO_2^- was depleted in others. In the presence of 7.5 mM NO_2^-, lactate was consumed at the same rate and growth was enhanced by 20% compared to cultures only containing lactate. In contrast, in cultures amended with higher concentrations of NO_2^- (15 and 30 mM), growth was almost completely inhibited and lactate consumption was suppressed until after the depletion of NO_2^-. These results suggested that NO_2^- suppresses lactate consumption if CO_2 is limiting. Nevertheless, because lactate is consumed at a low rate, once the NO_2^- was depleted (or the NO_2^--induced CO_2 limitation was relieved) growth was able to occur.

To determine if the repressive effect of NO_2^- on lactate consumption was caused indirectly by a CO_2 limitation, *N. hamburgensis* was grown heterotrophically on lactate in sealed culture bottles containing KOH to trap CO_2. In cultures without a CO_2 trap, all of the lactate (500μM) was consumed by 144 hrs (Figure 4.3B). When CO_2 was stripped from replicate cultures, the initial rate of growth and lactate consumption was not significantly different. By 72 h, the rate of growth and lactate consumption slowed, leading to a 20% decrease in growth yield (Figure 4.3A and 4.3B). In contrast, when NO_2^- was added in lieu of a CO_2 trap, *N. hamburgensis* did not grow at all and lactate consumption was supressed 75% over the same time course compared to sealed culture bottles only containing lactate.

1-^{14}C-Lactate Consumption. The consumption and distribution of radiolabeled 1-^{14}C- D-lactate was measured in resting cells from autotrophically, mixotrophically, and heterotrophically grown cultures of *N. hamburgensis* (Table 4.3). Regardless of how *N. hamburgensis* was grown, when cells were incubated with lactate in the absence of NO_2^-, most of the radiolabel from lactate was retrieved as CO_2 and only 31-42% of the ^{14}C was incorporated into the cells. In contrast, in the presence of NO_2^-, more lactate-C was assimilated (57-68%) and the percentage of ^{14}C retrieved as CO_2 decreased significantly. More lactate was consumed by heterotrophically grown cells compared to autotrophic and mixotrophically grown cells suggesting a physiological adaptation to growth on lactate. In the presence of NO_2^-, more ^{14}C was incorporated into cell material regardless of the growth condition, yet, the proportional distribution of ^{14}C in cellular material versus the CO_2 pool did not shift as drastically in cells that had been previously exposed to lactate. Furthermore, the total amount of lactate consumed by autotrophically grown cells was unaffected by the presence of NO_2^- whereas lactate consumption decreased 17% and 25% in mixotrophically and heterotrophically grown *N. hamburgensis*, respectively.

Respiration Measurements. Not only was 1) more lactate consumed by heterotrophically grown cells (Table 4.3), and 2) the rate of lactate consumption increased in mixotrophically grown cells compared to autotrophically grown cells (Table 4.1), other evidence of a physiological adaptation to lactate was obtained from measurements of respiration. Despite the fact the lactate was readily consumed by lithoautotrophically grown cells, D-lactate dependent O_2 uptake was significantly reduced in these cells (Table 4.4). In contrast, D-lactate supported significantly higher rates of respiration in heterotrophically and mixotrophically grown cells yet NO_2^--dependent respiration was reduced after heterotrophic growth on lactate. Mixotrophically grown cells had the same rate of NO_2^-- and lactate- dependent O_2 uptake as the autotrophically and heterotrophically grown cells, respectively.

DISCUSSION

Based on genome sequence analysis, the ability of *N. hamburgensis* to metabolize lactate, a previously unknown organic carbon source, was predicted given that multiple gene candidates were annotated as encoding LDHs. However, the ability of *N. hamburgensis* to grow on D- but not L-lactate was surprising given that both D- and L-LDHs were annotated in the genome. The gene thought to encode L-LDH , Nham_1112, shares 78% protein identity to a putative L-LDH in *N. hamburgensis*' close relative, *Rhodopseudomonas*, which can metabolize L-lactate (75, 107) and thus the function of Nham_1112 is unclear. Other candidate genes were identified which may be responsible for the metabolism of D-lactate. Nham_4010 encodes an FAD-dependent oxidase with an unspecified substrate. A putative glycolate oxidase (Nham_3202-4) could also be responsible for the metabolism of D-lactate in *N. hamburgensis* as the substrate range of glycolate oxidases includes D-lactate (102). With regard to transport, a truncated lactate permease gene (Nham_2174) was identified on the chromosome although it is likely a pseudogene given that the translated protein product spans two reading frames. Other candidate lactate transporters include a putative TDT-family transporter gene located in the operon which encodes the NO_2^- oxidoreductase. Similar TDT-family members can transport dicarboxylates(67). Further investigation is required to determine which gene(s) are responsible for the observed uptake and metabolism of lactate.

The metabolism of organic carbon in *N. hamburgensis* appears to differ from *N. winogradskyi* and our results also deviate from previous claims regarding heterotrophy in *N. hamburgensis*. For example, the rate of acetate assimilation by autotrophically and heterotrophically grown cells of *N. winogradskyi* was stimulated by NO_2^- (147) and organic compounds in general were suggested to positively influence the rate of NO_2^- oxidation (155, 165). These data suggest that in *N. hamburgensis*, organic carbon does not increase the rate of NO_2^- oxidation but instead reduces the NO_2^- requirement of the cell as evidenced by a faster growth rate and an increase in growth yield in mixotrophic versus lithoautotrophic conditions. In contrast to results obtained with acetate in *N. winogradskyi*, NO_2^- did not effect lactate consumption by autotrophically grown cells

and NO_2^- even slightly reduced the rate of lactate assimilation in cells grown mixotrophically and heterotrophically. Early descriptions of growth phenotypes of *N. hamburgensis* indicated that heterotrophic growth on organic substrates (pyruvate, acetate, and glycerol) was faster than lithoautotrophic growth (24). The generation time of cells grown solely on D-lactate was three fold slower than lithautotrophic cultures. Furthermore, the growth rate of *N. hamburgensis* on pyruvate or acetate in our laboratory was also slower than both lithoautotrophy and mixotrophy (S.R. Starkenburg, unpublished results). The differences in heterotrophic growth rates may be explained by the fact that in our experiments, a chemically defined minimal medium was used while many previous investigations of mixotrophy and heterotrophy in *N. hamburgensis* used undefined, complex media containing yeast extract and peptone (24, 70, 87, 159). At least with respect to nitrogen metabolism, ammonium amendments of NO_2^--containing cultures enhanced the growth rate and cell yield of lithoautotrophically grown *N. hamburgenisis* (S.R. Starkenburg, unpublished results), implying that reductant sparing in N assimilation is possible. Thus, the complex media components could have provided additional nutrients (nitrogen, vitamins, amino acids) that affected the mixotrophic and heterotrophic growth rates of *N. hamburgensis*.

Surprisingly, a lactate metabolic pathway in *N. hamburgensis* appears to be constitutively expressed. Cell suspensions of NO_2^- grown *N. hamburgensis* with no history of exposure to lactate readily consumed D-lactate in ≤ 60 minutes and, tracking the fate of lactate with ^{14}C, confirmed that 69% of the carboxyl carbon was respired as CO_2. Carbon fixation via the Calvin cycle is energetically expensive and thus, the constitutive expression of LDH may indicate a need for a constant supply of reductant. Due to the highly positive reduction potential of the NO_3^-/NO_2^- redox couple, reductant (NADH and $FADH_2$) must be generated via reversed flow of electrons through the electron transport chain. Reductant conserved from NO_2^- oxidation is likely to be quickly consumed by Calvin cycle reactions and other biosynthetic pathways, and thus, the ability to simultaneously conserve energy and gain carbon from organic substrates would provide a selective advantage. Constitutively expressing the required enzymatic machinery would further enable *N. hamburgensis* to respond to fluctuating supplies of

NO_2^- and organic carbon and compete with other heterotrophs that have a high affinity for the same organic carbon sources.

The results of these experiments also indicate that the physiology of *N. hamburgensis* changes in response to lactate, both in the presence and absence of NO_2^-. Although D-lactate was metabolized without induction, it was consumed at a faster rate by heterotrophically grown cells compared to autotrophically grown cells. Others have shown that a shift occurs in the cytochrome and FMN content of heterotrophically grown *N. hamburgensis* (23, 88). This agrees with the observation that lactate-dependent O_2 uptake rate was three fold higher in heterotrophically and mixotrophically grown cells compared to cells harvested from autotrophic cultures. Nonetheless, the physiological adaptations observed in response to lactate could not alleviate the dependence on CO_2 as a carbon source, since growth was completely inhibited in cultures containing lactate and NO_2^- in the absence of CO_2. When CO_2 was stripped from heterotrophic cultures, the rate of growth decreased yet the cells were still able to metabolize lactate (similar results were seen after NO_2^- depleted the CO_2 from mixotrophic cultures). Thus, it appears that the ability of *N. hamburgensis* to metabolize lactate at a higher rate under CO_2 limitation in the absence, but not in the presence of NO_2^-, may be explained by a shift in the redox state of the cell under these different conditions. Because NO_2^- is still consumed in a CO_2 limited environment, reverse flow of electrons could result in a build up of reductant. Consequently, lactate consumption might be inhibited due to the absence of oxidized electron acceptors. The same physiological constraints might not exist after adaptation to heterotrophic growth on lactate since the data suggest that electrons from lactate are used more effectively to support respiration.

The ability of *N. hamburgensis* to use organic carbon heterotrophically, yet still depend on CO_2 in the presence of NO_2^-, speaks to the true lithoautotrophic nature of this NO_2^- oxidizer. When other facultative lithoautotrophs encounter organic carbon, in many cases, a complete repression of autotrophic CO_2 fixation occurs (143, 161). On the other hand, in both *Thiobacillus intermedius* and *R. eutropha*, RuBisCO and other Calvin cycle enzymes are only partially repressed by the presence of some organic

carbon sources and fully repressed by others (143, 161). At least some repression of autotrophy in *Nitrobacter* does occur as RubisCO activity was reported to be suppressed 50-99% in heterotrophically grown cells (147, 154) and, the results presented here indicated that NO_2^--dependent O_2 uptake was also suppressed by 42 % after growth solely on organic carbon. These data suggest that *Nitrobacter* can take advantage of organic carbon if it is the only available source of energy yet, if NO_2^- is available, its heterotrophic potential is hampered by an inability to switch to an organic carbon source in the absence of CO_2. Further investigations of *N. hamburgensis* will help unravel the physiological and regulatory constraints of autotrophy during growth on inorganic energy sources and help elucidate the concurrent metabolism of mixed energy and carbon sources in facultative lithoautotrophs.

ACKNOWLEDGEMENTS

Funding was provided to S. R. Starkenburg by the Subsurface Biosphere Integrative Graduate Education and Research Traineeship (IGERT) grant 0114427-DGE at Oregon State University from the National Science Foundation's Division of Graduate Education. Special thanks to Norman Hommes for technical assistance, and many helpful scientific discussions.

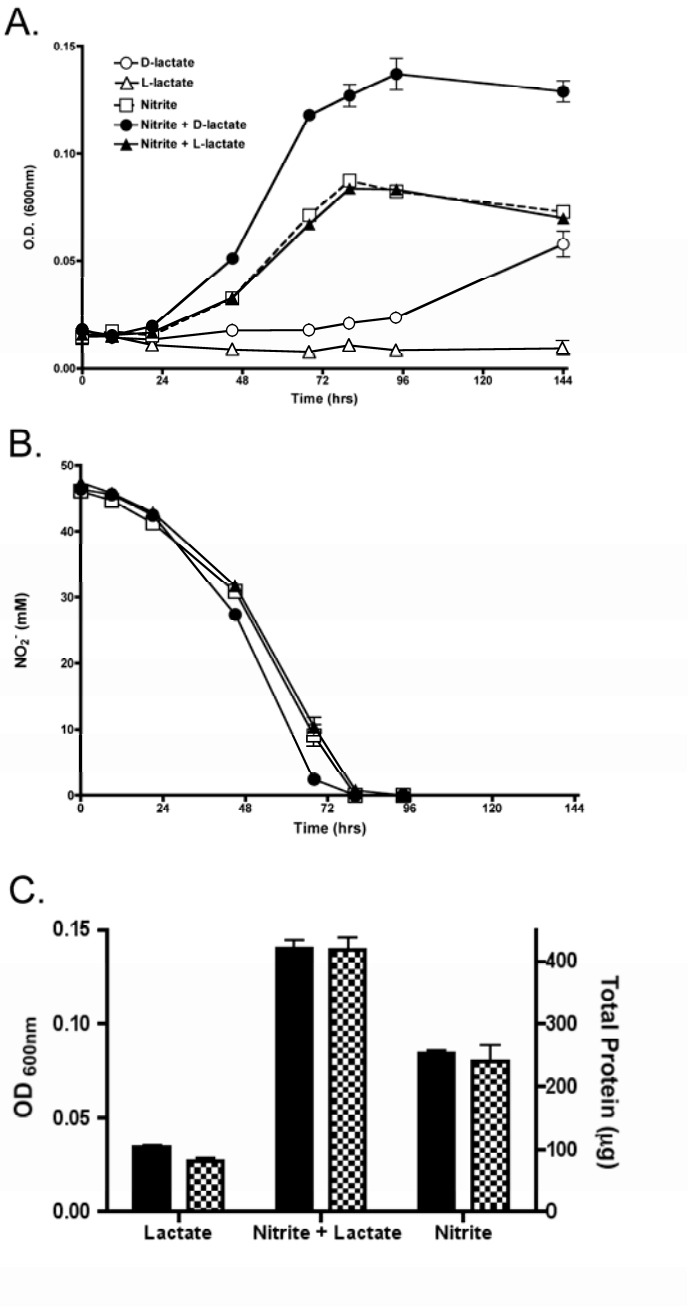


Figure 4.1. Growth Response of *N. hamburgensis* to D-lactate. Growth, NO_2^-, and the protein content of whole cells were measured in response to D- or L-lactate in Panels A, B, and C, respectively. Measurements depicted in Panel C were from a replicate experiment.

Table 4.1. Lactate Consumption Rates of Autotrophic and Mixotrophic Cells of *N. hamburgensis*

Growth Condition	Assay Condition	Lactate Consumed (μg lactate-C/mg protein/hr)	
NO_2^-	Lactate	764	± 63
NO_2^- + Lactate	Lactate	1181	± 62
NO_2^-	NO_2^- + Lactate	717	± 94
NO_2^- + Lactate	NO_2^- + Lactate	944	± 83

Table 4.2. Growth and NO_2^- Consumption in Response to Carbonate and D-lactate

CO_3^{2-} (μM)	D-lactate (μM)	O.D. (600 nm)*	NO_2^- (mM)*
0	0	0.016 ± 0.002	14.3 ± 0.3
187	0	0.023 ± 0.001	23.4 ± 0.3
750	0	0.047 ± 0.008	31.4 ± 2.4
0	500	0.016 ± 0.001	15.3 ± 0.3
187	500	0.027 ± 0.004	25.2 ± 0.3
750	500	0.068 ± 0.005#	33.0 ± 0.3

*Optical Density and NO_2^- consumed after 72 hrs of growth. The initial OD_{600nm} and concentration of NO_2^- was 0.015 and 35 mM, respectively.

#Statistically significantly different ($p \leq 0.005$) from cultures containing carbonate without lactate.

Table 4.3. 1-^{14}C-lactate Incorporation (nmol ^{14}C-lactate/mg protein)

Growth Condition	Assay Condition	Cells	%	CO_2	%	Total Consumed*
NO_2^-	Lactate	1.77 ± 0.04	31	3.90 ± 0.04	69	5.66 ± 0.42
	Lactate + NO_2^-	3.87 ± 0.07	61	2.49 ± 0.41	39	6.36 ± 0.36
NO_2^- + Lactate	Lactate	2.60 ± 0.09	42	3.61 ± 0.26	58	6.20 ± 0.35
	Lactate + NO_2^-	3.50 ± 0.37	68	1.61 ± 0.29	32	5.11 ± 0.21
Lactate	Lactate	3.54 ± 0.26	37	5.93 ± 0.34	63	9.47 ± 0.58
	Lactate + NO_2^-	4.49 ± 0.27	57	2.63 ± 0.44	43	7.11 ± 0.70

*represents the total amount of lactate consumed after 4 hrs.

Table 4.4. Respiration Rates of Auto-, Mixo-, and Heterotrophically grown *N. hamburgensis*

Growth Condition	O_2 uptake* (nmol/min/mg)	
	NO_2^-	Lactate
NO_2^-	630.9 ± 17.3	5.9 ± 1.0
NO_2^- + Lactate	685.9 ± 31.6	16.3 ± 0.8
Lactate	367.7 ± 10.3	16.2 ± 1.4

*Endogenous rates of O_2 uptake were subtracted from all reported values. Respiration rates were measured after a single passage in each respective growth condition.

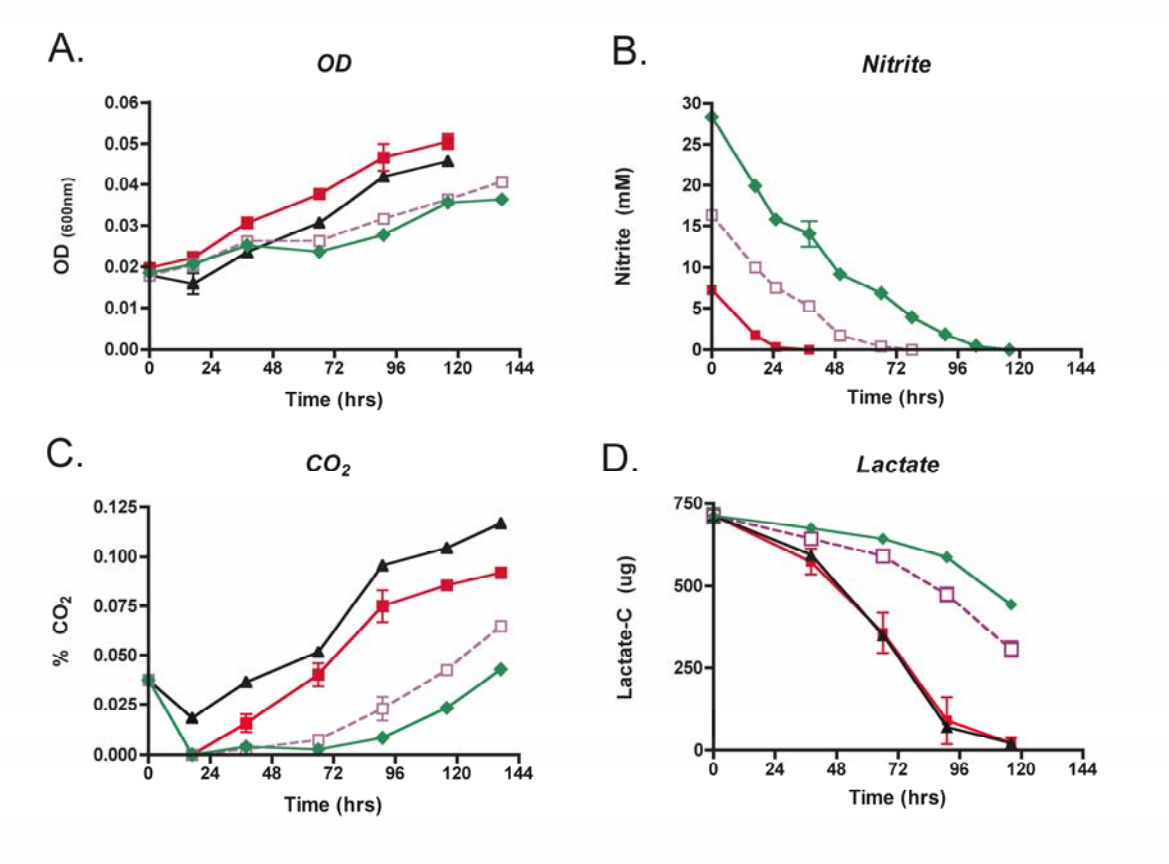


Figure 4.2. Effect of NO_2^- on Growth, CO_2, and Lactate Consumption. Data points indicate the mean from triplicate cultures for treatment. The experiment was conducted in sealed bottles and initially contained atmospheric amounts of CO_2 (≈0.038%) . Cultures were amended with 0 (red squares), 7.5 (black triangles), 15 (purple open square), and 30 mM NO_2^- (green diamond), respectively.

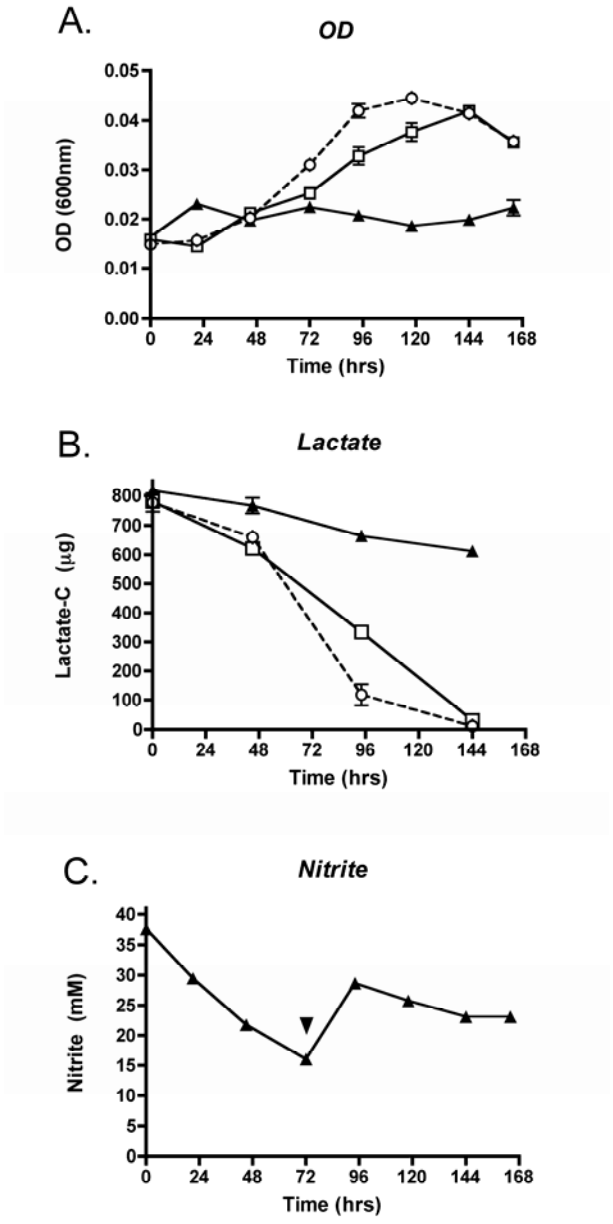


Figure 4.3. Effect of CO_2 limitation on heterotrophic growth on D-lactate. Growth (Panel A), D-lactate (Panel B), and NO_2^- (Panel C) were monitored under different types of CO_2 limitation. 50 mL cultures in sealed bottles were inoculated with NO_2^- grown cells to an initial OD_{600} of 0.015 into media containing 500 µM of D-lactate (open circles), 500 µM of D-lactate + KOH trap (open squares), or 35 mM NO_2^- and 500uM D-lactate (closed triangles). An additional supplement of 20mM NO_2^- was added (indicated by the inverted triangle in Panel C) to the NO_2^- containing culture to avoid NO_2^- limitation.

Chapter 5

Expression of a putative nitrite reductase and the reversible inhibition of nitrite-dependent respiration by nitric oxide in *Nitrobacter winogradskyi*

Shawn R. Starkenburg, Daniel J. Arp, and Peter J. Bottomley

Prepared for Publication in:
Applied and Environmental Microbiology
American Society for Microbiology

ABSTRACT

The nitrite oxidizing Alphaproteobacterium, *Nitrobacter winogradskyi*, primarily conserves energy from the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) through aerobic respiration. *N. winogradskyi* can also grow anaerobically using pyruvate and NO_3^- as the electron donor and acceptor, respectively. Intriguingly, a nitrite reductase, which reduces NO_2^- to nitric oxide (NO) was previously shown to be active under both anaerobic and aerobic conditions and NO-dependent NADH formation in aerobic or anaerobic cell suspensions was measured, suggesting that NO may play role in NO_2^- metabolism by *Nitrobacter*. Sequencing of the *N. winogradskyi* genome revealed that the chromosome contained a gene (Nwin_2648) which encodes a putative copper-containing nitrite reductase (NirK) that is similar to *nirK* found in ammonia oxidizing Betaproteobacterium. In this study, the putative *nirK* in *N. winogradskyi* was found to be expressed and maximally transcribed under low O_2 in the presence of NO_2^-. Transcription of *nirK* was not detected under anaerobic conditions in the presence of NO_3^- and pyruvate. Although production of NO under aerobic conditions was not detected, NO was consumed by viable cells and reversibly inhibited NO_2^--dependent O_2 uptake. Cyanide treated cells did not consume NO suggesting that NO was metabolized by, or electrons flowed through, cytochrome oxidase. The consumption of NO through both biological and abiological mechanisms resulted in the formation of NO_2^-. In light of this new information, the previously reported NO-dependent stimulation of NADH synthesis by *Nitrobacter* under aerobic and anaerobic conditions is more likely a result of the metabolism of NO_2^- produced from the auto-oxidation and metabolism of NO.

INTRODUCTION

As a chemolithoautotroph, *Nitrobacter winogradskyi* conserves energy from the oxidation of NO_2^- and fixes carbon dioxide as a carbon source (21, 23). NO_2^- oxidation is an aerobic process catalyzed by nitrite oxidoreductase (NXR), which converts NO_2^- to NO_3^- (187). The reaction catalyzed by NXR is reversible (159), and thus *N. winogradskyi* can also denitrify, converting NO_3^- back to NO_2^- under anaerobic conditions when given pyruvate or glycerol as an energy source (55). The major terminal products of denitrification in *N. winogradskyi* are reported to be nitric oxide (NO) and nitrous oxide (N_2O)(1, 55). Although the mechanism of N_2O production remains unknown, a protein isolated from *Nitrobacter vulgaris* that co-purified with NXR, was shown to reduce NO_2^- to NO (1). Freitag, et al (54) also measured NO dependent NADH formation in aerobic and anaerobic cell suspensions, suggesting that NO may play role in the NO_2^- oxidizing system of *Nitrobacter*.

Given the presence of NO_2^- reductase activity and NO-dependent NADH formation, it was hypothesized that NO, and not NO_2^-, might serve as the electron donor for biosynthesis (21, 126). Electrons from NO_2^- oxidation enter the respiratory chain at the level of complex III (187), therefore, a high energetic hurdle must be overcome to synthesize NADH via a reverse flow of electrons from NO_2^-. From an energetic perspective, NO should be a more favorable electron donor than NO_2^-, given the less positive redox potential of the NO_2^-/NO couple (E'_o = +387 mV). Nevertheless, further investigations into the metabolism of NO by this or any other NO_2^- oxidizer has not been completed.

The recent sequencing of the *N. winogradskyi* genome revealed that the chromosome contained a gene (Nwin_2648) encoding a putative *nirK* type nitrite reductase. NirK is a copper containing nitrite reductase which primarily reduces NO_2^- to NO during denitrification (17, 166, 195). Although a NirK is encoded in the genome of *N. winogradskyi's* close Alphaproteobacterial relative, *Rhodopseudomonas palustris* (96), the *N. winogradskyi nirK* was most similar to a homolog in the ammonia oxidizing Betaproteobacterium, *Nitrosomonas* (31). In recent years considerable effort has been expended to understand the role of *nirK* in *Nitrosomonas* (9-11, 31, 32, 138). Unlike an

archetypal nitrite reductase that is expressed under anaerobic conditions and functions in denitrification (17, 195), the expression of NirK in *N. europaea* is controlled by the concentration of NO_2^- and pH, but not O_2 (10). NirK is active and expressed under aerobic conditions and studies completed using a *nirK*-deficient strain of *N. europaea* demonstrated that *nirK* is required for optimal growth and cell yield during the aerobic oxidation of ammonia (138).

Investigations into *nirK* expression, and the production and consumption of NO by *N. winogradskyi* have not been completed. In this study, the putative *nirK* in *N. winogradskyi* was found to be expressed under aerobic conditions, and that factors which control its expression differ from the observed expression of *nirK* in *N. europaea*. Although NO_2^-dependent NO production was not detected, NO was rapidly consumed by *N. winogradskyi* and the mechanism of its metabolism was investigated.

MATERIALS AND METHODS

Culture Conditions. Stock cultures of *Nitrobacter winogradskyi* NB255 (ATCC 25391) were grown in chemically defined medium. The base medium contained 750 μM $MgSO_4$, 30 μM $FeSO_4$-EDTA, 200 μM $CaCl_2$, and 1 μM $CuSO_4$. One 500 μl of a trace metals stock solution (400 μM Na_2MoO_4, 1 mM $MnCl_2$, 350 μM $ZnSO_4$, 8.4 μM $CoCl_2$) was added per liter of base medium. After autoclaving, the base medium was amended with 20 ml/L of a phosphate buffer stock solution (480 mM KH_2PO_4, 42 mM NaH_2PO_4, pH 7.8). $NaNO_2$ (30-45mM) was added to the phosphate-buffered base medium as the energy and nitrogen source. Growth was monitored by determining the optical density at 600 nm. NO_2^- was measured spectrophotometrically or colorimetrically as previously described (68). Whole-cell protein content was assessed using the Bradford method as modified by Nelson (120).

NO, N_2O, and O_2 uptake measurements. *N. winogradskyi* cells were harvested by centrifugation from late log phase cultures, washed and re-suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 50 μM diethylenetriamine pentaacetic acid (DTPA). Rates of O_2 uptake by cell suspensions were measured with a Clark-type O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio)

mounted in an all-glass, water-jacketed reaction vessel (1.8-ml volume) held at 30ºC. NO was measured simultaneously in the same vessel with an amiNO-600 NO electrode attached to an inNO-T measuring system (Innovative Instruments, Tampa, FL).

NO, N_2O, and O_2 were also measured simultaneously using a membrane inlet system attached to a Prisma QMS-200 quadrapole mass spectrometer (Pfeiffer Vacuum, Nashua, NH, USA), with closed ion source and electron multiplier detector recording at mass/charge (*m/z*) ratios of 30 (NO), 32 (O_2), 44 (N_2O) and 40 (Ar). The membrane inlet system was composed of a 5 ml water-jacketed dubinsky chamber with a gas port drilled through the center. Samples were stirred using a magnetic stir bar. Temperature was maintained at 30°C. O_2 , N_2O, and NO signals were normalized to Ar to account for pressure changes during the sampling period. A dry ice cold trap was placed between the cell chamber and the detector to remove water vapor. When N_2O was measured, a salted ice bath (-5 to -7º C) was employed in place of the dry ice trap to allow N_2O, (but not water vapor) to pass to the detector.

Quantitative PCR detection of *nirK*. RNA was extracted from cell suspensions after 3 hrs of treatment using the RNeasy Mini kit(Qiagen Sciences, Maryland, USA) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturers instructions. *nirK* and 16s rRNA were amplified by quantitative PCR in 50 µl reactions using a Biorad quantitative PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the following cycle parameters; cycle 1: 95 ºC for 30 s, cycle 2-36: step 1-95 ºC for 30 s, step 2-55 ºC for 30 s, step 3-72 ºC for 40 s, cycle 37: 95 ºC for 60 s, cycle 38: 55 ºC for 1 min, cycle 39-120: initital set point temperature was 55ºC and was increased by 0.5 ºC every 10 s. The following primers were used for amplification*; NWnirK-R* 5'-FATTCTCGAAGATCTGATGC-3*'; NWnirk-F* 5'-AAGACCTTCTCCTACGTTCC-3'; *NW16s-975F* 5'-GGAGCATGGAGCACAGGT-3'*; NW16s-1157R* 5'-GTAAGGGCCATGAGGACTTG-3. Expression of *nirK* was normalized to 16s rRNA using the 2 ΔΔ CT method as previously described (101).

RESULTS

Effect of NO_2^- on anaerobic growth. Since some strains of *Nitrobacter* do not grow anaerobically (21), the growth response of *N. winogradskyi* NB255 under anoxic conditions were determined using pyruvate and NO_3^- as the electron donor and acceptor, respectively. A slow growth response was observed, resulting in a two-fold increase in optical density of the culture (Figure 5.1A). When NO_2^- was used in lieu of NO_3^-, growth did not occur implying that NO_2^- reduction could not support anaerobic growth (data not shown). Strikingly, when *N. winogradskyi* was cultured in the presence of NO_2^-, NO_3^-, and pyruvate, growth was also completely inhibited. In anaerobic cultures containing NO_3^- and pyruvate, NO_2^- did not accumulate during anaerobic growth but a measurable amount of NO_2^- was detected as cells reached stationary phase (Figure 5.1C). In summary, these data indicate that anaerobic growth of *N. winogradskyi* NB255 is possible, yet NO_2^- inhibits growth on NO_3^- and pyruvate in the absence of O_2, similar to what was observed during anaerobic growth studies of *N. winogradskyi* F83 (55).

Effect of O_2 and pH on *nirK* expression. Experiments were conducted to determine if *nirK* was expressed by *N. winogradskyi* and if the transcription of *nirK* was affected by pH, NO_2^-, and/or O_2. Cell suspensions of *N. winogradskyi* from aerobic lithoautotrophically grown batch cultures were exposed to different levels of O_2 in the presence of NO_2^- (Table 5.1). An effect of O_2 limitation was apparent since NO_2^- consumption decreased in response to lower amounts of O_2. For example, NO_2^- oxidizing cells exposed to 10% O_2 consumed 9.4 mM NO_2^- while cells given 2% O_2 only consumed 2.1 mM NO_2^-. With respect to *nirK,* the opposite pattern was observed. O_2-limited cells contained higher amounts of *nirK* mRNA than O_2 replete cells. Compared to cells exposed to 20% O_2, *nirK* expression increased 4.8, 20.3, and 34.3-fold when the O_2 concentration was reduced by 50%, 80% and 90%, respectively. In a separate experiment, the effect of pH on the expression of *nirK* under aerobic conditions was assessed (Table 5.1, Experiment 2). While pH 6.6 reduced the amount of NO_2^- consumed, *nirK* expression was not significantly different over a pH range of 6.6-8.2.

Because NO_2^- was not detected in the exponential stage of anaerobic growth and *nirK* expression increased under O_2 limitation, the expression of *nirK* under anaerobic conditions was assessed. Cell suspensions of *N. winogradskyi* incubated anaerobically in the presence of NO_3^- and pyruvate contained 3-50 fold lower amounts of *nirK* mRNA compared to cells exposed to 20% O_2 and 30 mM NO_2^- (Table 5.2). Yet, in cells exposed to NO_2^- in the absence of O_2, *nirK* was transcribed 20 fold more than cells exposed to both NO_2^- and 20% O_2, indicating that NO_2^-, in addition to O_2 limitation, controls expression of *nirK*.

NO consumption. Because the results indicated that *nirK* was transcribed under both aerobic and O_2 limited conditions, experiments were completed to determine if the increase in mRNA correlated with an increase in enzyme activity by measuring NO production. NO was not detected in the aqueous solutions of cells grown in fully aerobic conditions or in cells harvested from a 12hr O_2 limited culture (data not shown). Nevertheless, exogenously supplied NO (2 μM) was quickly consumed by cell suspensions of *N. winogradskyi* at a rate of 64.2 nmol/min/mg protein (Figure 5.2B). NO was partially consumed through abiotic mechanisms over time in phosphate buffer at a reduced rate (Figure 5.1A). Maximum consumption of NO was dependent upon viable cells as the rate of NO consumption in heat killed cell suspensions (Figure 5.2C) was reduced to the rate of NO consumption that occurred in phosphate buffer without cells ($\leq$ 27.6 nmol/min/mg protein).

Effect on O_2 Uptake. Experiments were conducted to determine if the observed consumption of NO was linked to respiration in *N. winogradskyi*. The rate of aerobic respiration on NO_2^- as the electron donor was monitored during exposure to NO. When NO_2^- was added to viable cell suspensions of *N. winogradskyi*, O_2 was quickly consumed (Figure 5.3B). In the presence of 2 μM NO, O_2 uptake was severely inhibited. However, as soon as NO was consumed, O_2 uptake was restored close to its initial rate ($\geq$ 90%). A NO concentration dependent arrest of O_2 consumption was observed over a concentration range of 0.5-32 μM NO, and O_2 uptake always resumed after the NO was consumed (data not shown). To verify these results, the simultaneous consumption of NO and O_2 in cell suspensions was measured using gas inlet mass

spectrometry. After the addition of 7 μM NO to cells respiring NO_2^-, O_2 uptake was inhibited for 7 minutes (Figure 5.4A). O_2 uptake resumed after NO was consumed. NO consumption was also observed under anaerobic conditions (Figure 5.4B). After O_2 was depleted by NO_2^--dependent O_2 consumption, 7 μM of NO was added to the chamber. NO was still consumed in the absence of O_2 yet the amplitude of the NO signal was 30% higher in the absence of O_2 and the rate of NO consumption decreased significantly suggesting that O_2 is involved in, or affects the consumption of NO.

Inhibition of NO consumption. NO reacts with many cellular targets, including heme, iron-sulfur, and copper containing proteins (194). Because O_2 uptake was arrested, experiments were conducted to determine if NO was binding to, or inhibiting, the major proteins involved in the NO_2^- oxidizing system. NXR contains Fe-S clusters to facilitate electron shuttling from NXR to the terminal oxidase via cytochrome c550. Since up to 30% of the cell protein is estimated to be NXR (21, 164), NO binding to the iron centers of NXR might be the cause of inhibition of NO_2^--dependent O_2 uptake. Thus, O_2 uptake and NO consumption were monitored in cells treated with azide, a known inhibitor of NXR. Although 20 μM azide reduced NO_2^--dependent O_2 uptake rate by 85% (Figure 5.3C), NO was still consumed at a rate comparable to untreated viable cells. In addition, when cells were treated with N, N diethyldithiocarbaminate (DDC), an inhibitor of copper-containing nitrite reductase (195), neither NO_2^--dependent O_2 uptake or the consumption of NO were affected (data not shown). Although azide and DDC were not effective inhibitors of NO consumption, NO_2^--dependent O_2 uptake and NO consumption were completely inhibited by 1 mM cyanide (Figure 5.4D). The NO consumption curve of cyanide treated cells mimicked the NO consumption curves observed in phosphate buffer alone (Figure 5.2A) and by suspensions of heat killed cells (Figures 5.2C; 5.3A).

End products of NO consumption. Since the data indicated that NO was consumed by viable cells in a cyanide sensitive process, an attempt was made to identify the products of NO metabolism. Using gas inlet mass spectrometry, the aqueous concentrations of NO_2, N_2O, N_2O_4, N_2O_3 were measured before and after NO exposure. The concentrations of the aformentioned gases did not change significantly

compared to abiotic controls. For example, immediately after the addition of NO, a small increase in the amount of N_2O was observed, yet, the N_2O signal did not continue to increase despite the fact that NO was consumed (Figure 5.4A and B). Furthermore, NO additions to phosphate buffer in the absence of cells also resulted in the same small increase in the N_2O signal (data not shown).

Since the presence of O_2 influenced the rate of NO consumption (Figure 5.4B), the abiological oxidative conversion of NO to NO_2^- and NO_3^- was assessed. NO was added to the head space of bottles containing cell suspensions of *N. winogradskyi*. After a 30 minute incubation, NO_2^- and NO_3^- were detected in the supernatant. As depicted in Figure 5.5, in the absence of cells, NO was auto-oxidized to NO_2^-. In the presence of viable cells, most of the NO derived NO_2^- was converted to NO_3^-. Since NO was not limiting, the total amount of NO_2^- and NO_3^--N produced in the presence of viable cells was greater than that observed in the abiotic control indicating that as NO_2^- was removed from solution by biological oxidation, the NO_2^- pool was replenished through accelerated conversion of NO to NO_2^-. When cells were treated with azide, less NO_3^- was produced compared to viable cells, indicating that azide was inhibiting NO_2^- oxidation. Supernatants from heat killed and cyanide treated cell suspensions did not contain NO_3^- and the level of NO_2^- was marginally higher (~15 %) than what was observed in phosphate buffer control.

DISCUSSION

In this study, Nwi_2648, the gene encoding a putative NirK-type nitrite reductase, was expressed by *N. winogradskyi*. The transcription of *nirK* in *N. winogradskyi* was unaffected over a pH range of 6.6-8.2 although similar shifts in pH affected NirK expression in *N. europaea* (10). *nirK* was maximally expressed under low O_2 conditions in the presence of NO_2^-, yet curiously, in the absence of NO_2^- and O_2, *nirK* expression was significantly repressed below the levels of *nirK* mRNA detected in aerobic, NO_2^- containing cells suspensions. These data suggest that nitrite reductase may be active in aerobic cultures and especially under low O_2 conditions, but suppressed during anaerobic growth. These observations are not consistent with a report

indicating that a copper-containing nitrite reductase isolated from anaerobically grown cells of *N. vulgaris* was active both *in vitro* and *in vivo* (1). On the other hand, the fact that, 1) NO_2^- accumulated during anaerobic growth (as reported here and by Freitag, et al (55)) and, 2) anaerobic growth did not occur if NO_2^- was present, suggests that the nitrite reductase is either not active, or the rate of NO_2^- reduction is slower than the rate of NO_3^- reduction by NXR. Although a previous report claimed that NO was formed by NO_3^- reduction under anaerobic conditions, the majority (~99%) of the NO_3^- was recovered as NO_2^- and ammonia indicating that NO was a minor product (55). Because NO_2^- appears to be required for expression of *nirK*, it is possible that *nirk* may be expressed and NO may be formed during anaerobic growth after NO_2^- accumulates to a critical level. Nevertheless, because NO_2^- accumulation inhibits anaerobic growth (presumably through an inhibition of NXR nitrate reductase activity), and NO_2^- could not serve as a terminal electron acceptor (S.R.Starkenburg, unpublished results), expression of *nirK* would be futile from an energetics perspective.

These data also suggest that the observed arrest of NO_2^--dependent O_2 uptake may be the result of NO binding to cytochrome oxidase. Cyanide treated cells did not consume NO, and cyanide was previously shown to inhibit the cytochrome aa_3 of *N. winogradskyi* strain agilis (188). The binding of NO to cytochrome oxidase is reversible (39) and was reported to be up to 8 orders of magnitude faster than the rate of dissociation (18, 136). This is consistent with the observation that μmolar concentrations of NO arrested NO_2^--dependent O_2 uptake within seconds, yet, the metabolism of NO and restoration of O_2 uptake required several minutes. The concentration of free NO in denitrifying bacterial cultures is kept low (1-30 nM) as a means to avoid the unwanted toxic interactions of NO with other cellular material (194, 195). Indeed, accumulation of NO via a mutation in the NO reductase was lethal to the denitrifier, *P. stutzeri* (194). Yet, even after *N. winogradskyi* was exposed to a 50% NO atmosphere for 90 minutes, the bacterium remained viable and NO_2^- was still oxidized (S.R. Starkenburg, unpublished results) suggesting than this bacterium is well equipped to mitigate any negative impacts of NO and may produce NO to control cytochrome

oxidase activity when environmental conditions become unfavorable for nitrite oxidation.

In summary, an examination of NO metabolism by NOB is a challenging task given the highly reactive nature of NO, especially in the context of aerobic respiration. NO was not detected during aerobic oxidation of NO_2^- because NO was readily consumed by *N. winogradskyi,* and NO abiotically reacted with O_2 that ultimately led to the formation of NO_2^-. The interpretation of the data is further complicated by the fact that metabolism of NO by cytochrome oxidase also results in the formation of NO_2^- (170). Nonetheless, in light of this information, the previously reported NO-dependent stimulation of NADH synthesis by *N. winogradskyi* and *N. vulgaris* under aerobic and anaerobic conditions (54) may be the result of metabolism of NO_2^-, chemically produced from the auto-oxidation of NO. Furthermore, Freitag and Bock (54) bubbled NO through aerobic cell suspensions and found most of the NO was recovered as NO_2^-, not NO_3^-, suggesting that NO was inhibiting NO_2^- oxidation and respiration, much like it did in these experiments. On the other hand, if the hypothesis that the cytochrome oxidase is inhibited (yet NXR is unaffected) by NO is correct, more electrons from NO_2^- oxidation via NXR would be channeled through reverse electron flow to reduce NAD^+ and be available for reductive biosynthesis of a storage product such as poly-β-hydroxybutyrate (Figure 5.6). Indeed, *N. hamburgensis* cells in the anaerobic zone of a biofilm contained significantly higher quantities of PHB granules compared to cells in the aerobic zone (55). Clearly, further investigation into the function and role of *nirk* and the production and metabolism of NO in *Nitrobacter* is warranted and remains a fascinating avenue of research.

ACKNOWLEDGEMENTS

Funding was provided to S. R. Starkenburg by the Subsurface Biosphere Integrative Graduate Education and Research Traineeship (IGERT) grant 0114427-DGE at Oregon State University from the National Science Foundation's Division of Graduate Education. Special thanks to Kim Halsey, Allen Milligan, and Mike Behrenfeld for technical assistance and use of their membrane-inlet mass spectrometer. Special thanks to Kristine Robinson for technical assistance with NO measurements and to Joseph Beckman for helpful scientific discussions and use of his equipment.

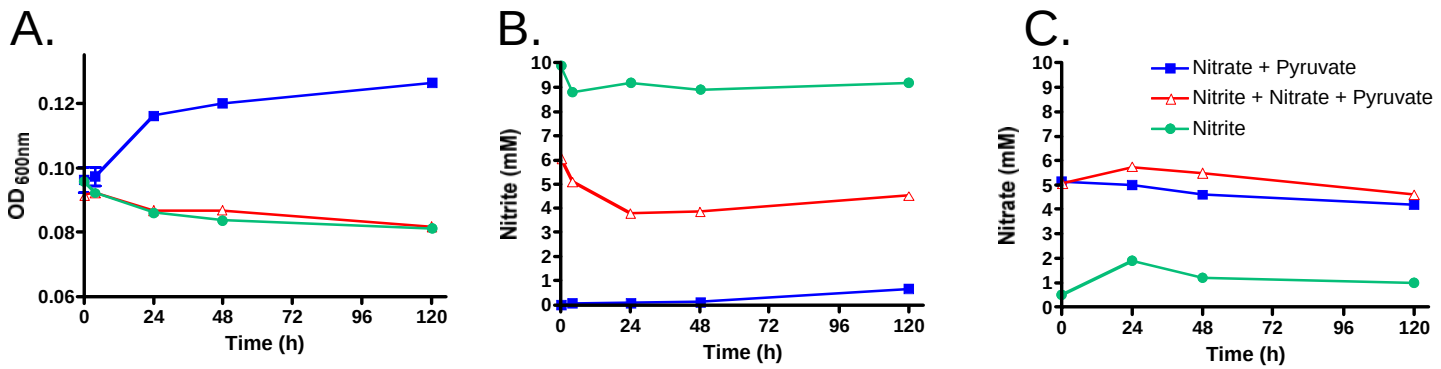


Figure 5.1. Anaerobic growth of *N. winogradskyi.* The optical density (Panel A) and the production/consumption of NO_2^- (Panel B) and NO_3^- (Panel C) were monitored during the growth period. Cells were inoculated into sealed bottles containing de-oxygenated medium. Air in the headspace was evacuated and replaced with nitrogen gas. An atmospheric concentration of CO_2 was also added back the headspace.

Table 5.1. Effect of O_2 and pH on *nirk* transcription.

	Treatment		NO_2^- consumed (mM)	*nirK* expression*
	Oxygen (%)	pH		
Experiment 1	2	7.2	2.1	34.3
	4	7.2	3.3	20.3
	10	7.2	9.4	4.8
	20	7.2	13.3	1.0
Experiment 2	20	6.6	14.0	1.0
	20	7.2	26.2	1.0
	20	8.2	23.3	0.9

*Values of *nirK* transcript were normalized to 16s rRNA in each treatment using the 2-ΔΔCT method and are expressed as the ratio of the amount of *nirk* measured in a given treatment versus standard culture conditions (20% O_2 treatment at pH 7.2 in experiments 1 and 2, respectively) vs each additional treatment. Cells were harvested after 3 hrs of treatment. The disparity in NO_2^- consumption rates between experiment 1 and 2 is a result of using different cell concentrations (Exp 1= $OD_{600} \approx 0.33$; Exp. 2 $OD_{600} \approx 0.5$). The initial concentration of NO_2^- in all treatments was 30mM.

Table 5.2. Effect of NO_2^- and O_2 limitation on *nirK* expression.

	Treatment			*nirK* expression*
	Oxygen (%)	NO_2- (mM)	NO_3-/ pyruvate (mM)	
Experiment 1	20	30	0	1.0
	0	0	5/5	0.3
	0	30	0	20.5
Experiment 2	20	10	0	1.00
	0	0	5/5	0.02

*Values of *nirK* transcript were normalized to 16s rRNA in each treatment using the 2-ΔΔCT method, and are expressed as the ratio of the amount of *nirk* measured in a given treatment relative to standard culture conditions (20% O_2, pH 7.2, in experiments 1 and 2, respectively).

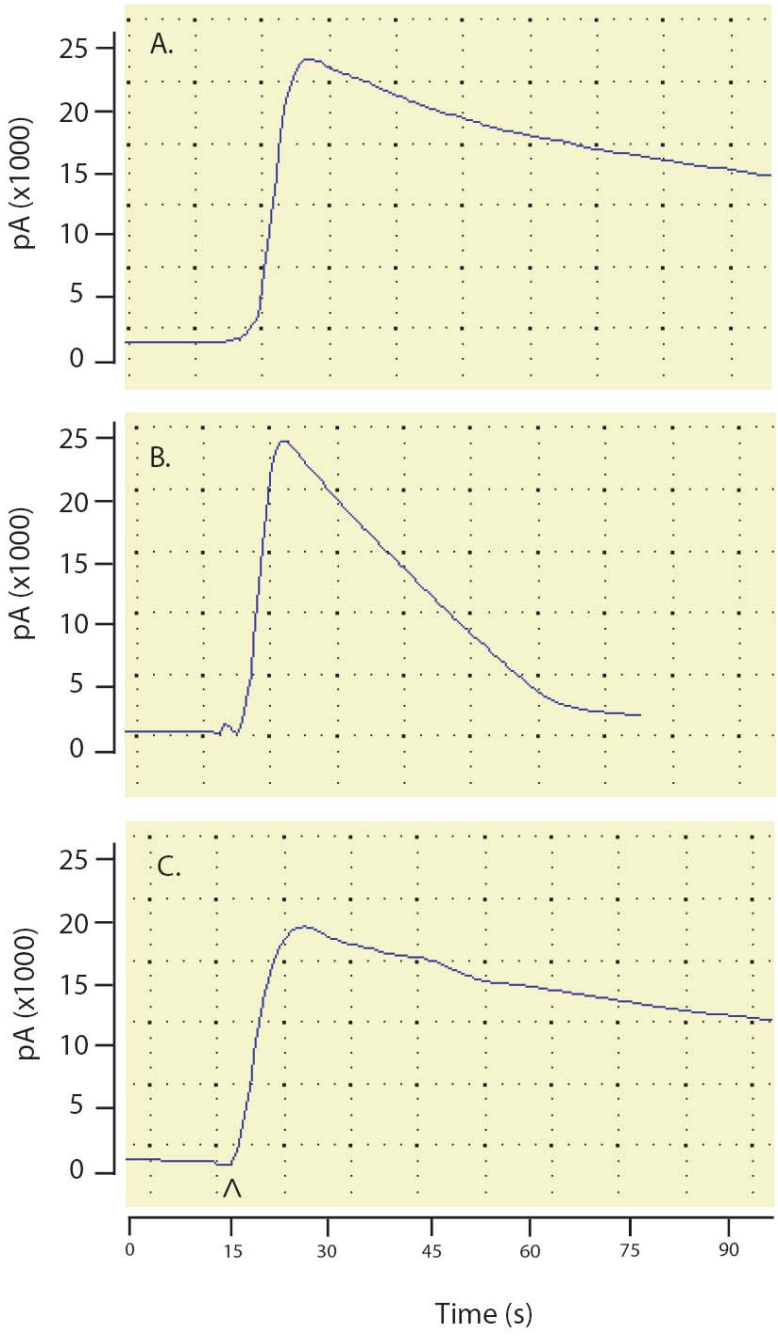


Figure 5.2. Consumption of NO by *N. winogradskyi***.** 3.6 nmoles of NO was added to a 1.8 ml reservoir containing phosphate buffer (Panel A), phosphate buffer plus viable *N. winogradakyi* (Panel B), or heat killed *N. winogradskyi* cells (Panel C).

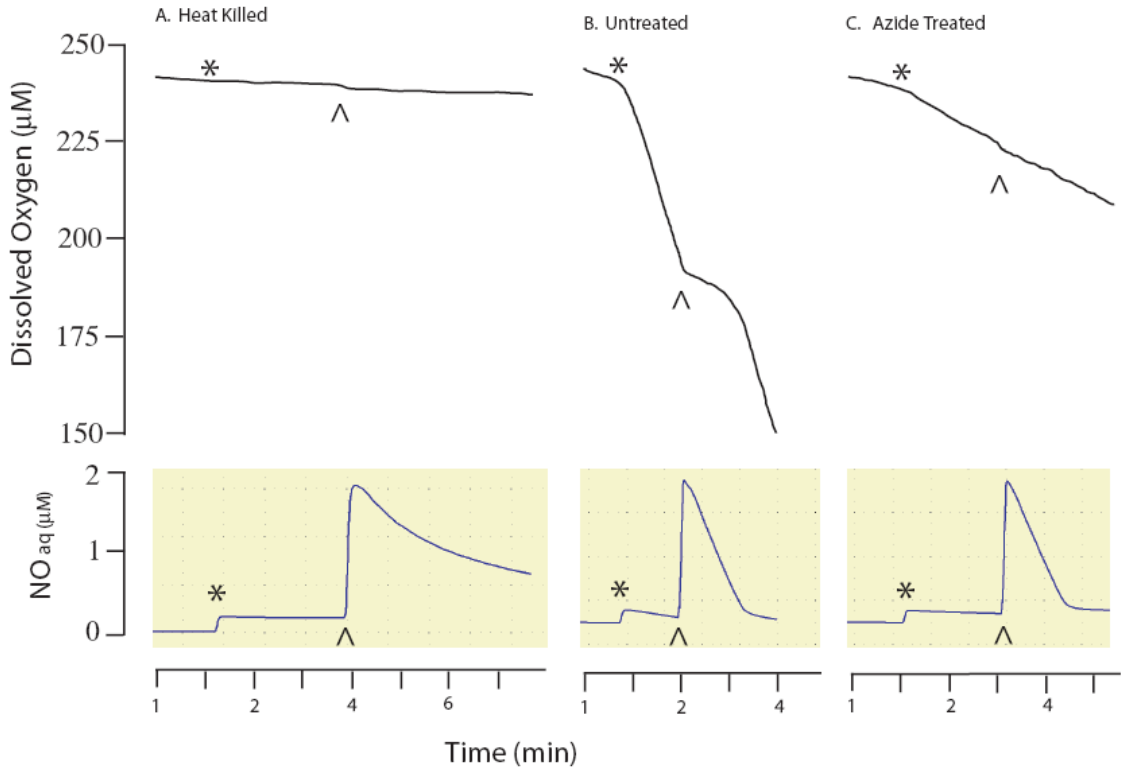


Figure 5.3. NO and O_2 consumption by *N. winogradskyi***.** 3.6 nmoles of NO was added to a 1.8 ml reservoir containing heat killed (Panel A), viable (B), or azide treated (Panel C) cells of *N. winogradskyi* suspended in phosphate buffer (pH 7.5). The star (*) and the caret (^) indicate the times of injection of NO_2^- and NO, respectively.

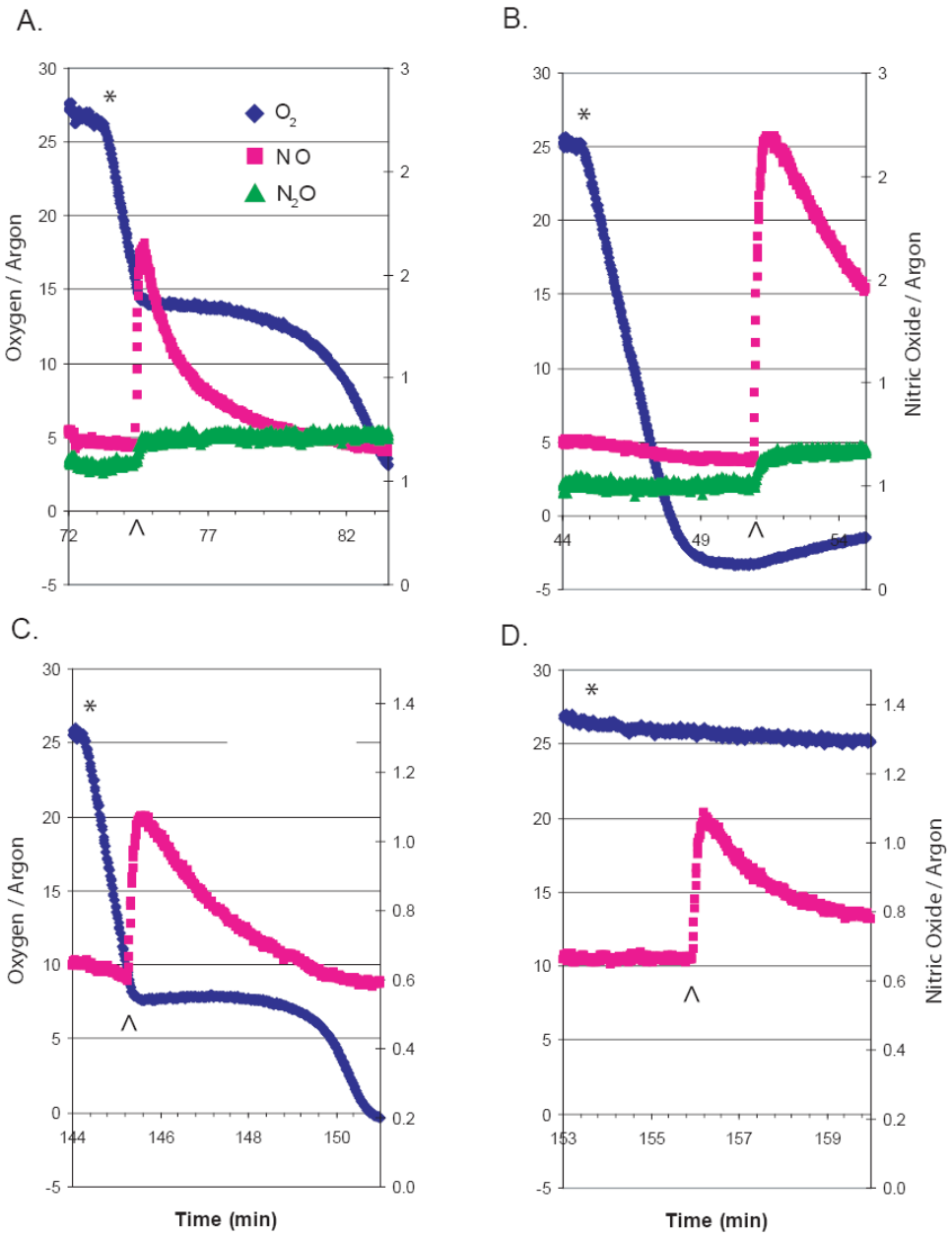


Figure 5.4. Effect of O_2 and cyanide on NO consumption by *N. winogradskyi*. O_2 (blue diamond), NO (pink square) and N_2O (green triangle) were measured in the aqueous phase in cell suspensions of *N. winogradskyi* during NO_2^--dependent respiration. NO was injected into the reservoir containing viable (Panels A,B,and C) or cyanide treated (Panel D) cells of *N. winogradsky*. NO was injected before (Panel A,C,D) and after (Panel B) the depletion of O_2. The graphs were generated from replicate injections of the same batch of cells. The initial concentrations of NO were 7 µM NO and 3.5 µM NO in Panels A/B and C/D, respectively.

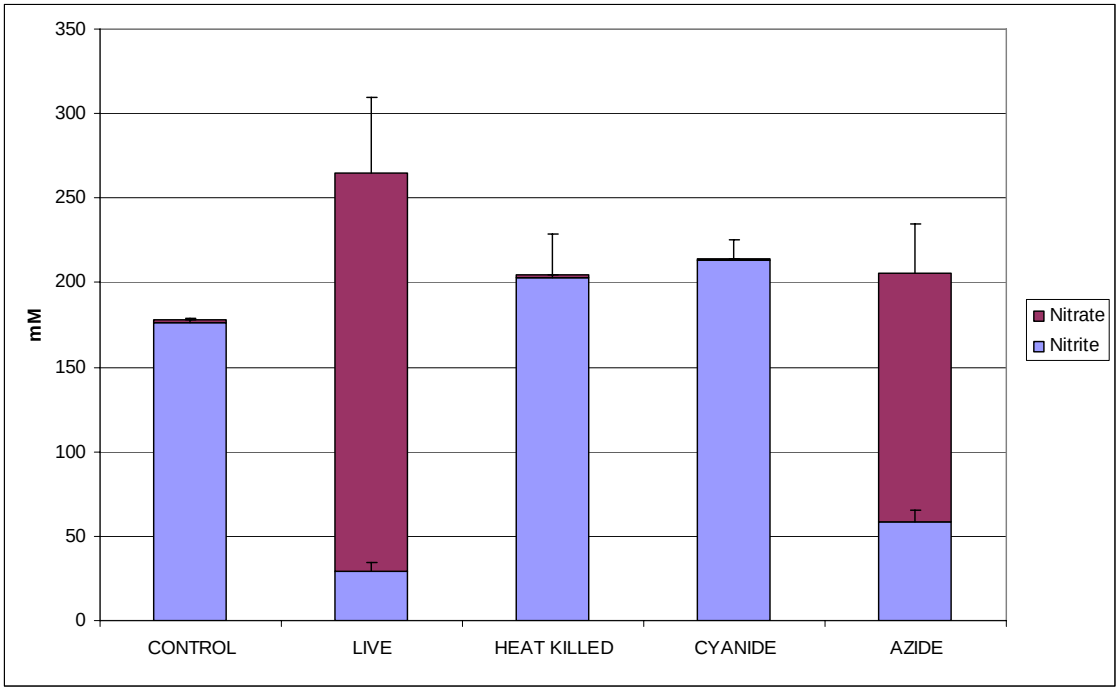


Figure 5.5. Chemical and biological transformation of NO to NO_2^- and NO_3^-. 25 µl of NO gas was added to the headspace of a 10 ml sealed vial containing 5 ml of phosphate buffer (control), or phosphate buffer with viable (LIVE), heat killed, cyanide (60 µM), or azide (60 µM) treated cells of *N. winogradskyi*.

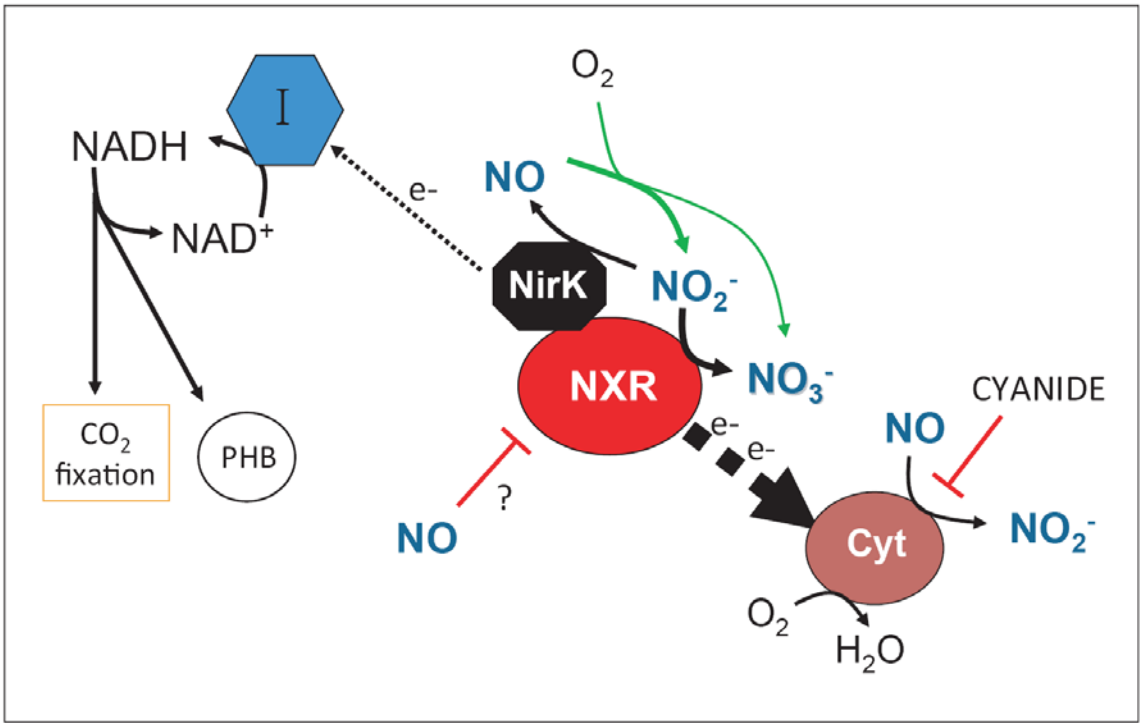


Figure 5.6. Model of NO metabolism. Black arrows indicate biological reactions. Green arrows indicate chemical transformations. (Abbreviations- PHB: polyhydroxybutyrate, Cyt: cytochrome aa3 oxidase, NXR: nitrite oxidoreductase, NirK: nitrite reductase, I: NADH dehydrogenase (Complex I)).

Chapter 6

Summary

Through an individual and comparative genomic analysis of *Nitrobacter,* new insights into the physiology of NOB were realized, particularly with respect to metabolism of lactate and the reduction of NO_2^- to NO. Generally, annotation of the *N. winogradskyi* genome revealed the genes encoding pathways for known modes of lithotrophic and heterotrophic growth, including multiple enzymes involved in anapleurotic reactions centered on the metabolism of C2 to C4 organic acids. Members of the Genus *Nitrobacter* do not appear to be well equipped to metabolize hexose sugars as a classical glycolysis pathway could not be reconstructed in *N. winogradskyi* due to the absence of a gene candidate for phosphofructokinase. Active transporters for sugars were also absent in *N. winogradskyi* and sparse in *N. hamburgensis* and NB311A. Nonetheless, the heterotrophic potential of *N. hamburgensis* may be greater than what was previously known as the genome harbors many genes not found in *N. winogradskyi*, including a complete glycolysis pathway, unique electron transport components (b-type cytochromes and alternative terminal oxidases), and putative pathways for the catabolism of aromatic (phenylacetate, homogentisate), organic (lactate, glycolate) and one-carbon compounds (formate, carbon monoxide). Several (2-3) FAD-dependent oxidases were identified in each *Nitrobacter* genome which suggested that lactate could be metabolized, providing reductant and carbon to the cell. Indeed, in this study, D-lactate enhanced the growth rate and yield of *N. hamburgensis* in the presence of NO_2^- and served as a sole energy and carbon source in the absence of NO_2^-. D-lactate consumption occurred constitutively in lithoautotrophically grown cells, yet a physiological adaptation to lactate was observed as D-lactate grown cells consumed and assimilated lactate at a faster rate than NO_2^- grown cells, and D-lactate-dependent O_2 uptake was significantly greater in cells grown heterotrophically or mixotrophically compared to cells grown lithoautotrophically. Nevertheless, D-lactate

could not substitute for CO_2 as the sole carbon source (lithoheterotrophy) during growth in the presence of NO_2^- and lactate assimilation appears to be suppressed by NO_2^- when *Nitrobacter* is limited for CO_2. The inability of *Nitrobacter* to maximally metabolize lactate during the NO_2^- induced CO_2 limitation may be indirectly caused by an absence of oxidized electron carriers. Normally, reduced electron carriers generated via NO_2^- oxidation are turned over by the high energy demands of the Calvin cycle as CO_2 serves as a strong reductant sink to help drive an energetically unfavorable reverse flow of electrons from NO_2^-. Yet, in the absence of CO_2 (or Calvin cycle activity), oxidized electron carriers may not be regenerated efficiently and could be unavailable to serve as electron acceptors to oxidize lactate via LDH. Use of CO_2 fixation as a reductant sink is not unprecedented as many Alphaproteobacteria require CO_2 as a reductant sink to balance their redox potential when growing photoheterotrophically (45, 143). This same mechanism may be at work in *Nitrobacter* although, unlike many facultative lithoautotrophs, the inability of *Nitrobacter* to stop fixing CO_2 in the presence of NO_2^- hinders its heterotrophic potential.

Through a comparative analysis of the *Nitrobacter* genomes with its close non-nitrite oxidizing alphaproteobacterial relatives, many genes that are potentially indicative of a nitrite-oxidizing lifestyle were identified. Several genes previously known to be involved in nitrogen metabolism were identified, including nitrite transport and regulation, the gene cluster encoding the subunits of the NXR, and the cytochromes and putative regulatory protein adjacent to the putative gene encoding a dissimilatory nitrite reductase (*nirK)*. Strikingly, the genomes of *Rhodopseudomonas palustris* and/or *Bradyrhizobium japonicum* contain homologs of several genes in the subcore inventory (PII, *nirK,* cytochromes, regulators), indicating that the subcore genes have diverged significantly from, or have origins outside of, the alphaproteobacterial lineage. Thus, the subcore appears to encode a collection of genes which are not indicative of its evolutionary origins, but instead reflects the ecological niche (nitrification, denitrification) of *Nitrobacter* achieved through assimilation, modification, and expression of genes acquired from more distant bacterial lineages.

While the function of many subcore genes is currently unknown, the co-occurrence of the *nirK* operon in distinct evolutionary lineages of nitrifying bacteria (betaproteobacterial AOB vs alphaproteobacterial NOB) may also indicate their global importance for nitrification. Indeed, in this study, the putative *nirK* in *N. winogradskyi* was found to be expressed and maximally transcribed under low oxygen in the presence of NO_2^-. Surprisingly, transcription of *nirK* was not detected under anaerobic conditions. Production of NO under aerobic conditions was not detected, yet, NO was consumed in a cyanide-sensitive process and reversibly inhibited NO_2^--dependent O_2 uptake, suggesting that NO is metabolized by, or electrons from NO flow through, cytochrome oxidase. Consumption of NO through abiotic mechanisms also occurred, and resulted in the formation of NO_2^- (metabolism of NO by cytochrome oxidase would also result in the formation of NO_2^- (170)). In light of this new information, the NO-dependent stimulation of NADH synthesis by *Nitrobacter* under aerobic and anaerobic conditions reported by Freitag and Bock (54) could simply be a result of the metabolism of NO_2^- produced from the auto-oxidation of NO. On the other hand, if cytochrome oxidase is inhibited (yet NXR is unaffected) by NO, more electrons from NO_2^- oxidation via NXR could be channeled through reverse electron flow to generate reducing equivalents for maintainence energy or for the reductive biosynthesis of storage products such as poly-β-hydroxybutyrate to manage the redox state of the cell. Indeed, *N. hamburgensis* contained significantly higher quantities of PHB granules in the anaerobic zone of a biofilm compared to cells in the aerobic zone (55). Thus, NO_2^- reduction to NO may be particularly important under low oxygen environments as a sufficient supply of oxidized electron acceptors would not be available. Further investigation into the function and role of *nirk*, and the production and metabolism of NO in aerobic and sub-oxic environments is warranted.

.

Bibliography

1. **Ahlers, B., Konig, W. and E. Bock.** 1990. Nitrite reductase activity in *Nitrobacter vulgaris*. FEM Microbiol. Lett **67:**121-126.

2. **Aleem, M. I. H., and D. L. Sewell.** 1984. Oxidoreductase systems in *Nitrobacter agilis*, p. 185-210. *In* W. R. Strohl and O. H. Tuovinen (ed.), Microbial Chemoautotrophy. Ohio State University Press, Columbus, OH.

3. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones.** 2003. Bacterial iron homeostasis. FEMS Microbiol Rev **27:**215-37.

4. **Arkin, I. T., H. Xu, M. O. Jensen, E. Arbely, E. R. Bennett, K. J. Bowers, E. Chow, R. O. Dror, M. P. Eastwood, R. Flitman-Tene, B. A. Gregersen, J. L. Klepeis, I. Kolossvary, Y. Shan, and D. E. Shaw.** 2007. Mechanism of Na^+/H^+ antiporting. Science **317:**799-803.

5. **Auran, T. B., and E. L. Schmidt.** 1976. Lipids of *Nitrobacter* and effects of cultural conditions on fatty acid composition. Biochim Biophys Acta **431:**390-8.

6. **Bardin, S. D., R. T. Voegele, and T. M. Finan.** 1998. Phosphate assimilation in *Rhizobium* (*Sinorhizobium*) *meliloti*: identification of a pit-like gene. J Bacteriol **180:**4219-26.

7. **Bartosch, S., C. Hartwig, E. Spieck, and E. Bock.** 2002. Immunological detection of *Nitrospira*-like bacteria in various soils. Microb Ecol **43:**26-33.

8. **Bartosch, S., I. Wolgast, E. Spieck, and E. Bock.** 1999. Identification of nitrite-oxidizing bacteria with monoclonal antibodies recognizing the nitrite oxidoreductase. Appl Environ Microbiol **65:**4126-33.

9. **Beaumont, H. J., N. G. Hommes, L. A. Sayavedra-Soto, D. J. Arp, D. M. Arciero, A. B. Hooper, H. V. Westerhoff, and R. J. van Spanning.** 2002. Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. J Bacteriol **184:**2557-60.

10. **Beaumont, H. J., S. I. Lens, W. N. Reijnders, H. V. Westerhoff, and R. J. van Spanning.** 2004. Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor. Mol Microbiol **54:**148-58.

11. **Beaumont, H. J., S. I. Lens, H. V. Westerhoff, and R. J. van Spanning.** 2005. Novel *nirK* cluster genes in *Nitrosomonas europaea* are required for NirK-dependent tolerance to nitrite. J Bacteriol **187:**6849-51.

12. **Belser, L. W.** 1977. Nitrate reduction to nitrite, a possible source of nitrite for growth of nitrite-oxidizing bacteria. Appl Environ Microbiol **34:**403-10.

13. **Bendtsen, J., H. Nielsen, D. Widdick, T. Palmer, and S. Brunak.** 2005. Prediction of twin-arginine signal peptides. BMC bioinformatics **6:**167-174.

14. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol **340:**783-795.

15. **Berben, G.** 1996. *Nitrobacter winogradskyi* cytochrome c oxidase genes are organized in a repeated gene cluster. Antonie Van Leeuwenhoek **69:**305-15.

16. **Bergmann, D. J., J. A. Zahn, A. B. Hooper, and A. A. DiSpirito.** 1998. Cytochrome P460 Genes from the Methanotroph *Methylococcus capsulatus*. Bath. Journal of Bacteriology **180:**6440-6445.

17. **Berks, B. C., S. J. Ferguson, J. W. Moir, and D. J. Richardson.** 1995. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim Biophys Acta **1232:**97-173.

18. **Blackmore, R. S., C. Greenwood, and Q. H. Gibson.** 1991. Studies of the primary oxygen intermediate in the reaction of fully reduced cytochrome oxidase. J Biol Chem **266:**19245-9.

19. **Blasco, F., J. P. Dos Santos, A. Magalon, C. Frixon, B. Guigliarelli, C. L. Santini, and G. Giordano.** 1998. NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of *Escherichia coli*. Molecular Microbiology **28:**435-447.

20. **Bock, E.** 1976. Growth of *Nitrobacter* in the presence of organic matter. II. Chemoorganotrophic growth of *Nitrobacter agilis*. Arch Microbiol **108:**305-12.

21. **Bock, E., H.P. Koops, H. Harms, and B. Ahlers.** 1991. The Biochemistry of Nitrifying Organisms, p. 171-200, Variations in Autotrophic Life. Academic Press, San Diego, California.

22. **Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert.** 1990. A new facultatively nitrite oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch. Microbiol. **153:**105-110.

23. **Bock, E., Koops, H.P., Harms, H.** 1986. Cell Biology of Nitrifiers, p. 17-38. *In* J. I. Prosser (ed.), Nitrification, vol. 20. IRL, Oxford ; Washington, D.C.

24. **Bock, E., Sundermeyer-Klinger, H., and E. Stackebrandt.** 1983. New facultative lithoautotrophic nitrite-oxidizing bacteria. Arch Microbiol **136:**281-284.

25. **Bock, E., P. A. Wilderer, and A. Freitag.** 1988. Growth of *Nitrobacter* in the absence of dissolved oxygen. Wat. Res. **22:**245-250.

26. **Boel, G., I. Mijakovic, A. Maze, S. Poncet, M. K. Taha, M. Larribe, E. Darbon, A. Khemiri, A. Galinier, and J. Deutscher.** 2003. Transcription regulators potentially controlled by HPr kinase/phosphorylase in Gram-negative bacteria. J Mol Microbiol Biotechnol **5:**206-15.

27. **Bottomley, P. J., A. E. Taylor, S. A. Boyle, S. K. McMahon, J. J. Rich, K. Cromack, Jr., and D. D. Myrold.** 2004. Responses of nitrification and ammonia-oxidizing bacteria to reciprocal transfers of soil between adjacent coniferous forest and meadow vegetation in the Cascade Mountains of Oregon. Microb Ecol **48:**500-8.

28. **Braun, V., and C. Herrmann.** 1993. Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli:* cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. Mol Microbiol **8:**261-8.

29. **Bren, A., and M. Eisenbach.** 2000. How signals are heard during bacterial chemotaxis: protein-protein interactions in sensory signal propagation. J Bacteriol **182:**6865-73.

30. **Cannon, G. C., Baker, S. H., Soyer, F., Johnson, D. R., Bradburne, C. E., Mehlman, J. L., Davies, P. S., Jiang, Q. L., Heinhorst, S., and J.M. Shively.** 2003. Organization of carboxysome genes in the *Thiobacilli*. Curr Microbiol **46:**115-9.

31. **Cantera, J. J., and L. Y. Stein.** 2007. Molecular diversity of nitrite reductase genes (*nirK*) in nitrifying bacteria. Environ Microbiol **9:**765-76.

32. **Cantera, J. J., and L. Y. Stein.** 2007. Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. Arch Microbiol **188:**349-54.

33. **Chain, P., J. Lamerdin, F. Larimer, W. Regala, V. Lao, M. Land, L. Hauser, A. Hooper, M. Klotz, J. Norton, L. Sayavedra-Soto, D. Arciero, N. Hommes, M. Whittaker, and D. Arp.** 2003. Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J Bacteriol **185:**2759-73.

34. **Cheesman, M. R., S. J. Ferguson, J. W. Moir, D. J. Richardson, W. G. Zumft, and A. J. Thomson.** 1997. Two enzymes with a common function but

different heme ligands in the forms as isolated. Optical and magnetic properties of the heme groups in the oxidized forms of nitrite reductase, cytochrome cd1, from *Pseudomonas stutzeri* and *Thiosphaera pantotropha*. Biochemistry **36:**16267-76.

35. **Chen, R., A. A. Bhagwat, R. Yaklich, and D. L. Keister.** 2002. Characterization of *ndvD*, the third gene involved in the synthesis of cyclic beta-(1 --> 3),(1 --> 6)-D-glucans in *Bradyrhizobium japonicum*. Can J Microbiol **48:**1008-16.

36. **Cho, C. M.-H., T. Yan, X. Liu, L. Wu, J. Zhou, and L. Y. Stein.** 2006. Transcriptome of *Nitrosomonas europaea* with a disrupted nitrite reductase (*nirK*) gene Appl. Environ. Microbiol. **72:**4450-4454.

37. **Clegg, S., F. Yu, L. Griffiths, and J. A. Cole.** 2002. The roles of the polytopic membrane proteins NarK, NarU and NirC in *Escherichia coli* K-12: two nitrate and three nitrite transporters. Molecular Microbiology **44:**143-155.

38. **Cobley, J. G.** 1984. Oxidation of nitrite and formate in *Nitrobacter* membrane preparations: evidence that both reactions are catalyzed by the same enzyme, p. 169-183. *In* W. R. Strohl and O. H. Tuovinen (ed.), Microbial Chemoautotrophy. Ohio State University Press, Ohio.

39. **Cooper, C. E.** 2002. Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? Trends Biochem Sci **27:**33-9.

40. **Daims, H., J. L. Nielsen, P. H. Nielsen, K. H. Schleifer, and M. Wagner.** 2001. In situ characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl Environ Microbiol **67:**5273-84.

41. **Daims, H., Nielsen, P.H., Nielsen, J.L., Juretschko, S. and M. Wagner.** 2000. Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology. Wat. Sci. Tech. **41:**85-90.

42. **de Vries, S., and I. Schröder** 2002. Comparison between the nitric oxide reductase family and its aerobic relatives, the cytochrome oxidases. Biochem Soc Trans **30:**662-7.

43. **Delwiche, C. C., and M. S. Feinstein.** 1965. Carbon and Energy Sources for the Nitrifying Autotroph *Nitrobacter*. Journal of Bacteriology **60:**102-107.

44. **Ditty, J. L., A. C. Grimm, and C. S. Harwood.** 1998. Identification of a chemotaxis gene region from *Pseudomonas putida*. FEMS Microbiol Lett **159:**267-73.

45. **Dubbs, J. M., and F. R. Tabita.** 2004. Regulators of nonsulfur purple phototrophic bacteria and the interactive control of CO_2 assimilation, nitrogen fixation, hydrogen metabolism and energy generation. FEMS Microbiol Rev **28:**353-76.

46. **Einsle, O., and P. M. H. Kroneck.** 2004. Structural basis of denitrification. Biol. Chem. **385:**875-883.

47. **Eisen, J. A., K. E. Nelson, I. T. Paulsen, J. F. Heidelberg, M. Wu, R. J. Dodson, R. Deboy, M. L. Gwinn, W. C. Nelson, D. H. Haft, E. K. Hickey, J. D. Peterson, A. S. Durkin, J. L. Kolonay, F. Yang, I. Holt, L. A. Umayam, T. Mason, M. Brenner, T. P. Shea, D. Parksey, W. C. Nierman, T. V. Feldblyum, C. L. Hansen, M. B. Craven, D. Radune, J. Vamathevan, H. Khouri, O. White, T. M. Gruber, K. A. Ketchum, J. C. Venter, H. Tettelin, D. A. Bryant, and C. M. Fraser.** 2002. The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc Natl Acad Sci U S A **99:**9509-14.

48. **Elmore, B. O., D. J. Bergmann, M. G. Klotz, and A. B. Hooper.** 2007. Cytochromes P460 and c'-beta; a new family of high-spin cytochromes c. FEBS Lett **581:**911-6.

49. **Erickson, R. H., and A. B. Hooper.** 1972. Preliminary characterization of a variant co-binding heme protein from *Nitrosomonas*. Biochim Biophys Acta **275:**231-44.

50. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res **8:**186-94.

51. **Ewing, B., L. Hillier, M. C. Wendl, and P. Green.** 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res **8:**175-85.

52. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, and et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496-512.

53. **Frankenberg, N., J. Moser, and D. Jahn.** 2003. Bacterial heme biosynthesis and its biotechnological application. Applied Microbiology and Biotechnology **63:**115-127.

54. **Freitag, A., and E. Bock.** 1990. Energy conservation in *Nitrobacter*. FEM Microbiol. Lett **66:**157-162.

55. **Freitag, A., M. Rudert, and E. Bock.** 1987. Growth of *Nitrobacter* by dissimilatoric nitrate reduction. FEMS Microbiol Lett **48:**105-109.

56. **Freitag, T. E., L. Chang, C. D. Clegg, and J. I. Prosser.** 2005. Influence of inorganic nitrogen management regime on the diversity of nitrite-oxidizing bacteria in agricultural grassland soils. Appl Environ Microbiol **71:**8323-34.

57. **Friedrich, C. G., F. Bardischewsky, D. Rother, A. Quentmeier, and J. Fischer.** 2005. Prokaryotic sulfur oxidation. Curr Opin Microbiol **8:**253-9.

58. **Friedrich, C. G., D. Rother, F. Bardischewsky, A. Quentmeier, and J. Fischer.** 2001. Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? Appl Environ Microbiol **67:**2873-82.

59. **Fuhrmann, S., M. Ferner, T. Jeffke, A. Henne, G. Gottschalk, and O. Meyer.** 2003. Complete nucleotide sequence of the circular megaplasmid pHCG3 of *Oligotropha carboxidovorans*: function in the chemolithoautotrophic utilization of CO, H-2 and CO2. Gene **322:**67-75.

60. **Galloway, J. N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., and B. Jack Cosby.** 2003. The Nitrogen Cascade. Bioscience **53:**341-356.

61. **Galloway, J. N., and E. B. Cowling.** 2002. Reactive nitrogen and the world: 200 years of change. Ambio **31:**64-71.

62. **Garcia-Estepa, R., M. Argandona, M. Reina-Bueno, N. Capote, F. Iglesias-Guerra, J. J. Nieto, and C. Vargas.** 2006. The *ectD* gene, which is involved in the synthesis of the compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic bacterium *Chromohalobacter salexigens*. J Bacteriol **188:**3774-84.

63. **Garcia-Estepa, R., D. Canovas, F. Iglesias-Guerra, A. Ventosa, L. N. Csonka, J. J. Nieto, and C. Vargas.** 2006. Osmoprotection of *Salmonella enterica* serovar *Typhimurium* by Nγ-acetyldiaminobutyrate, the precursor of the compatible solute ectoine. Syst Appl Microbiol **29:**626-33.

64. **Garvie, E. I.** 1980. Bacterial lactate dehydrogenases. Microbiol Rev **44:**106-39.

65. **Gieseke, A., L. Bjerrum, M. Wagner, and R. Amann.** 2003. Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. Environ Microbiol **5:**355-69.

66. **Gordon, D., C. Abajian, and P. Green.** 1998. Consed: a graphical tool for sequence finishing. Genome Res **8:**195-202.

67. **Grobler, J., F. Bauer, R. E. Subden, and H. J. Van Vuuren.** 1995. The mae1 gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. Yeast **11:**1485-91.

68. **Hageman, R. H., and D. P. Hucklesby.** 1971. Nitrate reductase in higher plants. Methods Enzymol **23:**491-503.

69. **Hancock, R. E., K. Poole, and R. Benz.** 1982. Outer membrane protein P of *Pseudomonas aeruginosa*: regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. J Bacteriol **150:**730-8.

70. **Harris, S., Ebert, A., Schutze, E., Diercks, M., Bock, E., and J.M. Shively.** 1988. Two different genes and gene products for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCOase) in *Nitrobacter hamburgensis*. FEMS Microbiol Lett **49:**267-271.

71. **Hendriks, J., Arthur Oubrie, Jose Castresana, Andrea Urbani, Sabine Gemeinhardt and Matti Saraste.** 2000. Nitric oxide reductases in bacteria. Biochim Biophys Acta **1459:**266-273.

72. **Hofmann, K., and W. Stoffel.** 1993. TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler **374:**166.

73. **Holtzendorff, J., D. Hung, P. Brende, A. Reisenauer, P. H. Viollier, H. H. McAdams, and L. Shapiro.** 2004. Oscillating global regulators control the genetic circuit driving a bacterial cell cycle. Science **304:**983-987.

74. **Hooper, A. B., D. M. Arciero, D. Bergmann, and M. P. Hendrich.** 2005. The oxidation of ammonia as an energy source in bacteria in respiration., p. 121-147. *In* D. Zannoni (ed.), Respiration in archaea and bacteria: Diversity of prokaryotic respiratory systems, vol. 16. Springer, Dordrecht, the Netherlands.

75. **Horikiri, S., Y. Aizawa, T. Kai, S. Amachi, H. Shinoyama, and T. Fujii.** 2004. Electron acquisition system constructed from an NAD-independent D-lactate dehydrogenase and cytochrome c2 in *Rhodopseudomonas palustris* No. 7. Biosci Biotechnol Biochem **68:**516-22.

76. **Horz, H. P., A. Barbrook, C. B. Field, and B. J. Bohannan.** 2004. Ammonia-oxidizing bacteria respond to multifactorial global change. Proc Natl Acad Sci U S A **101:**15136-41.

77. **Houghton, J., Y. Ding, D. Griggs, M. Noguer, and P. J. van der Linden.** 2001. Climate change 2001: the scientific basis. Cambridge University Press, Cambridge, UK.

78. **Hovanec, T. A., L. T. Taylor, A. Blakis, and E. F. Delong.** 1998. *Nitrospira*-Like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria. Appl Environ Microbiol **64:**258-264.

79. **Howell, M. L., E. Alsabbagh, J.-F. Ma, U. A. Ochsner, M. G. Klotz, T. J. Beveridge, K. M. Blumenthal, E. C. Niederhoffer, R. E. Morris, D. Needham, G. E. Dean, M. A. Wani, and D. J. Hassett.** 2000. AnkB, a Periplasmic Ankyrin-Like Protein in *Pseudomonas aeruginosa*, is Required for Optimal Catalase B (KatB) Activity and Resistance to Hydrogen Peroxide. J. Bacteriol. **182:**4545-4556.

80. **Ida, S., and M. Alexander.** 1965. Permeability of *Nitrobacter agilis* to Organic Compounds. J Bacteriol **90:**151-6.

81. **Jendrossek, D., and R. Handrick.** 2002. Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol **56:**403-32.

82. **Jormakka, M., B. Byrne, and S. Iwate.** 2003. Formate dehydrogenase - a versatile enzyme in changing environments. Curr. Opin. Struct. Biol. **13:**418-423.

83. **Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Roser, H. P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol **64:**3042-51.

84. **Kang, B. S., and Y. M. Kim.** 1999. Cloning and molecular characterization of the genes for carbon monoxide dehydrogenase and localization of molybdopterin, flavin adenine dinucleotide, and iron-sulfur centers in the enzyme of *Hydrogenophaga pseudoflava*. Journal of Bacteriology **181:**5581-5590.

85. **King, G. M.** 2006. Nitrate-dependent anaerobic carbon monoxide oxidation by aerobic CO-oxidizing bacteria. FEMS Microbiol. Ecol. **56:**1-7.

86. **King, G. M., and C. F. Weber.** 2007. Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. Nat. Rev. Microbiol. **5:**107-118.

87. **Kirstein, K., and E. Bock.** 1993. Close genetic relationship between *Nitrobacter hamburgensis* nitrite oxidoreductase and *Escherichia coli* nitrate reductases. Arch Microbiol **160:**447-53.

88. **Kirstein, K. O., Bock, E., Miller, D.J., and D.J.D. Nicholas.** 1986. Membrane-bound b-type cytochromes in *Nitrobacter*. FEMS Microbiol Lett **36:**63-67.

89. **Kobayashi, I.** 2001. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. Nucleic Acids Res **29:**3742-56.

90. **Kobayashi, I.** 1999. Homologous recombination and sex as a strategy against selfish genes attacking the genome. Ann NY Acad Sci **870:**354-6.

91. **Konneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl.** 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437:**543-6.

92. **Koper, T. E., A. F. El-Sheikh, J. M. Norton, and M. G. Klotz.** 2004. Urease-encoding genes in ammonia-oxidizing bacteria. Appl Environ Microbiol **70:**2342-8.

93. **Korshunov, S., and J. A. Imlay.** 2001. A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. Mol Microbiol **43:**95-106.

94. **Kraft, I., and E. Bock.** 1984. Plasmids in *Nitrobacter*. Arch. Microbiol. **140:**79-82.

95. **Kumar, S., and D.J.D. Nicholas.** 1982. Assimilation of Inorganic Nitrogen Compounds by *Nitrobacter agilis*. Journal of General Microbiology **128:**1795-1801.

96. **Larimer, F. W., P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. Do, M. L. Land, D. A. Pelletier, J. T. Beatty, A. S. Lang, F. R. Tabita, J. L. Gibson, T. E. Hanson, C. Bobst, J. L. Torres, C. Peres, F. H. Harrison, J. Gibson, and C. S. Harwood.** 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nat Biotechnol **22:**55-61.

97. **Leigh, J. A., and J. A. Dodsworth.** 2007. Nitrogen Regulation in Bacteria and Archaea. Annu. Rev. Microbiol. **61:**349-77.

98. **Li, L., Y. Jia, Q. Hou, T. C. Charles, E. W. Nester, and S. Q. Pan.** 2002. A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. Proc Natl Acad Sci U S A **99:**12369-74.

99. **Lipski, A., E. Spieck, A. Makolla, and K. Altendorf.** 2001. Fatty acid profiles of nitrite-oxidizing bacteria reflect their phylogenetic heterogeneity. Syst Appl Microbiol **24:**377-84.

100. **Liu, P., D. Wood, and E. W. Nester.** 2005. Phosphoenolpyruvate Carboxykinase Is an Acid-Induced, Chromosomally Encoded Virulence Factor in *Agrobacterium tumefaciens*. J Bacteriol **187:**6039-45.

101. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) Method. Methods **25:**402-8.

102. **Lord, J. M.** 1972. Glycolate oxidoreductase in *Escherichia coli*. Biochim Biophys Acta **267:**227-37.

103. **Lorite, M. J., J. Tachil, J. Sanjuán, O. Meyer, and E. J. Bedmar.** 2000. Carbon monoxide dehydrogenas activity in *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **66:**1871-1876.

104. **Macdonald, R. (ed.).** 1986. Nitrification in Soil: an introductory history, vol. 20. IRL Press, Oxford.

105. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annual Review in Genetics**:**131-158.

106. **Malavolta, E., C. C. Delwiche, and W. D. Burge.** 1962. Formate odixation by cell-free preparations from *Nitrobacter agilis*. Biochim Biophys Acta **57:**347-51.

107. **Markwell, J. P., and J. Lascelles.** 1978. Membrane-bound, pyridine nucleotide-independent L-lactate dehydrogenase of *Rhodopseudomonas sphaeroides*. J Bacteriol **133:**593-600.

108. **Matsumoto, S., A. Terada, Y. Aoi, S. Tsuneda, E. Alpkvist, C. Picioreanu, and M. C. van Loosdrecht.** 2007. Experimental and simulation analysis of community structure of nitrifying bacteria in a membrane-aerated biofilm. Water Sci Technol **55:**283-90.

109. **McFadden, B. a. S., JM.** 1991. Bacterial assimilation of carbon dioxide by the Calvin cycle, p. 25-49. *In* J. M. S. a. L. L. Burton (ed.), Variations in Autotrophic Growth. Harcourt Brace Jovanovich, London.

110. **Merrick, M. J., and J. R. Coppard.** 1989. Mutations in genes downstream of the *rpoN* gene (encoding sigma 54) of *Klebsiella pneumoniae* affect expression from sigma 54-dependent promoters. Mol Microbiol **3:**1765-75.

111. **Meyer, O., L. Gremer, R. Ferner, M. Ferner, H. Dobbek, M. Gnida, W. Meyer-Klaucke, and R. Huber.** 2000. The role of Se, Mo and Fe in the structure and function of carbon monoxide dehydrogenase. Biol Chem **381:**865-76.

112. **Meyer, O., K. Frunzke, D. Gadkari, S. Jacobitz, I. Hugendieck, and M. Kraut.** 1990. Utilization of carbon-monoxide by aerobes-recent advances. FEMS Microbiol. Rev. **87:**253-260.

113. **Min, B., J. T. Pelaschier, D. E. Graham, D. Tumbula-Hansen, and D. Soll.** 2002. Transfer RNA-dependent amino acid biosynthesis: an essential route to asparagine formation. Proc Natl Acad Sci U S A **99:**2678-83.

114. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol **62:**2156-62.

115. **Moeck, G. S., and J. W. Coulton.** 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol Microbiol **28:**675-81.

116. **Mogi, T., K. Saiki, and Y. Anraku.** 1994. Biosynthesis and Functional-Role of Heme-O and Heme-A. Molecular Microbiology **14:**391-398.

117. **Moreno-Vivian, C., and S. J. Ferguson.** 1998. Definition and distinction between assimilatory, dissimilatory and respiratory pathways. Mol Microbiol **29:**661-669.

118. **Moussa, M. S., D. U. Sumanasekera, S. H. Ibrahim, H. J. Lubberding, C. M. Hooijmans, H. J. Gijzen, and M. C. van Loosdrecht.** 2006. Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. Water Res **40:**1377-88.

119. **Naito, T., K. Kusano, and I. Kobayashi.** 1995. Selfish behavior of restriction-modification systems. Science **267:**897-9.

120. **Nelson, D. C., Waterbury, J.B., Jannasch, H.W.** 1982. Nitrogen fixation and nitrate utilization by marine and freshwater *Beggiatoa*. Archives of Microbiology **133:**172-177.

121. **Nomoto, T., Y. Fukumori, and T. Yamanaka.** 1993. Membrane-bound cytochrome c is an alternative electron donor for cytochrome aa3 in *Nitrobacter winogradskyi*. J Bacteriol **175:**4400-4.

122. **Numata, M., T. Saito, T. Yamazaki, Y. Fukumori, and T. Yamanaka.** 1990. Cytochrome P-460 of *Nitrosomonas europaea*: further purification and further characterization. J. Biochem (Tokyo) **108:**1016-21.

123. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol Mol Biol Rev **62:**1-34.

124. **Poole, K., and R. E. Hancock.** 1986. Phosphate-starvation-induced outer membrane proteins of members of the families Enterobacteriaceae and Pseudomonodaceae: demonstration of immunological cross-reactivity with an antiserum specific for porin protein P of *Pseudomonas aeruginosa*. J Bacteriol **165:**987-93.

125. **Poole, P., and D. Allaway.** 2000. Carbon and nitrogen metabolism in Rhizobium. Adv Microb Physiol **43:**117-63.

126. **Poughon, L., C. G. Dussap, and J. B. Gros.** 2001. Energy model and metabolic flux analysis for autotrophic nitrifiers. Biotechnol Bioeng **72:**416-33.

127. **Prosser, J. (ed.).** 1986. Nitrification, vol. 20. IRL Press, Washington DC.

128. **Qi, Z., I. Hamza, and M. R. O'Brian.** 1999. Heme is an effector molecule for iron-dependent degradation of the bacterial iron response regulator (Irr) protein. Proc Natl Acad Sci U S A **96:**13056-61.

129. **Rao, N. N., and A. Torriani.** 1990. Molecular aspects of phosphate transport in *Escherichia coli*. Mol Microbiol **4:**1083-90.

130. **Reijerse, E. J., M. Sommerhalter, P. Hellwig, A. Quentmeier, D. Rother, C. Laurich, E. Bothe, W. Lubitz, and C. G. Friedrich.** 2007. The unusal redox centers of SoxXA, a novel c-type heme-enzyme essential for chemotrophic sulfur-oxidation of *Paracoccus pantotrophus*. Biochemistry **46:**7804-10.

131. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146 (Pt 3):**551-71.

132. **Roberts, R. J., T. Vincze, J. Posfai, and D. Macelis.** 2005. REBASE--Restriction enzymes and DNA methyltransferases Nucl. Acids. Res. **33:**D230-232.

133. **Rolls, J. P., and E. S. Lindstrom.** 1967. Induction of a thiosulfate-oxidizing enzyme in *Rhodopseudomonas palustris*. J Bacteriol **94:**784-5.

134. **Romling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol **57:**629-39.

135. **Santiago, B., U. Schubel, C. Egelseer, and O. Meyer.** 1999. Sequence analysis, characterization and CO-specific transcription of the cox gene cluster on the megaplasmid pHCG3 of *Oligotropha carboxidovorans*. Gene **236:**115-124.

136. **Sarti, P., A. Giuffre, E. Forte, D. Mastronicola, M. C. Barone, and M. Brunori.** 2000. Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation. Biochem Biophys Res Commun **274:**183-7.

137. **Schmidt, E. L. (ed.).** 1982. Nitrification in Soil, vol. 22. American Society of Agronomy, Madison, WI.

138. **Schmidt, I., R. J. van Spanning, and M. S. Jetten.** 2004. Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants. Microbiology **150:**4107-14.

139. **Schramm, A., D. De Beer, M. Wagner, and R. Amann.** 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl Environ Microbiol **64:**3480-5.

140. **Scott, K. M., S. M. Sievert, F. N. Abril, L. A. Ball, C. J. Barrett, R. A. Blake, A. J. Boller, P. S. Chain, J. A. Clark, C. R. Davis, C. Detter, K. F. Do, K. P. Dobrinski, B. I. Faza, K. A. Fitzpatrick, S. K. Freyermuth, T. L. Harmer, L. J. Hauser, M. Hugler, C. A. Kerfeld, M. G. Klotz, W. W. Kong, M. Land, A. Lapidus, F. W. Larimer, D. L. Longo, S. Lucas, S. A. Malfatti, S. E. Massey, D. D. Martin, Z. McCuddin, F. Meyer, J. L. Moore, L. H. Ocampo, Jr., J. H. Paul, I. T. Paulsen, D. K. Reep, Q. Ren, R. L. Ross, P. Y. Sato, P. Thomas, L. E. Tinkham, and G. T. Zeruth.** 2006. The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. PLoS Biol **4:**e383.

141. **Seaver, L. C., and J. A. Imlay.** 2001. Hydrogen Peroxide Fluxes and Compartmentalization inside Growing *Escherichia coli*. J Bacteriol**:**7182-7189.

142. **Serrano, R.** 1996. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol **165:**1-52.

143. **Shively, J. M., G. van Keulen, and W. G. Meijer.** 1998. Something from almost nothing: carbon dioxide fixation in chemoautotrophs. Annu Rev Microbiol **52:**191-230.

144. **Simon, J.** 2002. Enzymology and bioenergetics of respiratory nitrite ammonification. Fems Microbiology Reviews **26:**285-309.

145. **Siripong, S., and B. E. Rittmann.** 2007. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Res **41:**1110-20.

146. **Skerker, J. M., and L. Shapiro.** 2000. Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. Embo J **19:**3223-34.

147. **Smith, A. J., and D. S. Hoare.** 1968. Acetate assimilation by *Nitrobacter agilis* in relation to its "obligate autotrophy". J Bacteriol **95:**844-55.

148. **Sockett, H., S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab.** 1992. Molecular analysis of the flagellar switch protein FliM of *Salmonella typhimurium*. J Bacteriol **174:**793-806.

149. **Spieck, E., J. Aamand, S. Bartosch, and E. Bock.** 1996. Immunocytochemical detection and location of the membrane-bound nitrite oxidoreductase in cells of *Nitrobacter* and *Nitrospira*. FEMS Microbiol Lett **139:**71-76.

150. **Spieck, E., Muller, S., Engel, A., Mandelkow, E., Patel, H, and E. Bock.** 1996. Two-Dimensional Structure of Membrane-Bound Nitrite Oxidoreductase from *Nitrobacter hamburgensis*. Journal of Structural Biology **117:**117-123.

151. **Starkenburg, S. R., P. S. Chain, L. A. Sayavedra-Soto, L. Hauser, M. L. Land, F. W. Larimer, S. A. Malfatti, M. G. Klotz, P. J. Bottomley, D. J. Arp, and W. J. Hickey.** 2006. Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255. Appl Environ Microbiol **72:**2050-63.

152. **Stein, L. Y., and Y. L. Yung. .** 2003. Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide. Annu. Rev. Earth Planet. Sci. **31:**329-356.

153. **Stein, L. Y., D. J. Arp, P. M. Berube, P. S. G. Chain, L. Hauser, M. S. M. Jetten, M. G. Klotz, F. W. Larimer, J. M. Norton, H. J. M. O. d. Camp, M. Shin, and X. Wei.** 2007. Comparison of key genes in the genome sequence of *Nitrosomonas eutropha* C91 to genome sequences of other aerobic and anaerobic ammonia-oxidizing bacteria. Environ. Microbiol. **in press**.

154. **Steinmuller, W., and E. Bock.** 1977. Enzymatic studies on autotrophically, mixotrophically and heterotrophically grown *Nitrobacter agilis* with special reference to nitrite oxidase. Arch Microbiol **115:**51-4.

155. **Steinmuller, W., and E. Bock.** 1976. Growth of *Nitrobacter* in the presence of organic matter. I. Mixotrophic growth. Arch Microbiol **108:**299-304.

156. **Stolz, J. F. B., P.** 2002. Evolution of Nitrate Reductase: Molecular and Structural Variations on a Common Function. Chembiochem **3:**198-206.

157. **Stout, J., G. Van Driessche, S. N. Savvides, and J. Van Beeumen.** 2007. X-ray crystallographic analysis of the sulfur carrier protein SoxY from *Chlorobium limicola f. thiosulfatophilum* reveals a tetrameric structure. Protein Sci **16:**589-601.

158. **Stroud, R. M., D. Savage, L. J. Miercke, J. K. Lee, S. Khademi, and W. Harries.** 2003. Selectivity and conductance among the glycerol and water conducting aquaporin family of channels. FEBS Lett **555:**79-84.

159. **Sundermeyer-Klinger, H., Meyer, W., Warninghoff, B., and E. Bock.** 1984. Membrane-bound nitrite-oxidoreductase of *Nitrobacter*: evidence for a nitrate reductase system. Arch Microbiol **140:**153-158.

160. **Svensson, B., and L. Hederstedt.** 1994. *Bacillus subtilis* CtaA is a heme-containing membrane protein involved in heme A biosynthesis. J Bacteriol **176:**6663-6671.

161. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol Rev **52:**155-89.

162. **Tanaka, Y., Y. Fukumori, and T. Yamanaka.** 1982. The complete amino acid sequence of *Nitrobacter agilis* cytochrome c-550. Biochim Biophys Acta **707:**14-20.

163. **Tanaka, Y., Fukumori, Y., and T. Yamanaka.** 1983. Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium. Arch Microbiol **135:**265-271.

164. **Tanaka, Y., Fukumori, Y., and T. Yamanaka.** 1983. Purification of cytochrome a1c1 from *Nitrobacter agilis* and characterization of the nitrite oxidation system of the bacterium. Arch. Microbiol. **135:**265-271.

165. **Tandon, S. P., and M. M. Mishra.** 1968. Effect of some organic acids on nitrification by *Nitrobacter agilis*. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg **122:**401-4.

166. **Tavares, P., A. S. Pereira, J. J. Moura, and I. Moura.** 2006. Metalloenzymes of the denitrification pathway. J Inorg Biochem **100:**2087-100.

167. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J Bacteriol **176:**6623-30.

168. **Tikhonovich, I. A.** 1995. Nitrogen fixation : fundamentals and applications : proceedings of the 10th International Congress on Nitrogen Fixation, St. Petersburg, Russia, May 28-June 3, 1995. Kluwer Academic Publishers, Dordrecht ; Boston.

169. **Todd, J. D., M. Wexler, G. Sawers, K. H. Yeoman, P. S. Poole, and A. W. Johnston.** 2002. RirA, an iron-responsive regulator in the symbiotic bacterium *Rhizobium leguminosarum*. Microbiology **148:**4059-71.

170. **Torres, J., Sharpe, M.A., Rosquist, A., Cooper, C.E., and M. Wilson.** 2000. Cytochrome c oxidase rapidly metabolizes nitric oxide to nitrite. FEBS Lett. **475:**263-266.

171. **Truper, H. G., and Johannes F. Imhoff.** 1989. Bergey's manual of systematic bacteriology, p. 1673. *In* J. T. Staley, M. P. Bryant, N. Pfennnig, and J. G. Holt (ed.), vol. 3. Williams & Wilkins, Baltimore.

172. **Van Gool, A., and H. Laudelout.** 1966. Formate utilization by *Nitrobacter winogradskyi*. Biochim Biophys Acta **127:**295-301.

173. **Vargas, C., M. Jebbar, R. Carrasco, C. Blanco, M. I. Calderon, F. Iglesias-Guerra, and J. J. Nieto.** 2006. Ectoines as compatible solutes and carbon and energy sources for the halophilic bacterium *Chromohalobacter salexigens*. J Appl Microbiol **100:**98-107.

174. **Vergnes, A., K. Gouffi-Belhabich, F. Blasco, G. Giordano, and A. Magalon.** 2004. Involvement of the molybdenum cofactor Biosynthetic machinery in the maturation of the *Escherichia coli* nitrate reductase A. J Biol Chem **279:**41398-41403.

175. **Voegele, R. T., S. Bardin, and T. M. Finan.** 1997. Characterization of the *Rhizobium* (*Sinorhizobium*) *meliloti* high- and low-affinity phosphate uptake systems. J Bacteriol **179:**7226-32.

176. **Von Heijne, G.** 1992. Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:** 487-494.

177. **Wagner, M., Rath, G., Koops, H.P., Flood, J. and R. Amman.** 1996. In Situ Analysis of Nitrifying Bacteria in Sewage Treatment Plants. Wat. Sci. Tech. **34:**237-244.

178. **Wanner, B. L.** 1993. Gene regulation by phosphate in enteric bacteria. J Cell Biochem **51:**47-54.

179. **Watson, S. W., Bock, E., Harms, H., Koops, H.P., and A.B. Hooper.** 1989. Genera of Nitrite-Oxidizing Bacteria, p. 1813-1822. *In* J. T. Staley (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams and Wilkins, Baltimore.

180. **Weidler, G. W., M. Dornmayr-Pfaffenhuemer, F. W. Gerbl, W. Heinen, and H. Stan-Lotter.** 2007. Communities of archaea and bacteria in a subsurface radioactive thermal spring in the Austrian Central Alps, and evidence of ammonia-oxidizing Crenarchaeota. Appl Environ Microbiol **73:**259-70.

181. **Withers, H., S. Swift, and P. Williams.** 2001. Quorum sensing as an intergral component of gene regulatory networks in Gram-negative bacteria. Curr Opin Microbiol **4:**186-193.

182. **Wood, N. J., T. Alizadeh, D. J. Richardson, S. J. Ferguson, and J. W. B. Moir.** 2002. Two domains of a dual-function NarK protein are required for nitrate uptake, the first step of denitrification in *Paracoccus pantotrophus*. Mol Microbiol **44:**157-170.

183. **Wrage, N., G. L. Velthof, M. L. van Beusichem, and O. Oenema.** 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biology and Biochemistry **36:**229-236.

184. **Wylie, J. L., and E. A. Worobec.** 1995. The OprB porin plays a central role in carbohydrate uptake in *Pseudomonas aeruginosa*. J Bacteriol **177:**3021-6.

185. **Yamanaka, T.** 1996. Mechanisms of oxidation of inorganic electron donors in autotrophic bacteria. Plant and Cell Physiology **37:**569-574.

186. **Yamanaka, T., and Y. Fukumori.** 1988. The nitrite oxidizing system of *Nitrobacter winogradskyi*. Fems Microbiology Reviews **54:**259-270.

187. **Yamanaka, T., and Y. Fukumori.** 1988. The nitrite oxidizing system of *Nitrobacter winogradskyi*. FEMS Microbiol Rev **4:**259-70.

188. **Yamanaka, T., Y. Fukumori, T. Yamazaki, H. Kato, and K. Nakayama.** 1985. A comparative survey of several bacterial aa3-type cytochrome c oxidases. J Inorg Biochem **23:**273-7.

189. **Yamanaka, T., Tanaka, Y. and Y. Fukumori.** 1982. *Nitrobacter agilis* cytochrome c-550: Isolation, physiochemical and enzymatic properties and primary structure. Plant Cell Physiol **23:**441-449.

190. **Yao, S. Y., L. Luo, K. J. Har, A. Becker, S. Ruberg, G. Q. Yu, J. B. Zhu, and H. P. Cheng.** 2004. *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both succinoglycan and flagellum production. J Bacteriol **186:**6042-9.

191. **Yu, X. C., and W. Margolin.** 2000. Deletion of the min operon results in increased thermosensitivity of an ftsZ84 mutant and abnormal FtsZ ring assembly, placement, and disassembly. J Bacteriol **182:**6203-13.

192. **Zart, D., and E. Bock.** 1998. High rate of aerobic nitrification and denitrification by *Nitrosomonas eutropha* grown in a fermentor with complete biomass retention in the presence of gaseous NO_2 or NO. Arch Microbiol **169:**282-6.

193. **Zhulin, I., B. Taylor, and R. Dixon.** 1997. PAS domain S-boxes in Archaea, bacteria and sensors for oxygen and redox. Tr Biochem Sci **22:**331-333.

194. **Zumft, W. G.** 1993. The biological role of nitric oxide in bacteria. Arch Microbiol **160:**253-264.

195. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev **61:**533-616.

196. **Zumft, W. G., and H. Korner.** 1997. Enzyme diversity and mosaic gene organization in denitrification. Antonie Van Leeuwenhoek **71:**43-58.