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Expression of Genes Linked to NOx Detoxification in Aerobic Bacteria

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Lynnie Shao Cua

December 2010

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University of California, Riverside

Acknowledgments

Dedication

I dedicate this dissertation to my parents, Virgilio and Chiu Ching Cua, for without their

love and support I would not be where I am today.

ABSTRACT OF THE DISSERTATION

Expression of Genes Linked to NOx Detoxification in Aerobic Bacteria

by

Lynnie Shao Cua

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, December 2010
Dr. Lisa Y. Stein, Co-Chairperson
Dr. Marylynn V. Yates, Co-Chairperson

Microorganisms capable of nitrifier denitrification and aerobic denitrification are of significant interest due to their release of NO and N_2O in the atmosphere. Studies have therefore focused on the physiology and genetic diversity in organisms capable of nitrogen oxide (NOx) metabolism in the production of these gases. Genomic analysis of ammonia-oxidizing bacteria (AOB) revealed the presence of NOx-related gene homologues normally present in denitrifying bacteria. Thought to serve in NO detoxification, these genes include nitrite reductase (*nir*K), nitric oxide reductases (*nor*B and *nor*S), and cytochromes P460 (*cyt*L) and *c'* beta (*cyt*S). The goal of the study in Chapter 2 was to determine regulation of these genes in three representative AOB grown in HEPES- or phosphate- buffered media in the presence of 0, 10, and 20mM $NaNO_2$. Results indicate that differential regulation of homologous genes occurs in each AOB in response to nitrite, suggesting different mechanisms in coping with nitrosative stress.

Sphingomonas wittichii RW1 contains genes involved in NOx metabolism, including *nir*K, *nor*B, *nor*Z, and *hmp* (encoding flavohemoglobin), but has no previous

history of denitrification. The goal of the study in Chapter 3 was to determine whether these genes were regulated in response to nitrite under initial 20% and 3% oxygen levels. *S. wittichii* RW1 was not capable of nitrite respiration. High basal mRNA levels of these genes indicate differential regulation by oxygen. Increased levels of *hmp* and *nor*B mRNA in NO_2^- -treated groups under low O_2 suggest hypoxic function and activity. Total nitrite consumption was observed, and aerobic production of N_2O was greatest in treated cells incubated under 3% O_2. Thus, *S. wittichii* is capable of aerobic denitrification, in the detoxification of nitrite.

A Δ*nor*Z mutant was constructed in *S. wittichii* for the study in Chapter 4 to determine the roles of the two *nor* genes with respect to aerobic N_2O production. Total nitrite consumption occurred indicating a functional activity of *nir*K. The Δ*nor*Z strain was able to produce N_2O, though significantly less compared to wildtype cells. An increase in *norB* mRNA levels in NO_2^- -treated cells was observed, suggesting compensation for the loss of NorZ function.

The studies presented show that nitrite differentially affected transcription of *nir*K, *amo*A, and *nor*S in the AOB. In *S. wittichii*, NOx- related genes are regulated by oxygen and collectively used to aerobically detoxify nitrite, resulting in N_2O production; additionally, *nor*B is an isofunctional NO reductase.

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Chapter 1

Introduction and Literature Review

Rationale and objectives of study

Nitrogen is an essential component for life, comprising ~78% of the atmosphere as inert N_2 and necessary in the production of nucleic acids and proteins. It is also one of the most important biogeochemical cycles in which nitrogen is a key element in controlling terrestrial and aquatic ecosystems as well as ecological diversity and dynamics within those environments (Vitousek et al., 1997). However, human activity such as the production and use of fertilizer as well as fossil fuel combustion has more than doubled the movement of nitrogen from the atmosphere to land. It has been estimated that between 1860 and 2000, the anthropogenic creation of reactive nitrogen (non-N_2 forms of nitrogen) increased from 15 Tg N yr^{-1} to ~165 Tg N yr^{-1}, with food production contributing more than five times the amount produced from energy production (Galloway et al., 2003). Of particular interest are the emissions of nitrogen and nitrous oxides (NO_x and N_2O). Nitrous oxide is a greenhouse gas involved in global warming and the depletion of stratospheric ozone through the generation of NO_x, while NO_x is involved in decreased air visibility (i.e. smog and aerosol formation). As a result, perturbations in the global nitrogen cycle, particularly in air and soil, are no doubt heavily influenced by the anthropogenic production of nitrogen.

Microorganisms play a major role in the global nitrogen cycle as they are largely responsible for the transformation of nitrogen. Thus, with the increase of anthropogenic

production and deposition of nitrogen, microbial metabolism of nitrogen is enhanced.

Transformation of nitrogen involves oxidation and reduction reactions by which N_2

fixation, nitrification, and denitrification occurs. Nitrogen fixation is the reduction of N_2

to NH_3 carried out by N_2-fixing microorganisms. Nitrification involves the overall

oxidation of NH_3 to nitrate (NO_3^-) involving ammonia-oxidizing bacteria and archaea and

nitrite-oxidizing bacteria. Denitrifying microorganisms then carry out the subsequent

reduction of NO_3^- to NO_2^-, NO, N_2O, and finally to N_2. Collectively, these

transformations of nitrogen are referred to as the microbial nitrogen cycle.

Microbial metabolism of nitrogen has been critical in the production of NO and

N_2O gases. While NO and N_2O are regular products of denitrification, studies have

shown that nitrifying bacteria- primarily ammonia-oxidizing bacteria- can likewise

produce these gases during the oxidation of NH_3 to NO_2^-, which has been termed

"nitrifier denitrification." It has been suggested that a significant proportion of NO and

N_2O emitted from soil are in fact produced through nitrifier denitrification (Bremner and

Blackmer, 1981; Tortoso and Hutchinson, 1990).

Genetic analyses of AOB have demonstrated the presence of gene homologues

found in the denitrifier gene inventory involved in NO_x metabolism, including: nitrite

reductase (*nirK*); nitric oxide reductase (*norB*); a cytochrome *c*-related nitric oxide

reductase (*norS*); cytochrome P460 (*cytL*); and cytochrome *c' β* (*cytS*). The function of

these nitrifier denitrification genes is believed to serve as a means of NO_x detoxification

in AOB. However, the exact physiological basis of such gaseous production is still

unclear and very limited knowledge exists concerning the regulation of gene expression

required for nitrifier denitrification by AOB (Conrad, 1996; Klotz and Stein, 2008). As a result, the objectives of research described in Chapter 2 were to:

- Compare the expression of nitrifier denitrification genes in three ecotypic AOB- *Nitrosomonas eutropha, Nitrosomonas europaea,* and *Nitrosospira multiformis* in response to nitrite

- Compare ammonia-oxidizing activities, as measured by changes in pH and nitrite production, in the three AOB

This body of work can therefore be added to the current state of knowledge concerning gene regulation of nitrifier denitrification genes as well as contribute to the field of nitrification as a whole.

Interestingly, phylogenetic analyses of full length sequences of the copper-containing nitrite reductase (NirK) revealed that the heterotroph *Sphingomonas wittichii* RW1 harbors a *nirK* gene homologous to those found in AOB. Further genomic analysis revealed the presence of genes encoding two nitric oxide reductases (*norZ* and *norB*) and flavohemoglobin (*hmp*), thus suggesting *S. wittichii* RW1 is capable of NOx metabolism and production of N_2O. This organism has no previous history with the nitrogen cycle and is commonly known for the degradation of dioxin-containing compounds. Thus, the research described in Chapter 3 determined whether these genes are involved in denitrification or serve a protective role during nitrosative stress (i.e. in the presence of nitrite). Specifically, the objectives of Chapter 3 were to:

- Determine whether *S. wittichii* RW1 is capable of nitrite consumption and aerobic production of nitrous oxide

• Determine the regulation of the NOx-related genes in *S. wittichii* RW1 treated

with nitrite under initial 20 and 3% oxygen

A subsequent study described in Chapter 4 was conducted in which a *ΔnorZ*

mutant was constructed to further determine the specific roles of the two nitric oxide

reductase genes with respect to N_2O production. The specific objectives of Chapter were

to:

• Construct a *norZ*-deficient strain of *S. wittichii*

• Compare expression of NOx-related genes in response to nitrite in both wildtype

and mutant strains

• Determine whether the $\Delta norZ$ strain is capable of N_2O production

The two studies in Chapters 3 and 4 illustrate the functional diversity of genes involved

in NOx metabolism in organisms previously uncharacterized with such abilities. Together,

these studies of nitrifier denitrification and aerobic denitrification characterize processes

which may have significant impact on the increasing atmospheric pool of NO and N_2O as

well as on the global nitrogen cycle.

Background

Global nitrogen cycle

Of the important chemical elements necessary for life (i.e. carbon, nitrogen,

oxygen, phosphorus, and sulfur), nitrogen has the greatest abundance in Earth's

atmosphere, hydrosphere, and biosphere (Galloway et al., 2003). In fact, the total amount

of nitrogen in the atmosphere, water, and soil is approximately 4×10^{21} grams- more than

the total amount of carbon, oxygen, phosphorus, and sulfur combined (Mackenzie, 1998, as referenced in Galloway et al., 2003). Despite the abundance of nitrogen, it is the element that is the least readily available to sustain life, with over 99% not available to more than 99% of living organisms, as almost all nitrogen is in an unreactive form of nitrogen- as dinitrogen gas, N_2. This form of nitrogen is unusable to most organisms due to the significant amount of energy necessary to break the triple bond between the two N atoms (Galloway et al, 2003; Erisman et al., 2007). In order for organisms to be able to use nitrogen, N_2 must first be converted to a usable form, such as ammonium, nitrate, or organic nitrogen (i.e. urea), which collectively are referred to as reactive nitrogen (Galloway et al., 2003).

Lightning, anthropogenic processes such as fossil fuel combustion and the production of nitrogen-based fertilizers, and to a large extent, biological nitrogen fixation are responsible for the transformation of these oxidized and reduced forms of nitrogen. The movement of such forms of nitrogen between air, soil, and water is referred to as the nitrogen cycle and is composed of four major processes: nitrogen fixation, mineralization, nitrification, and denitrification (Erisman et al., 2007). Alternatively, this movement of reactive nitrogen species, their associated transformations through environmental systems, and their link to ecological and human health effects has collectively been termed the nitrogen cascade (Galloway, 2003). According to this phenomenon, one atom of nitrogen can concurrently increase atmospheric ozone, increase fine particulate matter, alter forest productivity, acidify surface waters, increase productivity of coastal ecosystems and eutrophication, and increase greenhouse potential of the atmosphere (Galloway et al.,

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2004). This cycling of nitrogen therefore represents one of the most important and versatile biogeochemical cycles in nature (Figure 1.1).

Nitrogen in the atmosphere

While 78.08% of the earth's atmosphere is composed of the inert N_2, other atmospheric nitrogen compounds include nitrogen and nitrous oxides (NO_x and N_2O) and ammonia (NH_3). Considered the fourth major greenhouse gas behind water vapor, carbon dioxide (CO_2), and methane (CH_4), N_2O is a very effective greenhouse gas with each molecule absorbing about 200 times the outgoing radiation compared to CO_2, with a global warming potential of 296 over a 100-year span (Reay, 2007). N_2O has a residence time of 120 years and is the major source of ozone-depleting nitrogen oxides (NO_x- NO and NO_2) in the stratosphere through photolysis, which in turn is responsible for increasing tropospheric ozone concentrations (Galloway et al., 2003; Fields, 2004; Forster et al., 2007). Nitrogen oxides and ammonia, on the other hand, have a residence time of roughly 1 to 8 days. The presence of NO_x in the troposphere can also result in decreased visibility (i.e. smog) and adverse respiratory effects such as respiratory tract inflammation, reactive airways disease as well as complicate asthma, cough, and viral infections (Fields, 2004). Additionally, through its oxidation NH_3 can cause soil acidification and is a known precursor to atmospheric aerosols, comprising a significant portion of the fine particulate matter within the 2.5μm range ($PM_{2.5}$) in the United States (Anderson et al., 2003 and references therein; Erisman et al., 2007).

Global emission of NH_3 is estimated to be 53.7 Tg N, with the largest source attributed to livestock (i.e. animal waste) followed by fertilizer application, and to a minimal extent, automobile and industrial emissions, and wastewater treatment plants (Erisman et al., 2007; Anderson et al., 2003). While atmospheric N_2O emissions from transportation via fossil fuel combustion (including vehicles, watercraft, and aircraft) is considered the second largest source behind agroecosystems, such emissions account for about 1% of the estimated total N_2O budget which is estimated at 17.7 Tg N/year (Forster et al., 2007). Globally, atmospheric N_2O concentrations have risen to 319 ppb in 2005, with a linear increase of 0.26% yr^{-1} (Forster et al., 2007). Thus, in large part, NO_x and N_2O emissions have been attributed to nitrification and denitrification processes of microorganisms in soil, with the use of nitrogenous fertilizers greatly enhancing microbial metabolism of nitrogen (Conrad, 1996; Nevison and Holland, 1997).

Nitrogen in water

Nitrogen is present in water usually due to atmospheric deposition, surface and groundwater runoff, biological decay of organic matter, N_2 fixation, sewage effluent, and N-rich geological deposits (Camargo and Alonso, 2006 and references therein). The primary anthropogenic source of reactive nitrogen in groundwater is leaching from agricultural activities, where nitrate is the most common reactive nitrogen compound due to its high solubility in water (Galloway et al., 2003 and references therein; Fields, 2004). The presence of nitrate in groundwater poses a health risk in humans as ingestion of nitrate-contaminated water can lead to methemoglobinemia, or blue baby syndrome,

which results in the inability of hemoglobin to take up oxygen. However, the accumulation of nitrogen in groundwater is not considered a major sink as reactive nitrogen tends to be denitrified to N_2 and is lost to surface waters and the atmosphere. However, residence time in affected water bodies as well as fate and transformation of nitrogen are site-dependent (Galloway et al., 2003). Additionally, elevated concentrations of nitrate and other inorganic nitrogen compounds can lead to eutrophication, or nutrient loading, in freshwater, estuarine, and coastal marine ecosystems. Eutrophication can cause anoxia or hypoxia in stratified waters and is associated with algal blooms and eventually results in decreased aquatic biodiversity (Camargo and Alonso, 2006; Vitousek et al., 1997). It has been conservatively estimated that riverine and estuarine waters discharged ~59 Tg N yr^{-1}, with ~11 Tg N yr^{-1} transported to inland-receiving waters and drylands while coastal waters received ~48 Tg N yr^{-1} (Galloway et al., 2004).

In the open waters of the ocean, atmospheric transport and deposition of nitrogen is becoming an increasingly important problem due to increased emissions of anthropogenic nitrogen. Based on computer simulation and modeling data compiled from a number of studies detailing N_2 fixation, atmospheric nitrogen emission, and deposition to the surface ocean, Duce et al. (2008) estimated that total atmospheric deposition is 167 Tg N yr^{-1}, with anthropogenic nitrogen emissions contributing an average of 5 Tg N yr^{-1} as N_2O. However, the authors note that considerable uncertainty exists in their estimates as nitrogen fluxes, inputs, sources, and climate vary between oceans and calculations likewise vary between studies due to differences in modeling and extrapolation methods. Extrapolated data solely concerning marine contribution of global annual N_2 fixation of

the cyanobacterial *Trichodesmium* spp. (a N_2-fixing organism) was estimated at 80 Tg N yr^{-1} (Van Dommelen and Vanderleyden, 2007). Reactive nitrogen is usually removed from the ocean also through N_2 formation from denitrification, anaerobic ammonium oxidation, N_2O emissions, and sedimentation of organic matter (Duce et al., 2008).

Nitrogen in soil

Prior to the advent of the Haber-Bosch process in 1913, natural terrestrial reactive nitrogen production or N_2 fixation was ~100 Tg N yr^{-1} (with a range of 90-140 Tg N yr^{-1}) with ~15 Tg N yr^{-1} emitted to the atmosphere as either NH_3 or NO_x, while anthropogenic processes (legume/rice cultivation and fossil fuel combustion combined) totaled ~15 Tg N yr^{-1} (Vitousek et al., 1997; Galloway and Cowling, 2002; Galloway et al., 2003). By 1990, however, anthropogenic activities were responsible for ~140 Tg N yr^{-1} of reactive nitrogen in soil, of which ~85 Tg N yr^{-1} (~9-fold increase) was attributed to the Haber-Bosch process (Galloway and Cowling and references therein, 2002). This process, which involves the conversion of N_2 to NH_3 under high pressure and temperature, annually produces >500 million tons of nitrogen fertilizer worldwide to sustain food production. It is estimated that only a small amount (~4 Tg N yr^{-1}) actually accumulates in fertilized areas since the more reactive nitrogen used, the more loss through atmospheric and hydrologic pathways (Smil, 1999; Galloway et al., 2003). Volatilization of NH_3 is directly emitted from ammonia fertilizers as well as animal manure (Smil, 1999; Smil 2003). Even with the limited nitrogen accumulation from fertilizer use, microbial nitrification and denitrification are the main sources of NO, N_2O, and N_2 emitted from

soils and thus vastly contribute to atmospheric NOx loads. However, microbial nitrogen

processes are heavily influenced by rates of fertilization, crop varieties, and tillage

practices as well as climate (Smil, 1999; Smil 2003). As a result, quantification of

gaseous N loss is difficult and no standard methodologies exist (Munch and Velthof,

2007).

Forest and grassland soils can be a reservoir of reactive nitrogen, but may not

necessarily play an important role in the global nitrogen cycle in terms of NO_x and N_2O

soil emissions. In the absence of forest fires and anthropogenic input, reactive nitrogen

emissions in forest soil depend upon a number of factors such as forest history and type,

plant/root and overall microbial productivity, and previous nitrogen input (Galloway et

al., 2003 and references therein). It has been estimated that forest ecosystems globally

emit ~4 Tg N yr^{-1} as N_2O, of which ~3 Tg is attributed to tropical forests and ~1 Tg is

emitted from temperate/boreal forests (Ambus and Zechmeister-Boltenstern, 2007 and

references therein). Similarly, grasslands have low potential in the global nitrogen cycle

as soils tend to be nitrogen-limited due to subsurface biomass and slow decomposition

rates. Both forest and grassland soils primarily receive nitrogen through atmospheric

deposition and microbial metabolism which can result in substantial lag times on the

order of hundreds to thousands of years in terms of contribution to global nitrogen

cycling (Galloway et al., 2003).

Microbial nitrogen cycle

The global nitrogen cycle incorporates and links both anthropogenic and natural

processes in the production and movement of reactive nitrogen in the atmosphere, water,

and soil. As the previous sections established the ubiquitous presence of nitrogen in the

global environment, the key players directly involved in transformation of nitrogen are

microorganisms. Prokaryotes, bacteria and archaea, are predominantly responsible for

nitrogen fixation, nitrification, and denitrification, comprising the main processes in the

microbial (or biological) nitrogen cycle (Figure 1.2). Since the nitrogen cycle was

proposed over 100 years ago, significant findings have been made in elucidating the

organisms and pathways involved (Hayatsu et al., 2008). However, despite extensive

research, critical gaps remain in the knowledge concerning microbial composition,

population dynamics, and the possibility of novel pathways (Horner-Devine and Martiny,

2008). The following sections will provide an overview as to the microorganisms

involved in the microbial nitrogen cycle, as well as the mechanisms involved in

producing reactive nitrogen.

Nitrogen fixation

Only prokaryotes can perform biological nitrogen fixation and are collectively

known as diazotrophs, providing up to 60% of the total annual input of fixed-N to the

biosphere (Fisher and Newton, 2002; Newton, 2007). Diazotrophs are either free-living,

symbiotic, or in loose association with plant roots and can be found in aerobic,

microaerobic, or anaerobic conditions in terrestrial and marine environments. Commonly

studied free-living diazotrophs include the obligate aerobe *Azotobacter* species or the

phototrophic cyanobacteria while the model anaerobic, free-living diazotroph are

represented by the *Clostridia* species. Typical symbiotic N_2-fixing bacteria are the

Rhizobia which form symbiotic relationships with root nodules, usually of the legume

variety (Newton, 2007). The common feature found in these diverse N_2-fixing microbes

is the presence of the nitrogenase enzyme, which catalyzes the reduction of N_2 to NH_3.

With its structure resolved, the well-studied nitrogenase is composed of two

metallo-components- dinitrogenase and dinitrogenase reductase which contains a

molybdenum-iron (Mo-Fe) protein and an iron (Fe) protein, respectively (Cheng, 2008).

During the catalytic process of fixing N_2, the two proteins combine and electron-transfer

occurs from the Fe protein to the Mo-Fe protein where substrates are then bound and

reduced (Benton et al., 2002; Cheng, 2008; Newton, 2007; Postgate, 1998). Currently, it

is unknown as to how ATP hydrolysis controls electron transfer between the protein

components as well as how and where substrates bind within the Mo-Fe protein (Cheng,

2008).

Genetic analysis of the facultative anaerobe *Klebsiella pneumoniae* showed that

nitrogenase is encoded by twenty genes in a set of operons referred to as the *nif* cluster.

This suite of genes is organized into eight transcriptional units and includes regulatory

genes, structural genes, and other supplementary genes (Fisher and Newton, 2002;

Newton, 2007; Cheng, 2008). Significant differences do exist concerning the genetics of

nitrogenase as some organisms do not have the full complement of gene homologues and

others contain genes not found in the *nif* clusters of *K. pneumoniae* (i.e. *Azotobacter* and

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Rhizobia); however, there is high conservation among *nif* gene organization based on DNA sequence analysis among diazotrophic bacteria (Newton, 2007).

Denitrification

Denitrification is the key process responsible for the production of NO and N_2O through successive reduction of nitrate (NO_3^-) or nitrite (NO_2^-). It is also the major mechanism in creating N_2, thereby completing the nitrogen cycle (Ye et al., 1994). Approximately 10 to 15% of the bacterial population in soil, water, and sediment consists of denitrifying bacteria. These bacteria can also be found in the human digestive tract. While few archaea are capable of denitrification, very few studies have examined archaeal denitrification in natural ecosystems including soil ecosystems; thus, much work is needed in assessing their role in the nitrogen cycle (Hayatsu et al., 2008). Denitrifying bacteria are primarily heterotrophic and found in suboxic or anaerobic environments (Casella and Payne, 1996; Francis et al., 2007). However, as many of these organisms are facultative aerobes, denitrification can and does occur under aerobic conditions. Previously believed to be a rare process, aerobic denitrification is now considered a variant rather than the exception (Lloyd, 1993; Hayatsu et al., 2008).

Aerobic denitrification

Aerobic denitrification uses the same enzymatic machinery as denitrification (discussed in the following section); however, the terminal product is N_2O rather than N_2 due to inhibition of nitrous oxide reductase by oxygen (Lloyd, 1993; Baumann et al.,

1996; Otte et al., 1996; Takaya et al., 2003). This therefore results in the release of N_2O, further adding to the global N_2O pool. This is of great significance as many denitrifying organisms capable of aerobic denitrification are found in natural (i.e. soils and sediments) and artificial (i.e. wastewater treatment plants) environments (Robertson and Kuenen, 1983; Carter et al., 1995; Frette et al., 1997; Patureau et al., 2000). Many of the (anaerobic) denitrifiers belong to different genera and are mainly found in the *Proteobacteria* group, thus suggesting that aerobic denitrification likely evolved as a metabolic pathway parallel to anaerobic denitrification in species that are exposed to alternating aerobic-anaerobic conditions (Carter et al., 1995; Patureau et al., 2000; Gao et al., 2010).

Among the first organisms found capable of aerobic denitrification, *Thiosphaera pantotropha* was isolated from a sulfide-oxidizing, denitrifying effluent-treatment plant in Delft, The Netherlands (Robertson and Kuenen, 1983). Chemostat-grown *T. pantotropha* was able to aerobically denitrify nitrate at dissolved oxygen concentrations both below and above 30% (up to 80%) air saturation (Robertson et al., 1988). Strains largely from the *Pseudomonas* genus have been cultivated in batch culture from distinct soil types (i.e. sandy, fine, and coarse soils) and freshwater sediment, capable of nitrate respiration under dissolved oxygen concentrations ranging from 10 to 80% air saturation (Carter et al., 1995). Similarly, aerobic denitrifiers representing the *Paracoccus*, *Thiobacillus*, and *Enterobacter* genera have also been isolated under aerobic-anoxic conditions from various ecosystems including canals, ponds, mud-clay soil as well as

activated sludge (Patureau et al., 2000). Thus, nitrate respiration among aerobic

denitrifying bacteria is largely influenced by oxygen concentrations (Hayatsu et al., 2008).

The effects of oxygen in nitrate respiration were tested in eight denitrifying

bacterial isolates, including *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, and

Pseudomonas stutzeri (Lloyd et al., 1987). While the relative proportions of gaseous end

products varied depending on oxygen concentration and species, all isolates produced a

greater amount of N_2O than N_2 over a range of oxygen conditions (0 to 70 kPa), even

when under concentrations greater than the atmospheric saturation value (Lloyd et al.,

1987). Similarly, in the presence of low oxygen conditions (7 and 1 % O_2) N_2O

production (as much as ~25 and ~55 μmol N_2O, respectively) was greater in

Agrobacterium tumefaciens respiring nitrite compared to levels of cells incubated under

0% O_2 (~6 μmol N_2O) (Bergaust et al., 2008). In *Alcaligenes faecalis*, N_2O production

occurred under dissolved oxygen concentrations ranging from of 5 to 76% air saturation

in the presence of nitrite, with highest production occurring at 5% air saturation (Otte et

al., 1996). Additionally, it was observed that N_2O emissions were greatest during

transitions between alternating, dynamic aerobic-anaerobic conditions (Otte et al., 1996).

A similar finding was found in bacteria extracted from peat soil in which O_2-depleted

samples re-exposed to 5% O_2 experienced a decrease in N_2O reduction activity, and

hence an increase in N_2O overall compared to samples that weren't re-exposed to oxygen

(Morley et al., 2008). Thus, environments exposed to periods of anoxic or anaerobic

conditions such as transient flooding of soils or tidally-induced redox fluctuations in

permeable marine sediments can lead to unbalanced aerobic denitrification and enhanced N_2O production (Morley et al., 2008; Gao et al., 2010).

Emissions of N_2O and NOx are controlled by the expression and activity of the four denitrifying genes and their products- nitrate, nitrite, nitric oxide, and nitrous oxide reductases (Bergaust et al., 2008 and references therein). Studies concerning the regulation of these genes revealed that the absence of oxygen alone was not sufficient for denitrification, but required the presence of a nitrogenous oxide (i.e. NO_3^-, NO_2^-, N_2O) for enzyme synthesis (Körner and Zumft, 1989; Zennaro et al., 1993; Baumann et al., 1996; Philippot et al., 2001). Additionally, constitutive expression of denitrifying genes and lack of enzyme degradation observed under aerobic conditions in denitrifiers such as *P. denitrificans* and *A. tumefaciens* likely serve as an adaptation mechanism for survival in ecosystems that continually experience oxygen fluctuations (Baumann et al., 1996; Bergaust et al., 2008).

Transitions from anaerobic to aerobic conditions have resulted in the inhibition of nitrate and nitrous oxide reductases, thus resulting in the accumulation of N_2O and the decrease in production of N_2, while activities of nitrite and nitric oxide reductase were maintained during the first hours after transition as observed in *P. stutzeri* and *A. faecalis* (Körner and Zumft, 1989; Otte et al., 1996). Additionally, as seen in *P. denitrificans*, transcripts of denitrifying genes maintained anaerobic steady-state levels, experiencing only a very slight decrease, upon transition from anaerobic to aerobic conditions (Baumann et al., 1996). Thus, while de novo enzyme synthesis may have been inhibited during the switch to aerobic conditions, activities of existing enzymes such as nitrite

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reductase were not inactivated (Otte et al., 1996). On the other hand, the transition from aerobic to anaerobic conditions expectedly resulted in sequential induction of the denitrifying genes as well as the increase in N_2 production (Baumann et al., 1996; Otte et al., 1996; Philippot et al., 2001). Interestingly, greatest activity of denitrifying enzymes and maximum expression occurred at oxygen levels ranging from 0.6 to 4 mg/L as opposed to under complete anaerobiosis alone, suggesting differential O_2-dependent regulation and reversible O_2-inhibition of denitrifying genes (Otte et al., 1996; Körner and Zumft, 1989). Thus, it is likely that maximum N_2O production occurs during these transition states rather than solely under steady-state aerobic conditions.

Despite the well-studied aerobic denitrifying organisms such as *P. denitrificans*, *P. stutzeri*, and *A. faecalis* in which N_2O production was greater under aerobic conditions, there are species capable of further aerobic reduction of N_2O to N_2, as demonstrated with *T. pantotropha* (Robertson et al., 1988; Körner and Zumft, 1989; Otte et al., 1996; Baumann et al., 1996). In addition to *T. pantotropha*, other strains including *P. stutzeri* TR2, *Pseudomonas* sp. strain K50, and *Microvirgula aerodenitrificans*, have been isolated with the ability to also aerobically reduce nitrate to N_2, with very low N_2O production in comparison (Patureau et al., 1998; Takaya et al., 2003). Such organisms can therefore potentially be of use in wastewater treatment systems when N_2O emissions, predominantly emitted in aerated zones, result from incomplete aerobic denitrification. Thus, further studies are necessary in determining the effects of oxygen, nitrite, and carbon availability in these organisms as such factors can influence N_2O emissions and control strategies, and perhaps impact reduction of N_2O to N_2 (Takaya et al., 2003;

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Kampschreur et al., 2009). It should be noted that N_2O can also be emitted during nitrifier denitrification processes carried out by AOB which occur in these treatment systems as well.

As oxygen is much more energetically favorable than nitrate (or nitrite), it is believed that heterotrophic denitrifying organisms will respire oxygen when both are present and repress denitrification (Takaya et al., 2003). However, it appears that nitrate respiration occurs simultaneously with oxygen respiration, as observed in twenty-three strains isolated from soils and sediments, spanning three genera- *Pseudomonas*, *Aeromonas*, and *Moraxella* (Carter et al., 1995). In fact, a higher growth rate was observed in *T. pantothropha* cells co-respiring oxygen and nitrate (or nitrite) compared to those respiring oxygen alone, although cell yields were decreased (Robertson et al., 1984; Robertson et al., 1988). Increased growth rates were attributed to the contribution of aerobic denitrification towards overall respiration such that electrons were directed towards denitrifying enzymes to alleviate components of the electron transport chain (i.e. cytochromes) involved in shuttling electrons to the terminal oxidase in the reduction of oxygen (Robertson et al., 1984; Robertson et al., 1988). Thus, the prevailing theory is that nitrate (or nitrite) and oxygen are co-metabolized, simultaneously used as electron acceptors in a single organism in which denitrifying enzymes serves as an electron pool. In organisms capable of both (aerobic) denitrification and nitrification such as *T. pantotropha*, it has been suggested that aerobic denitrification can serve as NADH-oxidizing power for nitrification (Robertson et al, 1988). Alternatively, it is possible that aerobic denitrifying bacteria may co-exist with oxygen-respiring organisms in a

community, and thus have adapted their denitrifying abilities such that denitrification is no longer inhibited in the presence of oxygen and oxygen cannot compete for electrons (Gao et al., 2010).

Sphingomonas wittichii RW1- The potential for aerobic denitrification and production of N_2O

Sphingomonas sp. RW1, subsequently designated *S. wittichii* RW1, was isolated from the waters of the River Elbe, Germany (Wittich et al., 1992). *S. wittichii* RW1 contains the characteristic sphingoglycolipid glucuronosyl ceramide (SGL-1), but also contains the more uncommon galacturonosyl ceramide (SGL-1') found only in three other phenotypically-distinct *Sphingomonas* species (Yabuuchi et al., 2001). *S. wittichii* RW1 is the only known Sphingomonad capable of growing solely on polychlorinated dibenzo-*p*-dioxins and dibenzofurans which are in fact the preferred substrates for this organism (Wittich et al., 1992; Yabuuchi et al., 2001). *S. wittichii* RW1 is able to cooxidatively degrade mono- and di- chlorinated derivatives of dibenzofuran and dibenzo-*p*-dioxin as well as nitrodiphenyl ether pesticides (Wilkes et al., 1996; Hong et al., 2002; Nam et al, 2006; Keum et al., 2008).

Due to its ecological significance concerning bioremediation of the heterocyclic organic compounds dibenzo-*p*-dioxin and dibenzofuran and nitrodiphenyl ether pesticides there is no doubt as to the importance of *S. wittichii* RW1. Fortuitously, a recent finding further emphasized the versatility of this organism in relation to the nitrogen cycle, particularly in regards to (aerobic) denitrification. Previous work conducted by other lab personnel investigated the phylogenetic diversity of the nitrite reductase (NirK) protein in

nitrifying bacteria as it is a key enzyme in the nitrogen cycle (Cantera and Stein, 2007a). Interestingly, alignment of full-length NirK protein sequences revealed that *S. wittichii* RW1, along with two other heterotrophic bacteria (*Shewanella woodyi* and *Herminiimonas arsenicoxydans*) clustered with species from the ammonia- and nitrite-oxidizing bacteria *Nitrosomonas* and *Nitrobacter*. Analysis of the *nirK* operon structure further revealed the presence of a multicopper oxidase, two cytochrome *c* genes, and an upstream regulatory binding site NsrR, similar to the operon structures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (Stein, unpublished). The presence of the putative NsrR binding site may indicate that the operon is induced in the presence of nitrogen oxides (i.e. nitrite and nitric oxide). The genetic inventory of *S. wittichii* RW1 also included genes encoding a quinol- linked nitric oxide reductase (*nor*Z), a cytochrome *c*-linked nitric oxide reductase (*nor*B), and a flavohemoglobin (*hmp*). While *nor*Z is found within the genome of *S. wittichii* RW1, *nor*B and *hmp* are encoded on plasmid pSWIT01.

The presence of genes encoding *nir*K and two nitric oxide reductases suggest that *S. wittichii* RW1 may be capable of denitrification to N_2O. Hmp, also referred to as nitric oxide dioxygenase, has the ability to both aerobically oxidize NO to NO_3^- and anaerobically reduce NO to N_2O (Gardner et al., 1998; Gardner et al., 2006 and references therein). It is therefore predicted that in the event of nitrosative stress these genes encode for products that are involved in the detoxification of nitrogen oxides, resulting in the reduction of NO_2^- and subsequently, the production and release of N_2O to the atmosphere. Thus, this became the basis of Chapters 3 and 4. Because this suite of

genes has been found in *S. wittichii* RW1, an organism widely studied for its degradation abilities, this may therefore suggest that perhaps there are other uncharacterized organisms likewise capable of such processes which can ultimately impact the global nitrogen cycle.

Denitrification pathway

Denitrification is a form of respiration from which denitrifiying organisms generate energy, sharing respiratory chain components with the electron transport chain (van Spanning et al., 2007). Each step in the metabolic pathway of denitrification (NO_3^- → NO_2^- → NO → N_2O → N_2) is catalyzed by a specific reductase (i.e. nitrate, nitrite, nitric oxide, and nitrous oxide reductases) that transfers electrons from the chain to the particular intermediate of the denitrification pathway (Ingraham, 1981; van Spanning et al., 2007). Crystal structures have been resolved for these metalloenzymes, with the exception of nitric oxide reductase (Moura and Moura, 2001; van Spanning et al., 2007). However, NOR protein models have been engineered using sperm whale myoglobin that structurally and functionally mimic native NOR (Yeung et al., 2009).

Nitrate reductase

Of the reductases involved in denitrification, nitrate reductase has been the most studied especially in *Paracoccus denitrificans* and *Pseudomonas stutzeri* (Bryan, 1981; van Spanning et al., 2007). There are three forms of nitrate reductases in bacteria, all of which are molybdenum-dependent enzymes- the cytoplasmic assimilatory (Nas), the

membrane-bound (Nar), and the periplasmic dissimilatory (Nap) nitrate reductases

(Einsle and Kroneck, 2004). Many bacteria possess more than one type of nitrate

reductase; in fact, *Paracoccus* contains all three (Richardson et al., 2007). Of the three,

the best known nitrate reductase is the integral membrane-bound protein Nar, usually

referred to as the heterotrimer NarGHI. Interestingly, however, the first nitrate reductase

crystal structure resolved was that of a periplasmic nitrate reductase in the sulfate-

reducing bacteria *Desulfovibrio desulfuricans* by Dias et al. (1999), using

multiwavelength anomalous diffraction (MAD) methodology while the first three-

dimensional of Nar was derived from the non-denitrifier *E. coli* (Butler and Richardson,

2005; Richardson et al., 2007).

The membrane bound Nar is arranged in the *narGHJI* operon, induced under

anoxic conditions and in the presence of nitrate (Philippot, 2002). Through biochemical

studies using purified Nar extracts, it was determined that *narG* encodes a catalytic α-

subunit containing a molybdopterin cofactor and a 4Fe-4S cluster; *narH* encodes for a

soluble β-subunit and an additional Fe-S center; and *narI* encodes a γ-subunit containing

two *b* hemes. Additionally, *narJ* encodes a chaperone-like protein required for maturation

of the αβ complex and assembly of the αβγ complex (Philippot, 2002). In the Nar system,

the α and β subunits reside in the cytoplasmic domain while the γ subunit resides in the

membrane domain. Electron transport from quinol to the γ subunit, and subsequently to

the β subunit, results in the reduction of NO_3^- to NO_2^- and is coupled to the generation of

a proton motive force through an electrogenic redox loop (Philippot, 2002; Richardson

and Watmough, 1999).

Nitrite reductase

In continuing the reduction of NO_2^- to NO, nitrite reductase is the next enzyme in the denitrification pathway, of which there are two classes- the trimeric copper-containing enzyme (Cu-NIR) and the dimeric cytochrome cd_1 (cd_1NIR) enzyme. While evolutionarily unrelated, both Cu-NIR and cd_1NIR perform the same role in vivo, as determined through functional complementational studies. However, these two types of nitrite reductases are not found in the same bacterial species. Organisms containing cd_1NIR appear more abundant in nature, but those containing Cu-NIR are widely distributed in bacteria, occupying a wider range of ecological niches and exhibiting greater physiological diversity (Averill, 1996; Rinaldo and Cutruzzolà, 2007; Cantera and Stein, 2007a).

Copper nitrite reductases are periplasmic enzymes consisting of trimer proteins composed of three identical subunits in which each monomer contains two copper ions, forming type 1 and type 2 copper sites (Moura and Moura, 2001). Spectroscopic and structural studies of Cu-NIR in *Achromobacter cycloclastes* and *Alcaligenes xylosoxidans* show that in its oxidized state in both the presence and absence of NO_2^- suggest the type 2 copper centers are substrate binding sites, where after binding, reduction then occurs by electron transfer from the type 1 copper sites (Averill, 1996; Moura and Moura, 2001; Butler and Richardson, 2005).

Oxidized cd_1NIR enzymes resolved from *P. denitrificans* and *P. aeruginosa* show the reductase exists as dimers with each monomer consisting of two domains- a small

amino-terminal domain binding c-heme and a larger domain binding d_1 heme (Richardson and Watmough, 1999). While structural differences exist between the reductases of the two organisms, the proposed mechanism of nitrite reduction is thought to involve NO_2^- binding to the catalytic d_1 heme, forming NO while c-heme is the electron site, controlling the rate of electron transfer between the two hemes (Moura and Moura, 2001 and references therein; Rinaldo and Cutruzzolà, 2007). However, several issues remain unresolved including the exact role of d_1 heme during catalysis and the role of the redox state of c-heme as well as the residues responsible for NO displacement.

The genes encoding cd$_1$NIR and Cu-NIR are *nirS* and *nirK*, respectively. The *nirS* gene is part of a large gene cluster containing an array of genes, many of which encode for cytochromes and heme d$_1$ biosynthesis (i.e. *nirTBMCF* and *nirDLGHJEN*, respectively); however, gene arrangements vary between bacteria as well as within bacterial species (Philippot, 2002). While the *nirS* gene cluster has undergone extensive gene characterization in denitrifying bacteria, characterization of the *nirK* gene has relied on expression studies using pathogenic *Neisseria* despite the availability of resolved structures from *A. cycloclastes* and *Rhodobacter spaeroides* (Philippot, 2002). In *Neisseria*, nitrite reductase is encoded by the *aniA* gene, an outer membrane lipoprotein, necessary for anaerobic growth in potentially establishing virulence (Mellies et al., 1997). No gene other than *nirK* has been demonstrated to be involved in the synthesis of an active copper nitrite reductase in denitrifying bacteria (Philippot, 2002).

Nitric oxide reductase

Nitric oxide reductase further reduces NO to N_2O, forming the $N\equiv N$ bond. As with nitrite reductase, there are two forms of bacterial nitric oxide reductases, which are integral membrane complexes- a cytochrome *bc* complex (cNOR) that uses a *c*-type cytochrome as an electron donor and qNOR which lacks the cytochrome *c* component and accepts electrons from quinols (Hendriks et al., 2000). Additionally, qNOR can be found in archaea, and also in non-denitrifying pathogenic organisms including *N. gonorrhoea* and *Corynebacterium diphtheriae* where it is thought to serve a detoxification role (NO scavenger) from either self-produced NO or host macrophages (Hendriks et al., 2000; de Vries et al., 2007). As all bacteria that contain a cNOR are capable of denitrification, the cNOR form of nitric oxide reductases is briefly discussed here.

Purified cNOR is composed of two subunits- NorC which is a heme *c*-containing subunit and NorB which consists of a heme *b*-containing subunit. Electrons from *c*-type cytochromes are transferred to the heme *c* of NorC, where they are then transferred to heme *b* of NorB and eventually to the dinuclear non-heme Fe_b/b_3 site- the NO binding site (de Vries et al., 2007 and references therein). Using resonance Raman spectroscopy, Pinakoulaki and Varotsis (2008) proposed that NO activation occurs once bound NO reacts with two electrons transferred to heme b_3, allowing the formation of a Fe-N=O$^-$ species. The subsequent presence of a second NO molecule can attack the Fe-N complex

to yield an intermediate hyponitrite compound, where upon further cleavage can result in the N=N bond formation of N_2O.

Genetic analyses using *P. denitrificans* revealed that cNOR is encoded by a gene cluster consisting of six genes, *norCBQDEF* (de Boer et al., 1996). The authors determined that *norC* and *norB* encode the subunits of the NO reductase mentioned above while amino acid sequence alignments show *norQ* contains an ATP-binding motif and may serve as an activator for the reductase. The *norE* gene is thought to encode a protein similar to CoxIII, the third subunit of an aa_3-type cytochrome *c* oxidase. However, sequence alignments were not found for *norD* and *norF*, which are thought to be cytoplasmic and membrane-bound proteins, respectively. Both *norE* and *norF* are thought to serve in the stability, regulation, and assembly of the NO reductase (Philippot, 2002).

NO is rapidly reduced to N_2O immediately after it is generated due to its high reactivity in cells (Hendriks et al., 2000). While NO can bind to the Fe_b/b_3 active site of cNOR, NO can bind to and inhibit the activity of other metalloproteins as well such as cytochromes and hemoglobin, forming metal-nitrosyl complexes (Zumft, 1993). Nitrosyl species (i.e. HNO, $ONOO^-$) can interact with thiols, amines, and oxygen resulting in nitrosation of key thiol groups in enzymes, the deamination of amine groups in DNA, and oxidative damage to biological tissues (Hughes, 2008; Thomas et al., 2008). NO was also recently found to play a role in biofilm dispersal as Barraud et al. (2006) discovered that biofilm cells of *P. aeruginosa* became planktonic when exposed to a range of 25-300 nM of the NO-donor sodium nitroprusside, suggesting NO may be involved in regulating the

processes of differentiation within biofilms. On the contrary, in the presence of 5-50 ppm

NO, biofilm growth was induced in *Nitrosospira briensis*, *Nitrosolobus multiformis*, *N. eutropha*, and *N. europaea*, with down regulation of flagellar genes observed (Schmidt et al., 2004). In mammals, NO also serves as a signaling and defense molecule, where at approximately 10^{-7}M, NO controls blood pressure and is a messenger molecule in the central and peripheral nervous system (Poole, 2005).

Nitrous oxide reductase

The final enzyme of the denitrification pathway is nitrous oxide reductase, involved in the final reduction of N_2O to N_2. N_2O reductase is a periplasmic enzyme consisting of a homodimer, with each monomer containing two copper centers, Cu_A and Cu_Z (Einsle and Kroneck, 2004; Butler and Richardson, 2005). Cu_A contains binuclear copper atoms (Cu_1 and Cu_2) and is important for maintaining structural integrity of the reductase. The copper center of Cu_Z contains four copper ions and is the N_2O- binding site. Based on MAD phasing data from the nitrous oxide reductase of *Pseudomonas nautica*, studies indicate N_2O binds a single copper ion (CuIV) at the Cu_Z domain with the remaining copper ions serving as an electron reservoir (Moura and Moura, 2001; Butler and Richardson, 2005). It has been proposed that electron transfer occurs between the Cu_A of one monomer and the Cu_Z center of the second monomer in reducing to N_2O to N_2.

The catalytic subunit of nitric oxide reductase is encoded by *nosZ*. Further characterization of the gene revealed that *nosZ* resided in gene cluster of up to ten genes

involved in N_2O utilization. Though gene arrangements vary among organisms, the cluster *nosZDFYL* is found in every N_2O-reducing prokaryote (Zumft and Körner, 2007). The *nosD* gene was identified encoding for a Cu-processing periplasmic protein; *nosF* gene is predicted to encode an ABC transporter of ABC-type ATPase function; and *nosY* was identified as a membrane protein. The *nosL* gene is predicted to encode a lipoprotein that may be involved in the sequestration of copper in the periplasm for NosZ. According to taxonomic patterns, additional genes that may be in the *nos* cluster include *nosG, nosH, nosR,* and *nosX*. The *nosGH* genes are thought to encode for proteins involved in electron donation, while genes encoded by *nosR* and *nosX* are thought to play a role in either transport of copper to NosZ or insertion of copper into the metal binding site (Philippot, 2002, and references therein).

Anaerobic ammonium oxidation (Anammox)

Over the past decade, research has shown that denitrifying organisms are not the only organisms capable of producing N_2. Originally isolated from a wastewater treatment plant, there are currently five *Candidatus* genera related to the Planctomycetales order that can anaerobically oxidize NH_4^+ to N_2 with NO_2^- as the electron acceptor- Kuenenia, Brocadia, Anammoxoglobus, Scalindua, and Jettenia. While the biochemical mechanism of anaerobic NH_4^+ oxidation is not well understood, the production of hydrazine from hydroxylamine as an intermediate has led to the purification of hydrazine oxidoreductase in *B. anammoxidans* and the equivalent *hao* gene in *K. stuttgartiensis* (Jetten et al, 2005 and references therein; Op den Camp et al., 2007). Interestingly, immuno-labeling studies

revealed the enzyme is found within a membrane-bound organelle (the anammoxosome) composed of ladderane lipids.

Through phylogenetic analyses, use of fluorescently labeled probes and radio-labeled N isotopes, numerous studies have revealed that anammox bacteria are abundant in more than 30 freshwater and marine ecosystems all over the world (Op den Camp et al., 2006). In the anoxic waters of Golfo Dulce, Costa Rica, anammox activity has been attributed to 19 to 35% of N_2 formation (Dalsgaard et al., 2003). Kuypers et al. (2003) suggest that in the Black Sea, anammox bacteria could in theory consume more than 40% of the fixed nitrogen that sinks below depths of 80 meters. Thus, anammox bacteria may play a role in the nitrogen cycle in terms of oceanic nitrogen cycling. However, anammox activity has yet to be measured in soils, and on a global scale the overall contribution of anammox to global nitrogen loss remains unclear (Francis et al., 2007).

Nitrification

Nitrification is carried out in most soil ecosystems by the chemolithoautotrophic ammonia-oxidizing bacteria and archaea (AOB and AOA, respectively) and nitrite-oxidizing bacteria (NOB). These organisms are globally distributed in nearly every ecosystem on Earth. Nitrification is a two-step process in which NH_3 produced from N_2 fixation or mineralization is oxidized to nitrate (NO_3^-). The reaction carried out by AOB is the oxidation of NH_3 to NO_2^-, with the production of hydroxylamine (NH_2OH) as an intermediate. NOB are responsible for the subsequent oxidation of NO_2^- to NO_3^-. As with denitrification, nitrification allows these organisms to use the energy released from the

oxidation of NH_3 and NO_2^- for cell growth. Until recently, physiological and ecological

advances in these bacteria have been restricted due to the organisms' slow growth, low

yield, and difficulty of isolation (Hayatsu et al., 2008).

While heterotrophic nitrification exists, little is known as to the physiological role

and phylogenetic diversity of heterotrophic nitrifying microorganisms (Ferguson et al.,

2007; Hayatsu et al., 2008). As heterotrophic nitrifiers are incapable of using nitrification

to support growth and therefore energy generation, heterotrophic nitrification does not

appear to significantly contribute to nitrification in nature, though they have been linked

to the nitrification of acidic soils (Prosser, 2007; Hayatsu et al., 2008). Therefore, it is

generally accepted that autotrophic microorganisms are largely, if not entirely,

responsible for nitrification. (Bremner and Blackmer, 1981 and references therein;

Ferguson et al., 2007).

Currently, there are two major divisions into which AOB are classified, the γ-

proteobacteria (AOG) and the β-proteobacteria (AOB), as determined through

phylogenetic analyses of the 16S rRNA and the key functional gene ammonia

monooxygenase (*amoA*) (Prosser, 2007). Most cultivated strains fall within the AOB

division, under two major genera *Nitrosomonas* and *Nitrosospira*. Currently, published

genome sequences of AOB include those for *N. europaea*, *N. eutropha*, *Nitrosospira*

multiformis, and *Nitrosococcus oceani* (Chain et al., 2003; Stein et al., 2007; Norton et al.,

2008; Klotz et al., 2006). All bacterial soil NH_3 oxidizers appear to belong to the AOB,

with *Nitrosomonas europaea* used in biochemical and physiological studies as the model

strain (Prosser, 2007). However, phylogenetic surveys of soil and sediment indicate

Nitrosospira spp. may be the more dominant AOB in terrestrial environments (Stephen et al., 1996; Smith et al., 2001). *Nitrosospira spp.* has been identified in environments containing low amounts of nitrogen while *N. eutropha* and *N. europaea* are normally found in high and moderate nitrogen environments. *N. eutropha* and *N. europaea* can tolerate up to 600mM and 400mM NH_4, respectively (Stein et al., 2007 and references therein). Therefore, because of the ecological niches they occupy as well as the availability of their genome sequences, *N. multiformis*, *N. eutropha*, and *N. europaea* were selected for the study presented in Chapter 2 of this dissertation in the investigation of the role of nitrifier denitrifier genes in AOB.

AOB are usually found in communities also containing NOB. Classified under the α, γ, and δ-proteobacteria and the Nitrospira phylum, four genera have been identified based on phenotypic characterization: *Nitrobacter*, *Nitrospina*, *Nitrospira*, and *Nitrococcus*. Among the four, *Nitrobacter* has been used for biochemical and physiological analysis, with genome sequences available for *N. winograskyi* and *N. hamburgensis* X14 in 2006 and 2008, respectively (Starkenburg et al., 2006, Starkenburg et al., 2008).

Ammonia monooxygenase (*amoA*)

Bacterial ammonia monooxygenase is the membrane-bound, multi-subunit enzyme responsible for the oxidation of NH_3 to NH_2OH. Bacterial AMO is encoded by at least three genes, *amoA*, *amoB*, and *amoC*, found in duplicate operons (*amoCAB*) in the *Nitrosomonas* genome, with *amoA* and *amoB* encoding structural subunits of the enzyme

(Bergmann and Hooper, 1994; McTavish et al., 1993). The *amoA* gene is thought to

encode the subunit containing the catalytic site (AmoA), while *amoB* encodes for the

subunit AmoB. While the functional role of the *amoC* gene product has not been

established, it has been proposed that AmoC may aid in the localization of the AmoA and

AmoB subunits into the membrane and could play a role in recovery from starvation

(Klotz et al., 1997; Berube et al., 2007). Recently, two new genes in the *amo* operon were

identified in the marine NH_3 oxidizer *Nitrosococcus oceani*, *amoR* and *amoD* (El Sheikh

et al., 2008). The authors speculate that the *amoR* gene product may be involved in the

regulation of ammonia catabolism while *amoD* may encode a multicopper oxidase.

Because a purified AMO has never been obtained, very little is known regarding

its molecular structure (Hooper et al., 1997; Prosser, 2007). However, it has been

suggested that AMO contains copper, as sequences of the two subunits (AmoA and

AmoB) of the enzyme are homologous to a Cu-dependent methane monooxygenase

(Prosser, 2007 and references therein; Arp and Stein, 2003 and references therein).

Because AMO requires both molecular O_2 and electrons, it is thought that the ubiquinol

pool serves as the electron donor to AMO, which receives four electrons via cytochromes

relayed from hydroxylamine oxidoreductase and donates two electrons for each NH_3

molecule oxidized to NH_2OH (Whittaker et al., 2000).

Hydroxylamine oxidoreductase

Hydroxylamine oxidoreductase (HAO) is a periplasmic enzyme involved in the

oxidation of NH_2OH to NO_2^- in AOB, the reaction of which provide the electrons that are

supplied to AMO via the ubiquinone pool as previously mentioned. HAO is found in a gene cluster also containing *cycA* and *cycB*, encoding cytochrome c_{554} and a tetraheme *c* cytochrome (cytochrome c_{m552}), respectively, (Bergmann et al., 1994). Although not in an operon, three copies of *hao* and *cycA* and two copies of *cycB* are present in *N. europaea* (Hooper et al., 1997; Bergmann et al., 1994; McTavish et al., 1993).

With its crystal structure resolved, the HAO protein is a trimer consisting of eight hemes, seven *c*-type and one P460, in each monomer with the hemes localized in the bottom half of the molecule (Igarashi et al., 1997). The trimer form of HAO is necessary for stabilization and catalytic function, also providing putative binding clefts for cytochrome c_{554}. Located at the bottom of the catalytic pocket, the P460 heme is believed to be the binding site of NH_2OH. Clustered with P460 are two *c* hemes that can interact and remove electrons from NH_2OH and in turn transfer them to nearby heme clusters, and eventually to cytochrome c_{554} in the oxidation of NH_2OH to NO_2^-. However, many details remain unclear, especially regarding catalysis, as structures of redox partners coupled to HAO have not yet been resolved (Igarashi et al., 1997).

Nitrite oxidoreductase

Nitrite is oxidized to NO_3^- by the nitrite oxidoreductase (NXR) enzyme present only in NOB. The enzyme is a membrane-associated heterodimer consisting of a large (α) and small (β) subunit, encoded by the *nxrA* and *nxrB* genes, respectively (Starkenburg et al., 2006 and references therein). The two genes are located in an operon (*nxrAXB*) containing genes of putative accessory functions that may be involved in NXR

biosynthesis, electron transport, and NO_3^-/NO_2^- transport. NXR can act reversibly as well, reducing NO_3^- to NO_2^- which is thought to be part of the denitrification pathway in *N. winogradskyi* or perhaps may function in the presence of significant accumulated NO_3^- concentrations (Starkenburg et al., 2006; Prosser, 2007). However, the exact mechanism of NO_2^- oxidation to NO_3^- and electron transfer remains unclear.

Ammonia-oxidizing archaea

In addition to anaerobic ammonia oxidizing bacteria, ammonia oxidizing archaea (AOA) have also been of current interest as metagenomic analyses have revealed the presence of *amo*-like genes in in the Thaumarchaeota phylum of the Archaea. Thaumarchaeota have been found in distinct communities and in diverse environments ranging from the suboxic water columns of the Black Sea to the estuarine sediments of Bahía del Tóbari, Mexico (Francis et al., 2005). Though often found in extreme environments, a mesophilic marine thaumarchaeon has been isolated, *Nitrosopumilus maritimus*, which was found to grow chemolithoautotrophically with NH_3 as the sole energy source (Könneke et al., 2005). Comparison of *amo* gene clusters in soil and marine Thaumarchaeota with those of Proteobacteria reveals differences in gene arrangement and size (Nicol and Schleper, 2006 and references therein). While a *hao* homolog has not been found, it is assumed that Thaumarchaeota likely have either a variant form or process a different intermediate altogether; thus, further biochemical analyses are necessary in elucidating thaumarchaeal metabolism (Walker et al., 2010).

In terrestrial and aquatic environments, ~6% or more of the prokaryote population might be represented by Thaumarchaeota (Nicol and Schleper, 2006). After sampling a dozen sites of various soil types, Leininger et al. (2006) found that Thaumarchaeota *amoA* gene copy numbers were up to 3000-fold greater than *amoA* gene copies of AOB, suggesting that Thaumarchaeota may be the predominant ammonia oxidizer in soil ecosystems. However, Okano et al. (2004) found that kinetic parameters of ammonia oxidation (i.e. growth yields using *amoA* as genetic marker and rates of ammonia oxidation per cell) from agricultural soils amended with ammonium sulfate were similar to those of pure cultures of *Nitrosospira* spp. Therefore, while data may suggest that Thaumarchaeota are major contributors to nitrogen cycling, their quantitative contribution to nitrification and impact on the global nitrogen cycle has yet to be elucidated (Nicol and Schleper, 2006).

Nitrifier denitrification

While the role nitrifying and denitrifying bacteria play in the microbial nitrogen cycle may appear straightforward in terms of the transformation of nitrogen and the nitrogen products formed, research has shown that this is not the case. Previous literature has demonstrated that AOB produce NO and N_2O during nitrification. Indeed, production of both gases has been observed experimentally in both cells and cell-free extracts of *N. europaea* incubated with either NH_3 or NH_2OH under aerobic and anaerobic conditions (Hooper, 1968; Ritchie and Nicholas, 1972; Goreau et al., 1980). Thus far, two pathways have been proposed in which production of NO and N_2O are thought to occur: 1) through

biological activity of AOB- a phenomenon referred to as nitrifier denitrification and, to a lesser extent, 2) through incomplete oxidation of NH_2OH to NO_2^- (Colliver and Stephenson, 2000 and references therein). However, the physiological role of nitrifier denitrification remains unclear (Hayatsu et al., 2008).

The majority of NO and N_2O produced by AOB has been attributed to their biological activity. Sterile soil aerobically incubated with up to 1mg $(NH_4)_2SO_4$/g soil and inoculated with cultures of *Nitrosomonas* spp. produced significant amounts of N_2O (Blackmer et al., 1980). After 7 days of inoculation, N_2O emissions from *N. europaea* was 1820ng N_2O-N/g soil in the 1000µg $(NH_4)_2SO_4$/g soil treatment group, while after 28 days, 19,300ng N_2O-N/g soil was produced. Thus, a positive correlation was made between the increase of ammonium concentrations and N_2O production. N_2O emissions from well-aerated, field-moist soils are greatly enhanced by the addition of nitrifiable forms of nitrogen such as ammonium and urea, but are not significantly affected by the addition of nitrate. Hence, the majority of N_2O produced likely evolves from nitrifiable forms of nitrogen during nitrification rather than from denitrification (Bremner and Blackmer, 1981 and references therein). Lack of NO and N_2O emissions in nitrite-amended soils (without ammonium) containing *Nitrobacter* spp. as well as the lack of such contribution in soils amended with chlorate to inhibit nitrite oxidation in mixed cultures containing AOB and NOB further establishes AOB as the sole group of nitrifying organisms responsible for N_2O and NO production (Blackmer et al., 1980; Goreau et al., 1980; Tortoso and Hutchinson, 1990). There is no evidence that AOA are capable of producing N_2O.

While N_2O appears to be the predominant gas emitted by AOB in the presence of NH_2OH as the electron donor, the presence of both ammonium and nitrite and low O_2 favors NO and N_2O production in *Nitrosomonas* spp. (Anderson and Levine, 1986). In batch culture, it was observed that NO emissions were greater than those of N_2O, 1.6μL NO/L versus 0.8μL N_2O /L in the presence of 2.2mM NO_2^- and 0.5% oxygen after 28 hours (Anderson and Levine, 1986). Additionally, N_2O production by nitrifiers were inversely proportional to various oxygen tensions (0.5 to 10% O_2) while NO production was not affected, suggesting N_2O production is influenced by oxygen while production of NO is not. Remde and Conrad (1990) observed that a shift from aerobic to anaerobic conditions significantly stimulated N_2O production (~700-fold) though slightly increasing NO production (~3-fold) in *N. europaea* and a *Nitrosospira* spp. Seven other strains of the *Nitrosospira* genus were likewise capable of producing N_2O, though at a reduced capacity compared to *N. europaea*, suggesting nitrifier denitrification is a common trait among the AOB of the β-Proteobacteria class (Shaw et al., 2006). It is therefore assumed that AOB can likely produce NO over a wider range of soil conditions than N_2O, but production of N_2O is influenced by oxygen-limiting conditions while both gases evolve from the reduction of nitrite which serves as the electron acceptor (Anderson and Levine, 1986; Goreau et al., 1980; Remde and Conrad, 1990; Colliver and Stephenson, 2000 and references therein).

In AOB, NH_2OH is oxidized to NO_2^- by HAO, as mentioned previously. However, in the process, HAO itself carries out a two-step reaction wherein HNO is produced as an enzyme-bound intermediate. With the continuous flow of electrons, HAO allows further

oxidation to HNO_2 and eventually to NO_2^-. However, incomplete oxidation of HNO_2 or decomposition of NH_2OH results in the production of NO and N_2O, sometimes referred to as chemodenitrification (Igarashi et al., 1997; Wrage et al., 2001). In differentiating between chemodenitrification and nitrifier denitrification to the contribution of NO and N_2O, the use of nitrification inhibitors such as nitrapyrin have been employed. Used in the inhibition of ammonia oxidation to nitrite, nitrapyrin has been observed to suppress NO and N_2O emissions to near zero levels, suggesting both gases are metabolic products of AOB rather than intermediates of chemodenitrification (Tortoso and Hutchinson, 1990). While it was observed that about 15% to 20% of NO produced could be attributed to chemical decomposition of nitrite, biologically-mediated processes are responsible for the majority of NO and N_2O emissions in AOB (Remde and Conrad, 1990; Anderson and Levine, 1986).

Previous studies have suggested that nitrifier denitrification may be responsible for a significant proportion of NO and N_2O emissions from soil (Goreau et al., 1980; Bremner and Blackmer, 1981; Tortoso and Hutchinson, 1990). In fact, production rates of NO and N_2O under low oxygen tensions were significantly higher in *Nitrosomonas* spp. compared to three representative denitrifiers (*Pseudomonas fluorescens*, *Alcaligenes faecalis*, and *Rhizobium japonicum*) and a nitrate respirer (*Serratia marcescens*) grown in batch culture, with the exception of aerobic N_2O production from *P. fluorescens* (Levine and Anderson, 1986). It should be noted, however, that because a standard, simple method does not exist to specifically measure nitrifier denitrification, differentiating

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between nitrifier denitrification and denitrification remains a challenge (Wrage et al., 2001).

Nitrifier denitrification pathway

Enzymatic reduction of NO_2^- to produce NO and N_2O in *N. europaea* was demonstrated by Hooper (1968) using cell-free extracts. With several AOB genome sequences now available, genetic analyses have revealed the presence of denitrifier-type genes including nitrite and nitric oxide reductases as well as cytochrome P460 and cytochrome *c'*-beta. These genes are therefore thought to function during nitrifier denitrification and in the production of NO and N_2O. However, information concerning the physiological role and the regulation of these genes involved in nitrifier denitrification remain limited (Hayatsu et al., 2008; Klotz and Stein, 2008).

Nitrite reductase (NirK)

Nitrite reductase is the central enzyme of the denitrification pathway as it produces the first obligate, gaseous intermediate NO (Casciotti and Ward, 2001). Phylogenetic analyses and alignment of partial *nirK* sequences from denitrifying bacteria have revealed the presence of copper-containing nitrite reductase (*nirK*) genes in AOB (Casciotti and Ward, 2001; Cantera and Stein, 2007a; Garbeva et al., 2007). High amino acid sequence similarity (>59%) between nitrifying and denitrifying bacteria suggest lateral transfer of the gene while >58% sequence similarity between AOB likely indicates genetic diversity of *nirK* among this group of closely related bacteria (Casciotti and Ward,

2001; Garbeva et al., 2007). In fact, nitrifier *nirK* sequences were distributed into three distinct clusters: cluster 1 contained *Nitrosomonas* species from soil and sewage environments and the *Nitrobacter* species, cluster 2 contained *Nitrosospira* species, except for *Nitrosopira tenuis* which is grouped in cluster 3, along with marine species of *Nitrosomonas* (Cantera and Stein, 2007a).

Despite the prevalence of *nirK* in AOB, its presence does not necessarily indicate functionality (Casciotti and Ward, 2001; Garbeva et al., 2007). Thus, to determine whether the *nirK* gene encodes a functional nitrite reductase, a NirK-deficient strain of *N. europaea* was constructed (Beaumont et al., 2002). Interestingly, under aerobic conditions, N_2O production was actually enhanced in the NirK-mutant cells compared to wild-type cells (0.3 mM N_2O versus 0.11 mM N_2O) while NO production rates were similar between the two strains (Beaumont et al., 2002). In a similar study, Schmidt et al. (2004) also observed that N_2O as well as NO production were each ~6 times higher in NirK-deficient *N. europaea* cells than in wild-type cells; NH_2OH concentrations were ~7 times higher in NirK-deficient cells as well. Growth rates and ammonia oxidation activities remained similar (Beaumont et al., 2002; Schmidt et al., 2004). In the presence of increasing $NaNO_2$, growth was inhibited in NirK-deficient cells (Beaumont et al., 2002). Wild-type *N. europaea* were still able to grow in the presence of 100 mM $NaNO_2$, but NirK-deficient cells were not, unable to cope with NO_2^- produced from nitrification as well as added NO_2^-. This suggests NirK confers tolerance to nitrite by serving as a detoxifying enzyme since increased concentrations of nitrite can inhibit ammonia oxidation (Beaumont et al., 2002).

Under both high (23%) and low (3.3%) oxygen tensions, wild-type *N. europaea* cells produced twice as much N_2O as NirK-deficient cells when NH_2OH was used as the substrate (Cantera and Stein, 2007b). However, in the presence of NH_3 under fully oxic conditions, the NirK mutant produced a significantly greater amount of N_2O but exhibited a slower rate of conversion of NH_3 to NO_2^- compared to wild-type cells, similar to that observed previously (Beaumont et al, 2002). Together, the data suggest that HAO plays a role in N_2O production in conjunction with NirK. Additionally, use of N_2H_4 as an alternate substrate for HAO and $NaNO_2$ as the electron acceptor resulted in the same level of N_2O production in both wild-type and NirK-deficient cells regardless of the oxygen level, suggesting a NirK-independent pathway for N_2O production in *N. europaea*. Together, these results indicate the importance of NirK in supporting NH_3 oxidation to NO_2^-, particularly via NH_2OH, as well as the combined activities of both NirK and HAO in the formation of N_2O (Cantera and Stein, 2007b). The production of N_2O in the absence of NirK, as well as the production of NO and the presence of high concentrations of NH_2OH as seen in previous studies, can therefore be largely attributed to oxidation of NH_2OH (Beaumont et al., 2002; Schmidt et al., 2004; Cantera and Stein, 2007b). Furthermore, as dead cells did not produce N_2O in the presence of NH_2OH, it appears that formation of N_2O from NH_2OH must be an enzymatic rather than a chemical process.

In *N. europaea*, the *nirK* gene is clustered with three other genes (*ncgABC*). Sequence similarities suggest *ncgA* encodes for a periplasmic blue copper oxidase while *ncgB* and *ncgC* products are di- and mono-heme c-type cytochromes. However, the

physiological functions of the three genes which are upstream of *nirK* remain unknown (Beaumont et al., 2005). Mutagenesis experiments of each *ncg* gene resulted in reduced expression of *nirK*, suggesting the four genes are expressed in an operon. Additionally, introduction of a NirK expression vector in mutant strains enhanced NirK expression and activity, indicating *ncgABC* are not involved in the synthesis of NirK (Beaumont et al., 2005). Overall, growth was decreased among the *ncgABC* mutants compared to wild-type cells, with a more pronounced decrease in cell density in *ncgB* and *ncgC* mutants containing the NirK expression vector. In the presence of 10 mM $NaNO_2$, both *ncgA* and *nirK* mutants reached similar cell densities. The *nirK* mutant containing the NirK expression vector was able to regain a wild-type phenotype, while the *ncgA* mutant with the NirK expression vector was not, and showed a greater decrease in cell density. This negative effect on cell density was even more pronounced in the presence of 20 and 40 mM $NaNO_2$. Therefore, it is likely that NcgA plays a role in nitrite tolerance and that NcgABC is involved in scavenging NO produced by NirK (Beaumont et al., 2005), most likely by shuttling electrons between NirK and nitric oxide reductase, NorB.

Additionally, a gene was found divergently upstream of the *nirK* operon in *N. europaea*, homologous to genes of putative transcriptional regulators, and was thus designated as the *nsrR* gene (nitrite-sensitive repressor) (Beaumont et al., 2004a). In wild-type *N. europaea* cells, amount of NirK substantially increased as cell density reached late-log to stationary-phase, with an 18-fold increase compared to early exponentially growing cells. However, *nirK* was constitutively expressed in NsrR-deficient cells regardless of cell density. Results therefore suggest the involvement of

NsrR in nitrite-dependent regulation and expression of *nirK*. Studies using *E. coli* cells containing both the *nirK* promoter upstream of a *lacZ* gene and the NsrR expression vector showed decreased β–galactosidase activity, indicating repressive activity of NsrR. However, in the presence of increasing nitrite, repression of NsrR was reversed accordingly as indicated by increasing amounts of β–galactosidase activity, providing further support that NirK acts as a detoxifying enzyme during nitrification (Beaumont et al., 2004a).

N. eutropha shares the *nirK* gene cluster found in *N. europaea* as well as the *nsrR* regulatory gene (Stein et al., 2007). On the other hand, the *nirK* gene in *N. multiformis* is phylogenetically distinct, existing as a single gene and lacks the conserved binding motif necessary for a regulatory protein such as NsrR (Cantera and Stein, 2007a; Norton et al., 2008). Structurally different, NirK of *N. multiformis* consists of two regions, a copper binding motif related to plastocyanin proteins and conserved type 1 and type 2 copper binding motifs representative of copper-containing nitrite reductases (Cantera and Stein, 2007a). Thus, regulation and expression as well as the role and function of NirK, especially pertaining to nitrifier denitrification, in *N. multiformis* may be different than that of *N. europaea* and *N. eutropha* (Cantera and Stein, 2007a; Norton et al., 2008).

Nitric oxide reductase (NorB and NorS)

In addition to *nirK*, *N. europaea* also contains a cNOR-type nitric oxide reductase, encoded by the *norCBQD* gene cluster, similar to that found in denitrifying bacteria (Chain et al., 2003). *N. eutropha* and *N. multiformis* likewise have similar gene clusters in

their respective genomes (Stein et al., 2007). The gene cluster of *N. eutropha* shares

>70% amino acid sequence similarity compared with *N. europaea*, while only sharing

<56% similarity with *N. multiformis* (Stein et al., 2007). Interestingly, *Nitrosospira*

strains had 73-86% similarity to partial NorB sequences of denitrifying bacteria rather

than AOB (Garbeva et al., 2007). Thus far, functional studies concerning cNOR in AOB

have been carried out only in *N. europaea* (Beaumont et al., 2004b). While results can be

inferred as to the activity of cNOR in *N. eutropha* as the two AOB share high sequence

similarity, no studies have been conducted concerning the role of NOR in *Nitrosospira*

spp. in the metabolism of NO (Stein et al., 2007; Norton et al., 2008).

In *N. europaea*, the *norCBQD* cluster encodes a functional nitric oxide reductase;

however, unlike denitrifying bacteria, the cluster is flanked by uncharacterized open

reading frames and is separated from the *nirK* operon (Beaumont et al., 2004b). Growth

of NorB-deficient mutants was not affected as cell densities were identical to wild-type

cells under both aerobic and oxygen-limited conditions. NorB-deficient mutants showed

a marked decrease in growth rate and cell density in the presence of 200 μM SNP (NO-

releasing compound), however, at lower concentrations (≤100 μM SNP), both mutant and

wild-type cells exhibited similar growth characteristics (Beaumont et al., 2004b). Anoxic

NO consumption in NorB-deficient mutants was remarkably decreased compared to wild-

type cells, with consumption ceasing before NO was completely consumed. Wild-type

cells added at that time were able to consume remaining NO. Interestingly, membrane

fractions of mutants maintained NO consumption capabilities, albeit at a reduced capacity,

though it wasn't determined as to whether NO removal occurred enzymatically or

chemically (Beaumont et al., 2004b). In the presence of NO_2^-, mutants displayed comparable growth rates and cell density to wild-type *N. europaea*. The authors therefore concluded that NorCB does not play an important role in NO or NO_2^- tolerance during nitrification in *N. europaea* (Beaumont et al., 2004b).

NorB-deficient strains have been observed to produce N_2O. In one study, a mutant strain produced amounts comparable to that produced in wild-type *N. europaea*, with total N_2O amounts of 31± 5 and 40±10 μM, respectively (Beaumont et al., 2004b). Another study found that NH_2OH concentrations were ~7 times higher compared to wild-type cells, with NO and N_2O production nearly equally enhanced in NorB-deficient strains (Schmidt et al., 2004). The increased production of N_2O seen may be attributed to NH_2OH oxidation, or possibly an alternative NO-consuming mechanism may exist (Schmidt et al., 2004; Beaumont et al., 2004b).

N. europaea, *N. eutropha*, and *N. multiformis* have been found to contain the *norSY-SenC* (NorS) gene cluster in their respective genomes (Norton et al., 2008; Stein et al., 2007). This gene cluster encodes a novel cytochrome *c* oxidase of the heme copper oxidase family similar to the cytochrome *c* oxidase of nitric oxide reductase (NorB) and is thought to act as a nitric oxide reductase involved in the detoxification of NOx intermediates produced by AOB (Norton et al., 2008; Stein et al., 2007 and references therein). Transcriptome analysis comparing wild-type and NirK-deficient *N. europaea* showed mutant strains experienced a ~2.6 fold increase in genes encoding *norSY* (Cho et al., 2006). The up-regulation was observed as a response to the nitrosative stress conditions encountered by the cells due to the lack of NirK activity, suggesting NorS may

serve a protective role (i.e. a potential alternative nitric oxide reductase) in the presence of nitrogen oxides in AOB (Cho et al., 2006).

Cytochromes P460 and c'-beta

In *N. europaea*, a separate, periplasmic cytochrome P460 was found, encoded by a gene (*cyt*L) downstream of the *hao* genes (McTavish et al., 1993; Bergmann and Hooper, 1994). A dimer in form, this P460 is structurally different from that of P460 in HAO, sharing only a similar *c* heme binding motif (Numata et al., 1990; Pearson et al., 2007; Bergmann and Hooper, 1994). The motif lies within a novel binding fold consisting predominantly of β–sheets, with an exposed cleft thought to be involved with ligand binding and catalytic function (Pearson et al., 2007). P460 also contains a lysine-heme crosslink necessary for catalytic activity, unlike the tyrosine-heme crosslink found in the P460 of HAO. As with HAO, P460 can likewise oxidize NH_2OH to NO_2^-, but at a 40-fold lower rate (Numata et al., 1990). P460 can also bind hydrazine, CO, H_2O_2 as well as NO. However, the physiological role and function of P460 in AOB remains unclear (Numata et al., 1990; Pearson et al., 2007). The P460 gene has also been found in *N. eutropha*, but interestingly, not in *N. multiformis* (Stein et al., 2007; Norton et al., 2008).

A P460 of this nature usually can be found in denitrifying, N_2-fixing, and photosynthetic bacteria as well as in some Gammaproteobacteria methanotrophs. In these organisms, P460 has been associated with the oxidation/reduction of nitrogen oxides or in NO_x-ligation for detoxification and energy generation (Elmore et al., 2007 and references therein). Phylogenetic analysis of P460 protein sequences suggest cytochrome P460

evolved from ancestral cytochrome *c'*-beta genes. In the methanotroph *Methylococcus capsulatus* Bath, P460 has been co-purified with a cytochrome *c'* which is thought to be its redox partner (Pearson et al., 2007 and references therein). This may also be the case in AOB as a cytochrome *c'*-beta encoding gene (*cyt*S) has been found in the genomes of *N. europaea*, *N. eutropha*, and *N. multiformis*.

Also a dimer in form and containing the *c* heme binding motif, cytochrome *c'*-beta lacks the lysyl-heme crosslink and can reversibly bind, but not transform NO (Bergmann and Hooper, 2003). As a result, cytochrome *c'*-beta is also thought to play a role in NO detoxification, protecting against nitrosative stress as well as bind NO during denitrification (Cross et al., 2000; Choi et al., 2005). Photosynthetic purple bacterium *Rhodobacter capsulatus* expressing cytochrome *c'* were less susceptible to NO than in *c'*-mutant strains in terms of growth rate and oxygen respiration, suggesting that it likely serves to detoxify NO in vivo, providing increased resistance to NO (Cross et al., 2000; Cross et al., 2001). Recent studies suggest that cytochrome *c'*-beta is not a reductase but rather shuttles NO to a nitric oxide reductase (Mayburd and Kassner, 2002; Choi et al., 2005). However, the role and function of cytochrome *c'*-beta in AOB have not yet been elucidated.

References

Ambus P and Zechmeister-Boltenstern S. (2007). Denitrification and N-cycling in forest ecosystems. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Anderson IC and Levine JS. (1986). Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. *Applied and Environmental Microbiology*, **51(5)**, 938-945.

Anderson N, Strader R, Davidson C. (2003). Airborne reduced nitrogen: ammonia emissions from agriculture and other sources. *Environment International*, **29**, 277-286.

Armengaud J, Happe B, Timmis KN. (1998). Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: Catabolic genes dispersed on the genome. *Journal of Bacteriology*, **180(15)**, 3954-3966.

Armengaud J, Timmis KN, Wittich R-M. (1999). A functional 4-hydroxysalicylate/ hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-p-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *Journal of Bacteriology*, **181(11)**, 3452-3461.

Arp DJ and Stein LY. (2003). Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Critical Reviews in Biochemistry and Molecular Biology*, **38**, 471-495.

Averill BA. (1996). Dissimilatory nitrite and nitric oxide reductases. *Chemical Reviews*, **96**, 2951-2964.

Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S, Webb JS. (2006). Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, **188(21)**, 7344-7353.

Basta T, Keck A, Klein J, Stolz A. (2004). Detection and characterization of conjugative degradative plasmids in xenobiotic-degrading *Sphingomonas* strains. *Journal of Bacteriology*, **186(12)**, 3862-3872.

Baumann B, Snozzi M, Zehnder AJB, van der Meer JR. (1996). Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. *Journal of Bacteriology*, **178(15)**, 4367-4374.

Beaumont HJE, Hommes NG, Sayavedro-Soto LA, Arp DJ, Arciero DM, Hooper AB, Westerhoff HV, van Spanning RJM. (2002). Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *Journal of Bacteriology*, **184(9)**, 2557-2560.

Beaumont HJE, Lens SI, Reijnders WNM, Westerhoff HV, van Spanning RJM. (2004a). Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor. *Molecular Microbiology*, **54(1)**, 148-158.

Beaumont HJE, van Schooten V, Lens SI, Westerhoff HV, van Spanning RJM. (2004b). *Nitrosomonas europaea* expresses a nitric oxide reductase during nitrification. *Journal of Bacteriology*, **186(13)**, 4417-4421.

Beaumont HJE, Lens SI, Westerhoff HV, van Spanning RJM. (2005). Novel *nirK* cluster genes in *Nitrosomonas europaea* are required for NirK-dependent tolerance to nitrite. *Journal of Bacteriology*, **187(19)**, 6849-6851.

Benton PMC, Sen S, Peters JW. (2002). Nitrogenase structure. *In: Nitrogen Fixation at the Millennium* [Leigh GJ (ed.)] Elsevier, B.V., Amsterdam, The Netherlands.

Bergmann DJ, Arciero DM, Hooper AB. (1994). Organization of the *hao* gene cluster of *Nitrosomonas europaea*: Genes for two tetraheme *c* cytochromes. *Journal of Bacteriology*, **176(11)**, 3148-3153.

Bergmann DJ and Hooper AB. (1994). Sequence of the gene, *amoB*, for the 43-kDa polypeptide of ammonia monoxygenase of *Nitrosomonas europaea*. *Biochemical and Biophysical Research Communications*, **204(2)**, 759-762.

Bergman DJ and Hooper AB. (1994). The primary structure of cytochrome P460 of *Nitosomonas europaea*: presence of a *c*-heme binding motif. FEBS Letters, **353**, 324-326.

Bergmann DJ and Hooper AB. (2003). Cytochrome P460 of *Nitrosomonas europea*. Formation of the heme-lysine cross-link in a heterologous host and mutagenic conversion to a non-cross-linked cytochrome *c'*. *Eur. J. Biochem*, **270**, 1935-1941.

Berube PM, Samudrala R, Stahl DA. (2007). Transcription of all *amoC* copies is associated with recovery of *Nitrosomonas europaea* from ammonia starvation. *Journal of Bacteriology*, **189(11)**, 3935-3944.

Beyer S, Gilch S, Meyer O, Schmidt I. (2008). Transcription of genes coding for metabolic key functions in *Nitrosomonas europaea* during aerobic and anaerobic growth. *Journal of Molecular Microbiology and Biotechnology*, 1-11. Published online: July 1, 2008.

Blackmer AM, Bremner JM, Schmidt EL. (1980). Production of nitrous oxide by ammonia-oxidizing chemoautotrophic microorganisms in soil. *Applied and Environmental Microbiology*, **40(6)**, 1060-1066.

Bollman A, Schmidt I, Saunders AM, Nicolaisen MH. Influence of starvation on potential ammonia-oxidizing activity and *amoA* mRNA levels of *Nitrosospira briensis*. *Applied and Environmental Microbiology*, **71(3)**, 1276-1282.

Bremner JM and Blackmer AM. (1981). Terrestrial nitrification as a source of atmospheric nitrous oxide. *In: Denitrification, nitrification, and atmospheric nitrous oxide* [Delwiche CC. (ed)] Wiley & Sons, Inc., New York.

Bryan BA. (1981). Physiology and biochemistry of denitrification. *In: Denitrification, nitrification, and atmospheric nitrous oxide* [Delwiche CC. (ed)] Wiley & Sons, Inc., New York.

Bünz PV and Cook AM. (1993). Dibenzofuran 4, 4a-dioxygenase from *Sphingomonas* sp. strain RW1: Angular dioxygenation by a three-component enzyme system. *Journal of Bacteriology*, **175(20)**, 6467-6475.

Butler CS and Richardson DJ. (2005). The emerging molecular structure of the nitrogen cycle: an introduction to the proceedings of the 10th annual N-cycle meeting. *Biochemical Society Transactions*, **33(1)**, 113-117.

Cantera JJL and Stein LY. (2007a). Molecular diversity of nitrite reductase genes (*nirK*) in nitrifying bacteria. *Environmental Microbiology*, **9(3)**, 765-776.

Cantera JJL and Stein LY. (2007b). Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. *Archives of Microbiology*, **188(4)**, 349-354.

Carmargo JA and Alonso Á. (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic systems: A global assessment. *Environment International*, **32**, 831-849.

Carter JP, Hsiao YH, Spiro S, Richardson DJ. (1995). Soil and sediment bacteria capable of aerobic nitrate respiration. *Applied and Environmental Microbiology*, **61(8)**, 2852-2858.

Casciotti KL and Ward BB. (2001). Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, **67(5)**, 2213-2221.

Casella S and Payne WJ. (1996). Potential of denitrifiers for soil environment protection. *FEMS Microbiology Letters*, **140**, 1-8.

Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz M, Norton J, Sayavedra-Soto L, Arciero D, Hommes N, Whittaker M, Arp D. (2003). Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithautotroph *Nitrosomonas europaea*. *Journal of Bacteriology*, **185(9)**, 2759-2773.

Cheng Q. (2008). Perspectives in biological nitrogen fixation research. *Journal of Integrative Plant Biology*, **50(7)**, 786-798.

Cho CM-H, Yan T, Liu X, Wu L, Zhou J, Stein LY. (2006). Transcriptome of a *Nitrosomonas europaea* mutant with a disrupted nitrite reductase gene (*nirK*). *Applied and Environmental Microbiology*, **72(6)**, 4450-4454.

Choi PS, Grigoryants VM, Abruña HD, Scholes CP, Shapleigh JP. (2005). Regulation and function of cytochrome *c'* in *Rhodobacter sphaeroides* 2.4.3. *Journal of Bacteriology*, **187(12)**, 4077-4085.

Clesceri LS, Greenberg AE, Eaton AD (1998) Standard methods for the examination of water and wastewater, 20th edn. American Public Health Assoc, Washington, D.C.

Colliver BB and Stephenson T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, **18**, 219-232.

Conrad R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiological Reviews*, **60(4)**, 609-640.

Cross R, Aish J, Paston SJ, Poole RK, Moir JWB. (2000). Cytochrome *c'* from *Rhodobacter capsulatus* confers increased resistance to nitric oxide. *Journal of Bacteriology*, **182(5)**, 1442-1447.

Cross R, Lloyd D, Poole RK, Moir JW. (2001). Enzymatic removal of nitric oxide catalyzed by cytochrome *c'* in *Rhodobacter capsulatus*. *Journal of Bacteriology*, **183(10)**, 3050-3054.

Dalsgaard T, Canfield DE, Petersen J, Thamdrup, Acuňa-Gonzalez J. (2003). N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature*, **422**, 606-608.

de Boer APN, Van der Oost J, Reijnders WNM, Westerhoff HV, Stouthamer AH, van Spanning RJM. (1996). Mutational analysis of the nor gene cluster which encodes nitric-oxide reductase from Paracoccus denitrificans. *Eur. J. Biochem*, **242**, 592-600.

de Vries S, Pouvreau LAM. (2007). Nitric oxide reductase: Structural variations and catalytic mechanism. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Dias JM, Than ME, Humm A, Huber R, Bourenkov GP, Bartunik HD, Bursakov S, Calvete J, Caldeira J, Carneiro C, Moura JJG, Moura I, Ramão MJ. (1999). Crystal structure of the first dissimilatory nitrate reductase at 1.9Å solved by MAD methods. *Structure*, **7**, 65-79.

Duce RA, LaRoche J, Altieri K, Arrigo KR, Baker AR, Capone DG, Cornell S, Dentener F, Galloway J, Ganeshram RS, Geider RJ, Jickells T, Kuypers MM, Langlois R, Liss PS, Liu SM, Middelburg JJ, Moore CM, Nickovic S, Oschlies A, Pedersen T, Prospero J, Schlitzer R, Seitzinger S, Sorensen LL, Uematsu M, Ulloa O, Voss M, Ward B, Zamora L. (2008). Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science*, **320**, 893-897.

Einsle O and Kroneck PMH. (2004). Structural basis of denitrification. *Biological Chemistry*, **385**, 875-883.

Elmore BO, Bergmann DJ, Klotz MG, Hooper AB. (2007). Cytochromes P460 and *c'*-beta; A new family of high-spin cytochromes *c*. *FEBS Letters*, **581**, 911-916.

El Sheikh AF. Poret-Peterson AT, Klotz MG. (2008). Characterization of two new genes, *amoR* and *amoD*, in the *amo* operon of the marine oxidizer *Nitrosococcus oceani* ATCC 19707. *Applied and Environmental Microbiology*, **74(1)**, 312-318.

Erisman JW, Bleeker A, Galloway J, Sutton MS. (2007). Reduced nitrogen in ecology and the environment. *Environmental Pollution*, **150**, 140-149.

Fahlgren C, Hagström Å, Nilsson D, Zweifel UL. (2010). Annual variations in the diversity, viability, and origin of airborne bacteria. *Applied and Environmental Microbiology*, **76(9)**, 3015-3025.

Ferguson SJ, Richardson DJ, van Spanning RJM. (2007). Biochemistry and molecular biology of nitrification. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Fields S. (2004). Global nitrogen cycling out of control. *Environmental Health Perspectives*, **112(10)**, A557-A563.

Fisher K and Newton WE. (2002). Nitrogen Fixation- a general overview. *In: Nitrogen Fixation at the Millennium* [Leigh GJ. (ed)] Elsevier Science B.V., Amsterdam, The Netherlands.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey DW, Haywood J, Lean J, Lowe DC, Myhre J, Nganga J, Prinn R, Raga G, Schulz M, Van Dorland R. (2007). Changes in Atmospheric Constituents and in Radiative Forcing. *In: Climate Change 2007: The Physical Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Francis CA, Beman JM, Kuypers MMM. (2007). New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archael ammonia oxidation. *The ISME Journal*, **1**, 19-27.

Francis CA, Robrets KJ, Beman M, Sasntoro AE, Oakley BB. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *PNAS*, **102(41)**, 14683-14688.

Frette L, Gejlsbjerg B, Westermann P. (1997). Aerobic denitrifiers isolated from an alternating activated sludge system. *FEMS Microbiology Ecology*, **24**, 363-370.

Gabriel FLP, Giger W, Guenther K, Kohler H-PE. (2005). Differential degradation of nonylphenol isomers by *Sphingomonas xenophaga* Bayram. *Applied and Environmental Microbiology*, **71(3)**, 1123-1129.

Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB, Cosby BJ. (2003). The Nitrogen Cascade. *Bioscience*, **53(4)**, 341-356.

Galloway JN and Cowling EB. (2002). Reactive nitrogen and the world: 200 years of change. *Ambio*, **31(2)**, 64-71.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vörösmarty CJ. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry*, **70**, 153-226.

Gao H, Schreiber F, Collins G, Jensen MM, Kostka JE, Lavik G, de Beer D, Zhou H-Y, Kuypers MMM. (2010). Aerobic denitrification in permeable Wadden Sea sediments. *The ISME Journal*, **4**, 417-426.

Garbeva P, Baggs EM, Prosser JI. (2007). Phylogeny of nitrite reductase (*nirK*) and nitric oxide reductase (*norB*) genes from *Nitrosospira* species isolated from soil. *FEMS Microbiol. Lett.*, **266**, 83-89.

Gardner PR, Gardner AM, Brashear WT, Suzuki T, Hvitved AN, Setchell KDR, Olson JS. (2006). Hemoglobins dioxygenate nitric oxide with high fidelity. *Journal of Inorganic Biochemistry*, **100**, 542-550.

Gardner PR, Gardner AM, Martin LA, Salzman AL. (1998). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proceedings of the National Academy of Sciences*, **95**, 10378-10383.

Goreau TJ, Kaplan WA, Wofsy SC, McElroy MB, Valois FW, Watson SW. (1980). Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Applied and Environmental Microbiology*, **40(3)**, 526-532.

Hayatsu M, Tago K, Saito M. (2008). Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Science and Plant Nutrition*, **54**, 33-45.

Hendriks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S, Saraste M. (2000). Nitric oxide reductases in bacteria. *Biochimica et Biophysica Acta*, **1459**, 266-273.

Hong H-B, Chang Y-S, Nam I-H, Fortnagel P, Schmidt S. (2002). Biotransformation of 2, 7 dichloro- and 1, 2, 3, 4- tetrachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* RW1. *Applied and Environmental Microbiology*, **68(5)**, 2584-2588.

Hooper AB. (1968). A nitrite-reducing enzyme from *Nitrosomonas europaea*. Preliminary characterization with hydroxylamine as electron donor. *Biochimica et Biophysica Acta*, **162**, 49-65.

Hooper AB, Vannelli T, Bergmann DJ, Arciero DM. (1997). Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie van Leeuwenhoek*, **71**, 59-67.

Horner-Devine MC and Martiny AC. (2008). News about nitrogen. *Science*, **320**, 575-758.

Hughes MN. (2008). Chemistry of nitric oxide and related species. *Methods in Enzymology*, **436**, 3-19.

Hyman MR and Arp DJ. (1995). Effects of ammonia on the de novo synthesis of polypeptides in cells of *Nitrosomonas europaea* denied ammonia as an energy source. *Journal of Bacteriology*, **177(17)**, 4974-4979.

Igarashi N, Moriyama H, Fujiwara T, Fukumori Y, Tanaka N. (1997). The 2.8Å structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nature Structural Biology*, **4(4)**, 276-284.

Ingraham JL. (1981). Microbiology and genetics of denitrifiers. *In: Denitrification, nitrification, and atmospheric nitrous oxide* [Delwiche CC. (ed)] Wiley & Sons, Inc., New York.

Jetten MSM, Cirpus I, Kartal B, van Niftrik L, van de Pas-Schoonen KT, Sliekers O, Haaijer S, van der Star W, Schmid M, van de Vossenberg J, Schmidt I, Harhangi H, van Loosdrecht M, Kuenen JG, Op den Camp H, Strous M. (2005). 1994-2004: 10 years of research on the anaerobic oxidation of ammonium. *Biochemical Society Transactions*, **33(1)**, 119-123.

Kampschreur MJ, Temmink H, Kleerebezem R, Jetten MSM, van Loosdrecht MCM. (2009). Nitrous oxide emission during wastewater treatment. *Water Research*, **43**, 4093-4103.

Keum YS, Lee YJ, Kim J-H. (2008). Metabolism of nitrodiphenyl ether herbicides by dioxin-degrading bacterium *Sphingomonas wittichii* RW1. *Journal of Agricultural and Food Chemistry*, **56**, 9146-9151.

Klotz MG, Alzerreca J, Norton JM. (1997). A gene encoding a membrane protein exists upstream of the *amoA/amoB* genes in the ammonia oxidizing bacteria: a third member of the *amo* operon. *FEMS Microbiology Letters*, **150**, 65-73.

Klotz MG, Arp DJ, Chain PSG, El-Sheikh AF, Hauser LJ, Hommes NG, Larimer FW, Malfatti SA, Norton JM, Poret-Peterson AT, Vergez LM, Ward BB. (2006). Complete genome sequence of the marine, chemolithoautotrophic ammonia-oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. *Applied and Environmental Microbiology*, **72(9)**, 6299-6315.

Klotz MG and Stein LY. (2008). Nitrifier genomics and evolution of the nitrogen cycle. *FEMS Microbiol. Lett.*, **278**, 146-156.

Könneke M, Bernhard AE, de la Torre JR, Walker CH, Waterbury JB, Stahl DA. (2005). Isolation of an autrotrophic ammonia-oxidizing marine archaeon. *Nature*, **437**, 543-546.

Körner H and Zumft WG. (1989). Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Applied and Environmental Microbiology*, **55(7)**, 1670-1676.

Kuypers MMM, Sliekers AO, Lavik G, Schmid M, Jørgensen BB, Kuenen JG, Damsté JSS, Strous M, Jetten MSM. (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature*, **422**, 608-611.

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*, **442**, 806-809.

Lloyd D. (1993). Aerobic denitrification in soils and sediments: From fallacies to facts. *Trends in Ecology & Evolution*, **8(10)**, 352-355.

Lloyd D, Boddy L, Davies KJP. (1987). Persistence of bacterial denitrification capacity under aerobic conditions: The rule rather than the exception. *FEMS Microbiology Ecology*, **45**, 185-190.

Mayburd AL and Kassner RJ. (2002). Mechanism and biological role of nitric oxide binding to cytochrome *c'*. *Biochemistry*, **41**, 11582-11591.

McTavish H, Fuchs JA, Hoooper AB. (1993). Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. *Journal of Bacteriology*, **175(8)**, 2436-2444.

McTavish H, LaQuier F, Arciero D, Logan M, Mundfrom G, Fuchs JA, Hooper AB. (1993). Multiple copies of genes coding for electron transport proteins in the bacterium *Nitrosomonas europaea*. *Journal of Bacteriology*, **175(8)**, 2445-2447.

Mellies J, Jose J, Meyer TF. (1997). The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase. Mol Gen Genet, **256**, 525-532.

Morley N, Baggs EM, Dörsch P, Bakken L. (2008). Production of NO, N_2O and N_2 by extracted soil bacteria, regulation by NO_2^- and O_2 concentrations. *FEMS Microbiology*, *Ecology*, **65**, 102-112.

Moura I and Moura JJG. (2001). Structural aspects of denitrifying enzymes. *Current Opinion in Chemical Biology*, **5**, 168-175.

Munch JC and Velthof GL. (2007). Denitrification and agriculture. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Nam I-H, Kim Y-M, Schmidt S, Chang Y-S. (2006). Biotransformation of 1, 2, 3,- tri- and 1, 2, 3, 4, 7, 8- hexachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* strain RW1. *Applied and Environmental Microbiology*, **72(1)**, 112-116.

Nevison C and Holland E. (1997). A reexamination of the impact of anthropogenically fixed nitrogen on atmospheric N_2O and the stratospheric O_3 layer. *Journal of Geophysical Research*, **102(21)**, 25.519-25.536.

Newton WE. (2007). Physiology, biochemistry, and molecular biology of nitrogen fixation. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Nicol GW and Schleper C. (2006). Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends in Microbiology*, **14(5)**, 207-212.

Nolan T, Hands RE, Bustin SA. (2006) Quantification of mRNA using real-time PCR. *Nature Protocols*, **1(3)**, 1559-1582.

Norton JM, Klotz MG, Stein LY, Arp DJ, Bottomley PJ, Chain SG, Hauser LJ, Land ML, Larimer FW, Shin MW, Starkenburg. (2008). Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. *Applied and Environmental Microbiology*, **74(11)**, 3559-3572.

Numata M, Saito T, Yamazaki T, Fukumori Y, Yamanaka T. (1990). Cytochrome P-460 of *Nitrosomonas europaea*: Further purification and further characterization. *Journal of Biochemistry*, **108(6)**, 1016-1021.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM. (2004). Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Applied and Environmental Microbiology*, **70(2)**, 1008-1016.

Op den Camp HJM, Jetten M, Strous M. (2007). Anammox. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Op den Camp HJM, Kartal B, Guven D, van Niftrik LAMP, Haaijer SCM, van der Star WRL, van de Pas-Schoonen KT, Cabezas A, Ying Z, Schmid MC, Kuypers MMM, van de Vossenberg J, Harhangi HR, Picioreanu C, van Loosdrecht MCM, Kuenen JG, Strous M, Jetten MSM. (2006). Global impact and application of the anaerobic ammonium- oxidizing (anammox) bacteria. *Biochemical Society Transactions*, **34(1)**, 174-178).

Otte S, Grobben NG, Robertson LA, Jetten MSM, Kuenen JG. (1996). Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. *Applied and Environmental Microbiology*, **62(7)**, 2421-2426.

Patureau D, Godon J-J, Dabert P, Bouchez T, Bernet N, Delgenes JP, Moletta R. (1998). *Microvirgula aerodenitrificans* gen. nov., sp. nov., a new Gram-negative bacterium exhibiting co-respiration of oxygen and nitrogen oxides up to oxygen-saturated conditions. *International Journal of Systematic Bacteriology*, **48**, 775-782.

Patureau D, Zumstein E, Delgenes JP, Moletta R. (2000). Aerobic denitrifiers isolated from diverse natural and managed ecosystems. *Microbial Ecology*, **39**, 145-152.

Pearson AR, Elmore BO, Yang C, Ferrara JD, Hooper AB, Wilmot CM. (2007). The crystal structure of cytochrome P460 of *Nitrosomonas europaea* reveals a novel cytochrome fold and heme-protein cross-link. *Biochemistry*, **46(28)**, 8340-8349.

Philippot L. (2002). Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysica Acta*, **1577**, 355-376.

Philippot L, Mirleau P, Mazurier S, Siblot S, Hartmann A, Lemanceau P, Germon JC. (2001). Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar*, *nir*, *nor*, and *nos* genes. *Biochimica et Biophysica Acta*, 1517, 436-440.

Pinakoulaki E and Varotsis C. (2008). Nitric oxide activation and reduction by heme-copper oxidoreductases and nitric oxide reductase. *Journal of Inorganic Biochemistry*, **102**, 1277-1287.

Poole RK. (2005). Nitric oxide and nitrosative stress tolerance in bacteria. *Biochemical Society Transactions*, **33(1)**, 176-180.

Poret-Peterson A, Graham JE, Gulledge J, Klotz MG. (2008). Transcription of nitrification genes by the methane-oxidizing bacterium, *Methylococcus capsulatus* strain Bath. *The ISME Journal*, 1-8.

Postgate J. (1998). Nitrogen Fixation, 3rd ed. Cambridge University Press, Cambridge, UK.

Prosser JI. (2007). The ecology of nitrifying bacteria. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Reay, Dave (Lead Author); Lori Zaikowski (Topic Editor). 2007. "Nitrous oxide." *In: Encyclopedia of Earth.* Eds. Cutler J. Cleveland (Washington, D.C.: Environmental Information Coalition, National Council for Science and the Environment). [First published in the Encyclopedia of Earth July 9, 2007; Last revised July 10, 2007; Retrieved August 25, 2008]. <http://www.eoearth.org/article/Nitrous_oxide>

RemdeA and Conrad R. (1990). Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. *Archives of Microbiology*, **154**, 187-191.

Richardson DJ, van Spanning RJM, Ferguson SJ. (2007). The prokaryotic nitrate reductases. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Richardson DJ and Watmough NJ. (1999). Inorganic nitrogen metabolism in bacteria. *Current Opinion in Chemical Biology*, **3**, 207-219.

Rinaldo S and Cutruzzolà F. (2007). Nitrite reductases in denitrification. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Ritchie GAF and Nicholas DJD. (1972). Identification of the sources if nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. *Biochem. J.*, **126**, 1181-1191.

Robertson LA and Kuenen JG. (1983). *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. *Journal of General Microbiology*, **129**, 2847-2855.

Robertson LA and Kuenen JG. (1984). Aerobic denitrification: A controversy revived. *Archives of Microbiology*, **139**, 351-354.

Robertson LA, van Niel EWJ, Torremans RAM, Kuenen JG. (1988). Simultaneous nitrification and denitrification in aerobic chemostat cultures of *Thiosphaera pantotropha*. *Applied and Environmental Microbiology*, **54(11)**, 2812-2818.

Rozen S and Skaletsky HJ. (2000). Primer3 on the WWW for general users and for biologist programmers. *In: Bioinformatics Methods and Protocols: Methods in Molecular Biology* [Krawetz S and Misener S (eds.)]. Humana Press, Totowa, NJ, pp 365-386.

Sayavedra-Soto LA, Hommes NG, Russell SA, Arp DJ. (1996). Induction of ammonia monooxygenase and hydroxylamine oxidoreductase mRNAs by ammonium in *Nitrosomonas europaea*. *Molecular Microbiology*, **20(3)**, 541-548.

Schmidt I, Steenbakkers PJM, op den Camp HJM, Schmidt K, Jetten MSM. (2004). Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. *Journal of Bacteriology*, **186(9)**, 2781-2788.

Schmidt I, van Spanning RJM, Jetten MSM. (2004). Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants. *Microbiology*, **150**, 4107-4114.

Shaw LJ, Nicol GW, Smith Z, Fear J, Prosser JI, Baggs EM. (2000). *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology*, **8(2)**, 214-222.

Smil V. (1999). Nitrogen in crop production: an account of global flows. *Global Biogeochemical Cycles*, **13(2)**, 647-662.

Smil V. (2002). Nitrogen and food production: proteins for human diets. *Ambio*, **31(2)**, 126-131.

Smith Z, McCaig AE, Stephen JR, Embley TM, Prosser JI. (2001). Species diversity of uncultured and cultured populations of soil and marine ammonia oxidizing bacteria. *Microbial Ecology*, **42**, 228-237.

Starkenburg SR, Chain PSG, Sayavedra-Soto LA, Hauser L, Land ML, Larimer FW, Malfatti SA, Klotz MG, Bottomley PJ, Arp DJ, Hickey WJ. (2006). Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium *Nitrobacter winogradskyi* Nb-255. *Applied and Environmental Microbiology*, **72(3)**, 2050-2063.

Starkenburg SR, Larimer FW, Stein LY, Klotz MG, Chain PSG, Sayavedra-Sota LA, Poret-Peterson AT, Gentry ME, Arp DJ, Ward B, Bottomley PJ. (2008). Complete genome sequence of *Nitrobacter hamburgensis* X14 and comparative genomic analysis of species within the genus *Nitrobacter*. *Applied and Environmental Microbiology*, **74(9)**, 2852-2863.

Stein LY and Arp DJ. (1998a). Ammonium limitation results in the loss of ammonia-oxidizing activity in *Nitrosomonas europaea*. *Applied and Environmental Microbiology*, **64(4)**, 1514-1521.

Stein LY and Arp DJ. (1998b). Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Applied and Environmental Microbiology*, **64(10)**, 4098-4102.

Stein LY and Yung YL. (2003). Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide. *Annu. Rev. Earth Planet. Sci.*, **31**, 329-356.

Stein LY, Arp DJ, Berube PM, Chain PSG, Hauser L, Jetten MSM, Klotz MG, Larimer FW, Norton JM, op den Camp JHM, Shin M, Wei X. (2007). Whole-genome analysis of the ammonia oxidizing bacterium, *Nitrosomonas eutropha* C91: implications for niche adaptation. *Environmental Microbiology*, **9(12)**, 2993-3007.

Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM. (1996). Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, **62(11)**, 4147-4154.

Stolz A. (2009). Molecular characteristics of xenobiotic-degrading Sphingomonads. *Applied Microbiology and Biotechnology*, **81**, 793-811.

Takaya N, Catalan-Sakairi MAB, Sakaguchi Y, Kato I, Zhou Z, Shoun H. Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Applied and Environmental Microbiology*, **69(6)**, 3152-3157.

Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. (2008). The chemical biology of nitric oxide: Implications in cellular signaling. *Free Radical Biology and Medicine*, **45**, 18-31.

Tortorso AC and Hutchinson GL. (1990). Contributions of autotrophic and heterotrophic nitrifiers to soil NO and N_2O emissions. *Applied and Environmental Microbiology*, **56(6)**, 1799-1805.

Van Dommelen A and Vanderleyden J. (2007). Associative nitrogen fixation. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

van Spanning RJM, Richardson, Ferguson SJ. (2007). Introduction to the biochemistry and molecular biology of denitrification. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications*, **7(3)**, 737-750.

Walker CB, de la Torre JR, Klotz MG, Urakawa H, Pinel N, Arp DJ, Brochier-Armanet C, Chain PSG, Chan PP, Gollabgir A, Hemp J, Hügler M, Karr EA, Könneke M, Shin M, Lawton TJ, Lowe T, Martens-Habbena W, Sayavedra-Soto LA, Lang D, Sievert SM, Rosenzweig AC, Manning G, Stahl DA. (2010). *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proceedings of the National Academy of Sciences*, **107(19)**, 8818-8823.

Wei X, Yan T, Hommes NG, Liu X, Wu L, McAlvin C, Klotz MG, Sayavedra-Soto LA, Zhou J, Arp DJ. (2006). Transcript profiles of *Nitrosomonas europaea* during growth and upon deprivation of ammonia and carbonate. *FEMS Microbiology Letters*, **257**, 76-83.

Whittaker M, Bermann D, Arciero D, Hooper AB. (2000). Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. *Biochimica et Biophysica Acta*, **1459**, 346-355.

Wilkes H, Wittich R-M, Timmis KN, Fortnagel P, Francke W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Applied and Environmental Microbiology*, **62(2)**, 367-371.

Wittich R-M, Wilkes H, Sinnwell V, Francke W, Fortnagel P. (1992). Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. *Applied and Environmental Microbiology*, **58(3)**, 1005-1010.

Wrage N, Velthof GL, van Beusichem ML, Oenema O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*, **33**, 1723-1732.

Yabuuchi E, Yamamoto H, Terakubo S, Okamura N, Naka T, Fujiwara N, Kobayashi K, Kosako Y, Hiraishi A. (2001). Proposal of *Sphingomonas wittichii* sp. nov. for strain RW1, known as a dibenzo-p-dioxin metabolizer. *International Journal of Systematic and Evolutionary Microbiology*, **51**, 281-292.

Ye RW, Averill BA, Tiedje JM. (1994). Denitrification: Production and consumption of nitric oxide. *Applied and Environmental Microbiology*, **60(4)**, 1053-1058.

Yeung N, Lin Y-W, Gao Y-G, Zhao X, Russell BS, Lei L, Miner KD, Robinson H, Lu Y. (2009). Rational design of a structural and functional nitric oxide reductase. *Nature*, **462**, 1079-1082.

Zennaro E, Ciabatti I, Cutruzzola F, D'Alessandro R, Silvestrini MC. (1993). The nitrite reductase gene of *Pseudomonas aeruginosa*: Effect of growth conditions on the expression and construction of a mutant by gene disruption. *FEMS Microbiology Letters*, **109**, 243-250.

Zumft WG. (1993). The biological role of nitric oxide in bacteria. *Archives of Microbiology*, **160**, 253-264.

Zumft WG and Körner H. (2007). Nitrous oxide reductases. *In: Biology of the Nitrogen Cycle* [Bothe H, Ferguson SJ, Newton WE (eds.)] Elsevier B.V., Oxford, UK.
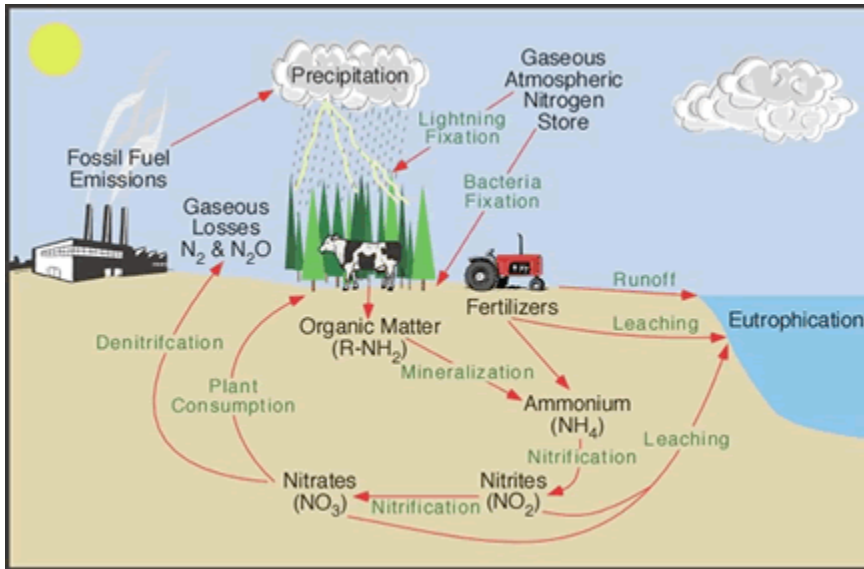
Figure 1.1. The global nitrogen cycle. http://sbi.oregonstate.edu/about/images/cycle.gif
Retrieved Aug. 25, 2008.
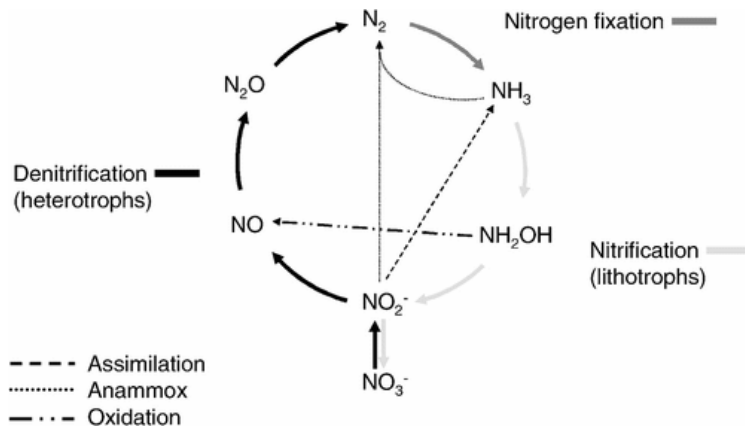
Figure 1.2. The microbial nitrogen cycle (Stein and Yung, 2003).
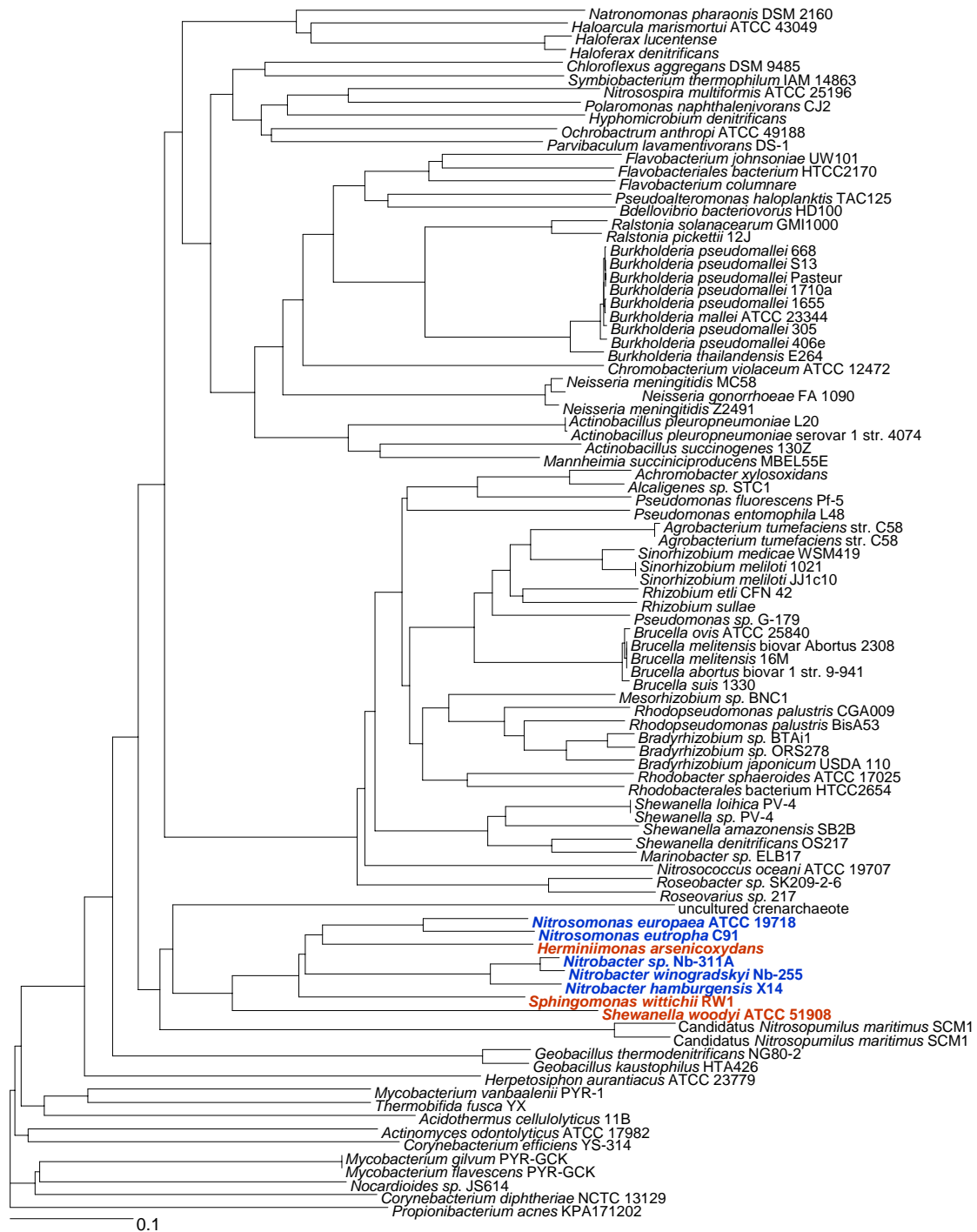
Figure 1.3. Neighbor- joining tree based on an alignment of full-length NirK protein sequences. After retrieval from Genbank, sequences were aligned and used for phylogenetic inference (MEGA v. 3.1). In the clade of interest, names in blue are nitrifiers, and those in red are heterotrophs. This NirK clade has been previously designated as "Nitrifier Cluster 1" (Cantera and Stein, 2007a).

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Chapter 2

Differential regulation of N-cycle genes by nitrite in cultivated ammonia-oxidizing bacteria

Abstract

Nitrite (NO_2^-) is the main product of ammonia-oxidizing bacteria (AOB), but its effects on AOB physiology have not been broadly explored. Effects of NO_2^- on activity and expression of genes for N-metabolism were compared in two soil (*Nitrosomonas europaea* ATCC 19718, *Nitrosospira multiformis* ATCC 25196) and one sewage (*Nitrosomonas eutropha* C-91) AOB strain. Medium buffered with HEPES or phosphate was supplemented with 0, 10, or 20 mM $NaNO_2$. Rates and amounts of NO_2^- production decreased most significantly in NO_2^- -amended cultures of *N. eutropha* C-91 followed by *N. europaea* ATCC 19718 and *N. multiformis* ATCC 25196. Incubations amended with 20 mM $NaNO_2$ negatively affected *amoA* (ammonia monooxygenase) expression in *N. multiformis* and *norS* (cytochrome c nitric oxide reductase) in *N. europaea* ATCC 19718 and *N. eutropha* C-91 in HEPES-buffered medium. Only *nirK* (copper-containing nitrite reductase) of *N. europaea* ATCC 19718 showed increased expression in $NaNO_2$ amended medium. NO_2^- amendment had no significant effect on *norB* (cytochrome c nitric oxide reductase), *cytL* (cytochrome P460) or *cytS* (cytochrome *c*'-beta) mRNA levels in any AOB. Basal levels of mRNA were more variable in mid-log phase *N. eutropha* C-91 than *N. europaea* ATCC 19718 in HEPES- versus phosphate-buffered medium, suggesting greater sensitivity of *N. eutropha* C-91 to culture conditions. Together, the data show

high resilience of the AOB strains to NO_2^-, with *N. eutropha* C-91 showing greatest sensitivity. Differential responses to NO_2^- at both activity and gene expression levels suggest that the AOB have acquired different mechanisms with varying effectiveness to resist NO_2^- toxicity.

Introduction

Nitrification is carried out in soil ecosystems by the chemolithoautotrophic ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively) and the ammonia-oxidizing Crenarchaea (AOA). It is well documented that cultivated AOB produce and release both nitric (NO) and nitrous oxide (N_2O) during ammonia oxidation (Shaw et al., 2006; Wrage et al., 2001); to date there is no published evidence that AOA do the same. Both the reduction of nitrite (NO_2^-) and incomplete oxidation of hydroxylamine (NH_2OH) to N_2O via NO (or nitroxyl; HNO) have been demonstrated in cultivated *Nitrosomonas* and *Nitrosospira* spp. as well as in soils and wastewater (Avrahami and Bohannan, 2009; Bremner et al., 1981; Colliver and Stephenson, 2000; Goreau et al., 1980; Hayatsu et al., 2008; Kampschreur et al., 2009; Shaw et al., 2006; Tortoso and Hutchinson, 1990; Wrage et al., 2001). These studies show that production of NO and N_2O from nitrification is predominantly controlled by substrate (NH_4^+, NH_2OH or NO_2^-) availability, pH, moisture, and O_2 level; however, many of the participating enzymes and their regulation have only been functionally characterized in *Nitrosomonas europaea* strain ATCC 19718 (Beaumont et al., 2004a; Beaumont et al., 2004b; Beyer et al., 2009; Elmore et al., 2007; Schmidt et al., 2004; Upadhyay et al., 2006; Yu and Chandran, 2010).

Previous studies of *N. europaea* ATCC 19718 showed that Cu-containing nitrite reductase (NirK) and heme-copper nitric oxide reductase (NorB) catalyze the reduction of NO_2^- to N_2O via NO (Beaumont et al., 2002; Beaumont et al., 2004b; Schmidt et al., 2004). Both *nirK* and *norCBQD* genes have been identified in multiple *Nitrosomonas* and *Nitrosospira* isolates (Cantera and Stein, 2007b; Casciotti and Ward, 2005; Casciotti and Ward, 2001; Garbeva et al., 2007). However, altered *N. europaea* ATCC 19718 strains defective in NirK or NorB activity remain capable of producing N_2O, suggesting that other enzymes are also responsible (Beaumont et al., 2002; Beaumont et al., 2004b). Although proposed alternate nitrite reductases have yet to be verified for AOB (Stein and Klotz, 2010), candidate genes encoding alternate nitric oxide reductases identified in genome sequences of *Nitrosomonas europaea* ATCC 19178, *Nitrosomonas eutropha* C-91, *Nitrosospira multiformis* ATCC 25196, and *Nitrosococcus oceani* ATCC 19707 include *norS* (encoding a cytochrome c nitric oxide reductase) (Norton et al., 2008; Stein et al., 2007) and *cytS* (encoding cytochrome *c'*-beta) (Bergmann et al., 2000; Elmore et al., 2007; Poret-Peterson et al., 2008) (Figure 1). In addition, *N. europaea* ATCC 19178, *N. eutropha* C-91, and *N. oceani* ATCC 19707 encode the *cytL* gene for cytochrome P460, which has been implicated in detoxifying NH_2OH and NO to NO_2^- (Elmore et al., 2007; Numata et al., 1990; Pearson et al., 2007). Together, this group of genes along with those encoding ammonia monooxygenase (*amoCAB*) and hydroxylamine oxidoreductase (*haoA*) represent much of the inventory found in cultivated AOB to metabolize ammonia to NO_2^- as the major product, N_2O as a minor product, and NH_2OH and NO as intermediates (Figure 2.1).

Studies of aerobically grown cultures of *N. europaea* ATCC 19718 have shown

that expression of *amoA* and *haoA* genes is largely regulated by availability of ammonia

(Sayavedra-Soto et al., 1996) and oxygen (Yu and Chandran, 2010), expression of the

ncgABC-nirK operon is predominantly regulated by NO_2^- as controlled by a NsrR

repressor (Beaumont et al., 2004a), and *norB* is constitutively expressed (Beaumont et al.,

2004b). There is also evidence that expression of *nirK* and *norB* is induced in anaerobic

cultures of *N. europaea* ATCC 19718 growing on ammonia or pyruvate, NO_2^-, and NO_2

(Beyer et al., 2009). Although nearly all physiological and genomic information about

AOB has been derived from studying cultivated isolates, AOB in culture experience

significantly different nutrient loads and physicochemical conditions than AOB living in

natural environments. Nevertheless, culture studies have demonstrated how specific

molecules might affect AOB function to influence rates of nitrification and nitrogen

oxide production. For instance, in correspondence to pure culture studies of *N. europaea*

ATCC 19718 (Stein et al., 1997), ecological studies have correlated increases in bacterial

amoA transcripts and nitrification activity with increases in soil-N levels (Di et al., 2009;

Jia and Conrad, 2009).

While *N. europaea* ATCC 19718 is the best studied AOB isolate, phylogenetic

surveys of 16S rRNA and *amoA* genes indicate that members of *Nitrosospira* spp. are

dominant AOB in terrestrial ecosystems (Koops et al., 2007). In contrast, relatives of

Nitrosomonas eutropha are apparently restricted to environments with very high N-load,

while relatives of *N. europaea* are generally found in ecosystems with moderate to high

N-load (Koops et al., 2007). Some variables that affect AOB such as oxygen level

(Bodelier et al., 1996), pH (Nicol et al., 2008; Pommerening-Roser and Koops, 2005), availability and response to ammonium (Bollmann et al., 2002; Bollmann et al., 2005), and tolerance to NO_2^- (Tan et al., 2008), have all been hypothesized as factors leading to niche differentiation. The goal of the present study was to further our understanding of gene regulation in cultivated AOB by NO_2^-, the terminal product of ammonia oxidation and substrate for N_2O production, at concentrations relevant to that produced in aerobic batch cultures of AOB isolates. We hypothesized that homologous genes involved in N-metabolism would be differentially regulated by NO_2^- as a reflection of the environments from which the bacteria were originally isolated.

Materials and Methods

Growth of ammonia-oxidizing strains

Cultures of *Nitrosospira multiformis* strain ATCC 25196, *Nitrosomonas europaea* strain ATCC 19718, and *Nitrosomonas eutropha* strain C-91 were grown in medium containing per L: 10 mM $(NH_4)_2SO_4$, 0.4 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM KCl, 0.05% Phenol red, 1 mL of trace element solution (per L distilled water: 11.5 mM Na_2-EDTA, 10 mM $FeCl_2 \cdot 4H_2O$, 0.5 mM $MnCl_2 \cdot 2H_2O$, 0.1 mM $NiCl_2 \cdot 6H_2O$, 0.1 mM $CoCl_2 \cdot 6H_2O$, 0.1 mM $CuCl_2 \cdot 2H_2O$, 0.5 mM $ZnCl_2$, 0.1 mM $Na_2MoO_4 \cdot 2H_2O$, 1 mM H_3BO_3), and 15 mM HEPES buffer pH 7.5 (Figure 2.2). The pH was maintained at ca. 7.5 during culturing using 5% sodium bicarbonate (Na_2CO_3), which was added daily to the cultures following the first 48 h of growth. *N. europaea* ATCC 19718 and *N. eutropha* C-91 were also grown in the same media, but buffered

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with phosphate (per L: 5.47 g KH_2PO_4 and 0.47 g NaH_2PO_4, pH 8) in place of HEPES

buffer (Figure 2.2). Cultures were maintained in a rotary shaker (180 r.p.m.) at 28°C.

The average doubling times of the AOB in HEPES-buffered medium were similar at 20.6

h (±1.73) (*N. eutropha*), 24 h (± 1.90) (*N. europaea*), and 22.1 h (± 1.71) (*N. multiformis*)

and all cultures produced 13-15 mM NO_2^- by late-log phase. Doubling times were 7.1 (±

0.68) and 9.2 (± 1.38) hours for *N. europaea* ATCC 19718 and *N. eutropha* C-91,

respectively, when grown in phosphate-buffered medium and both produced ca. 18 mM

NO_2^- (± 0.04) by late-log phase.

Experimental incubations

Cells were harvested from cultures at mid-log phase when NO2- levels were at ca.

10 mM (± 0.76) for *N. multiformis* ATCC 25196 and ca. 13 mM (± 0.23) for both *N.*

europaea ATCC 19718 and *N. eutropha* C-91. Cells were collected by centrifugation at

8,000 rpm for 10 minutes at 4°C, washed three times in buffer (HEPES or phosphate),

and resuspended in 10 mL fresh medium to 109 cells ml^{-1} as enumerated in a Petroff

Hausser counting chamber and phase-contrast light microscopy. Medium was amended

with 10 or 20 mM $NaNO_2$, an amount of NO_2^- either exceeded by or in excess of that

produced from ammonia oxidation by these strains during growth in batch cultures.

Controls included incubations of cells in medium without $NaNO_2$ amendment and cells

harvested directly from growth cultures without additional incubation. Flasks were

incubated on a rotary shaker (180 rpm) at 28 °C in the dark. Samples (2 mL) were taken

at t = 0, 0.5, 2, 4, and 6 hours and cells were collected by centrifugation at 13,200 rpm for

2 minutes. The supernatant was used for pH and NO_2^- measurements (Hageman and

Hucklesby, 1971) and the cell pellets were immediately treated with 500 μL of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) for storage at -80°C prior to RNA extraction. Three to seven replicates of each incubation condition using batches of cells grown on separate days were used for statistical comparison.

Dot-blot hybridization

Gene sequences identified in the genome sequences of each strain were used to design specific primers using Primer3 Input 0.4.0 software (Rozen and Skaletsky, 2000), with the exception of universal 27F/1492R 16S rRNA primers used for *N. europaea* (Table 2.1). PCR reactions included standard reagents for Taq polymerase and genomic DNA isolated from each bacterium by AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA) as template. Amplification conditions were: 95°C for 5 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 50 seconds, with an additional extension cycle of 72°C for 7 minutes (iCycler, Biorad, Hercules, CA). PCR products were checked by agarose gel to verify single products of appropriate size. Amplification products were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA). Amplification products were labeled using the Prime-a-Gene labeling system (Promega, Madison, WI) with [α- ^{32}P]-dCTP (3000 Ci mmol-1; Perkin-Elmer Inc., Waltham, MA) and random hexamers. The dynamic range of detection for each probe was tested using a concentration series of mRNA from 0.25 to 3 μg from the control incubations of each AOB strain in both media formulations. The r^2 values for the slope of hybridization intensity/μg RNA was between 0.92 to 1.00 for all probes and all strains.

Total RNA was extracted from cell pellets using the Aurum Total RNA Mini kit

(Bio-Rad Laboratories, Hercules, CA). Nucleic acid concentration was determined using

the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington,

DE). Two μg total RNA from each sample was blotted onto a Zeta-Probe GT nylon

membrane (Bio-Rad Laboratories, Hercules, CA) using a Minifold microsample filtration

manifold (Dot-Blot System, Schleicher & Schuell, Keene, NH). RNA from mid-log

phase cells harvested directly from culture was blotted onto the same membrane as RNA

from cells subjected to short-term incubations to ensure comparability of the

hybridization signals. Membranes were allowed to dry overnight and UV-crosslinked

(FB-UVXL-1000, Fisher Scientific, Pittsburgh, PA). Prehybridization, hybridization, and

washing of Zeta-Probe nylon membranes were done according to manufacturer's

instructions at 30°C. To allow re-probing, membranes were stripped of radioactivity by

washing twice in a 0.1X SSC/0.5% SDS solution at 95-100°C for 20 minutes. All blots

were hybridized to both gene-specific and 16S rRNA probes to normalize hybridization

signals to the 16S rRNA pool. Hybridization intensity was analyzed using a Typhoon

Phosphorimager and Imagequant software (Amersham, Piscataway, New Jersey).

Data Analysis

Background and signal from non-specific binding was subtracted, after which the

relative hybridization intensity of specific probes was normalized by dividing by the

signal from gene-specific to 16S rRNA probe hybridizations. The fold difference in

levels of mRNA for each gene, time point, and organism from NO_2^- exposure was

determined by dividing hybridization intensities from dot blots of RNA extracted from

NO_2^- amended to those from unamended cultures. A two fold change in transcript level was considered a significant effect of NO_2^- on gene expression. Student's t-test ($p < 0.05$) was performed to verify significant differences between treatments.

Results

Effect of NO_2^- amendment on activity of AOB

The effect of NO_2^- amendment on ammonia-oxidizing activity of the three AOB strains was assessed by measuring changes in pH and NO_2^- concentration over the 6 h time course (Table 2.2). The final pH was higher in NO_2^- -amended than in unamended HEPES-buffered cultures for all three AOB, indicating less acidification and thus reduced ammonia oxidation. However, only *N. eutropha* C-91 showed significantly slower rates and lower net NO_2^- production in all NO_2^- -amended cultures. Rates of NO_2^- production were unaffected in NO_2^- -amended HEPES-buffered cultures of *N. europaea* ATCC 19718 and net NO_2^- production was only lower in cultures amended with 20 mM NO_2^-. Interestingly, NO_2^- amendment did not significantly alter the rates or net amounts of NO_2^- produced in cultures of *N. multiformis* ATCC 25196.

N. eutropha C-91 incubated in NO_2^- -amended phosphate-buffered medium showed significantly slower rates and lower net amounts of NO_2^- production than did identical cultures of *N. europaea* ATCC 19718, particularly in incubations amended with 20 mM NO_2^- (Table 2.2). Together, the data indicate that among the three AOB, *N. eutropha* C-91 was the most sensitive to NO_2^- followed by *N. europaea* and last by *N. multiformis* ATCC 25196. Furthermore, the two *Nitrosomonas* spp. had significantly

higher rates of NO_2^- production in phosphate- than in HEPES-buffered medium, most likely due to its stronger buffering capacity (i.e. less net acidification) and hence greater availability of NH_3, the substrate of ammonia monooxygenase.

Effect of NO_2^- amendment on expression of selected genes

Amendment of 20 mM NO_2^- to HEPES-buffered medium had a significant negative effect on levels of *amoA* mRNA of *N. multiformis* ATCC 25196 and *norS* mRNA of *N. europaea* ATCC 19718 and *N. eutropha* C-91 (Figure 2.3). Only *nirK* mRNA of *N. europaea* ATCC 19718 showed a significant increase in medium amended with either 10 or 20 mM NO_2^-, although the increase was short lived (Figure 2.4). No significant change was detected in levels of *norB*, *cytL*, or *cytS* mRNA of any AOB incubated in HEPES-buffered medium (Figure 2.5), suggesting that these genes were not regulated by NO_2^-. In phosphate-buffered medium, only levels of *nirK* mRNA of *N. europaea* ATCC 19718 showed increased expression with similar trends to that observed in HEPES-buffered medium. Levels of *nirK* mRNA in *N. europaea* ATCC 19718 peaked at 4 and 10 fold in 10 and 20 mM NO_2^- -amended relative to non-amended phosphate-buffered medium, respectively. Unlike in HEPES-buffered medium, levels of *norS* mRNA remained below a 2 fold difference for *N. europaea* and *N. eutropha* incubated in NO_2^- -amended phosphate-buffered medium (data not shown).

Basal levels of gene expression were compared in mid-log phase *Nitrosomonas* spp. cells harvested directly from culture to further examine differential effects of HEPES- versus phosphate-buffered medium on the bacteria (Figure 2.6). Although the growth and NO_2^- production rates were faster for both *Nitrosomonas* spp. when grown in

phosphate- relative to HEPES-buffered medium, cells were harvested at similar density ($\sim 7 \times 107$ cells ml^{-1}) and NO_2^- level (11.64 ± 0.43). Overall, mRNA levels of specific genes in *N. eutropha* C-91 were more variable between the two media than mRNA levels in *N. europaea* ATCC 19718, suggesting greater sensitivity of *N. eutropha* C-91 to the different buffer formulations. In cultures of *Nitrosospira multiformis* ATCC 25196, mRNA levels of the specific genes were essentially the same (ca. 0.1% of the 16S rRNA pool), except for *norB* mRNA, which was significantly less (ca. 0.03% of the 16S rRNA pool). These differences in basal levels of gene-specific mRNA between the three AOB strains further demonstrate that homologous inventory is not similarly regulated and that *N. europaea* ATCC 19718 and *N. eutropha* C-91 have different sensitivities to culturing conditions as evidenced by differences in growth (Table 2.2) and gene expression characteristics (Figure 2.6).

No significant differences were found between hybridization intensities of mRNA extracted from cells immediately harvested from culture versus those taken at t = 0 from the short-term incubations, indicating no immediate effects from resuspending cells into fresh medium with or without NO_2^- amendment (data not shown).

Discussion

The hypothesis tested in this study was that homologous gene inventory (Figure 2.1) would not be similarly regulated by NO_2^- in three cultivated AOB as a reflection of the environments from which the bacteria were originally isolated. *N. europaea* ATCC 19718 was famously isolated from soil by Sergei Winogradsky in 1892, *N. eutropha* C-

91 was isolated from a sewage disposal plant in 1971 and *N. multiformis* ATCC 25196 was isolated from soil near Paramaribo, Surinam in 1971. Based on this information alone, one might expect *N. eutropha* C-91 to be the most NO_2^- tolerant as sewage environments experience higher levels and frequent fluxes of NO_2^- relative to soils. However, the activity of *N. multiformis* ATCC 25196 was the least affected by NO_2^- exposure, followed by *N. europaea* ATCC 19718 and lastly by *N. eutropha* C-91 (Table 2.2). This suggests that *N. multiformis* ATCC 25196 is highly resistant to NO_2^- toxicity, a feature that perhaps allowed this strain to be relatively easily isolated into pure culture.

The decrease in *amoA* transcript in *N. multiformis* ATCC 25196 both over time and with increased NO_2^- concentration (Figure 2.3) is logical as energy-generating metabolism should decrease as nutrients diminish and end products accumulate. However, the decrease in *amoA* mRNA did not immediately translate to a decreased rate of ammonia conversion to NO_2^- (Table 2.2). In prior work, *N. europaea* ATCC 19718 specifically decreased its ammonia-oxidizing activity through physiological changes at the post-transcriptional level in response to high NO_2^- and low ammonium concentrations (Stein and Arp, 1998). This may well have been the mechanism for measurable decreases in both rate and amount of ammonia conversion to NO_2^- in the two *Nitrosomonas* spp. over 6 h (Table 2.2); although a similar mechanism of posttranslational control of ammonia-oxidizing activity has not been investigated in *N. eutropha* C-91.

Increased levels of *nirK* mRNA upon exposure to NO_2^- in *N. europaea* ATCC 19718 confirmed previous observations of *nirK* regulation (Beaumont et al., 2004a), although the transcript was relatively short lived (Figure 2.4). As the *ncgABC-nirK*

operon, promoter-proximal NsrR binding motif, and NsrR repressor share high sequence identity between *N. europaea* ATCC 19718 and *N. eutropha* C-91 (Cantera and Stein, 2007b), it was surprising that the level of *nirK* mRNA in *N. eutropha* was not similarly increased by NO_2^-. The lack of enhanced NirK expression in *N. eutropha* C-91 may explain its sensitivity to NO_2^- relative to *N. europaea* ATCC 19718, as NirK has been associated with NO_2^- tolerance (Beaumont et al., 2005; Cantera and Stein, 2007a). The orphan *nirK* gene of *N. multiformis* ATCC 25196 is structurally distinct from that of the two *Nitrosomonas* spp. and lacks a promoter-proximal regulatory binding motif (Cantera and Stein, 2007b); hence, the lack of regulation by NO_2^- was expected. The data suggest that while NirK is obviously important for NO_2^- reduction (Schmidt et al., 2004) and tolerance (Beaumont et al., 2005; Cantera and Stein, 2007a) in *N. europaea* ATCC 19718 it may play a lesser role in *N. eutropha* C-91 and *N. multiformis* ATCC 25196. However, detailed studies of NirK activity and its role in AOB other than *N. europaea* ATCC 19718 have yet to be performed.

A functional role for NorS has yet to be determined in any AOB, although it may be involved in reducing NO arising from NH_2OH in the two *Nitrosomonas* sp. (Figure 2.1) as suggested by decreased *norS* mRNA levels as conditions became less favorable for ammonia oxidation, i.e. low pH and high NO_2^- (Table 2.2 & Figure 2.3). This result supports prior studies showing up-regulation of *norS* in NirK-deficient *N. europaea* ATCC 19718 under conditions where NH_2OH oxidation to N_2O was favored (Cantera and Stein, 2007a; Cho et al., 2006). In contrast, levels of *norB* mRNA were unchanged in response to NO_2^- in all three strains, suggesting constitutive expression. The possible

roles of CytL and CytS remain unclear as transcript levels did not appear regulated by nitrite, possibly suggesting constitutive expression as well (Figure 2.5).

A previous study showed that incubating *N. europaea* and *N. multiformis* isolates in phosphate- versus HEPES-buffered medium resulted in a significant increase in N_2O production (Jiang and Bakken, 1999), indicating a physiological response to a change in buffering agent. In the present study, growth characteristics and basal mRNA (Figures 2.2 and 2.6) levels of the two *Nitrosomonas* spp. showed significant differences when cultivated in HEPES- versus phosphate-buffered medium. Furthermore, levels of *norS* mRNA in these two AOB were only negatively affected when incubated in HEPES-buffered medium. The results indicate that the buffering agent selected for physiological and gene expression experiments have significant effects on AOB that must be recognized prior to extrapolating functional data from pure culture to natural AOB populations.

A main conclusion of this work is that unlike bacteria that do not create NO_2^- for a living, the three AOB strains used in this study, two cultivated from soils and one cultivated from sewage, all demonstrated high levels of resiliency to NO_2^-. Unlike bacteria that experience nitrosative stress at 2.5 to 5 mM $NaNO_2$ (He et al., 2006; Schlag et al., 2007), neither activity nor gene regulation in these cultivated AOB was particularly affected by significantly higher NO_2^- concentrations, perhaps as an adaptation to the pure culture environment where NO_2^- accumulates unabated. Although this information can not be directly extrapolated to AOB in the natural environment where they interact in complex communities, it is possible that resistance of AOB to NO_2^- toxicity is beneficial

when relationships to nitrite-oxidizers are less-than-robust. The greater tolerance of the soil than the sewage AOB strains to NO_2^- was surprising given the lack of appreciable NO_2^- accumulation in soils, although this indicates that *N. europaea* ATCC 19718 and *N. multiformis* ATCC 25196 have excellent defense mechanisms against NO_2^- toxicity that *N. eutropha* C-91 apparently lacks. Differential responses to NO_2^- at the activity and gene expression levels are important observations for comparing to other cultivated AOB to determine whether those isolated from similar environments share similar adaptations or whether each individual strain adapts with unique responses to high NO_2^- loads. If the former is true, then NO_2^- tolerance and its effect on specific gene expression is likely an important mechanism for survival, competition, and niche differentiation of AOB. If the latter is true, then NO_2^- and adaptations to its toxicity are not significant or tractable features that can differentiate AOB populations.

References

Avrahami S and Bohannan BJM. (2009). N$_2$O emission rates in a California meadow soil are influenced by fertilizer level, soil moisture and the community structure of ammonia-oxidizing bacteria. *Global Change Biology*, **15(3)**, 643-655.

Beaumont HJE, Hommes NG, Sayavedra-Soto LA, Arp DJ, Arciero DM, Hooper AB, Westerhoff HV, van Spanning RJM. (2002). Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *Journal of Bacteriology*, **184(9)**, 2557-2560.

Beaumont HJE, Lens SI, Reijnders WNM, Westerhoff HV, van Spanning RJM. (2004a). Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite sensitive transcription repressor. *Molecular Microbiology*, **54(1)**, 148-158.

Beaumont HJE, Lens SI, Westerhoff HV, van Spanning RJM. (2005). Novel *nirK* cluster genes in *Nitrosomonas europaea* are required for NirK-dependent tolerance to nitrite. *Journal of Bacteriology*, **187(19)**, 6849-6851.

Beaumont HJE, van Schooten B, Lens SI, Westerhoff HV, van Spanning RJM. (2004b). *Nitrosomonas europaea* expresses a nitric oxide reductase during nitrification. *Journal of Bacteriology*, **186(13)**, 4417-4421.

Bergmann DJ, Zahn JA, DiSpirito AA. (2000). Primary structure of cytochrome c' of *Methylococcus capsulatus* Bath: evidence of a phylogenetic link between P460 and *c'*-type cytochromes. *Archives of Microbiology*, **173(1)**, 29-34.

Beyer S, Gilch S, Meyer O, Schmidt I. (2009). Transcription of genes coding for metabolic key functions in Nitrosomonas europaea during aerobic and anaerobic growth. *Journal of Molecular Microbiology & Biotechnology*, **16**, 187-197.

Bodelier PLE, Libochant JA, Blom CWPM, Laanbroek HJ. (1996). Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. *Applied and Environmental Microbiology*, **62(11)**, 4100-4107.

Bollmann A, Bär-Gilissen M-J, Laanbroek HJ. (2002). Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, **68(10)**, 4751-4757.

Bollmann A, Schmidt I, Saunders AM, Nicolaisen MH. (2005). Influence of starvation on potential ammonia-oxidizing activity and amoA mRNA levels of *Nitrosospira briensis*. *Applied and Environmental Microbiology*, **71(3)**, 1276-1282.

Bremner JM, Breitenbeck GA, Blackmer AM. (1981). Effect of anhydrous ammonia fertilization on emission of nitrous oxide from soils. *Journal of Environmental Quality*, **10**, 77-80.

Cantera JJL and Stein LY. (2007a). Interrelationship between nitrite reductase and ammonia-oxidizing metabolism in *Nitrosomonas europaea*. *Archives of Microbiology*, **188(4)**, 349-354.

Cantera JJL and Stein LY. (2007b). Molecular diversity of nitrite reductase (*nirK*) genes in nitrifying bacteria. *Environmental Microbiology*, **9(3)**, 765-776.

Casciotti K and Ward BB. (2005). Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria. FEMS Microbiology Ecology, **52(2)**, 197-205.

Casciotti KL and Ward BB. (2001). Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, **67(5)**, 2213-2221.

Cho CM-H, Yan T, Liu X, Wu L, Zhou J, Stein LY. (2006). Transcriptome of *Nitrosomonas europaea* with a disrupted nitrite reductase (*nirK*) gene. *Applied and Environmental Microbiology*, **72(6)**, 4450-4454.

Colliver BB and Stephenson T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, **18**, 219-232.

Di HJ, Cameron KC, Shen JP, Winefield CS, O'Callaghan M, Bowatte S, He JZ. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience*, **2(9)**, 621-624.

Elmore BO, Bergmann DJ, Klotz MG, Hooper AB. (2007). Cytochromes P460 and c'-beta; A new family of high-spin cytochromes c. *FEBS Letters*, **581(5)**, 911-916.

Garbeva P, Baggs EM, Prosser JI. (2007). Phylogeny of nitrite reductase (*nirK*) and nitric oxide reductase (*norB*) genes from *Nitrosospira* species isolated from soil. *FEMS Microbiology Letters*, **266**, 83-89.

Goreau TJ, Kaplan WA, Wofsy SC, McElroy MB, Valois FW, Watson SW. (1980). Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Applied and Environmental Microbiology*, **40(3)**, 526-532.

Hageman RH and Hucklesby DP. (1971). Nitrate reductase in higher plants. *Methods in Enzymology*, **23**, 491-503.

Hayatsu M, Tago K, Saito M. (2008). Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Science and Plant Nutrition*, **54(1)**, 33-45.

He Q, Huang KH, He Z, Alm EJ, Fields MW, Hazen TC, Arkin AP, Wall JD, Zhou J. (2006). Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Applied and Environmental Microbiology*, **72(6)**, 4370-4381.

Jia ZJ and Conrad R. (2009). Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology*, **11(7)**, 1658-1671.

Jiang QQ and Bakken LR. (1999). Nitrous oxide production and methane oxidation by different ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, **65(6)**, 2679-2684.

Kampschreur MJ, Poldermans R, Kleerebezem R, van der Star WRL, Haarhuis R, Abma WR, Jetten MSM, van Loosdrecht MCM. (2009). Emission of nitrous oxide and nitric oxide from a full-scale single-stage nitritation-anammox reactor. *Water Science and Technology*, **60(12)**, 3211-3217.

Koops H-P, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M. (2007). The lithotrophic ammonia-oxidizing bacteria. In, The Prokaryotes: A Handbook on the Biology of Bacteria. Vol. 5: Proteobacteria: Alpha and Beta Subclass [M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt (eds.)]. Springer-Verlag, New York.

Nicol GW, Leininger S, Schleper C, Prosser JI. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environmental Microbiology*, **10(11)**, 2966-2978.

Norton JM, Klotz MG, Stein LY, Arp DJ, Bottomley PJ, Chain PSG, Hauser LJ, Land ML, Larimer FW, Shin MW, Starkenburg SR. (2008). Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. *Applied and Environmental Microbiology*, **74(11)**, 3559-3572.

Numata M, Saito T, Yamazaki T, Fukumori Y, Yamanaka T. (1990). Cytochrome P-460 of *Nitrosomonas europaea*: Further purification and further characterization. *Journal of Biochemistry*, **108(6)**, 1016-1021.

Pearson AR, Elmore BO, Yang C, Ferrara JD, Hooper AB, Wilmot CM. (2007). The crystal structure of cytochrome P460 of *Nitrosomonas europaea* reveals a novel cytochrome fold and heme-protein cross-link. *Biochemistry*, **46(28)**, 8340-8349.

Pommerening-Roser A and Koops HP. (2005). Environmental pH as an important factor for the distribution of urease positive ammonia-oxidizing bacteria. *Microbiological Research*, **160(1)**, 27-35.

Poret-Peterson AT, Graham JE, Gulledge J, Klotz MG. (2008). Transcription of nitrification genes by the methane-oxidizing bacterium, *Methylococcus capsulatus* strain Bath. *ISME Journal*, **2**, 1213-1220.

Rozen S and Skaletsky HJ. (2000). Primer2 on the WWW for general users and for biologist programmers. In, Bioinformatics Methods and Protocols: Methods in Molecular Biology [S. Krawetz & S. Misener (eds.)]. Humana Press, Totowa, NJ.

Sayavedra-Soto LA, Hommes NG, Russell SA, Arp DJ. (1996). Induction of ammonia monooxygenase and hydroxylamine oxidoreductase mRNAs by ammonium in *Nitrosomonas europaea*. *Molecular Microbiology*, **20(3)**, 541-548.

Schlag S, Nerz C, Birkenstock TA, Altenberend F, Gotz F. (2007). Inhibition of staphylococcal biofilm formation by nitrite. *Journal of Bacteriology*, **189(21)**, 7911-7919.

Schmidt I, van Spanning RJM, Jetten MSM. (2004). Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants. *Microbiology*, **150**, 4107-4114.

Shaw LJ, Nicol GW, Smith Z, Fear J, Prosser JI, Baggs EM. (2006). *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology*, **8(2)**, 214-222.

Stein LY and Arp DJ. (1998). Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Applied and Environmental Microbiology*, **64(10)**, 4098-4102.

Stein LY, Arp DJ, Berube PM, Chain PSG, Hauser L, Jetten MSM, Klotz MG, Larimer FW, Norton JM, Op den Camp HJM, Shin M, Wei X. (2007). Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas eutropha* C91: Implications for niche adaptation. *Environmental Microbiology*, **9(12)**, 2993-3007.

Stein LY, Arp DJ, Hyman MR. (1997). Regulation of the synthesis and activity of ammonia monooxygenase in *Nitrosomonas europaea* by altering pH to affect NH_3 availability. *Applied and Environmental Microbiology*, **63(11)**, 4588-4592.

Stein LY and Klotz MG. (2010). Towards a new nomenclature for microbial metabolism of nitrogen oxides. *In review*.

Tan NCG, Kampschreur MJ, Wanders W, van der Pol WLJ, van de Vossenberg J, Kleerebezem R, van Loosdrecht MCM, Jetten MSM. (2008). Physiological and phylogenetic study of an ammonium-oxidizing culture at high nitrite concentrations. *Systematic and Applied Microbiology*, **31(2)**, 114-125.

Tortoso AC and Hutchinson GL. (1990). Contributions of autotrophic and heterotrophic nitrifiers to soil NO and N_2O emissions. *Applied and Environmental Microbiology*, **56(6)**, 1799-1805.

Upadhyay AK, Hooper AB, Hendrich MP. (2006). NO reductase activity of the tetraheme cytochrome c554 of *Nitrosomonas europaea*. *Journal of the American Chemical Society*, **128**, 4330-4337.

Wrage N, Velthof GL, van Beusichem ML, Oenema O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*, **33**, 1723-1732.

Yu R and Chandran K. (2010). Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiology*, **10**, 70.

Table 2.1 Genes and PCR primers used in this study.

Organism	Locus Tag	Gene	Forward primer	Reverse primer	Probe size
Nitrosomonas europaea ATCC 19718	NE2063	*amoA*	ccctctggaaagccttcttc	actttatgctgctggcaggt	687 bp
	NE0924	*nirK*	ccatttgttctgcatcatgg	gcaattactgaggggcatgt	526 bp
	NE2004	*norB*	acccagaagcttgcttaccc	tgttcggtgacgatgacact	1315 bp
	NE0684	*norS*	ataacgttttcattttgatccag	ccgtgcacagtcataatctgata	1075 bp
	NE0011	*cytL*	ggcgtgtcaggaacatagaaa	agcagcctgattctttcagc	413 bp
	NE0824	*cytS*	cactggtcggtttgacactg	gcgcacaagtcagatcgtta	423 bp
	RNA_45	16S rRNA	agagtttgatcmtggctcag	agrtaccttgttacgactt	1437 bp
Nitrosomonas eutropha C-91	NEUT2077	*amoA*	tacaaacgcagcgaagaatg	ccatatgtcacgcctgattg	625 bp
	NEUT1403	*nirK*	aaattatttaatgcaagctttgctg	tctgaccaggatattgtcacc	930 bp
	NEUT0520	*norB*	taaatcggctgcccgtaata	gccagattgagcatgacgta	1494 bp
	NEUT1874	*norS*	acgccattacactcgtaggg	tcttgaattttccgggtttg	444 bp
	NEUT0132	*cytL*	ttacttcggtgcaaccacac	tggtcggtactcaggtgaca	452 bp
	NEUT1345	*cytS*	aacgggttatgacattgtgc	aggtttgtctgcactgatcg	450 bp
	NEUT_R0005	16S rRNA	taaaggagcggctgatgtct	ttacgtgtgaagccctaccc	1015 bp
Nitrosospira multiformis ATCC 25196	Nmul_A2765	*amoA*	caaatgctgtcacgtcgttc	cttctggcttgactggaagg	638 bp
	Nmul_A1998	*nirK*	gcgagacaaagagcttcacc	gcaccgtgagataacctgct	573 bp
	Nmul_A1255	*norB*	cacgcccacccagtagtagt	gccatccacaccaatcttct	585 bp
	Nmul_A2667	*norS*	caatcagacgcaagtcagga	gatgatcacgtttggtgtcg	344 bp
	Nmul_A2484	*cytS*	aaggtgtgacacgggaagtc	gcggcaggtattgagatcat	488 bp
	Nmul_AR0012	16S rRNA	gataacgcaccgaaaggtgt	ttacgtgtgaagccctaccc	1087 bp

Table 2.2. Measurements of pH, net NO_2^- production, and rates of NO_2^- production for incubations of AOB strains in medium amended with 0, 10, or 20 mM $NaNO_2$. Values in parentheses indicate statistical error (n = 3 to 7). Bold values indicate a significant difference between measurements for the same organism incubated in medium with NO_2^- amendment relative to medium without NO_2^- amendment. Superscript letters indicate measurements within the same statistical grouping in each treatment for all organisms. Significant differences were determined by Student's t-test with $p < 0.05$. Initial pH: 7.58 ± 0.02 for HEPES-buffered medium and 8.09 ± 0.03 for phosphate-buffered medium. Rates of NO_2^- production were linear over the 6 hr time course.

Organism	pH at 6 hours			Net NO_2^- (mM) produced at 6 hours			Rate of NO_2^- production (mM · hr^{-1})		
	No amendment	10 mM NO_2^-	20 mM NO_2^-	No amendment	10 mM NO_2^-	20 mM NO_2^-	No amendment	10 mM NO_2^-	20 mM NO_2^-
N. europaea ATCC 19718 (HEPES)	5.80 (0.03)[a]	**5.95 (0.06)**[a]	**6.04 (0.07)**[a]	4.55 (0.17)[a]	3.98 (0.42)[a]	3.33 (0.92)[a]	1.83 (0.14)[a]	1.80 (0.10)[a]	1.57 (0.21)[a]
N. eutropha C91 (HEPES)	5.57 (0.04)[b]	**5.72 (0.02)**[b]	**5.81 (0.02)**[b]	4.84 (0.07)[a]	**4.61 (0.08)**[a]	**4.43 (0.14)**[a]	2.26 (0.02)[b]	**2.15 (0.03)**[b]	**2.03 (0.02)**[b]
N. multiformis ATCC 25196 (HEPES)	5.57 (0.12)[ab]	**5.90 (0.07)**[a]	**6.07 (0.06)**[a]	4.49 (0.19)[a]	4.34 (0.43)[a]	4.89 (0.53)[a]	1.62 (0.33)[a]	1.80 (0.50)[ab]	1.50 (0.57)[ab]
N. europaea ATCC 19718 (phosphate)	6.56 (0.04)[c]	6.59 (0.05)[c]	6.60 (0.04)[c]	17.09 (0.28)[b]	**15.74 (0.32)**[b]	**13.30 (0.84)**[b]	5.50 (0.59)[c]	5.01 (0.52)[c]	4.28 (0.43)[c]
N. eutropha C-91 (phosphate)	6.50 (0.02)[c]	6.51 (0.02)[c]	6.52 (0.02)[c]	15.25 (0.63)[c]	**12.85 (0.63)**[c]	**5.16 (0.70)**[a]	4.56 (0.35)[c]	4.10 (0.19)[c]	**2.74 (0.25)**[d]
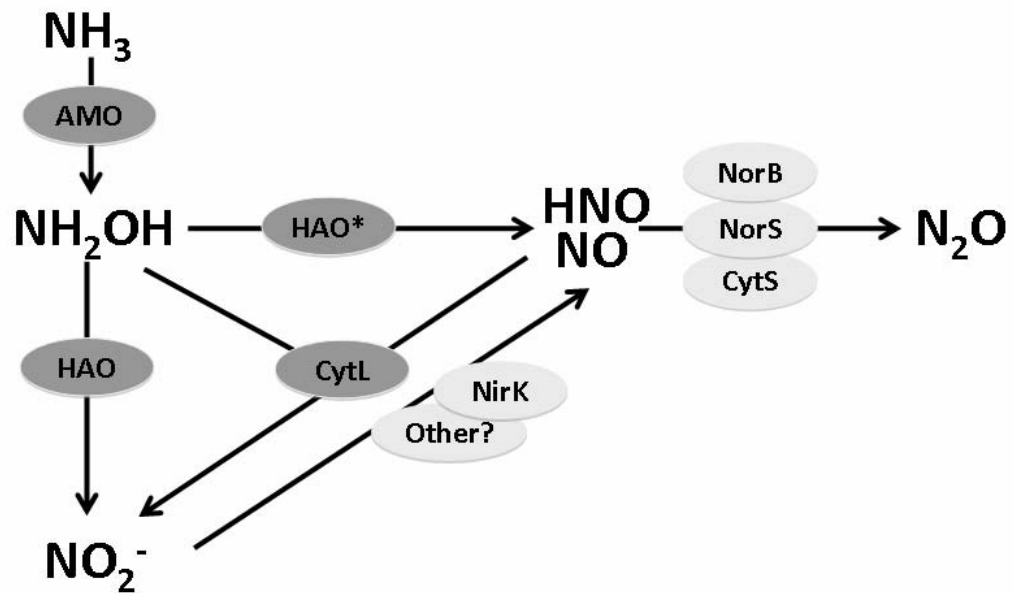
Figure 2.1. Model of nitrogen oxide metabolism and putative enzyme function in ammonia-oxidizing bacteria. Incomplete oxidation of NH_2OH to NO, perhaps via HNO, is likely triggered by interaction between HAO and an additional factor (Martin Klotz, personal communication).
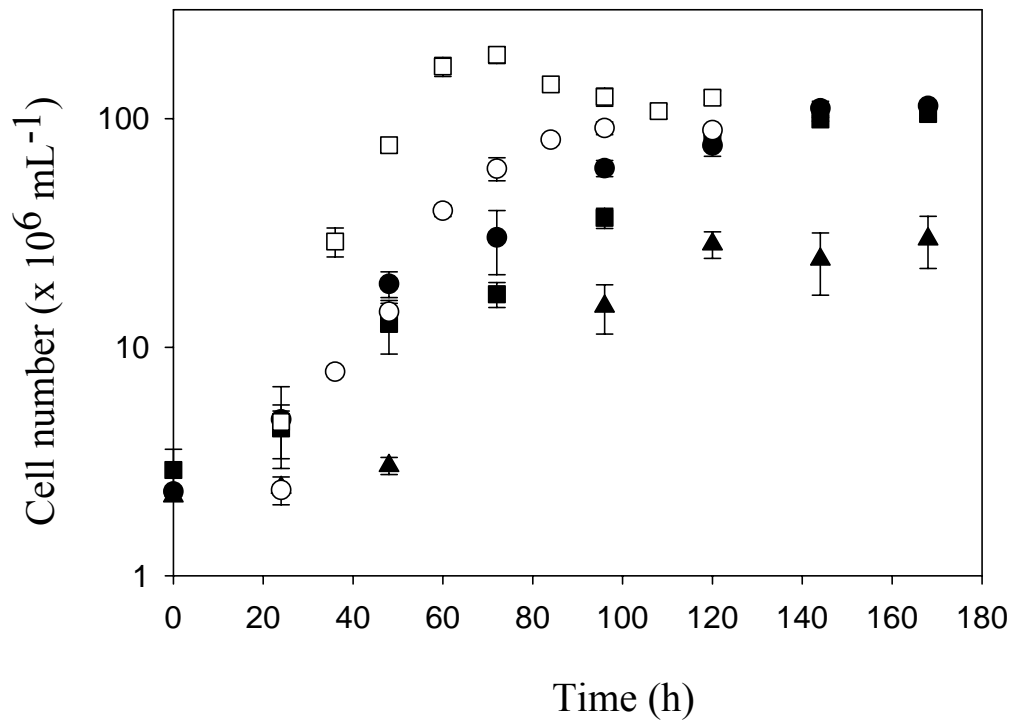
Figure 2.2. Growth curves of AOB in HEPES- (closed symbols) and phosphate- (open symbols) buffered medium. *N. europaea* ATCC 19718 (squares), *N. eutropha* C-91 (circles), *N. multiformis* ATCC 25196 (triangles). Error bars represent standard error for three to seven replicate cultures grown on different days.
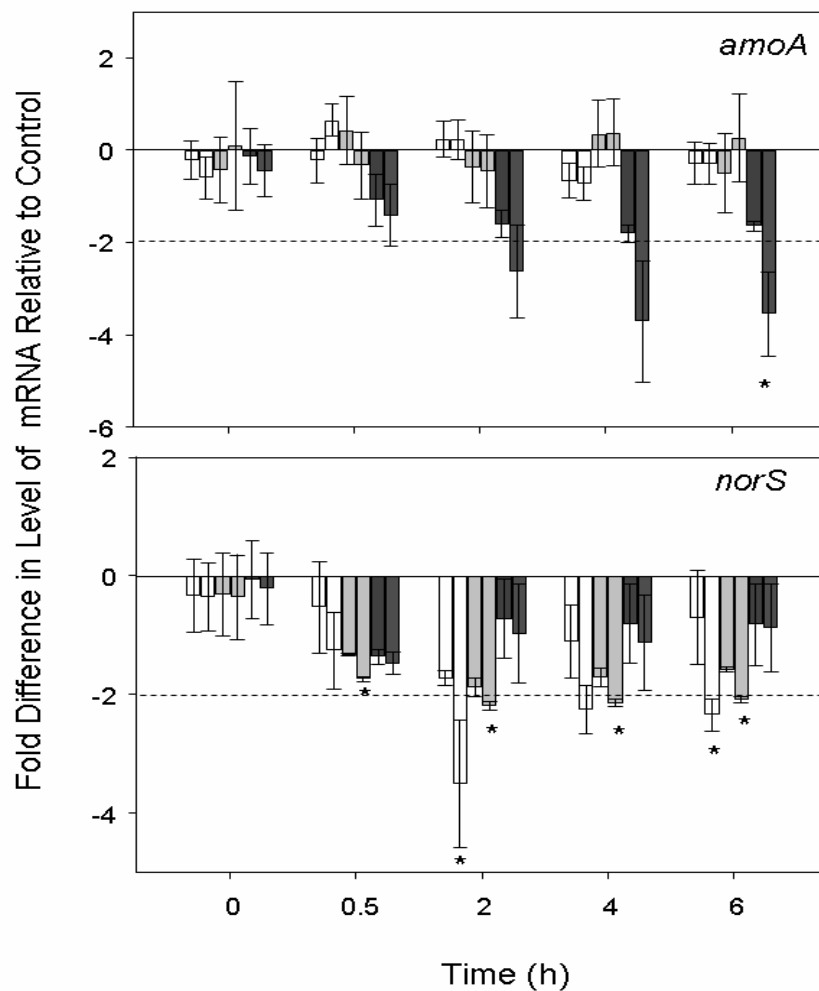
Figure 2.3. Fold difference in *amoA* and *norS* mRNA levels over a 6 h time course for cultures of *N. europaea* (white bars), *N. eutropha* (light gray bars) and *N. multiformis* (dark gray bars) incubated with 10 mM (first bar of each couple) or 20 mM (second bar of each couple) additional nitrite relative to a control culture with no nitrite addition. (*) indicates a significant difference in level of mRNA between the 10 mM and 20 mM nitrite treatment. Error bars represent standard error for three to seven replicate experiments with cells from different cultures grown on different days.
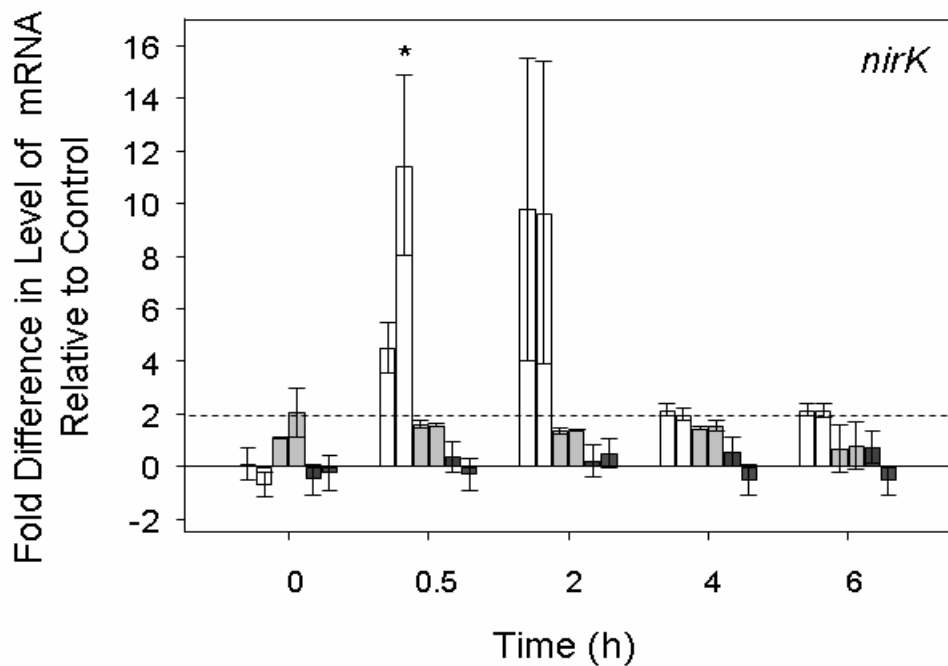
Figure 2.4. Fold difference in *nirK* mRNA levels over a 6 h time course for cultures of *N. europaea* (white bars), *N. eutropha* (light gray bars) and *N. multiformis* (dark gray bars) incubated with 10 mM (first bar of each couple) or 20 mM (second bar of each couple) additional nitrite relative to a control culture with no nitrite addition. (*) indicates a significant difference in level of mRNA between the 10 mM and 20 mM nitrite treatment. Error bars represent standard error for three to seven replicate experiments with cells from different cultures grown on different days.
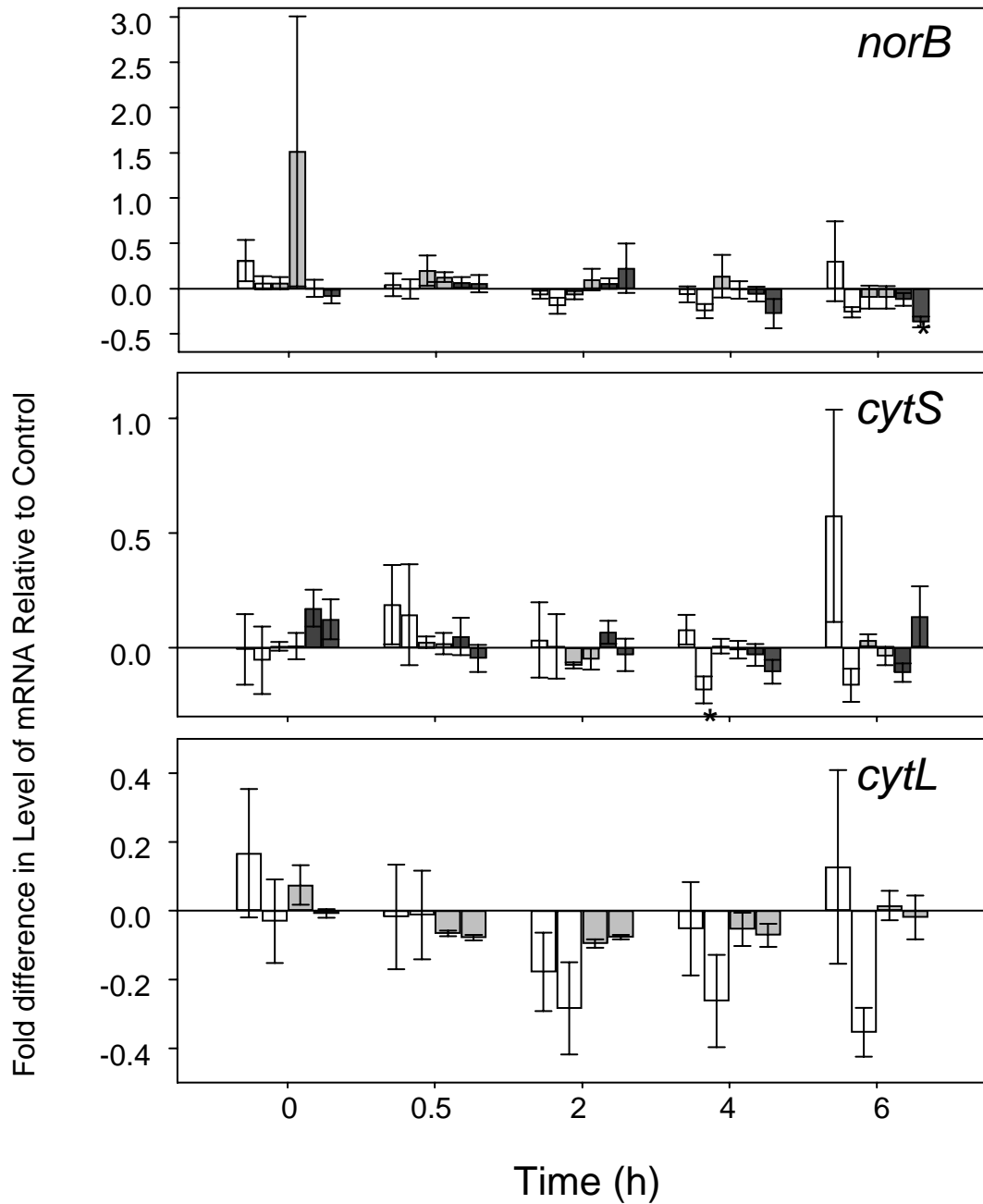
Figure 2.5. Fold difference in *nor*B, *cyt*S, and *cyt*L mRNA levels over a 6 h time course for cultures of *N. europaea* (white bars), *N. eutropha* (light gray bars) and *N. multiformis* (dark gray bars) incubated with 10 mM (first bar of each couple) or 20 mM (second bar of each couple) additional nitrite relative to a control culture with no nitrite addition. (*) indicates a significant difference in level of mRNA between the 10 mM and 20 mM nitrite treatment. Error bars represent standard error for three to seven replicate experiments with cells from different cultures grown on different days.
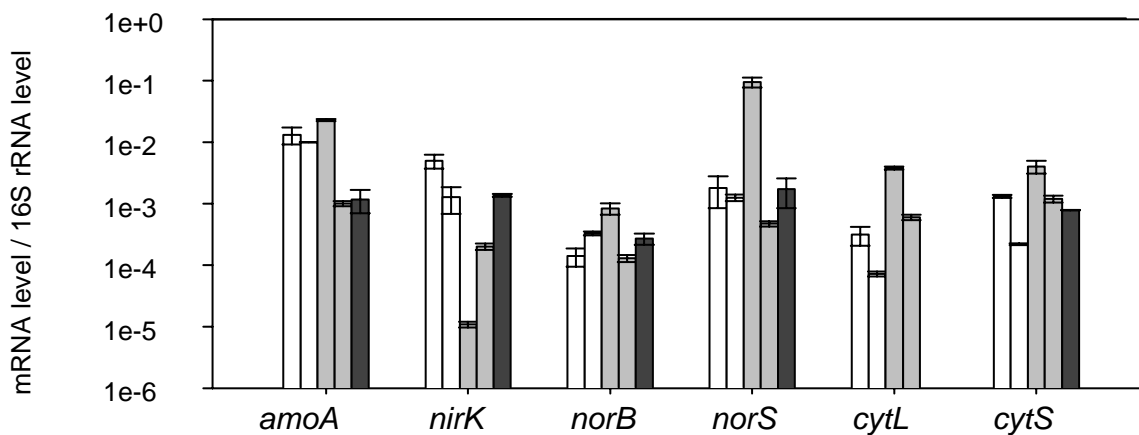
Figure 2.6. mRNA levels of select genes relative to 16S rRNA levels in mid-log phase cells of *N. europaea* ATCC 19713 (white bars), *N. eutropha* C-91 (light gray bars) and *N. multiformis* ATCC 25196 (dark gray bars). The two bars for each of the *Nitrosomonas* cultures represent mRNA levels for cells grown in HEPES- (first bar) or phosphate- (second bar) buffered media.

Chapter 3

Regulation of Genes Involved in NOx Metabolism in *Sphingomonas wittichii* RW1

Abstract

Recent genomic analyses of *Sphingomonas wittichii* RW1 have revealed the presence of genes involved in NOx metabolism, including a copper-containing nitrite reductase (*nirK*), quinol- and cytochrome *c*- linked nitric oxide reductases (*norZ* and *norB*) and flavohemoglobin (*hmp*). The presence of these genes may therefore likely serve a detoxification role in the presence of NOx. Using dot blot hybridization, relative mRNA levels were determined for *nirK*, *norZ, norB*, and *hmp* during growth of *S. wittichii* under initial 20% and 3% oxygen conditions in response to 0, 0.3, and 1mM NaNO$_2$. Levels of *nirK* mRNA in NO$_2^-$ -treated cells were highest during the stationary phase of growth under initial 20% O$_2$ relative to mRNA levels of untreated controls. Greater fold increases in levels of *hmp* mRNA occurred in cells incubated under 3% initial O$_2$. mRNA levels of *norZ* did not vary among the treatment groups and were similar to control levels regardless of O$_2$ conditions; however basal levels were highest of all genes examined. Levels of *norB* mRNA in NO$_2^-$ -treated cells were greater under 3% initial O$_2$, suggesting greater NorB activity under low O$_2$. Total nitrite consumption was observed in nitrite- treated cultures initially under 20% O$_2$. N$_2$O production was greatest in cells incubated under 3% initial O$_2$ with up to 54.62 and 101.54 nmol N$_2$O $\cdot$ OD^{-1} in 0.3 and 1mM NaNO$_2$ treatment groups, respectively, compared to cells incubated under 20% initial O$_2$, with 12.04 and 45.95 nmol N$_2$O $\cdot$ OD^{-1}, at those same concentrations.

Thus, increased N$_2$O production and transcription of *nirK*, *norB*, and *hmp* show that *S. wittichii* RW1 is capable of aerobic denitrification but that this activity is enhanced under hypoxic conditions.

Introduction

Denitrification is considered the main process by which nitric (NO) and nitrous (N$_2$O) oxides are produced as they are formed in the successive reduction of nitrite (or nitrate) to N$_2$ by denitrifying organisms (Ye et al., 1994). Under anaerobic conditions, denitrification proceeds to N$_2$ production; however, in the presence of oxygen, denitrification can be incomplete due to oxygen inhibition of nitrous oxide reductase, leading to N$_2$O as the terminal product (Takaya et al., 2003 and references therein; Morley et al., 2008). This is of significant consequence as oxygen fluctuations often occur in natural and controlled (i.e. wastewater treatment plants) environments, which can result in the release of considerable amounts of N$_2$O during aerobic denitrification (Lloyd, 1993; Takaya et al., 2003). Additionally, previous studies have demonstrated that these gases can also be produced aerobically during nitrification, in the oxidation of ammonia to nitrite (Goreau et al., 1980; Remde and Conrad, 1990; Tortoso and Hutchinson, 1990; Colliver and Stephenson, 2000 and references therein; Wrage et al., 2001). Both heterotrophic denitrifying bacteria and chemolithoautotrophic nitrifying bacteria can be found in similar environments including soil, wastewater, and sewage. Their collective roles in the nitrogen cycle have therefore been the subject of much investigation, especially with respect to the bacterial diversity, physiology, and genetic

inventory involved in the production of NO and N_2O. Emissions of NO and N_2O are of global significance due to their involvement in the depletion of stratospheric ozone, decreased air visibility and quality, and adverse respiratory effects (Galloway et al., 2003; Fields, 2004).

Recent studies have investigated the diversity of the copper- containing nitrite reductase enzyme (NirK) in nitrifying bacteria (Cantera and Stein, 2007a; Garbeva et al., 2007). Using full length NirK protein sequences, phylogenetic analysis (Figure 1.3 in Chapter 1) have shown that *Sphingomonas wittichii* RW1 possesses NirK as well, the gene arrangement of which is similar to that observed in the ammonia-oxidizer *Nitrosomonas europaea* and the nitrite-oxidizer *Nitrobacter winogradskyi*. Isolated from the River Elbe, Germany, *Sphingomonas wittichii* RW1 has largely been studied for the bioremediation of dioxin- containing compounds such as dibenzo-*p*-dioxin and dibenzofuran and chlorinated congeners as well as herbicides (Wittich et al., 1992; Wilkes et al., 1996; Keum et al., 2008; Stolz, 2009 and references therein). Further genetic analysis revealed that *S. wittichii* RW1 also contains genes encoding two nitric oxide reductases (both quinol- linked and cytochrome *c* dependent NOR, herein referred to as *norZ* and *norB*, respectively (Cramm et al., 1997)) and flavohemoglobin (*hmp*). In *S. wittichii* RW1, both *norB* and *hmp* are plasmid-encoded genes. A *Pseudomonas* sp. (R-25208) has been found to contain both *norB* and *norZ* genes; however, further studies are needed to determine functionality and whether the genes are chromosomally- or plasmid-encoded (Heylen et al., 2007). An isolate closely resembling *Sphingomonas adhaesiva* was previously observed with aerobic nitrate reduction to nitrite (Patureau et al., 2000).

However, the organism was unable to denitrify further and production of N_2O was absent, thus, it was not recognized as a true aerobic denitrifier as nitrite produced could be due to assimilatory reduction of nitrate (Patureau et al., 2000).

Of the dissimilatory denitrifying enzymes, nitrite reductase (NIR) is considered the key enzyme as it produces the obligate denitrification intermediate NO through the reduction of nitrite (NO_2^-). Due to the toxicity of NO, nitric oxide reductases (NOR) are involved in the subsequent reduction of NO to N_2O. The presence of cNOR in an organism is indicative of denitrification capabilities, while those containing qNOR are typically not involved in denitrification (i.e. as a form of respiration), but rather use the enzyme in the detoxification of NO (Hendriks et al., 2000). Though not involved in the anaerobic respiration of NOx, flavohemoglobin (Hmp) can be found in both denitrifying and non-denitrifying organisms also capable of NO reduction to N_2O. Hmp is able to oxidize NO to NO_3^- as well, thus functioning as either a NO dioxygenase or NOR depending on oxygen conditions. Due to its functional versatility, flavohemoglobin is involved in nitrosative and oxidative stress response in prokaryotes (Membrillo-Hernández et al., 1999; Zumft, 2005 and references therein). Because this suite of genes can be found within the genetic inventory of *S. wittichii* RW1, it was therefore hypothesized that they likewise function to serve a protective role in the presence of NOx, possibly resulting in aerobic N_2O production, as shown in Figure 3.1. Thus, the objective of this study was to investigate the regulation of these genes in response to nitrite under 20% and 3% O_2 and determine whether *S. wittichii* RW1 is capable of nitrite consumption and aerobic N_2O production.

Materials and Methods

Bacterial strain and culture maintenance

The bacterial strain used in this study was *Sphingomonas wittichii* RW1. Cultures were grown in 5 mLs Luria-Bertani Broth (LB) media in sterilized 15 mL capped-polystyrene tubes containing 1% tryptone, 0.5% yeast extract, and 1% NaCl in a rotary shaker (180 r.p.m.) at 28°C. Cultures were periodically streaked and grown on LB agar plates containing the above formulation with 15 g/L agar at 28°C for ~3 days and then stored at 4°C. Single colonies were used to inoculate fresh LB media to ensure and maintain pure cultures of *S. wittichii* RW1.

Growth experiments

For growth experiments initially under 20% O_2, exponentially-growing *S. wittichii* RW1 cells (0.5%) were inoculated into 100 mLs LB media containing 0, 0.3, or 1 mM $NaNO_2$ in glass bottles crimp-sealed with rubber septa. Upon inoculation, 2 mL samples were extracted every 4 hours using a sterile 1 mL (0.8mM x 25mM) PrecisionGlide™ needle with syringe with which to measure growth. Growth was determined by measuring the absorbance of the optical density of the culture at 600nm using a Spectronic 20 Genesys spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). For growth experiments conducted under initial 3% O_2, sealed bottles were first purged with N_2 and O_2 was added back prior to inoculation of *S. wittichii* RW1. Cells harvested for RNA extraction were immediately treated with 500 μL RNAprotect™ Bacteria Reagent, following the manufacturers' instructions (Qiagen, Valencia, CA) to

prevent degradation of RNA and kept at -80°C prior to extraction. Experiments consisted of five independent trials at each nitrite concentration under both O_2 conditions.

Nucleic acid extraction

Genomic DNA was isolated using the Wizard® SV Genomic DNA Purification System kit, following the manufacturers' instructions (Promega Corp., Madison, WI). Total RNA was extracted using the Aurum™ Total RNA Mini kit, following the manufacturers' instructions (Bio-Rad Laboratories, Hercules, CA). Nucleic acid concentration was determined using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE). DNA and RNA samples were kept at -20°C and -80°C, respectively, prior to probe construction and dot blot hybridization.

Dot-blot hybridization

DNA sequences of each gene of interest were found using the National Center for Biotechnology Information (NCBI) and Joint Genome Institute (JGI) databases. Gene-specific primers were designed using Primer3 Input 0.4.0 software (Rozen and Skaletsky, 2000) (Table 3.1). PCR reactions included standard reagents for Taq polymerase and genomic DNA as template. Using a thermocycler (iCycler, BioRad, Hercules, CA), amplification conditions were: 95°C for 5 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 50 seconds, with an additional extension cycle of 72°C for 7 minutes. PCR products were checked by agarose gel (1%) to verify single products of appropriate size. Amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI) according to the manufacturers' instructions. Amplification products were labeled using the Prime-a-Gene

labeling system (Promega Corp., Madison, WI) with [α-^{32}P]-dCTP (3000 Ci mmol^{-1}; Perkin-Elmer Inc., Waltham, MA) and random hexamers. The dynamic range of detection for each probe was tested using a concentration series of mRNA from 0.1 to 3 μg from the control incubations (0mM NaNO$_2$). The r^2 values for the slope of hybridization intensity/μg RNA was between 0.94 to 0.9998 for all probes.

Two μg total RNA from each sample was blotted onto a Zeta-Probe® GT nylon membrane (Bio-Rad Laboratories, Hercules, CA) using a Minifold® microsample filtration manifold (Dot-Blot System, Schleicher & Schuell, Keene, NH) following the Zeta-Probe® protocol. Membranes were allowed to dry overnight and UV-crosslinked (FB-UVXL-1000, Fisher Scientific, Pittsburgh, PA). Prehybridization, hybridization, and washing of Zeta-Probe® nylon membranes were done according to manufacturer's instructions at 30°C. To allow re-probing, membranes were stripped of radioactivity by washing twice in a 0.1X SSC/0.5% SDS solution at 95-100°C for 20 minutes. All blots were hybridized to gene-specific probes to normalize hybridization signals to the 16S rRNA pool. Hybridization intensity was analyzed using a Typhoon Phosphorimager and Imagequant software (Amersham, Piscataway, New Jersey).

Data Analysis

Background and signal from non-specific binding was subtracted, after which the relative hybridization intensity of specific probes was normalized by dividing by the signal from gene-specific to 16S rRNA probe hybridizations. The fold difference in levels of mRNA for each gene and time point from NO$_2^-$ exposure, under 20% and 3% initial O$_2$, was determined by dividing hybridization intensities from dot blots of RNA

extracted from NO_2^- amended to those from unamended cultures. Student's t-test ($p <$ 0.05) was performed to verify significant differences between treatments.

Physiological measurements

Nitrite consumption was measured using a colorimetric assay in which 1mL of a sulfanilamide-HCl solution and 1mL of a NNEQ-HCl (N-(1-Naphthyl) ethylenediamine dihydrochloride) solution was mixed with 20 μL of supernatant from harvested samples. The mixture was allowed to incubate at room temperature for at least 30 minutes and the absorbance was read at 540nm using a Spectronic 20 Genesys spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). Nitrate production was measured using a Standard Range Lab Nitrate Test kit (NECi, Lake Linden, Michigan) according to the manufacturers' instructions. However, nitrate production was not observed. Ammonia was measured colorimetrically using the phenate method (Clesceri et al., 1995). Oxygen and N_2O were measured daily from the headspace of inoculated bottles using a gas chromatograph equipped with a thermal conductivity detector and Molesieve 5A (80/100) and HAYESEP Q (80/100) columns, respectively (GC-8AIT, Shimadzu, Columbia, MD).

Results

Effect of nitrite on the growth of *S. wittichii* RW1 under 20% and 3% oxygen

Growth of *S. wittichii* RW1 was characterized under initial 20% and 3% oxygen in sealed bottles in the presence of 0, 0.3, and 1 mM $NaNO_2$ (Figure 3.2). The doubling times were 3.78 (±0.10), 3.7 (±0.005), and 3.89 (±0.003) hours for cells grown initially under 20% O_2 in the presence of 0, 0.3, and 1 mM $NaNO_2$, respectively. Under initial 3%

O_2, the doubling times of *S. wittichii* RW1 were 5.72 (±0.30), 5.70 (±0.43), and 6.19 (±0.06) hours at the corresponding $NaNO_2$ concentrations. The exponential phase of growth ranged from 10 to 24 hours in cells initially exposed to 20% O_2, with late log/ stationary phase occurring by ~28 hours. Under initial 3% O_2, exponential phase of cells is roughly between 10 and 16 hours, with stationary phase occurring by 20 hours. It was important to identify the exponential and stationary phases during the growth of *S. wittichii* RW1 as sampling for the expression work was targeted towards these two growth phases.

Regardless of O_2 condition, cell growth decreased with respect to increasing concentrations of nitrite. In fact, cell densities were comparable in the first 12 hours of growth in untreated and nitrite-treated groups; however growth was severely inhibited in cells grown initially incubated under 3% O_2. In fact, overall cell density was approximately 3 times less compared to cells grown initially under 20% O_2. Under both O_2 conditions, cell growth was similar in untreated and 0.3 mM $NaNO_2$ treated cells until late exponential/early stationary phase, where a statistically significant ($p < 0.05$) decrease in growth was observed from 28 hours until 48 hours. Additionally, nitrite significantly ($p < 0.05$) inhibited growth in the 1 mM $NaNO_2$ treatment group much earlier, after 12 hours of growth until 48 hours compared to the untreated group. A difference was also observed between the two nitrite- treated groups (0.3 mM and 1 mM $NaNO_2$), with inhibition of growth occurring from 16 to 48 hours in cells initially at 20% O_2, while the decrease in growth occurred earlier at 12 hours in cells at the lower oxygen

condition. Growth was significantly ($p < 0.05$) impaired in cells initially incubated under 3% O_2, regardless of nitrite treatment.

Nitrite consumption and nitrous oxide production

O_2 consumption (Table 3.2) in *S. wittichii* RW1 cells was indicative of aerobic respiration. Measured daily, average O_2 levels after the first 24 hours, were 11.91, 11.47, and 12.81% in cells treated with 0, 0.3, and 1mM $NaNO_2$ incubated under 20%, and 1.05, 0.96, and 0.83% in cells similarly treated incubated under 3% O_2, respectively (data not shown). Despite a decrease in growth, *S. wittichii* RW1 was able to consume nitrite, though to a much lesser extent in cells incubated under 3% O_2 (Table 3.2). Complete nitrite consumption occurred by 96 and 120 hours, respectively in cells amended with 0.3mM and 1mM $NaNO_2$ incubated under initial 20% O_2 (Table 3.2) However, less than half of the added nitrite was consumed in treated cells incubated under 3% O_2 after 120 hours (Table 3.2). Production of N_2O was detected after 48 hours, likely resulting from nitrite consumption (i.e. reduction of NO_2^- to NO). Nitrite-treated cells incubated under low O_2 produced significantly more ($p < 0.05$) N_2O on a per-cell basis than similarly treated cells incubated under 20% O_2. Peak N_2O production occurred after 120 hours of growth in treated cells incubated under both O_2 conditions (Table 3.2). Approximately 25% of $NaNO_2$-N accounted for the N_2O-N pool in both nitrite-treated groups (0.3 and 1mM $NaNO_2$) incubated under 20% O_2 after 120 hours (peak production), and 22% and 17% in similarly treated cells incubated under 3% O_2 at that same time point. Consumption of $NaNO_2$ and O_2 and production of N_2O were not observed in heat-killed controls.

Effects of nitrite on RNA levels

For all genes, changes in mRNA levels $\geq$ 2-fold upon NO_2^--treatment were considered significant. Levels of 16S rRNA in untreated and treated cells were consistent throughout the sampling period (data not shown) suggesting no change in viable cell number. While variable, basal mRNA levels of *nirK, norZ,* and *hmp* in the untreated group (0 mM $NaNO_2$) increased during the late exponential phase and into the stationary phase of growth (28 to 36 hours) in cells incubated initially under 20% O_2, relative to the transcription level of the 16S rRNA gene (Figure 3.3). Levels of *norZ* mRNA were highest among the four genes, with as much as a 12-fold increase relative to 16S RNA levels. Interestingly, *norB* mRNA levels were substantially (<2 fold) downregulated relative to 16S rRNA levels, suggesting lack of NorB activity under aerobic conditions. mRNA levels of all four genes in cells grown initially under 3% O_2 were markedly lower relative to the 16S rRNA level, although *nirK* mRNA levels increased over time. Under low O_2, *hmp* mRNA levels were significantly lower than 16S rRNA levels during early to late stationary phase of growth (10 to 20 hours), indicating greater use of the Hmp gene product under high O_2 conditions.

For nitrite-treated cells, the fold difference in mRNA levels were calculated relative to the control or untreated group, where mRNA levels $\geq$2 fold relative to levels of the untreated control group were considered significant. Overall, levels of *nirK* mRNA increased in response to nitrite, particularly in the 1 mM $NaNO_2$ treatment group, relative to the untreated group under both O_2 conditions (Figure 3.4). As activity of NirK reduces NO_2^- to NO, this may be responsible for some of the nitrite consumption and N_2O

production observed (Table 3.2) since nitrate produced from putative Hmp activity was not detected and ammonia concentrations from nitrite reduction to ammonia did not differ between treatment groups (data not shown).

Levels of *norZ* mRNA were similar between the treatment groups, regardless of O_2 condition (Figure 3.5). Overall, relative mRNA levels of treated cells were similar to those observed in the untreated group. However as earlier mentioned, basal mRNA levels of *norZ* relative to the 16S rRNA level were especially high compared to mRNA levels of the other genes under initial 20% O_2 (Figure 3.3). This suggests that because *norZ* is regularly expressed at high levels, it does not need further induction to handle up to 1 mM $NaNO_2$ and the subsequent NO produced from NirK activity, and may therefore be the dominant nitric oxide reductase in *S. wittichii* RW1. The data also imply that *norZ* is not regulated in response to decreased O_2 levels or nitrite, unlike *nirK*.

Relative mRNA levels of *hmp* were elevated in both nitrite treatment groups only significant under low O_2 conditions (Figure 3.6); however, cells exposed to the higher nitrite treatment (1 mM $NaNO_2$) under low O_2 exhibited a significantly greater fold increase in mRNA levels. Peak mRNA levels occurred during mid-exponential to stationary phase. This suggests that transcription of *hmp*, and therefore Hmp activity, likely occur under low O_2 conditions, thereby implying involvement in production of N_2O as an additional NO reductase in *S. wittichii* RW1.

Levels of *norB* mRNA were relatively similar between the nitrite treatment groups under initial 20% O_2, which remained consistent during the sampling period. Interestingly, relative mRNA levels of *norB* were modestly increased, and considered

statistically significant ($p < 0.05$), in cells initially incubated under 3% O_2 compared to cells initially incubated under 20% O_2 (Figure 3.7). It is therefore likely that this gene and its product are active under low O_2 conditions in addition to *hmp*, though not regulated by nitrite, unlike *hmp*.

The presence of N_2O (Table 3.2) suggests the activity of one or more of these gene products- Hmp, NorZ, and NorB, especially at the lower O_2 condition as N_2O production was greatest per unit cells initially incubated at 3% O_2. By 120 hours, 54.62 and 101.54 nmol $N_2O \cdot OD^{-1}$ were produced in 0.3 and 1 mM NaNO$_2$-treated cells, respectively, under 3% initial O_2, a 4.5- and 2.2- fold increase compared to cells treated at those same concentrations (12.04 and 45.95 nmol $N_2O \cdot OD^{-1}$) initially under 20% O_2. Thus, the increase in N_2O production in nitrite-treated cells initially under 3% O_2 suggests greater activity of multiple NO-reductases at lower oxygen levels.

Discussion

Nitrite inhibited growth of *S. wittichii* RW1 under both O_2 conditions. It is therefore likely the presence of nitrite interfered with O_2 consumption, as was similarly observed in the denitrifier *Agrobacterium tumefaciens* grown in the presence of 0, 0.2, 1, and 2 mM KNO$_2$ initially incubated under 7, 1, and 0% O_2 (Bergaust et al., 2008). In *A. tumefaciens*, and perhaps in this study, O_2 consumption, and hence cell growth, was determined to be dependent upon nitrite and O_2 concentrations as well as on NO production and concentration. The inhibition of growth observed in *S. wittichii* RW1 cells

at the lower O_2 condition suggested the cells were not capable of further, or comparable, growth under near-anaerobic conditions by denitrification.

Generation times calculated in this study were more rapid, if not agreeable, compared to *S. wittichii* RW1 grown with either 5 mM dibenzo-*p*-dioxin or 5 mM dibenzofuran as sole carbon and energy sources, which exhibited doubling times of 8 and 5 hours, respectively (Wittich et al., 1992). In both studies, cells were grown at 28°C on a rotary shaker; however, use of rich LB media as carbon and energy sources in this study as opposed to the biaryl ethers amended in mineral salts media likely contributed to the faster generation time observed here. Interestingly, *S. aromaticivorans* F199 required microaerobic (40- 80 μM O_2, equivalent to 3.5- 7% O_2) conditions for growth on mineral salts media containing various aromatic compounds (i.e. toluene, naphthalene, xylene, etc) as sources of carbon and energy; however, such conditions were not necessary for growth in rich media such as PTYG or tryptic soy agar (Fredrickson et al., 1991).

In this study, with the exception of *nor*B, mRNA levels of *nir*K, *nor*Z, and *hmp* were ≥ 3-fold relative to 16S rRNA levels in untreated cells under initial 20% O_2 and maintained during late log/early stationary phase. This suggests that regulation of these genes may be responsive to oxygen especially as transcripts of the denitrifying genes were not as numerous in cells incubated under 3% initial O_2. As 16S rRNA levels did not change throughout the sampling period, production of mRNA transcripts was therefore greater than degradation. It is possible *S. wittichii* RW1 cells regularly (i.e. constitutively) maintain these levels of transcript. In fact, maintaining a set of already-synthesized denitrification enzymes would prove beneficial to denitrifying organisms especially

during oxygen fluctuations (Baumann et al., 1996; Morley et al., 2008). In a similar manner, *nirK* (and nitrous oxide reductase) are constitutively expressed in *Alcaligenes faecalis*, an organism capable of heterotrophic nitrification and aerobic denitrification, such that induction will occur under low oxygen conditions in the absence of a nitrogenous oxide, triggering the sequential induction of the other denitrifying enzymes (Otte et al., 1996).

However, in other (aerobic) denitrifiers such as *Pseudomonas stutzeri, Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Paracoccus denitrificans,* anaerobiosis alone was insufficient to induce gene expression, as basal levels of denitrifying genes were very low with no appreciable differences in expression regardless of O_2 concentration, but required the added presence of a nitrogenous oxide for the activation and promotion of enzyme synthesis (Körner and Zumft, 1989; Zennaro et al., 1993; Baumann et al., 1996; Philippot et al., 2001). Under fully aerobic denitrifying conditions (in the presence of nitrate), *P. aeruginosa* expressed high basal levels of nitrite reductase, just slightly less than observed under anaerobic conditions (Zennaro et al., 1993). On the other hand, *P. denitrificans* expressed very low basal levels of denitrifying enzymes (nitrate, nitrite, and nitrous oxide reductases) aerobically when also in the presence of nitrate (Baumann et al., 1996). Thus, differential basal regulation of denitrifying enzymes does occur among denitrifying bacteria under different oxygen conditions in the presence of a nitrogenous oxide (Bergaust et al., 2008).

In the presence of nitrite, fold increases of *nir*K mRNA levels occurred within 15 hours following cell growth, with peak levels occurring by 24 hours when the overall

average of O_2 was 12% and 0.95% in cells incubated initially under 20% and 3% O_2, respectively. In *A. tumefaciens* grown in media amended with 1 mM KNO_2 incubated under 1% O_2, *nir*K mRNA was detected almost immediately (< 5 hours) after exposure with levels gradually increasing before reaching a steady state within 20 hours (~3 fold increase relative to the 16S rRNA levels) (Bergaust et al., 2008), similar to the present study on *S. wittichii* RW1.

Activity of nitric oxide reductase (NOR) has often been linked with nitrite reductase (NIR) as NIR produces the obligate denitrification intermediate NO. The combination of low O_2 and NO produced from nitrite reduction are likely responsible for the modest increase seen in mRNA levels of *norB* in nitrite- treated cells incubated under 3% O_2 compared to those incubated under 20% O_2 (Fig. 3.7). In semi-automated batch cultures of *A. tumefaciens* grown in the presence of 1mM KNO_2 under 1% O_2, *norB* mRNA levels were detected only during active denitrification with nondetectable levels prior to and after that period (Bergaust et al., 2008). In fact, detection was delayed and lower compared to detection of *nirK* mRNA, and concomitant with NO levels (Bergaust et al., 2008). The short-lived induction and overall decrease in mRNA levels of *norB* under diminishing oxygen conditions, especially during stationary phase of growth, is therefore not unprecedented despite the possibility of accumulating NO produced from NirK (Bergaust et al., 2008).

In this study, levels of *norZ* mRNA levels did not vary among the treatment groups regardless of O_2 conditions; however, as basal mRNA levels were substantially high in cells under 20% O_2, the corresponding gene products were perhaps sufficient to

cope with NO produced from nitrite reduction. Similar to *S. wittichii* RW1, *R. eutropha* possesses two nitric oxide reductases, both quinol-linked NORs, of which one is also encoded on a plasmid (Cramm et al., 1997). Mutational studies revealed that the chromosomally-encoded *norZ* gene product was needed to maintain denitrification during nitrate respiration and prevent cell death due to NO accumulation; however, the plasmid-encoded *nor* gene was isofunctional in the absence of *norZ* and could substitute for the activity of NorZ (Cramm et al., 1997). Based on NO reductase activity measurements, it was determined that the chromosomally-encoded *norZ* was the more active and therefore dominant NO reductase, and thus expressed to a greater extent than the plasmid-encoded *nor* gene (Cramm et al., 1997). With the importance of *norZ* with respect to growth and NO detoxification and the presence of two isofunctional nitric oxide reductases in *R. eutropha*, it is plausible that *norZ* serves a similar role in *S. wittichii* RW1 and that perhaps the plasmid-encoded *norB* could likewise serve as an isofunctional NOR. Thus, this hypothesis was further pursued as indicated in Chapter 4.

Since nitrate production was not observed in this study, it is likely Hmp functioned solely as a nitric oxide reductase rather than a nitric oxide dioxygenase. However, Hmp may also serve an alternative, unidentified function in *S. wittichii* RW1 especially as basal mRNA levels were lower at the lower O_2 condition than levels under 20% initial O_2, suggesting differential regulation in response to oxygen, as was observed in *Mycobacterium tuberculosis* and *Bacillus subtilis* (Hu et al., 1999; Nakano, 2006). *Hmp* has been shown to protect cells from NO toxicity under microaerobic conditions, serving as a protective mechanism against nitrosative stress, and is involved in cell

survival during prolonged anaerobiosis (Hu et al., 1999; Nakano, 2006). Additionally, *hmp* has been linked to N_2O production under denitrifying conditions, as demonstrated in *Ralstonia eutropha* (Cramm et al., 1994). Similarly, in this study, it is possible *hmp* contributed to the protection of cells in the presence of nitrite and survival under diminishing O_2 conditions since elevated mRNA levels and N_2O production were greatest in NO_2^--treated cells at the lower O_2 condition. Additionally, since cells at the lower O_2 condition were at a significantly lower density compared to cells initially incubated at 20% O_2, this implies that cells at the lower O_2 condition were likely more sensitive to nitrite (or rather NO produced from nitrite reduction) and required a greater need for NO detoxification, thus resulting in increased N_2O production on a per-cell basis. This therefore supports the role of Hmp as an NO reductase under low O_2 conditions in the presence of nitrogenous oxides. However, further studies are necessary in determining the full extent of the role *hmp* plays in *S. wittichii* RW1 with respect to oxygen fluctuations and nitrosative stress conditions.

In this study, N_2O production accounted for $\leq25\%$ of the NO_2^--N in treated *S. wittichii* RW1 cells. While it is possible N_2O consumption may have occurred to account for the remaining nitrogen pool, the genome of *S. wittichii* RW1 does not appear to contain a gene encoding nitrous oxide reductase, however N_2 was not measured in this study. The decrease in N_2O could in part be due to leakage of N_2O from the bottles through the rubber septa as the septa were perforated while taking N_2O and O_2 measurements. However, loss of N_2O cannot solely be due to leakage as this would mean a loss of over 75% N_2O and an observable increase in O_2, which is highly unlikely.

Nitrate production was measured, but not detected. While the genome of *S. wittichii* RW1 contains a gene (*nirBD*) encoding an assimilatory nitrite reductase, involved in the reduction of nitrite to ammonia, ammonia concentrations were measured but did not vary between treatment groups suggesting nitrite was not reduced to ammonia. However, mRNA levels of *nirBD* were not examined in this study. It is possible LB media used in the study masked ammonia production. Thus, further detailed studies are needed, perhaps using radiolabelled N, in accounting for the assimilation and conversion of $NaNO_2$-N products.

Nitrite consumption and N_2O production continued well into stationary phase, suggesting that the corresponding gene products (i.e. reductase enzymes) were retained in the cells. In *P. denitrificans,* the nitrite reductase enzyme was maintained in the cells despite changing oxygen conditions, retaining enzymatic activity (Baumann et al., 1996). Thus, perhaps this is true for *NirK* in this study as well and can also be extended to other genes involved in the aerobic denitrification pathway in *S. wittichii* RW1. Permanent or constitutive expression of denitrifying enzymes of aerobic bacteria prevents reinduction, and is likely a result of rapid adaptation and response to aeration changes (Patureau et al., 2000). Under transient anoxic conditions, N_2O generation is perhaps more dependent upon enzymatic activity and metabolic levels rather than controlled at the transcript level, as observed in *P. stutzeri* (Körner and Zumft, 1989).

Co-respiration of oxygen and nitrate (or nitrite) in aerobic denitrifiers, while not energetically favorable, is thought to serve as a mechanism allowing electrons to flow to denitrifying enzymes so as not to overwhelm components (i.e. cytochromes) of the

electron transport chain (Robertson et al., 1988). Consequently, upon depletion of oxygen, denitrification would then be the main form of respiration. However, irrespective of respiration, the genes and their products involved in the aerobic denitrifying pathway can also function to protect cells from NOx as observed in non-denitrifying organisms such as *Nitrosomonas europaea* and *Neisseria gonorrhea* (Householder et al, 2000; Beaumont et al., 2002; Cantera and Stein, 2007b). As nitrite inhibited growth of *S. wittichii* RW1, which was exacerbated under low O_2 conditions, it is likely that aerobic denitrification follows the latter case, in the protection of cells from nitrosative stress during and following cell growth, as evidenced by the production of N_2O although nitrate respiration was not examined.

In summary, this study shows that *S. wittichii* RW1 is capable of aerobic denitrification as demonstrated by nitrite consumption and aerobic production of N_2O. Based on basal mRNA levels, *nirK, norZ, norB*, and *hmp* appear to be regulated by oxygen, with *nirK*, *norB*, and *hmp* expression altered in the presence of nitrite. Increased mRNA levels of *nirK* supports its involvement in the reduction of nitrite while the combined activities of *norZ, norB*, and *hmp* gene products were implicated in the reduction of NO to N_2O, following the proposed model, with the exception of the *hmp* gene product serving as a NO dioxygenase (Figure 3.1). Enhanced transcription of *norB* and *hmp* under low O_2 in the presence of nitrite is likely attributable to increased NO sensitivity; the subsequent increase in the concomitant production of N_2O further suggests greater activity of the corresponding gene products under low O_2. Though not among the suite of genes characteristically involved in aerobic denitrification, *hmp* was

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nonetheless important in the protection against nitrosative stress in *S. wittichii* RW1.

Despite lack of apparent upregulation in the presence of nitrite, high basal transcription of

*nor*Z and its importance in an organism (*R. eutropha*) likewise possessing two NO

reductases proved intriguing for further study (Chapter 4). The combined function of

these genes therefore serves to protect the cells from nitrite, thus supporting the proposed

hypothesis of this study. Thus, *S. wittichii* RW1 may be among the first in the

Sphingomonas genus to be classified as an aerobic denitrifying organism, aerobically

producing N_2O in the detoxification of nitrite.

References

Baumann B, Snozzi M, Zehnder AJB, van der Meer JR. (1996). Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. *Journal of Bacteriology*, **178(15)**, 4367-4374.

Beaumont HJE, Hommes NG, Sayavedro-Soto LA, Arp, DJ, Arciero DM, Hooper AB, Westerhoff HV, van Spanning RJM.. (2002). Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *Journal of Bacteriology*, **184(9)**, 2557-2560.

Bergaust L, Shapleigh J, Frostegård Å, Bakken L. (2008). Transcription and activities of NO_x reductases in *Agrobacterium tumefaciens*: the influence of nitrate, nitrite and oxygen availability. *Environmental Microbiology*, **10(11)**, 3070-3081.

Cantera JJL and Stein LY. (2007a). Molecular diversity of nitrite reductase genes (*nirK*) in nitrifying bacteria. *Environmental Microbiology*, **9(3)**, 765-776.

Cantera JJL and Stein LY. (2007b). Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. *Archives of Microbiology*, **188(4)**, 349-354.

Colliver BB and Stephenson T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, **18**, 219-232.

Clesceri LS, Greenberg AE, Eaton AD. (1998). Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington, D.C.

Cramm R, Siddiqui RA, Friedrich B. (1994). Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. *The Journal of Biological Chemistry*, **269(10)**, 7349-7354.

Cramm R, Siddiqui RA, Friedrich B. (1997). Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. *Journal of Bacteriology*, **179(21)**, 6769-6777.

Fields S. (2004). Global nitrogen cycling out of control. *Environmental Health Perspectives*, **112(10)**, A557-A563.

Fredrickson JK, Brockman FJ, Workman DJ, Li SW, Stevens TO. (1991). Isolation and characterization of a subsurface bacterium capable of growth on toluene, naphthalene, and other aromatic compounds. *Applied and Environmental Microbiology*, **57(3)**, 796-803.

Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB, Cosby BJ. (2003). The Nitrogen Cascade. *Bioscience*, **53(4)**, 341-356.

Garbeva P, Baggs EM, Prosser JI. (2007). Phylogeny of nitrite reductase (*nirK*) and nitric oxide reductase (*norB*) genes from Nitrosospira species isolated from soil. *FEMS Microbiology Letters*, **266**, 83-89.

Goreau TJ, Kaplan WA, Wofsy SC, McElroy MB, Valois FW, Watson SW. (1980). Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Applied and Environmental Microbiology*, **10(3)**, 526-532.

Hendriks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S, Saraste M. (2000). Nitric oxide reductases in bacteria. *Biochimica et Biophysica Acta*, **1459**, 266-273.

Heylen K, Vanparys B, Gevers D, Wittebolle L, Boon N, De Vos P. (2007). Nitric oxide reductase (*norB*) gene sequence analysis reveals discrepancies with nitrite reductase (*nir*) gene phylogeny in cultivated denitrifiers. *Environmental Microbiology*, **9(4)**, 1072-1077.

Householder TC, Fozo EM, Cardinale JA, Clark VL. (2000). Gonococcal nitric oxide reductase is encoded by a single gene, *norB*, which is required for anaerobic growth and is induced by nitric oxide. *Infection and Immunity*, **68(9)**, 5241-5246.

Hu Y, Butcher PD, Mangan JA, Rajandream M-A, Coates ARM. (1999). Regulation of *hmp* gene transcription in Mycobacterium tuberculosis: effects of oxygen limitation and nitrosative and oxidative stress. *Journal of Bacteriology*, **181(11)**, 3486-3493.

Keum YS, Lee YJ, Kim J-H. (2008). Metabolism of nitrodiphenyl ether herbicides by dioxin-degrading bacterium *Sphingomonas wittichii* RW1. *Journal of Agricultural and Food Chemistry*, **56**, 9146-9151.

Körner H and Zumft WG. (1989). Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Applied and Environmental Microbiology*, **55(7)**, 1670-1676.

Lloyd D. (1993). Aerobic denitrification in soils and sediments: From fallacies to facts. *Trends in Ecology and Evolution*, **8(10)**, 352-356.

Membrillo-Hernández J, Coopamah MD, Anjum MF, Stevanin TM, Kelly A, Hughes MN, Poole RK. (1999). The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is

essential for transcriptionaly responses to oxidative stress. *The Journal of Biological Chemistry*, **274(2)**, 748-754.

Morley N, Baggs EM, Dörsch P, Bakken L. (2008). Production of NO, N_2O, N_2 by extracted soil bacteria, regulation by NO_2^- and O_2 concentrations. *FEMS Microbiology Ecology*, **65**, 102-112.

Nakano MM. (2006). Essential role of flavohemoglobin in long-term anaerobic survival of *Bacillus subtilis*. *Journal of Bacteriology*, **188(17)**, 6415-6418.

Otte S, Grobben NG, Robertson LA, Jetten MSM, Kuenen JG. (1996). Nitrous oxide production by *Alcaligene faecalis* under transient and dynamic aerobic and anaerobic conditions. *Applied and Environmental Microbiology*, **62(7)**, 2421-2426.

Patureau D, Zumstein E, Delgenes JP, Moletta R. (2000). Aerobic denitrifiers isolated from diverse natural and managed ecosystems. *Microbial Ecology*, **39**, 145-152.

Philippot L, Mirleau P, Mazurier S, Siblot S, Hartmann A, Lemanceau P, Germon JC. (2001). Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar*, *nir*, *nor*, and *nos* genes. *Biochimica et Biophysica Acta*, **1517**, 436-440.

Remde A and Conrad R. (1990). Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. *Archives of Microbiology*, **154**, 187-191.

Stolz A. (2009). Molecular characteristics of xenobiotic-degrading Sphingomonads. *Applied Microbiology and Biotechnology*, **81**, 793-811.

Takaya N, Catalan-Sakairi MAB, Sakaguchi Y, Kato I, Zhou Z, Shoun H. (2003). Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Applied and Environmental Microbiology*, **69(6)**, 3152-3157.

Tortoso AC and Hutchinson GL. (1990). Contributions of autotrophic and heterotrophic nitrifiers to soil NO and N_2O emissions. *Applied and Environmental Microbiology*, **56(6)**, 1799-1805.

Wilkes H, Wittich R-M, Timmis KN, Fortnagel P, Francke W. (1996). Degradation of chlorinated dibenzofurans dibenzo-*p*-dioxins by *Sphingomonas* sp. Strain RW1. *Applied and Environmental Microbiology*, **62(2)**, 367-371.

Wittich R-M, Wilkes H, Sinnwell V, Francke W, Fortnagel P. (1992). Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. Strain RW1. *Applied and Environmental Microbiology*, 58(3), 1005-1010.

Wrage, N., Velthof G. L., van Beusichem ML, Oenema O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*, **33,** 1723-1732.

Ye RW, Averill BA, Tiedje JM. (1994). Denitrification: Production and consumption of nitric oxide. *Applied and Environmental Microbiology*, **60(4)**, 1053-1058.

Zennaro E, Ciabatti I, Cutruzzola F, D'Alessandro R, Silvestrini MC. (1993). The nitrite reductase gene of *Pseudomonas aeruginosa*: Effect of growth conditions on the expression and construction of a mutant by gene disruption. *FEMS Microbiology Letters*, **109**, 243-250.

Zumft WG. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *Journal of Inorganic Biochemistry*, **99**, 194-215.
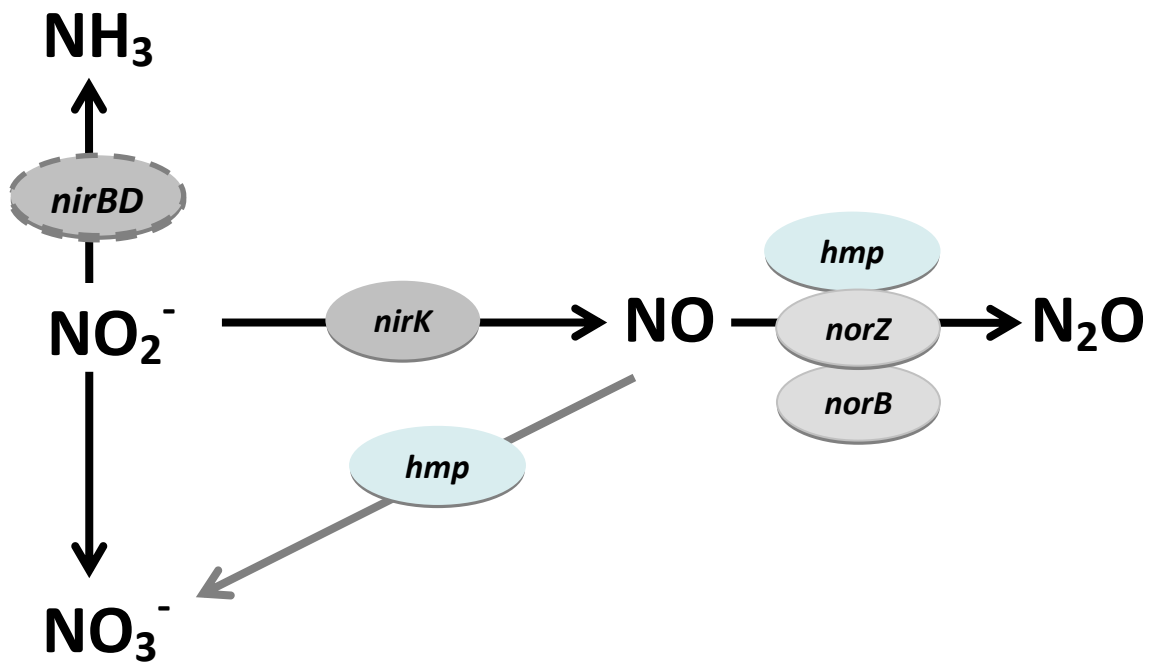
Figure 3.1. Proposed roles of N-metabolism genes in *Sphingomonas wittichii* RW1. The gene encoding an assimilatory nitrite reductase (*nir*BD) was not studied in this investigation. Ammonia was not measurable and nitrate was not detected.
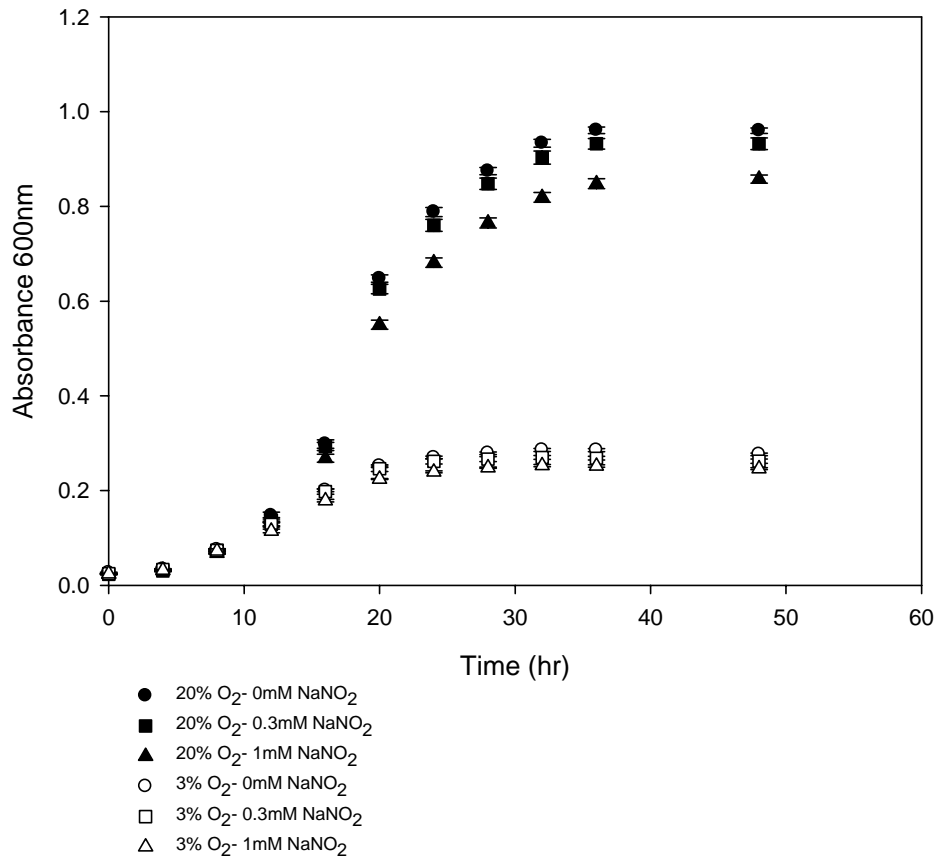
Figure 3.2. Growth curves of *S. wittichii* RW1 grown in LB media under initial 20% and 3% O_2 in the presence of 0, 0.3, and 1 mM $NaNO_2$. Error bars represent the standard error of 5 independent experiments.
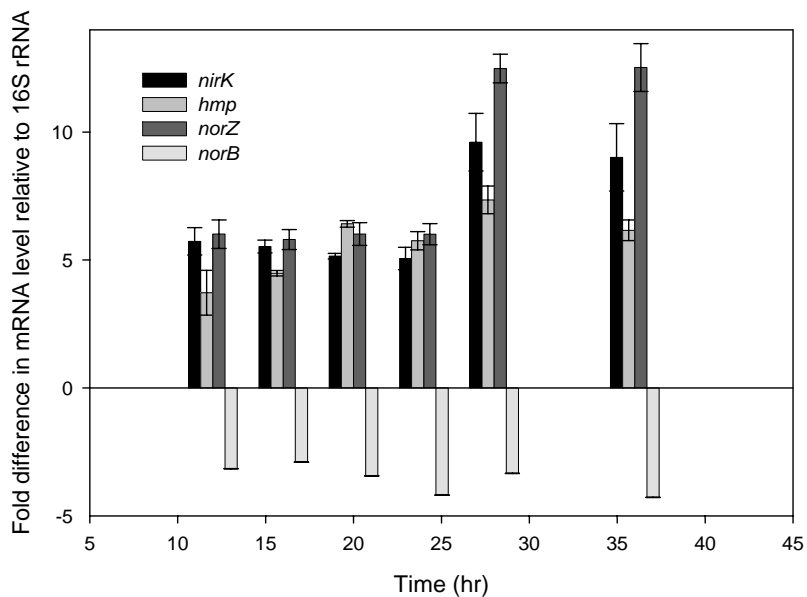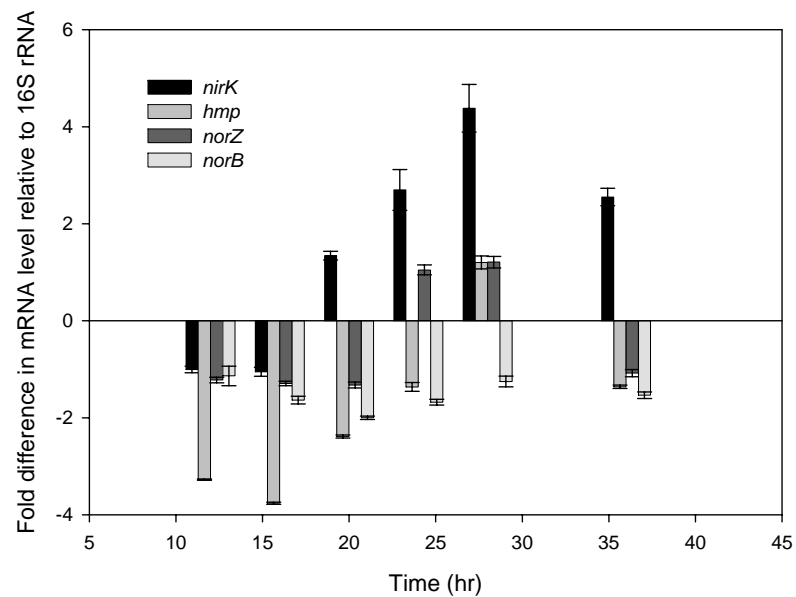
A. Fold difference in cells incubated under 20% O_2 B. Fold difference in cells incubated under 3% O_2

Figure 3.3. Fold difference in mRNA levels of genes involved in NOx metabolism relative to the 16S rRNA level in *S. wittichii* RW1 under initial 20% (A) and 3% (B) oxygen, as determined by dot blot hybridization. Error bars represent the standard error of 5 independent experiments.

Figure 3.4. Fold difference in mRNA levels of *nirK* relative to the control (untreated, 0mM NaNO$_2$) group in *S. wittichii* RW1 treated with 0.3 and 1mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.

Figure 3.5. Fold difference in mRNA levels of *norZ* relative to the control (untreated, 0 mM NaNO$_2$) group in *S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.
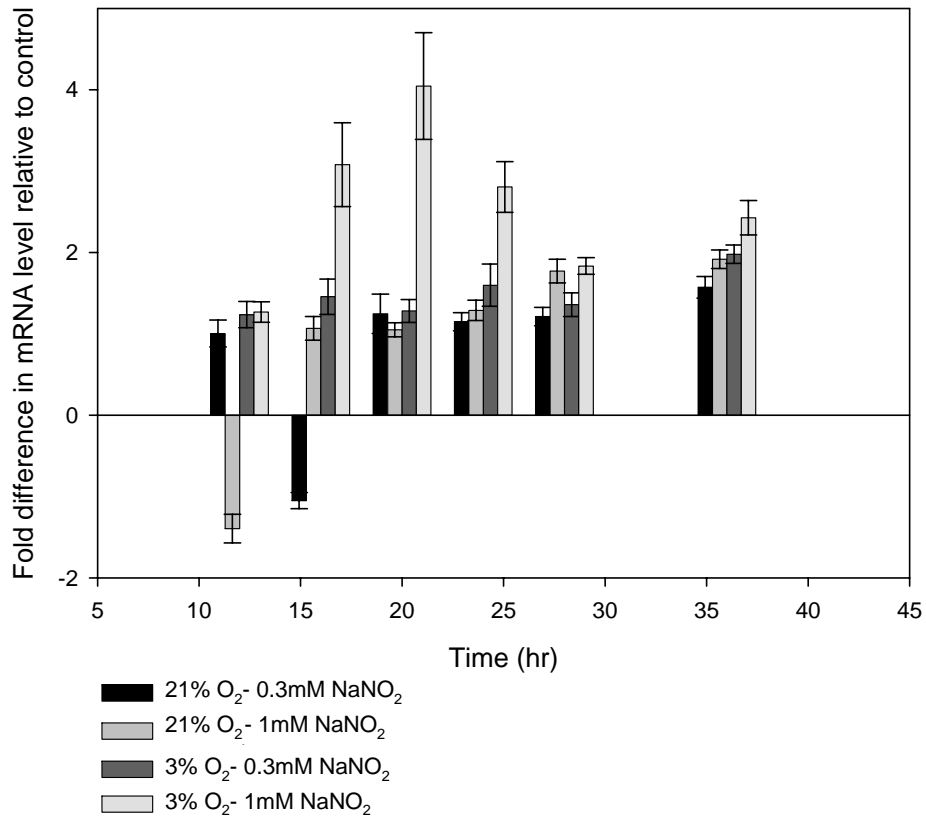
Figure 3.6. Fold difference in mRNA levels of *hmp* relative to the control (untreated, 0 mM NaNO$_2$) group in *S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.
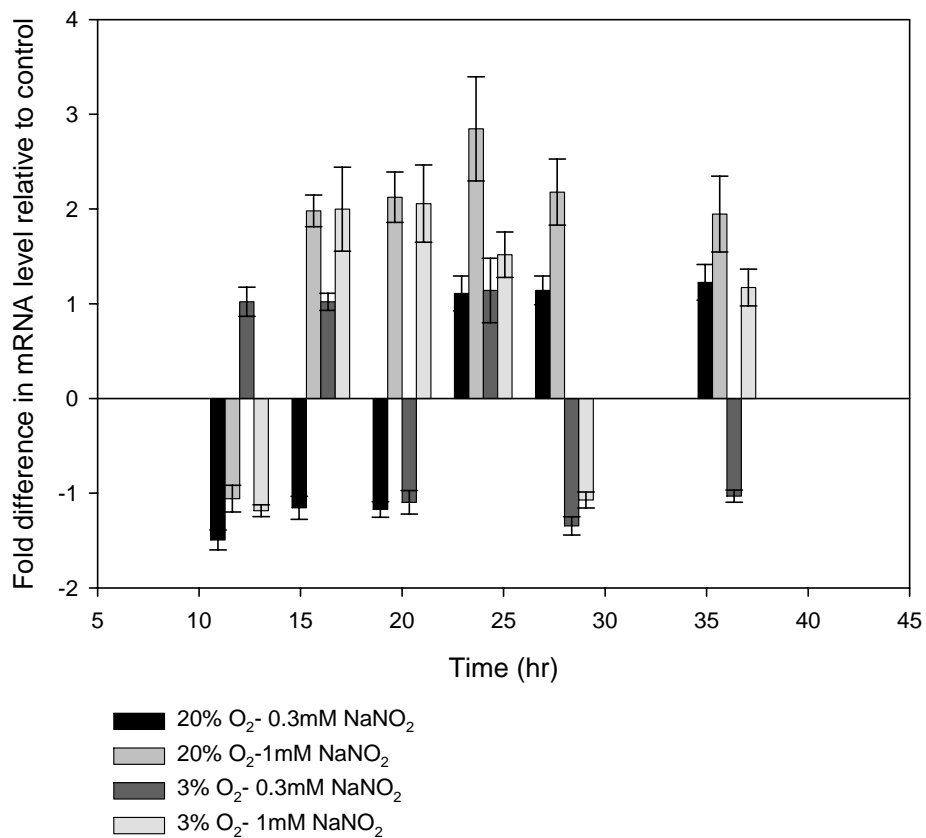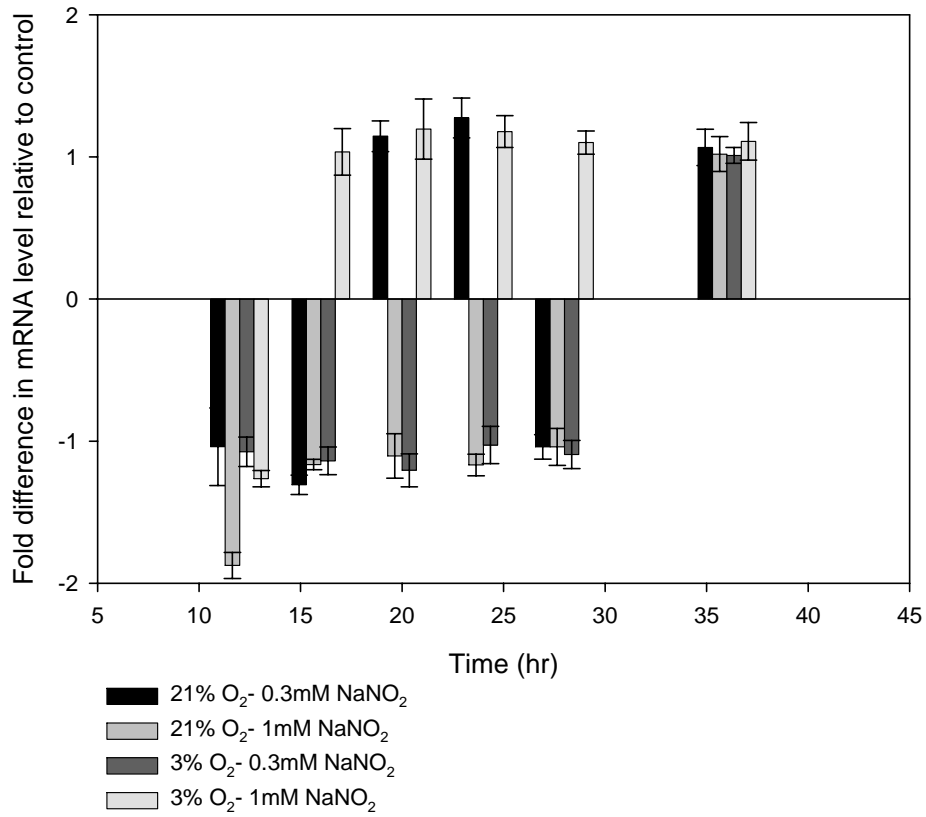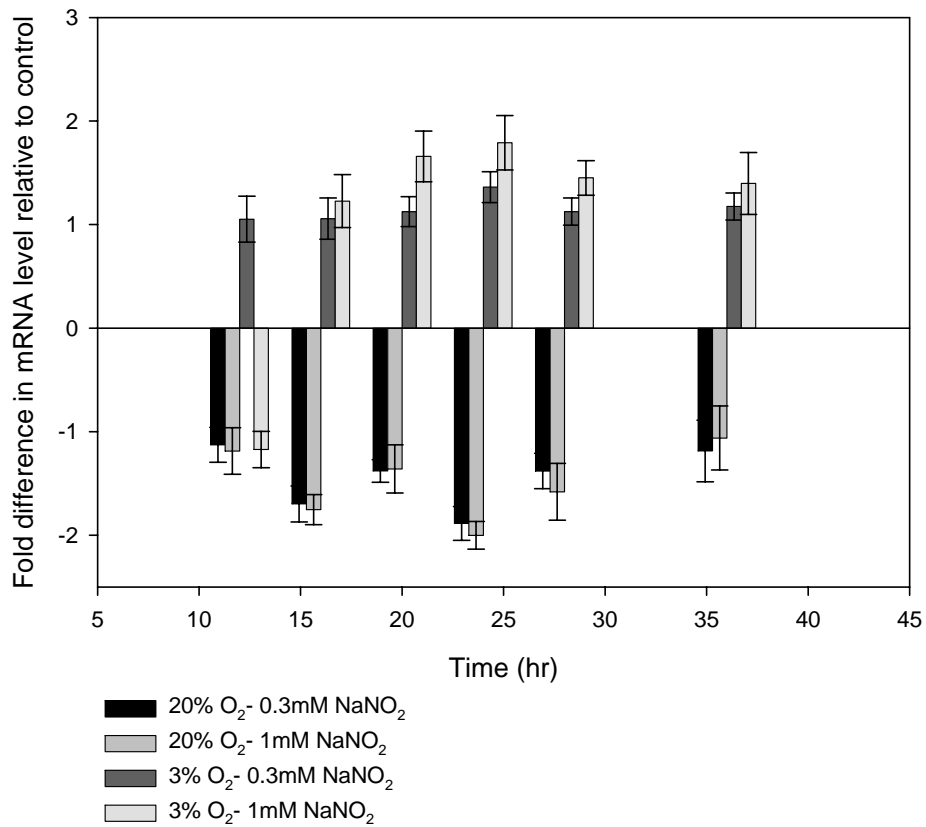
Figure 3.7. Fold difference in mRNA levels of *norB* relative to the control (untreated, 0 mM NaNO$_2$) group in *S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.

Table 3.1. *S. wittichii* RW1 primers used in this study, as designed using Primer3 Input 0.4.0 software.

Locus Tag	Gene	F primer	R primer	PCR product
Swit_1793	*nirK*	ctgaccgcgaaggaagtatc	catggtcgacgatcacattg	742 bp
Swit_5203	*hmp*	ccaacgccaatactcaacct	cagcatttctacggcatcaa	210 bp
Swit_R0031	*16S*	gtacaaggcctgggaacgta	tttatcgcctgaggatgagc	1159 bp
Swit_4614	*norZ*	gtggtgcccgagaaatagag	gccagagcttctacggtgtc	703 bp
Swit_5200	*norB*	tcgagcttgtccacattctg	attgtctccccaaaccatga	513 bp

Table 3.2. Nitrite and O_2 consumption and N_2O production in *S. wittichii* RW1 under initial 20% and 3% O_2. The standard error of 5 independent experiments are indicated in parentheses. $NaNO_2$ and nmol N_2O produced · OD^{-1} measured activities: [a] signifies statistical significance ($p < 0.05$) between 0.3 and 1mM $NaNO_2$ treatment groups at either O_2 level; [b] signifies statistical significance ($p < 0.05$) between cells initially under 20% and 3% O_2 at each concentration. For O_2 consumption, [a] and [b] are as previously indicated, [c] signifies statistical significance ($p < 0.05$) between 0 and 0.3mM $NaNO_2$ treatments at either O_2 level; [d] signifies statistical significance ($p < 0.05$) between 0 and 1mM $NaNO_2$ treatments at either O_2 level.

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NaNO₂ amendment to LB media and measured activities	Time (hr) and Oxygen (%)							
	48		72		96		120	
	20%	3%	20%	3%	20%	3%	20%	3%
mM NaNO₂ Remaining								
0 mM	0	0	0	0	0	0	0	0
0.3 mM	0.20 (0.003)[ab]	0.29 (0.003)[ab]	0[ab]	0.23 (0.005)[ab]	0[b]	0.18 (0.007)[ab]	0[b]	0.16 (0.008)[ab]
1.0 mM	0.86 (0.017)[ab]	0.93 (0.026)[ab]	0.41 (0.036)[ab]	0.84 (0.03)[ab]	0.05 (0.02)[b]	0.73 (0.03)[ab]	0[b]	0.68 (0.038)[ab]
nmol N₂O Produced · OD⁻¹								
0 mM	0	0	0	0	0	0	0	0
0.3 mM	4.41 (0.09)[ab]	13.05 (1.01)[ab]	10.41 (0.14)[ab]	29.05 (1.67)[ab]	11.56 (0.11)[ab]	48.33 (3.05)[ab]	12.04 (0.25)[ab]	54.62 (2.78)[ab]
1.0 mM	7.72 (1.03)[ab]	23.09 (4.42)[ab]	27.53 (1.80)[ab]	51.03 (5.30)[ab]	39.66 (2.39)[ab]	90.17 (8.98)[ab]	45.95 (3.26)[ab]	101.54 (8.36)[ab]
% O₂ Remaining								
0 mM	5.49 (0.09)[bcd]	0.41 (0.01)[bd]	4.68 (0.15)[bd]	0.29 (0.005)[bd]	4.66 (0.19)[bd]	0.29 (0.004)[bd]	--	0.29 (0.003)[cd]
0.3 mM	5.16 (0.09)[abc]	0.41 (0.006)[b]	4.52 (0.16)[b]	0.28 (0.007)[b]	4.49 (0.19)[ab]	0.28 (0.003)[b]	--	0.27 (0.003)[c]
1.0 mM	4.71 (0.23)[abcd]	0.39 (0.002)[bd]	3.96 (0.36)[bd]	0.28 (0.002)[bd]	3.41 (0.86)[abd]	0.28 (0.003)[bd]	--	0.27 (0.003)[d]

Chapter 4

Isofunctional *norB* and Regulation of NOx-related Genes in Wildtype and Δ*norZ* *Sphingomonas wittichii* RW1

Abstract

Sphingomonas wittichii RW1 contains genes involved in nitrogen oxide metabolism including nitrite reductase (*nir*K), two nitric oxide reductases (*nor*Z and *nor*B), and flavohemoglobin (*hmp*). Work in the previous chapter demonstrated that these genes are involved in aerobic denitrification of nitrite and the production of N_2O. In this chapter, a *nor*Z mutant of *S. wittichii* RW1 was constructed by disruption of the *nor*Z gene with a gentamycin resistance gene cassette. Using dot blot hybridization, mRNA levels of the four genes were determined in wildtype and Δ*nor*Z *S. wittichii* RW1 cells in response to 0, 0.3, and 1mM $NaNO_2$ incubated under initial 20% O_2 conditions. Relative to 16S rRNA levels, basal mRNA levels of *nirK*, *hmp*, and *norB* were significantly increased in Δ*norZ* *S. wittichii* RW1 cells compared to wildtype levels. Levels of *nirK* and *hmp* mRNA did not vary between nitrite-treated wildtype and Δ*norZ* cells while levels of *norB* mRNA were increased in the Δ*norZ* strain. Highest levels of N_2O production and total nitrite consumption were observed by 120 hours in both cell types. Approximately 18.21 and 72.80 nmol $N_2O \cdot OD^{-1}$ were produced in wildtype cells treated with 0.3 and 1mM $NaNO_2$, respectively; however, N_2O production was significantly decreased in the Δ*norZ* strain, with 16.72 and 61.95 nmol $N_2O \cdot OD^{-1}$ produced in similarly treated cells. Results therefore indicate that in the absence of *norZ*, *norB* serves

as an isofunctional *nor* in *S. wittichii* RW1, compensating for the loss of NO-reductase activity of NorZ in the presence of NOx.

Introduction

 Sphingomonas wittichii RW1 was first isolated from water extracted from the River Elbe and was shown to degrade the biaryl ethers dibenzo-*p*-dioxin and dibenzofuran and mono- and di- chlorinated congeners as well as the nitrodiphenyl ether herbicides nitrofen, chlomethoxyfen, and oxyfluorfen (Wittich et al., 1992; Wilkes et al., 1996; Keum et al., 2008). *S. wittichii* RW1 in distinct among *Sphingomonas* species in that it contains the sphingoglycolipid galacturonosyl ceramide in addition to the characteristic gluruconosyl ceramide, a feature found only in three other phenotypically distinct *Sphingomonas* species (Yabuuchi et al., 2001). Genetic and enzymatic work in *S. wittichii* RW1 has primarily focused on the degradative pathways of dibenzo-*p*-dioxin and dibenzofuran and the associated enzymes (i.e. dioxin dioxygenase) as well as their structural organizations within the genome (Bünz and Cook, 1993; Armengaud et al., 1998; Armengaud et al., 1999; Basta et al., 2004).

 Recent phylogenetic work investigating the bacterial diversity of the copper-containing nitrite reductase protein (NirK) in non-denitrifying organisms revealed a homologous *nir*K-encoding gene in the genome of *S. wittichii* RW1 (Fig. 1.3 in Chapter 1). Additional genes found in the genome of *S. wittichii* RW1 associated with NOx metabolism and denitrification include nitric oxide reductases (*nor*Z and *nor*B) and flavohemoglobin (*hmp*). The gene product of *nor*Z is a quinol- linked nitric oxide

reductase while the gene product of *nor*B is cytochrome *c*-dependent. Both *nor*B and *hmp* are plasmid-encoded genes. Nitric oxide reductases and flavohemoglobin have also been shown to confer resistance and protection from nitrosative stress (i.e. NO) in non-denitrifying organisms (Beaumont et al., 2004; Membrillo-Hernández et al., 1999; Zumft, 2005; Bonamore and Boffi, 2008).

The reductive pathway of the microbial nitrogen cycle, denitrification is a process in which microorganisms use nitrogen oxides (i.e. NO_3^-, NO_2^-) as electron acceptors for energy production in the absence of oxygen (Philippot, 2002). Denitrification entails the successive reduction of NO_3^- or NO_2^- to N_2, with microorganisms gaining energy at each step as catalyzed by reductases (i.e. Nir and Nor). Denitrification, especially under aerobic conditions, is a major source of NO and N_2O, gases which are of great importance to the destruction of atmospheric ozone and global warming (Ye et al., 1994).

It was observed in Chapter 3 that these genes in *S. wittichii* RW1 participate in aerobic denitrification, in the detoxification of nitrite and aerobic production of N_2O. Production of N_2O can be attributed to activity of one or more gene products encoded by *norZ, norB*, and *hmp*. Previous studies have demonstrated that deletion of *norB* results in increased NO sensitivity, diminished NO consumption and N_2O production, and decreased growth of denitrifying bacteria under anoxic conditions (Householder et al., 2000; Büsch et al., 2002; Beaumont et al., 2004). Interestingly, *Ralstonia eutropha*, formerly known as *Alcaligenes eutrophus* H16, possesses two independent quinol- linked nitric oxide reductases (Cramm et al., 1997). Deletion of either gene resulted in the lack of phenotypic change under aerobic and anaerobic growth in the presence of nitrate or

nitrite, suggesting either gene product could substitute for the other physiologically, and were therefore isofunctional and instrumental in denitrification (Cramm et al., 1997).

The objective of this study was to determine the roles of *nirK, norZ, norB*, and *hmp* in the aerobic production of N_2O in response to nitrite in wildtype *S. wittichii* RW1 and a *norZ-* deficient strain. A Δ*norZ S. wittichii* RW1 mutant was therefore constructed in which to compare *nirK*, *norZ*, *norB*, and *hmp* transcript levels as well as nitrite consumption and N_2O production against wildtype levels and activity. It was hypothesized that expression of *norB* and/or *hmp* in the Δ*norZ* mutant would increase in which to compensate for the lack of *norZ* function and activity, and as a result, growth and N_2O production would remain relatively the same compared to wildtype levels. Alternatively, it is possible that deletion of *norZ* in *S. wittichii* RW1 could be detrimental to the organism likely due to accumulating NO produced from NirK, resulting in decreased growth and decreased N_2O production.

Materials and Methods

Bacterial strain and culture maintenance

The bacterial strain used in this study was *Sphingomonas wittichii* RW1. Cultures were grown in 5mLs Luria-Bertani Broth (LB) media in sterilized 15mL capped-polystyrene tubes containing 1% tryptone, 0.5% yeast extract, and 1% NaCl in a rotary shaker (180 r.p.m.) at 28°C. Cultures were periodically streaked and grown on LB agar plates containing the above formulation with 15g/L agar at 28°C for 3 days and then

stored at 4°C. Single colonies were used to inoculate fresh LB media to ensure and maintain pure cultures of *S. wittichii* RW1.

Construction of Δ*norZ S. wittichii* RW1 mutant

In its entirety, the *norZ* gene is 2.271 kb in length. Due to difficulty amplifying the entire gene for mutagenesis, a 573 bp fragment spanning base pairs 203 to 776 and containing a BclI restriction site was amplified (PCR conditions as described below) using gene-specific primers designed by Primer3 Input 0.4.0 software (Table 4.1) (Rozen and Skaletsky, 2000). The gene product was purified and ligated to the pGEM®-T Vector (containing an ampicillin-resistance cassette) according to the manufacturers' instructions (Promega Corp., Madison, WI). The ligation mixture was transformed into *dam⁻/ dcm⁻* competent *E. coli* cells (New England BioLabs Inc., Ipswich, MA) as cleavage with BclI is *dam* sensitive, and transformants were selected via blue-white screen on LB agar plates containing 0.5 mM IPTG, 80 μg/mL X-Gal, and 100 μg/mL ampicillin, and verified using PCR (with PCR products sequenced). Plasmids from positive transformants were purified using Wizard® *Plus* SV Minipreps DNA Purification System kit according to the manufacturers' instructions (Promega Corp., Madison, WI), digested with the BclI restriction enzyme (New England BioLabs Inc., Ipswich MA) according to the manufacturers' instructions. The digest was run on a 0.8% agarose gel in which to isolate the linearized vector from non- or partial digested fragments. The linearized vector was then gel-purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI), and stored at -20°C until use.

A gentamycin-resistance cassette (871 bp) was digested from the pUCGM vector

using BamHI restriction enzyme (New England BioLabs Inc., Ipswich MA) according

the manufacturers' instructions. The digest was run on a 0.8% agarose gel, gel-purified,

and subsequently ligated to the previously BclI-digested pGEM-T-*norZ* vector, as

cleaved ends resulting from BclI digestion can be ligated to DNA fragments generated by

BamHI, thus disrupting the *norZ* gene product. The ligation mixture was then

transformed into competent *E. coli* JM109 cells. Transformed cells were plated onto LB

agar plates containing 100 μg/mL ampicillin and 10 μg/mL gentamycin and positive

transformants were verified by PCR (with PCR products sequenced). Plasmids from

transformants were purified as described above, again verified by PCR (with PCR

products sequenced), and electroporated into prepared *S. wittichii* RW1 cells using an *E.*

coli Pulser™ Transformation Apparatus (BioRad Laboratories, Hercules, CA) following

the manufacturers' protocol. Transformed cells were plated onto LB agar plates

containing 10 μg/mL gentamycin. The Δ*norZ* strain, containing the *norZ* gene disrupted

with the gentamycin resistance cassette via homologous recombination, was checked by

PCR using primers in Table 4.1, with PCR products purified and sequenced for further

verification, and maintained in LB media supplemented with 50 μg/mL gentamycin

(Table 4.1). To prepare *S. wittichii* RW1 cells for electroporation, cells were grown in 50

mLs LB and harvested by centrifugation during exponential phase of growth (OD ~0.7).

Cells were subsequently washed three times with 20 mLs ice-cold, sterile nuclease-free

water, then washed twice with 2 mLs ice-cold, sterile 10% glycerol, and finally

resuspended in 10% glycerol in a final volume of 100 to 250 µLs. A schematic diagram of the ΔnorZ construction is shown in Figure 4.1.

Growth experiments

Exponentially-growing wildtype and ΔnorZ *S. wittichii* RW1 cells were inoculated (0.5%) into 100 mLs LB media containing 0, 0.3, or 1 mM $NaNO_2$ in glass bottles crimp-sealed with rubber septa. Prior to inoculation of ΔnorZ *S. wittichii* RW1 cells, 50 µg/mL gentamycin was added to LB media. Upon inoculation, 2 mL samples were extracted every 4 to 6 hours using a sterile 1mL (0.8 mM x 25 mM) PrecisionGlide™ needle with syringe with which to measure growth. Growth was determined by measuring the absorbance of the optical density of the culture at 600 nm using a Spectronic 20 Genesys spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). Cells harvested for RNA extraction were immediately treated with 500 µL RNAprotect™ Bacteria Reagent, following the manufacturers' instructions (Qiagen, Valencia, CA) to prevent degradation of RNA and kept at -80°C prior to extraction. Experiments consisted of five independent trials at each nitrite concentration for both wildtype and ΔnorZ strains.

Nucleic acid extraction

Genomic DNA was isolated using the Wizard® SV Genomic DNA Purification System kit, following the manufacturers' instructions (Promega Corp., Madison, WI). Total RNA was extracted using the Aurum™ Total RNA Mini kit, following the manufacturers' instructions (Bio-Rad Laboratories, Hercules, CA). Nucleic acid concentration was determined using a NanoDrop® ND-1000 Spectrophotometer

(Nanodrop Technologies, Inc., Wilmington, DE). DNA and RNA samples were kept at -20°C and -80°C, respectively, prior to probe construction and dot blot hybridization.

Dot-blot hybridization

DNA sequences of each gene of interest were found using the National Center for Biotechnology Information (NCBI) and Joint Genome Institute (JGI) databases. Gene-specific primers were designed using the software Primer3 Input 0.4.0 software (Rozen and Skaletsky, 2000), as previously mentioned (Table 4.1). PCR reactions included standard reagents for Taq polymerase and genomic DNA as template. Using a thermocycler (iCycler, BioRad, Hercules, CA), amplification conditions were: 95°C for 5 minutes, 30 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 50 seconds, with an additional extension cycle of 72°C for 7 minutes. PCR products were checked by agarose gel (1%) to verify single products of appropriate size. Amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI) according to the manufacturers' instructions. Amplification products were labeled using the Prime-a-Gene labeling system (Promega Corp., Madison, WI) with [α-^{32}P]-dCTP (3000 Ci mmol^{-1}; Perkin-Elmer Inc., Waltham, MA) and random hexamers. The dynamic range of detection for each probe was tested using a concentration series of mRNA from 0.1 to 3 μg from the control incubations (0mM NaNO$_2$). The r^2 values for the slope of hybridization intensity/μg RNA was between 0.94 to 1 for all probes.

Two μg total RNA from each sample was blotted onto a Zeta-Probe® GT nylon membrane (Bio-Rad Laboratories, Hercules, CA) using a Minifold® microsample filtration manifold (Dot-Blot System, Schleicher & Schuell, Keene, NH) following the

Zeta-Probe® protocol. Membranes were allowed to dry overnight and UV-crosslinked (FB-UVXL-1000, Fisher Scientific, Pittsburgh, PA). Prehybridization, hybridization, and washing of Zeta-Probe® nylon membranes were done according to manufacturer's instructions at 30°C. To allow re-probing, membranes were stripped of radioactivity by washing twice in a 0.1X SSC/0.5% SDS solution at 95-100°C for 20 minutes. All blots were hybridized to gene-specific probes to normalize hybridization signals to the 16S rRNA pool. Hybridization intensity was analyzed using a Typhoon Phosphorimager and Imagequant software (Amersham, Piscataway, New Jersey).

Data Analysis

Background and signal from non-specific binding was subtracted, after which the relative hybridization intensity of specific probes was normalized by dividing by the signal from gene-specific to 16S rRNA probe hybridizations. The fold difference in levels of mRNA for each gene and time point from NO_2^- exposure was determined by dividing hybridization intensities from dot blots of RNA extracted from NO_2^- -treated to those from untreated cultures. Student's t-test ($P<0.05$) was performed to verify significant differences between treatments.

Physiological measurements

Nitrite consumption was measured using a colorimetric assay in which 1mL of a sulfanilamide-HCl solution and 1mL of a NNEQ-HCl (N-(1-Naphthyl) ethylenediamine dihydrochloride) solution was mixed with 20 μL of supernatant from harvested samples. The mixture was allowed to incubate at room temperature for at least 30 minutes and the absorbance was read at 540nm using a Spectronic 20 Genesys spectrophotometer

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(Thermo Fisher Scientific, Inc., Waltham, MA). Nitrate production was measured using a Standard Range Lab Nitrate Test kit (NECi, Lake Linden, Michigan) according to the manufacturers' instructions. However, nitrate production was not observed. Ammonia was measured colorimetrically using the phenate method (Clesceri et al., 1995). Oxygen and N_2O were measured daily from the headspace of inoculated bottles using a gas chromatograph equipped with a thermal conductivity detector and Molesieve 5A (80/100) and HAYESEP Q (80/100) columns, respectively (GC-8AIT, Shimadzu, Columbia, MD).

Results

Effect of nitrite on the growth of wildtype and Δ*norZ S. wittichii* RW1

Over a 72 hour period, growth was nearly comparable between wildtype and Δ*norZ S. wittichii* RW1 cells (Figure 4.2). Interestingly, doubling times were faster in the Δ*norZ* strain, with averages of 5.16 (±0.43), 5.22 (±0.44), and 5.21 (±0.38) hours in cells amended with 0, 0.3, and 1 mM $NaNO_2$ compared to 6.56 (±1.12), 7.02 (±1.32), and 6.82 (±1.13) hours in similarly treated wildtype cells. Increasing concentrations of nitrite inhibited growth of both wildtype and Δ*norZ* cells. In wildtype cells, a significant decrease ($p < 0.05$) in growth was observed in 0.3 mM $NaNO_2$- treated cells compared to untreated cells from 32 hours to 72 hours (from late exponential to stationary phase of growth). The decrease was much more pronounced in 1 mM $NaNO_2$- treated cells, with growth inhibited as early as 16 hours until 72 hours (early exponential to stationary phase of growth). Additionally, wildtype cells amended with 1 mM $NaNO_2$ experienced

impaired growth by 24 hours, lasting until the end of the 72 hour sampling period, compared to those treated with 0.3 mM $NaNO_2$.

A similar trend was observed in $\Delta norZ$ cells between untreated and nitrite-treated groups; however, significant differences in growth weren't seen until after 38 and 32 hours (until 72 hours) in 0.3 mM and 1 mM $NaNO_2$ treatment groups, respectively, compared to the 0mM $NaNO_2$ treatment group. Cell growth of the $\Delta norZ$ strain was similar between the nitrite-treated groups; however, from 38 to 72 hours growth was significantly decreased ($p < 0.05$) in the 1 mM $NaNO_2$ treatment group compared to the 0.3 mM $NaNO_2$ treatment group. While wildtype and $\Delta norZ$ S. wittichi RW1 cells experienced similar growth trends overall, a significant decrease ($p < 0.05$) was observed in untreated and nitrite- treated $\Delta norZ$ cells at each time point from 12 hours (late lag phase) to 32 hours (mid-exponential phase) when compared to their wildtype counterparts. Thus, lack of norZ and its activity may have been linked to the growth impairment observed during that critical phase of growth. Despite a slightly more rapid doubling time in $\Delta norZ$ cells, it is possible cell size decreased and resulted in a lower optical density reading; however, after 38 hours, cell density of $\Delta norZ$ cells were comparable to those observed in wildtype cells.

Nitrite consumption and aerobic N_2O production in wildtype and $\Delta norZ$ S. wittichii RW1

Wildtype and $\Delta norZ$ S. wittichii RW1 cells were able to consume 0.3 and 1 mM $NaNO_2$ after 48 hours, with complete consumption occurring by 120 hours (Table 4.2). A subtle, but statistically significant difference ($p < 0.05$) was observed in $NaNO_2$

consumption between wildtype and $\Delta norZ$ cells after 48 hours in both 0.3 mM $NaNO_2$-treated groups, and after 72 hours in both 1mM $NaNO_2$- treated groups.

N_2O production was observed in both wildtype and $\Delta norZ$ cells within 48 hours with peak production occurring by 120 hours (Table 4.2). Production of N_2O was significantly increased ($p < 0.05$) in the 1mM $NaNO_2$- treated group compared to the 0.3mM $NaNO_2$- treated group in both cell types beginning by 72 hours until the end of the 144 hour sampling period. The $\Delta norZ$ strain produced slightly less N_2O, though statistically significant ($p <0.05$), than wildtype cells throughout the entire sampling period. The N_2O-N pool accounted for 38 and 42% of reduced NO_2^--N in wildtype cells treated with 0.3 and 1 mM $NaNO_2$ (after 120 hours), respectively, and 34 and 36% in similarly treated $\Delta norZ$ strains. Nitrate production was not observed in this study, ammonia concentrations did not vary between treatment groups, and N_2 was not measured.

Oxygen consumption indicated aerobic respiration (Table 4.2). No discernible pattern regarding O_2 consumption was detected though minor but statistically significant differences ($p < 0.05$) occurred between treatment groups within either cell type, as well as between wildtype and $\Delta norZ$ cells. The slight increase in O_2 measurements at 144 hours is likely attributed to O_2 introduced during sampling. Consumption of $NaNO_2$ and O_2 and production of N_2O were not observed in heat-killed controls.

Effect of nitrite on mRNA levels of wildtype and $\Delta norZ$ S. wittichii RW1

Prior to investigating the effects of nitrite on widtype and $\Delta norZ$ S. wittichii RW1 cells, basal transcription levels of the genes of interest were determined relative to the

transcription level of the 16S rRNA gene during the early exponential and stationary phases of growth, from 24 to 66 hours (Figure 4.3). For all genes, mRNA levels $\geq$ 2-fold higher than16S rRNA levels were considered significant. Levels of 16S rRNA in wildtype and $\Delta norZ$ strains were consistent throughout the sampling period (data not shown). Similar to the patterns in mRNA levels observed in the previous study (Chapter 3), basal transcription of *nirK* and *norZ* were highest among the four genes. Overall, mRNA levels increased over time in wildtype cells, but to a lesser extent in $\Delta norZ$ cells, suggesting activity of the gene products occur during stationary phase when oxygen levels have diminished. A significant increase in basal mRNA levels ($p < 0.05$) was observed in *nirK*, *hmp*, and *norB* in the $\Delta norZ$ strain across all time points compared to levels seen in wildtype cells. Thus, disruption of *norZ* and subsequent lack of functional activity affected transcription of not only *hmp* and *norB*, but also *nirK* as well, such that an increase in transcription occurred.

Levels of *nirK* mRNA were higher in the 1 mM $NaNO_2$- treatment groups of both wildtype and $\Delta norZ$ *S. wittichii* RW1 cells compared to cells treated with 0.3 mM $NaNO_2$ (Figure 4.4). This was expected since higher nitrite exposure would presumably induce a higher response in *nirK* transcription. The increase in mRNA levels further corroborates nitrite consumption data resulting from NirK activity, as observed in Table 4.2. Wildtype and $\Delta norZ$ strains exhibited similar trends in *nirK* transcription which is as expected as nitrite consumption and nitrous oxide production occurred for both cell types.

With *norZ* disrupted with a gentamycin cassette, mRNA levels of the $\Delta norZ$ strain were expectedly indiscernible regardless of nitrite amendments (Figure 4.5). Considering

that basal mRNA levels were high among the four genes relative to 16S rRNA levels (Figure 4.3) and the lack of substantial increase overall in wildtype strains in the presence of nitrite, it is likely existing *norZ* gene products were sufficient in protecting cells from NO produced from the reduction of nitrite by NirK. Subsequently, activity of NorZ would therefore contribute to the production of N_2O in wildtype cells as previously detected (Table 4.2).

With *S. wittichii* RW1 lacking a functional *norZ*, it was presumed either *hmp* or *norB* (or both) may be upregulated in the Δ*norZ* strain in the presence of nitrite. In this study, there was no apparent difference in *hmp* expression between nitrite- treated groups in wildtype cells (Figure 4.6). Similar to *nirK*, levels of *hmp* mRNA were similar in treated wildtype and Δ*norZ* strains. As previously mentioned, Hmp can also oxidize NO to NO_3^-; however, nitrate production was not observed. Thus, while *hmp* was not induced to a large extent overall in Δ*norZ*- deficient cells compared to wildtype cells, activity of Hmp likely resulted in the production of N_2O, thereby adding to the overall N_2O pool detected in this study, as previously observed in Chapter 3.

Though patterns of *hmp* transcription may have been subtle between wildtype and Δ*norZ* cells, transcription of *norB* yielded the most interesting findings. Levels of *norB* mRNA between treatment groups of wildtype cells yielded no apparent differences (Figure 4.7). Subtle differences were observed in *norB* mRNA levels between treated cells of the Δ*norZ* strain, with the exception of levels at 42 and 48 hours; however, levels of *norB* mRNA in the Δ*norZ* strain were statistically significant ($p < 0.05$) compared to levels in wildtype cells at all time points. Thus, such induction likely served as a

compensatory mechanism in the absence of a functional *nor*Z, contributing to N_2O production, albeit at a lower amount compared to wildtype cells (Table 4.2).

Discussion

Aerobic respiration occurred as indicated by O_2 consumption in Table 4.2; however, nitrite adversely affected respiration as remaining O_2 levels were higher overall but not significant in treated groups, both in wildtype and ΔnorZ strains. *Sphingomonas* species are strictly aerobic organisms; however, depending on growth substrate, they can grow under low O_2 conditions. *S. aromaticivorans* F199 can grow microaerobically in the presence of 40 to 80 μM O_2 (equivalent to 3.5 -7% O_2) (Fredrickson et al., 1991). While *S. aromaticivorans* F199 required fully aerobic conditions for growth in rich media (i.e. PTYG or tryptic soy agar) regardless of carbon source, suboxic conditions are necessary for its growth in mineral media amended with hydrocarbons (i.e. toluene and naphthalene), glucose, acetate, or lactate (Fredrickson et al., 1991). While it is possible that under the proper conditions, *Sphingomonas* species may be capable of anoxic growth, *S. wittichii* RW1 grew modestly under initial 3% O_2 conditions as observed in the previous study (Chapter 3).

Basal mRNA levels of *nor*Z show it was the second most highly expressed gene after *nirK*, which was also observed in the previous study (Chapter 3). This is not uncommon as the gene encoding the quinol- linked nitric oxide reductase in the nondentrifying cyanobacterium *Synechocystis* sp. strain PCC6803 was likewise expressed in the absence of NO (Büsch et al., 2002). This is thought to enable the cells to respond

immediately to the toxic compound if encountered in the environment (Büsch et al., 2002). Similarly, the constitutive, basal levels of genes observed here while substantially increased relative to 16S rRNA levels, especially in the $\Delta norZ$ strain, may serve as an immediate defense response in the event *S. wittichii* RW1 cells suddenly experience nitrosative stress. Additionally, these basal levels could therefore serve to compensate for the basal activity of *norZ*, which suggests that these genes may play a role in the general physiology of the organism in addition to their collective roles in NOx metabolism.

As nitrite inhibited growth of *S. wittichii* RW1, even under low O_2 conditions (Chapter 3), it was determined that the organism cannot use denitrification as an alternative form of respiration. However, nitrite consumption was observed in this study suggesting activity of NirK. NO-reducing abilities were maintained in the $\Delta norZ$ strain as evident by the production of N_2O, thus, such production is likely attributable to activity of the second NO reductase (NorB) since *nor*B mRNA levels of treated $\Delta norZ$- deficient cells showed statistically significant upregulation.

As anticipated, disruption of the quinol-linked *norZ* in *S. wittichii* RW1 with a gentamycin resistance gene cassette resulted in lack of *norZ* transcription. Intensity signals observed are likely attributed to residual background hybridization signals from multiple probing and stripping of the dot blots. Lack of *norZ*, and therefore a functional gene product, resulted in a decrease in N_2O production, but did not inhibit growth as the $\Delta norZ$ strain exhibited faster growth compared to wildtype cells. This is in contrast to other organisms such as *Synechocystis* sp. strain PCC6803 and *Bradyrhizobium japonicum* in which lack of the NO reductase resulted in growth inhibition and the

inability to carry out denitrification and NO reduction (Büsch et al., 2002; Mesa et al., 2002). However, this is likely attributable to the fact that *S. wittichii* RW1 possesses two NO reductases and Hmp, which is physiologically advantageous for the organism as additional enzymes can serve as an enzymatic backup system for NO reduction, similar to *R. eutropha* (Cramm et al., 1997). While the proportion of the N_2O-N pool in *S. wittichii* RW1 does not fully account for the total reduction of nitrite, possible explanations are as previously mentioned in Chapter 3.

While the physiological function of flavohemoglobins remains unclear, there have been numerous findings in bacteria, fungi, and plants linking the function of Hmp to oxygen sensing, scavenging, or delivery, as well as protection from reactive oxygen species; ferrisiderophore reduction, heme sequestration; and cell survival due to anaerobic reduction and aerobic oxidation of NO to N_2O and NO_3^-, respectively (Gardner et al., 1998 and references therein; Kim et al., 1999 and references therein; Membrillo-Hernandez et al., 1999; Gardner and Gardner, 2002; Nakano, 2006). In addition to possessing two nitric oxide reductases, *R. eutropha* also contains flavohemoglobin which has been physiologically linked to N_2O production during nitrite respiration (Cramm et al., 1994). However, in this study, the modest response in *hmp* mRNA levels suggests that the lack of *norZ* function and activity in the Δ*norZ* strain did not put increased selective pressure on the cells to induce *hmp* in the presence of nitrite. Additionally, nitrate production was not observed implying Hmp did not serve as an NO dioxygenase in *S. wittichii* RW1.

The two nitric oxide reductases (NorZ and NorB) found in the genetic inventory of *R. eutropha* likely occurred from a gene duplication event, and represents the first prokaryotic example of isofunctional NO reductases (Cramm et al., 1997). Deletion studies revealed there was no phenotypically detectable difference between either *nor* mutant and wildtype cells under aerobic or anaerobic growth, leading the authors to conclude that one isofunctional Nor could compensate for the other physiologically, and produce N_2O. Wildtype *R. eutropha* H16 consumed 430 nmol NO min^{-1} mg protein^{-1} while the ΔnorB mutant consumed less, 348 nmol NO min^{-1} mg protein^{-1} (Cramm et al., 1997). On the other hand, disruption of *nor*Z revealed a substantial decrease in NO consumption, with ΔnorZ *R. eutropha* H16 cells consuming 195 nmol NO min^{-1} mg protein^{-1}. This led to the conclusion that the chromosomally-encoded enzyme is either slightly more active or expressed to a greater extent than the plasmid-encoded Nor (Cramm et al., 1997). However, deletion of both resulted in lethality due to accumulation and toxicity of NO produced from nitrite reductase (NirS), which was further confirmed by the construction of a triple mutant (ΔnorB/*nor*Z/*nir*S). Both *nor* gene products were therefore directly linked to the reduction of NO produced from NirS, as opposed to abiotic chemical processes that may occur during denitrification (Cramm et al., 1997). Thus, it can similarly be assumed that expression and function of the chromosomally-encoded *nor*Z in *S. wittichii* RW1 is greater than that of the plasmid- encoded *nor*B**,** and is therefore the dominate *nor* gene and largely responsible for N_2O. However, further mutational studies in *S. wittichii* RW1 are required for accurate determination.

Because preferential expression of the chromosomally-encoded *nor* gene likely occurs, it is possible that the *nor* genes are differentially regulated. Interestingly, however, both *nor* genes are regulated by the NorR regulator in *R. eutropha*, along with sigma factor RpoN in response to NO (Pohlmann et al., 2000). In fact, NorR is also present in two isofunctional copies- both in the chromosome and plasmid, wherein one copy was sufficient to allow expression of the *nor* genes (Pohlmann et al., 2000). In *S. wittichii* RW1, a regulator has not been identified for *nor*Z, thus the regulation of *nor*B as well as other genes involved in NOx metabolism in *S. wittichii* RW1 merit further investigation.

Results in this study show that *nor*Z- deficient cells retain aerobic NO consumption and N_2O- producing capabilities, though N_2O production was at slightly decreased levels compared to wildtype cells. Also, complete nitrite consumption was observed in both cell types and O_2 consumption was detected, implying aerobic respiration and aerobic reduction of NO_2^- and NO. While mRNA levels of *hmp* were modestly increased in Δ*nor*Z strains treated with nitrite, the increase in *nor*B mRNA levels in similarly NO_2^--treated cells suggests this gene and its product likely serve as a compensatory mechanism in the absence of a functional *nor*Z. Increased transcription of *nor*B likely resulted in the subsequent increase of the activity of the corresponding gene product, thus responsible for the observed production of N_2O. The function and activity of *nor*B can therefore serve as a physiological substitute for *nor*Z in *S. wittichii* RW1 in the presence of NOx.

References

Armengaud J, Happe B, Timmis KN. (1998). Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: Catabolic genes dispersed on the genome. *Journal of Bacteriology*, **180(15)**, 3954-3966.

Armengaud J, Timmis KN, Wittich R-M. (1999). A functional 4-hydroxysalicylate/ hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *Journal of Bacteriology*, **181(11)**, 3452-3461.

Basta T, Keck A, Klein J, Stolz A. (2004). Detection and characterization of conjugative degradative plasmids in xenobiotic-degrading *Sphingomonas* strains. *Journal of Bacteriology*, **186(12)**, 3862-3872.

Beaumont HJE, van Schooten B, Lens SI, Westerhoff HV, van Spanning RJM. (2004). *Nitrosomonas europaea* expresses a nitric oxide reductase during nitrification. *Journal of Bacteriology*, **186(13)**, 4417-4421.

Bonamore A and Boffi A. (2008). Flavohemoglobin: Structure and reactivity. *IUBMB Life*, **60(1)**, 19-28.

Bünz PV and Cook AM. (1993). Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: Angular dioxygenation by a three-component enzyme system. *Journal of Bacteriology*, **175(20)**, 6467-6475.

Büsch A, Friedrich B, Cramm R. (2002). Characterization of the *norB* gene, encoding the nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. strain PCC6803. *Applied and Environmental Microbiology*, **68(2)**, 668-672.

Clesceri LS, Greenberg AE, Eaton AD. (1998). Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington, D.C.

Cramm R, Pohlmann A, Friedrich B. (1999). Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16. *FEBS Letters*, **460**, 6-10.

Cramm R, Siddiqui RA, Friedrich B. (1994). Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. *The Journal of Biological Chemistry*, **269(10)**, 7349-7354.

Cramm R, Siddiqui RA, Friedrich B. (1997). Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. *Journal of Bacteriology*, **179(21)**, 6769-6777.

Gardner AM and Gardner PR. (2002). Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*. *The Journal of Biological Chemistry*, **277(10)**, 8166-8171.

Gardner PR, Gardner AM, Martin LA, Salzman AL. (1998). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proceedings of the National Academy of Sciences*, **95**, 10378-10383.

Hendricks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S, Saraste M. (2000). Nitric oxide reductases in bacteria. *Biochimica et Biophysica Acta*, **1459**, 266-273.

Keum YS, Lee YJ, Kim J-H. (2008). Metabolism of nitrodiphenyl ether herbicides by dioxin-degrading bacterium *Sphingomonas wittichii* RW1. *Journal of Agricultural and Food Chemistry*, **56**, 9146-9151.

Kim SO, Orii Y, Lloyd D, Hughes MN, Poole RK. (1999). Anoxic function for the *Escherichia coli* flavohemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. *FEBS Letters*, **445**, 389-394.

Membrillo-Hernández J, Coopamah MD, Anjum MF, Stevanin TM, Kelly A, Hughes MN, Poole RK. (1999). The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. *The Journal of Biological Chemistry*, **274(2)**, 748-754.

Mesa S, Velasco L, Manzanera ME, Delgado MJ, Bedmar EJ. (2002). Characterization of the *norBCQD* genes, encoding nitric oxide reductase, in the nitrogen fixing bacterium *Bradyrhizobium japonicum*. *Microbiology*, **148**, 3553-3560.

Philippot L. (2002). Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysica Acta*, **1577**, 355-376.

Pohlmann A, Cramm R, Schmelz K, Friedrich B. (2000). A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Molecular Biology*, **38(3)**, 626-638.

Wilkes H, Wittich R-M, Timmis KN, Fortnagel P, Francke W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. Strain RW1. *Applied and Environmental Microbiology*, **62(2)**, 367-371.

Wittich R-M, Wilkes H, Sinnwell V, Francke W, Fortnagel P. (1992). Metabolism of dibenzo-p-dioxin by Sphingomonas sp. Strain RW1. *Applied and Environmental Microbiology*, **58(3)**, 1005-1010.

Yabuuchi E, Yamamoto H, Terakubo S, Okamura N, Naka T, Fujiwara N, Kobayashi K, Kosako Y, Hiraishi A. (2001). Proposal of *Sphingomonas wittichii* sp. nov. for strain RW1, known as a dibenzo-*p*-dioxin metabolizer. *International Journal for Systematic and Evolutionary Microbiology*, **51**, 281-292.

Ye RW, Averill BA, Tiedje JM. (1994). Denitrification: Production and consumption of nitric oxide. *Applied and Environmental Microbiology*, **60(4)**, 1053-1058.

Zumft WG. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *Journal of Inorganic Biochemistry*, **99**, 194-215.
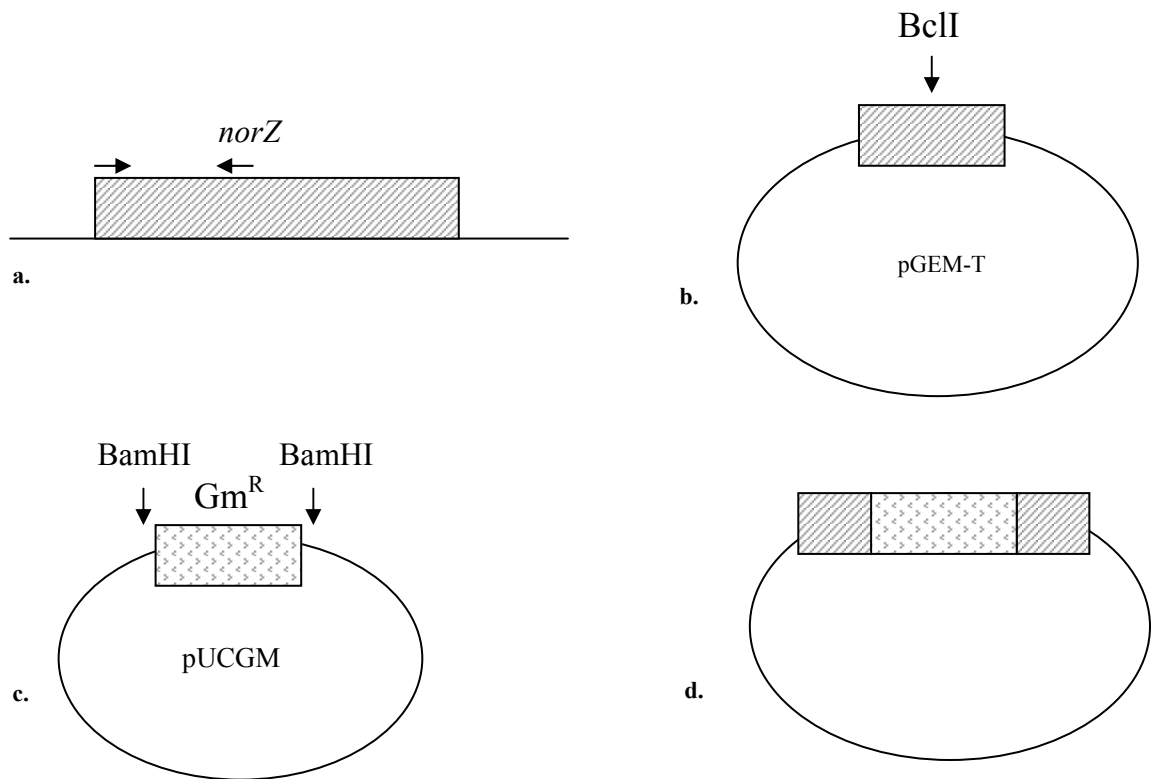
Figure 4.1. Schematic diagram of the construction of the Δ*norZ* recombinant plasmid. A 573 bp gene product of *norZ* was amplified (a), ligated into the pGEM-T vector and digested with BclI restriction enzyme (b). A gentamycin resistance cassette was cut from the pUCGM vector by digestion with BamHI restriction enzyme (c). The gentamycin resistance cassette was then ligated to the digested pGEM-T- *norZ* vector, thus disrupting the *norZ* gene product (d), and subsequently transformed into *S. wittichi* RW1 cells.
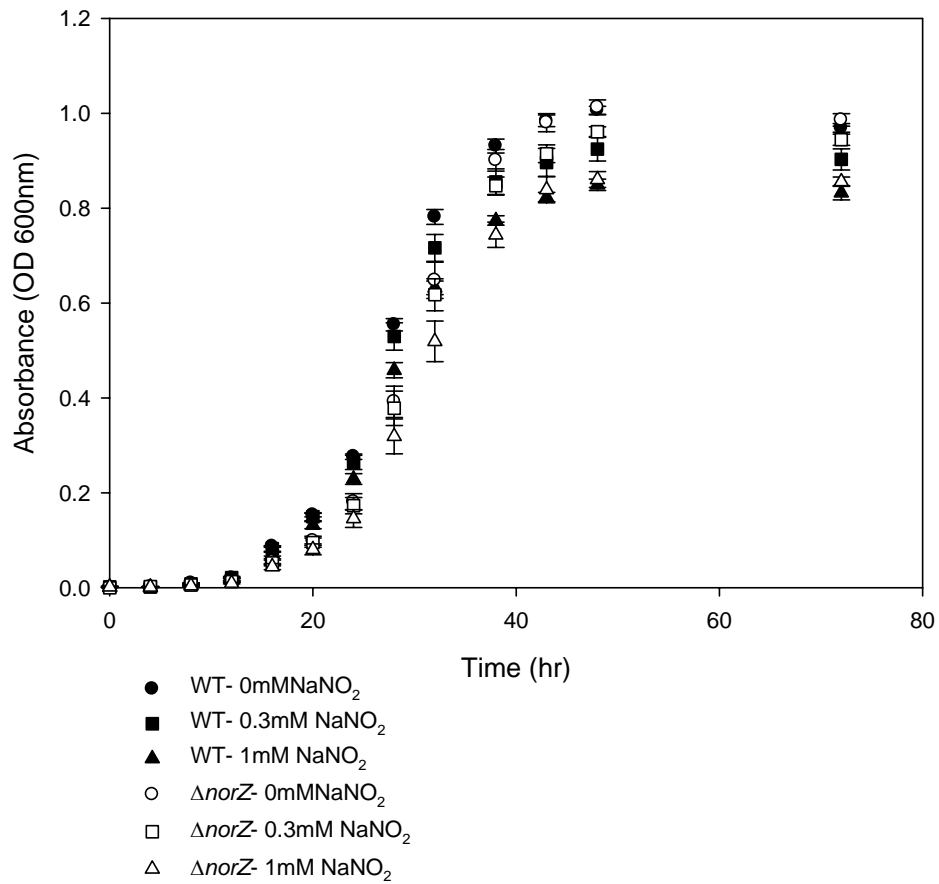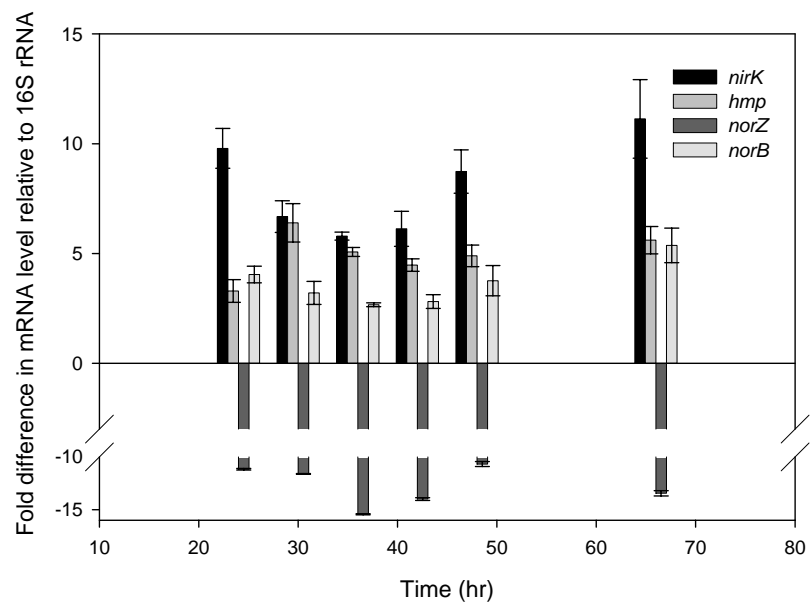
Figure 4.2. Growth curves of wildtype (WT) and Δ*norZ S. wittichii* RW1 grown in LB media under initial 20% O_2 conditions in the presence of 0, 0.3, and 1 mM $NaNO_2$. Error bars represent the standard error of 5 independent trials.
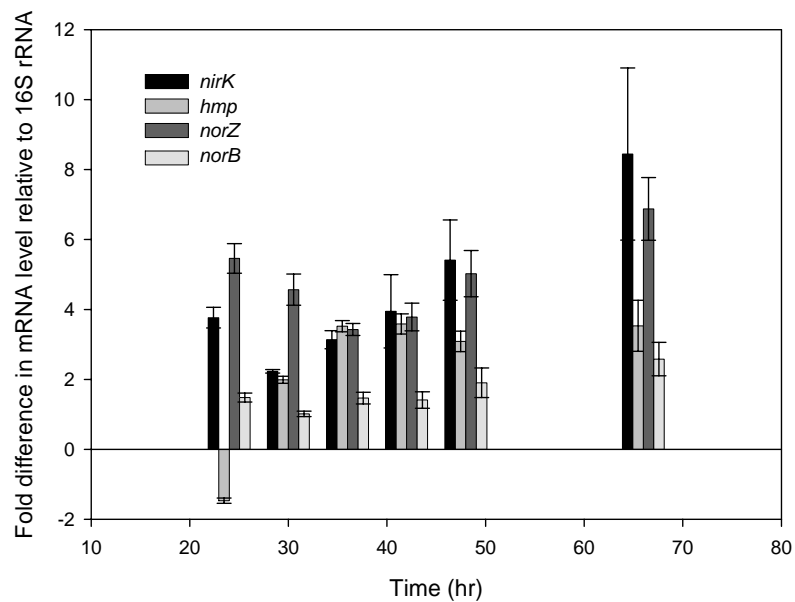
A. Wildtype *S. wittichii* RW1

B. Δ*norZ S. wittichii* RW1

Figure 4.3. Fold difference in mRNA levels of genes involved in NOx metabolism relative to 16S rRNA levels in wildtype and Δ*norZ S. wittichii* RW1 under initial 20% O_2 conditions. Levels of mRNA were determined by dot blot hybridization. Error bars represent the standard error of 5 independent experiments.
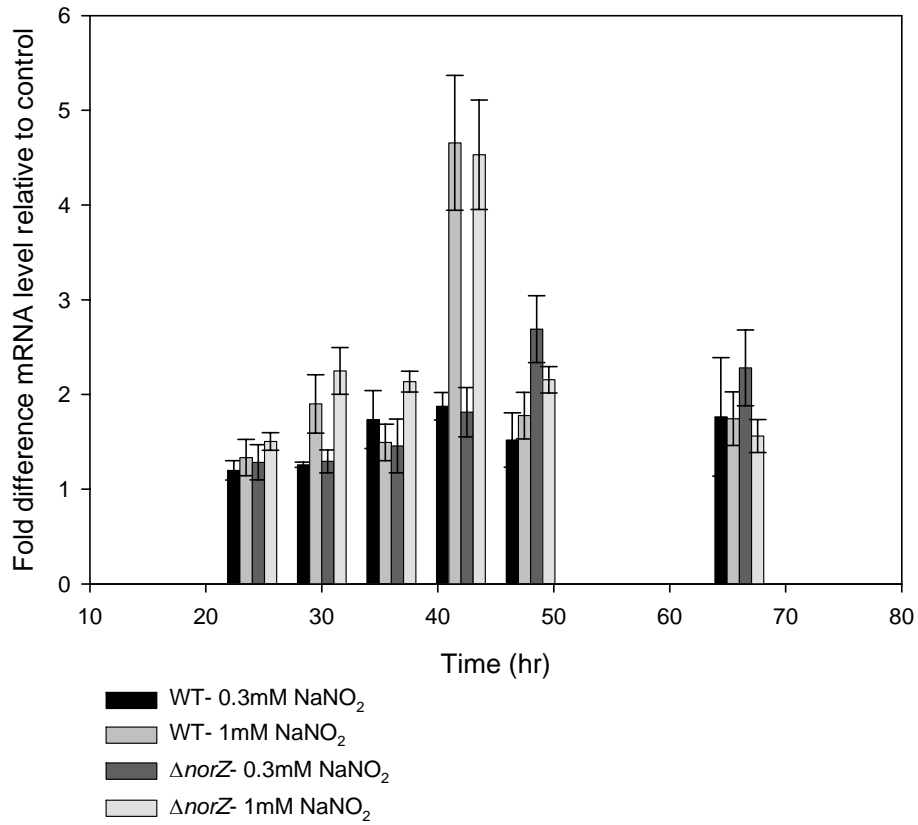
Figure 4.4. Fold difference in mRNA levels of *nirK* relative to the control (untreated, 0 mM NaNO$_2$) group in wildtype (WT) and Δ*norZ S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.
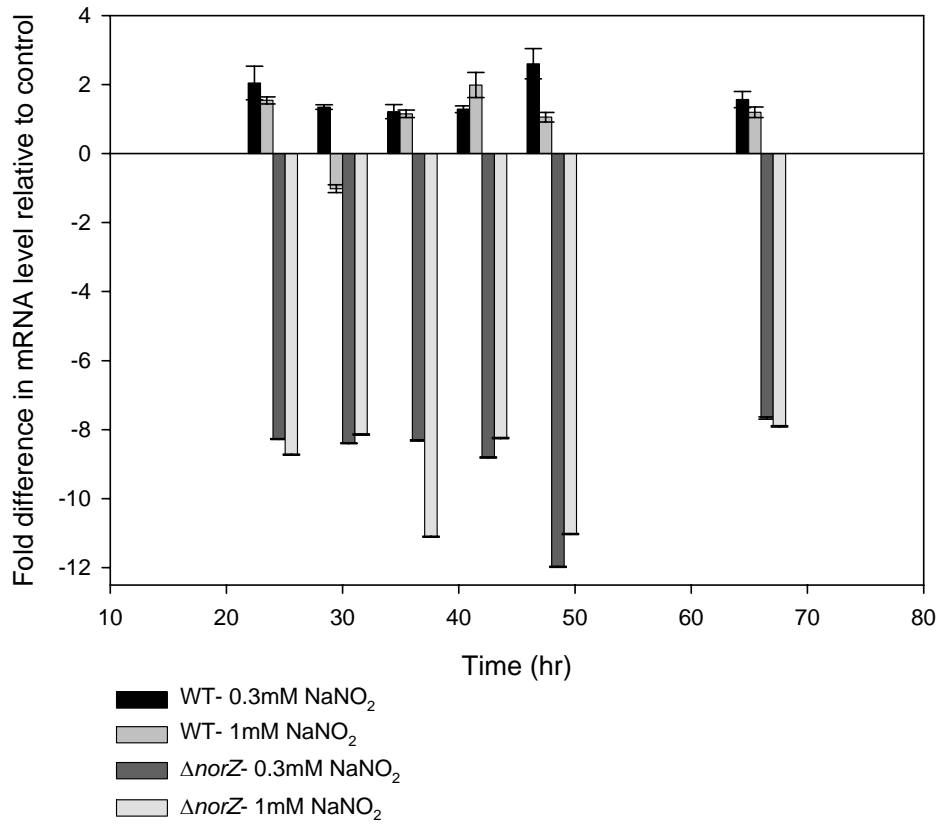
Figure 4.5. Fold difference in mRNA levels of *norZ* relative to the control (untreated, 0 mM NaNO$_2$) group in wildtype (WT) and Δ*norZ S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.
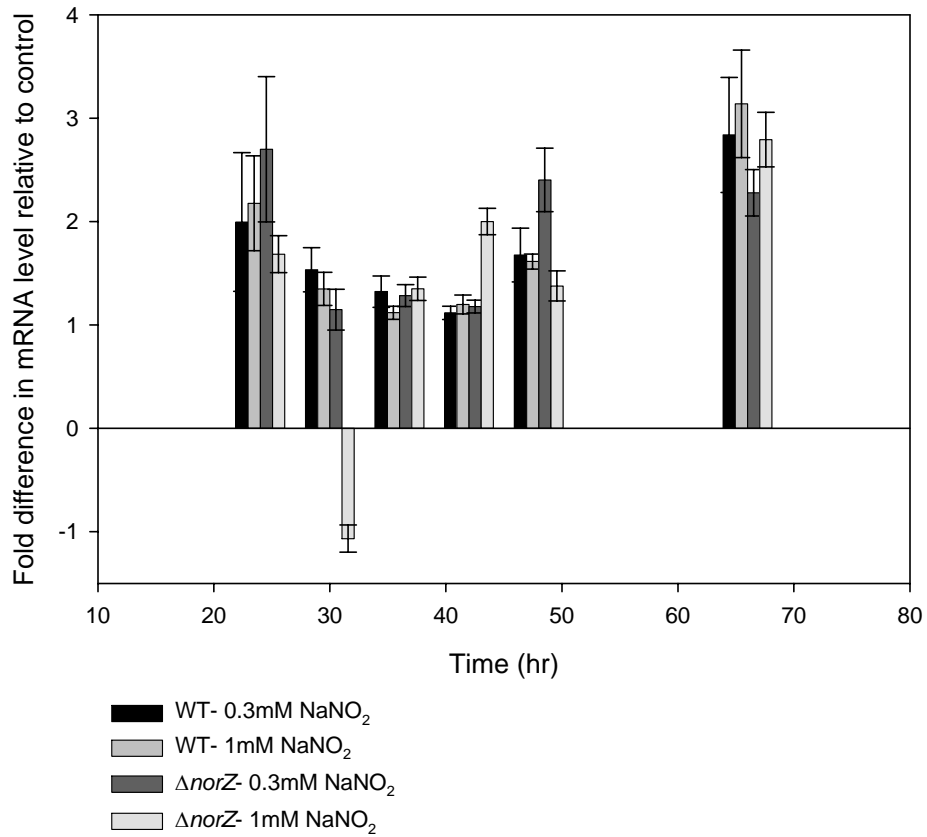
Figure 4.6. Fold difference in mRNA levels of *hmp* relative to the control (untreated, 0 mM NaNO$_2$) group in wildtype (WT) and Δ*norZ S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.
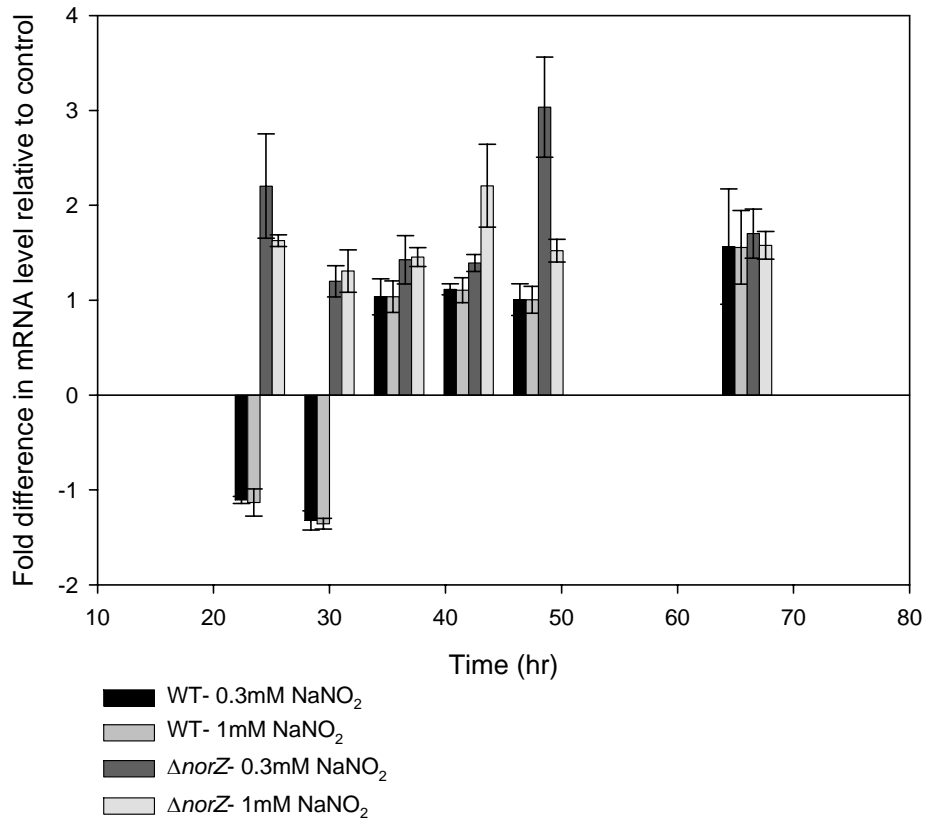
Figure 4.7. Fold difference in mRNA levels of *norB* relative to the control (untreated, 0 mM NaNO$_2$) group in wildtype (WT) and Δ*norZ S. wittichii* RW1 treated with 0.3 and 1 mM NaNO$_2$. Error bars represent the standard error of 5 independent experiments.

Table 4.1. Primers used in this study were designed using Primer3 Input 0.4.0 software.

Locus Tag/Name	Gene	F primer	R primer	PCR product
Swit_1793	*nirK*	ctgaccgcgaaggaagtatc	catggtcgacgatcacattg	742 bp
Swit_5203	*hmp*	ccaacgccaatactcaacct	cagcatttctacggcatcaa	210 bp
Swit_R0031	*16S*	gtacaaggcctgggaacgta	tttatcgcctgaggatgagc	1159 bp
Swit_4614	*norZ*	gtggtgcccgagaaatagag	gccagagcttctacggtgtc	703 bp
Swit_5200	*norB*	tcgagcttgtccacattctg	attgtctccccaaaccatga	513 bp
pUCGM173F		tgcctcgggcatccaagcagca		
pUCGM514R			gagagcgccaacaaccgcttct	
pUCGM519F		cttacgttctgcccaggttt		
Swit_4614_203F	*norZ*	aactggaacaggccgatg		
Swit_4614_854R	*norZ*		tcaccgtcatggaatattgg	
Swit_4614_45F	*norZ*	agagacccaggaccacgac		
Swit_4614_776R	*norZ*		cgatcgccttcatcttcg	
Swit_4614_1048R	*norZ*			

Table 4.2. Nitrite and O_2 consumption and N_2O production in wildtype and $\Delta norZ$ *S. wittichii* RW1. The standard error of 5 independent experiments are indicated in parentheses. $NaNO_2$ consumption and nmol N_2O production $\cdot$ OD^{-1}: [a] signifies statistical significance (p < 0.05) between 0.3 and 1mM $NaNO_2$ treatment groups of wildtype (WT) or $\Delta norZ$ cells at each time point; [b] signifies statistical significance (p < 0.05) between wildtype and $\Delta norZ$ *S. wittichii* RW1 cells treated with 0.3mM $NaNO_2$ or 1mM $NaNO_2$. For O_2 consumption data, [a] and [b] signify statistical significance (p < 0.05) as indicated above; [c] signifies statistical significance (p < 0.05) between 0 and 0.3mM $NaNO_2$ treatment groups of wildtype or $\Delta norZ$ cells at each time point; [d] signifies statistical significance (p < 0.05) between 0 and 1mM $NaNO_2$ treatment groups of wildtype or $\Delta norZ$ cells at each time point.

NaNO$_2$ amendment to LB media and measured activities	Time (hr)					
	48		72		96	
	WT	$\Delta norZ$	WT	$\Delta norZ$	WT	$\Delta norZ$
mM NaNO$_2$ Remaining						
0 mM	0	0	0	0	0	0
0.3 mM	0.24 (0.005)[ab]	0.27 (0.006)[ab]	0.01 (0.008)[a]	0.04 (0.015)[a]	0	0
1.0 mM	0.78 (0.013)[a]	0.79 (0.01)[a]	0.24 (0.024)[ab]	0.33 (0.025)[ab]	0.01 (0.005)	0.02 (0.014)
nmol N$_2$O Produced $\cdot$ OD^{-1}						
0 mM	0	0	0	0	0	0
0.3 mM	2.66 (0.20)[ab]	1.76 (0.18)[ab]	10.30 (0.34)[ab]	8.80 (0.18)[ab]	17.69 (0.31)[ab]	14.79 (0.42)[ab]
1.0 mM	3.19 (0.08)[ab]	2.40 (0.21)[ab]	34.68 (1.32)[ab]	24.99 (1.91)[ab]	63.62 (2.70)[ab]	49.31 (4.22)[ab]
% O$_2$ Remaining						
0 mM	7.37 (0.19)	7.70 (0.14)	4.39 (0.11)[d]	4.57 (0.08)	4.07 (0.07)	4.14 (0.12)[c]
0.3 mM	7.24 (0.08)[ab]	7.82 (0.08)[b]	4.50 (0.09)[b]	4.71 (0.06)[b]	4.19 (0.08)[b]	4.42 (0.02)[cb]
1.0 mM	7.62 (0.17)[a]	7.55 (0.43)	4.65 (0.07)[d]	4.63 (0.16)	4.12 (0.20)	4.45 (0.24)

Table 4.2. (continued) Nitrite and O_2 consumption and N_2O production in wildtype and $\Delta norZ$ *S. wittichii* RW1. The standard error of 5 independent experiments are indicated in parentheses. $NaNO_2$ consumption and nmol N_2O production $\cdot$ OD^{-1}: [a] signifies statistical significance ($p < 0.05$) between 0.3 and 1mM $NaNO_2$ treatment groups of wildtype (WT) or $\Delta norZ$ cells at each time point; [b] signifies statistical significance ($p < 0.05$) between wildtype and $\Delta norZ$ *S. wittichii* RW1 cells treated with 0.3mM $NaNO_2$ or 1mM $NaNO_2$. For O_2 consumption data, [a] and [b] signify statistical significance ($p < 0.05$) as indicated above; [c] signifies statistical significance ($p < 0.05$) between 0 and 0.3mM $NaNO_2$ treatment groups of wildtype or $\Delta norZ$ cells at each time point; [d] signifies statistical significance ($p < 0.05$) between 0 and 1mM $NaNO_2$ treatment groups of wildtype or $\Delta norZ$ cells at each time point.

NaNO₂ amendment to LB media and measured activities	Time (hr)			
	120		144	
	WT	$\Delta norZ$	WT	$\Delta norZ$
mM NaNO₂ Remaining				
0 mM	0	0	--	--
0.3 mM	0	0	--	--
1.0 mM	0	0	--	--
nmol N₂O Produced · OD⁻¹				
0 mM	0	0	0	0
0.3 mM	19.28 (0.16)[ab]	16.83 (0.19)[ab]	18.21 (0.11)[ab]	16.72 (0.11)[ab]
1.0 mM	73.06 (0.67)[ab]	59.91 (3.99)[ab]	72.80 (0.59)[ab]	61.95 (3.16)[ab]
% O₂ Remaining				
0 mM	4.13 (0.06)	4.06 (0.11)[c]	4.17 (0.04)[c]	4.19 (0.09)[c]
0.3 mM	4.31 (0.16)	4.48 (0.03)[c]	4.45 (0.03)[c]	4.50 (0.08)[c]
1.0 mM	3.89 (0.40)	4.33 (0.28)	3.96 (0.47)	4.39 (0.31)

Chapter 5

Conclusion

Nitrous oxide (N_2O) has been of global significance as it is involved in global warming and the destruction of stratospheric ozone through the generation of nitrogen oxides (NOx- NO and NO_2). Additionally, NO(x) can lead to decreased air quality and adverse respiratory effects. Emissions and run-offs from anthropogenic activities such as automobile, industrial, and agriculture have increased the presence of reactive nitrogen. This in turn has impacted the microbial transformations of nitrogen as the majority of N_2O and NO emissions have been linked to nitrification and denitrification processes in terrestrial environments.

Nitrification and denitrification are two major processes, along with mineralization and nitrogen fixation, which compose the microbial nitrogen cycle. From the successive reduction of NO_2^-/NO_3^-, the terminal product of denitrification is inert N_2; however, as these anaerobic reactions can be incomplete, intermediate products such as NO and N_2O can be released into the atmosphere. In addition, aerobic denitrification can likewise occur, in which case, the terminal end product is N_2O. Nitrification, the oxidation of NH_3 to NO_3^-, can also result in the aerobic production of NO and N_2O. Genomic and phylogenetic analyses have revealed that nitrifying organisms (i.e. ammonia- and nitrite- oxidizing bacteria) contain homologous genes found in denitrifying organisms that participate in the metabolism of NOx. The studies presented here are therefore an investigation of the regulation of denitrifying genes found in ammonia-

oxidizing bacteria (AOB) and in an organism with no prior involvement in the nitrogen cycle with respect to NOx metabolism.

The first study investigated the regulation of "nitrifier denitrification" genes in three ecologically diverse AOB- *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosospira multiformis* exposed to 0, 10, and 20mM NaNO$_2$ in HEPES- and phosphate-buffered media. Genes studied included *nirK*- encoding a copper-containing nitrite reductase; *norB* and *norS*- encoding nitric oxide reductases; *cytS*- encoding cytochrome *c'* beta; and *cytL*- encoding cytochrome P460. In denitrifying organism, these genes are involved in the reduction of NO$_2$$^-$ to NO, NO to N$_2$O, and in the binding of NO. Results indicate differential regulation of these genes among the three AOB in response to nitrite. Use of buffering systems also affected transcriptional and physiological changes, thus, buffering capacities should therefore be considered when conducting such experiments. As nitrite did not appear to induce expression of *cytL* and *cytS*, their roles in NOx metabolism in AOB are unclear and merit further study. Rates and net nitrite production were likewise variable in the three AOB. It is therefore evident that regulation and physiological diversity exists among AOB, even within species of the same genus, with respect to NOx metabolism and tolerance to nitrite.

Similar to the first study, the following two projects presented here also investigated genes involved in NOx metabolism but in the heterotroph *Sphingomonas wittichi* RW1. While this organism is commonly studied for its biodegradative capabilities, genomic analyses revealed that *S. wittichii* RW1 possesses *nirK*, two nitric oxide reductases (*norZ* and *norB*), and flavohemoglobin (*hmp*). Results indicate that *S.*

wittichii RW1 is capable of aerobic denitrification in the detoxification of nitrite, as corroborated by nitrite consumption and aerobic production of N_2O. High basal mRNA levels of genes indicate differential regulation in the presence of oxygen; additionally, the elevated basal mRNA levels of *nirK* and *norZ* may indicate alternative roles in the central physiology of *S. wittichii* RW1. Increased mRNA levels of *norB* and *hmp* under low O_2 in the presence of nitrite suggest hypoxic function and activity of these genes and their gene products. Significantly greater under low O_2, concomitant N_2O production additionally implies such production occurred to protect cells from nitrosative stress. As *S. wittichii* RW1 has no previous connection to the nitrogen cycle, this study therefore demonstrates the functional diversity and prevalence of these genes in previously uncharacterized organisms. This suggests that there are likely other uncharacterized organisms likewise harboring homologous genes, capable of such activities which can further impact the global nitrogen cycle in terms of NO and N_2O production.

As *S. wittichii* RW1 was capable of aerobic N_2O production, the third study focused on this ability in wildtype and a constructed $\Delta norZ$ strain with respect to regulation of the same suite of genes. Results indicate that growth did not differ between the two cells types and the $\Delta norZ$ strain retained the ability to produce N_2O. In the absence of nitrite, basal transcription levels of *nir*K, *nor*B, and *hmp* were significantly higher in the $\Delta norZ$ strain than in wildtype cells. Levels of *nirK* and *hmp* mRNA did not differ among nitrite-treated wildtype and *norZ*- deficient cells. The observed N_2O gas production as a function of nitrite concentration was attributed to NorB activity as indicated by the significant increase in mRNA levels of *norB*, but not *hmp* or *nirK*, in the

presence of increasing nitrite. Thus, it appears that regulation of *norB* can serve as a compensatory mechanism wherein function and activity of *norB* can substitute for *norZ* in the presence of NOx.

It is clear that the presence of these denitrifying genes serve a diverse and protective role in AOB and *S. wittichii* RW1; however, transcriptional regulation of these genes must also be considered as they are typically under the control of regulatory proteins. In fact, many denitrifying genes (and related operons) often share common regulatory systems, induced in the presence of NOx. Thus, further studies are warranted in elucidating the specific regulator(s) and effector molecule(s) that control transcription of genes involved in NOx metabolism in these organisms. Future studies are also necessary in investigating nitrosative stress responses of *S. wittichii* RW1 at the transcriptome level in the attempt to fully understand the intricacies of the metabolic and physiological capabilities of this organism and thus its relevance and impact on the global nitrogen cycle.
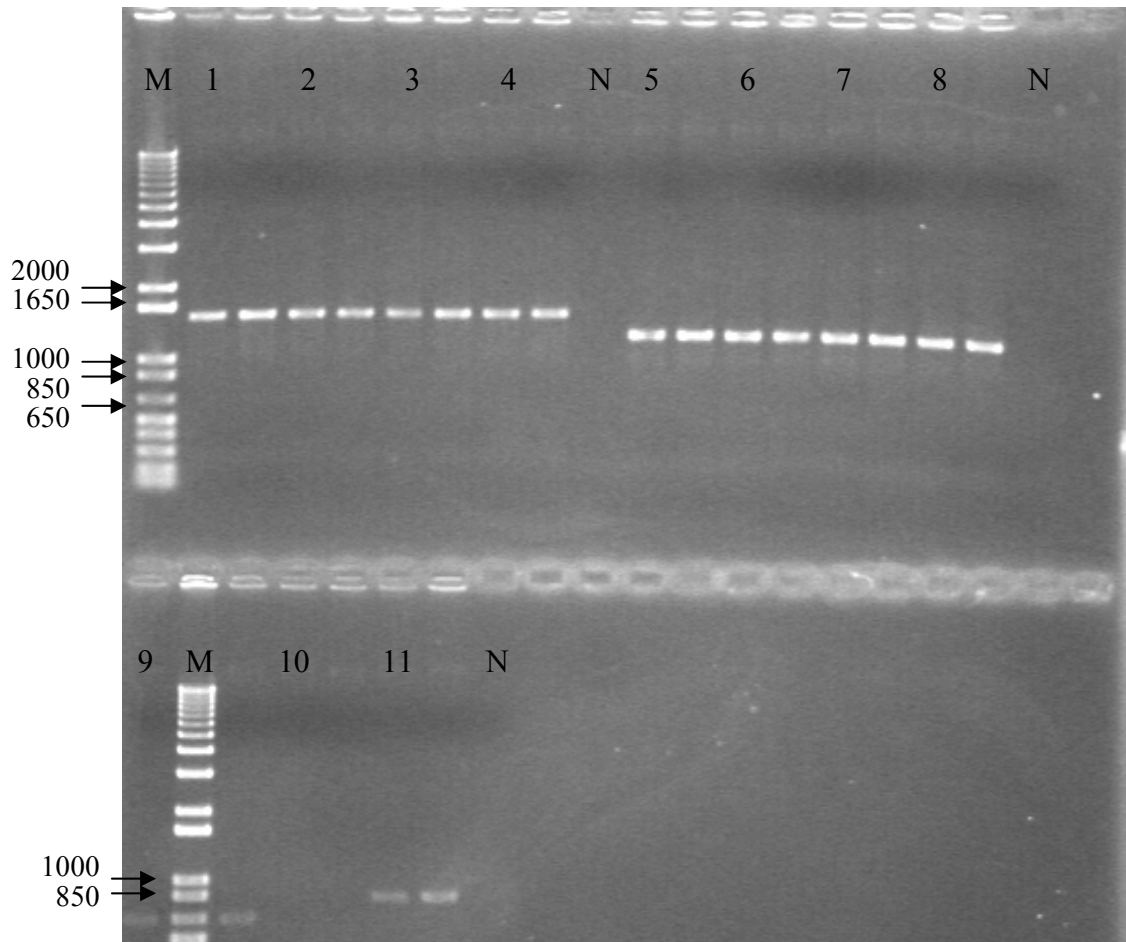
APPENDICES

Appendix A
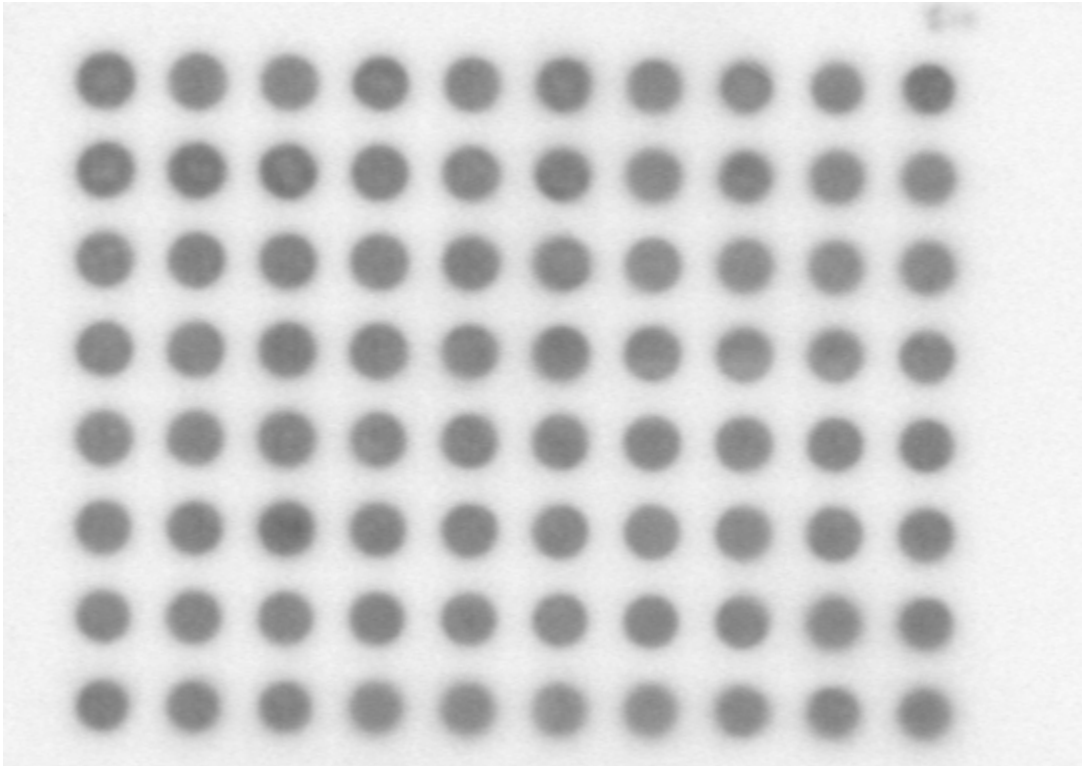
Representative image of a dot blot after membrane hybridization with a gene-specific radiolabelled probe. In this particular image, the membrane contains *S. wittichii* RW1 RNA hybridized to the 16S rRNA probe.

Appendix B

Representative gel picture of PCR confirmations of ΔnorZ strain (Chapter 4). M: 1 Kb plus marker; lanes 1-4 (in duplicate): PCR reaction with gDNA from ΔnorZ strain using Swit_4614_203F and Swit_4614_854R primers, expected size of 1522 bp (including Gm^R cassette); lanes 5-8 (in duplicate): PCRs with gDNA from ΔnorZ strain using Gm 519F and Swit_4614_1343R primers, expected size of 1255 bp; N: negative control- no DNA added; W is wildtype gDNA; Lane 9 (in duplicate): wildtype gDNA using Swit_4614_203F and Swit_4614_854R primers, expected size of 651 bp; Lane 10 (in duplicate): wildtype gDNA using Gm 519F and Swit_4614_1343R primers; Lane 11 (in duplicate): wildtype gDNA using Swit_4614_45F and Swit_4614_854R primers, expected size of 809 bp.