Prokaryotes (2006) 2:769–792
DOI: 10.1007/0-387-30742-7_23

CHAPTER 1.23

The Denitrifying Prokaryotes

JAMES P. SHAPLEIGH

Introduction

One of the hallmarks of bacterial physiology is the ability to use a wide variety of substrates as oxidants or terminal oxidants for respiration. Because of its high redox potential and prevalence, oxygen is generally the preferred reductant for respiratory bacteria. However, bacteria can utilize many other compounds as terminal respiratory oxidant. One of these compounds is nitrate. Nitrate respiration occurs via two dissimilar pathways that utilize the same initial substrate but produce different end products. One of these pathways, termed ammonification, is carried out by bacteria such as *Escherichia coli*, and is marked by reduction of nitrate to nitrite and then to ammonia.

The second pathway of nitrate respiration is denitrification, which is the reduction of nitrate to gaseous nitrogen oxides, principally nitrogen gas (Fig. 1). The initial step in denitrification is the reduction of nitrate to nitrite, as occurs in ammonification. In the next step, the defining reaction, nitrite is reduced to nitric oxide, a gaseous nitrogen oxide. This conversion of a fixed, non-gaseous form of nitrogen to gaseous forms has led this respiratory process to be termed "denitrification" because biologically preferred forms of nitrogen are lost. Once nitric oxide is produced, it is further reduced to nitrous oxide and then to nitrogen gas. The production of nitrogen gas connects denitrification to the nitrogen cycle via nitrogen fixation. The ammonia produced by nitrogen fixation can be converted by nitrifying bacteria to nitrite and nitrate, the substrates of denitrification. This series of reductions and oxidation reactions constitute the nitrogen cycle (Fig. 2).

Gayon and Dupetit carried out the first systematic study of nitrate conversion to gaseous forms of nitrogen in 1882 (Gayon, 1882). Noting the loss of nitrate from decomposing sewage, they called it "denitrification" and were the first to isolate denitrifying bacteria (Gayon, 1886), which they dubbed *Bacterium denitrificans* α and β. In the early stages of the study of denitrification, it was erroneously assumed that nitrate was releasing and thus supplying elemental oxygen to organisms that subsequently carried out a reaction equivalent to oxygen respiration. The observation of denitrification, although biologically significant, was disquieting to agronomists who soon realized that the addition of organic matter to soils could lead to the loss of fixed nitrogen. The agricultural importance of the process provided the impetus for much of the early work on denitrification, and by the end of the 19th century, denitrification had been reasonably well-defined.

In the 20th century, a significant interest in the agricultural consequences of denitrification has continued. However, with the realization that nitric oxide and nitrous oxide play important roles in atmospheric and biological chemistry, research emphases have shifted to the environmental consequences of denitrification and the molecular mechanisms of enzymes and gene regulation.

Defining the Denitrifiers

Prokaryotes (mostly Bacteria, but a few Archaea) constitute the vast majority of organisms capable of denitrification. A number of fungal isolates carry out reduction of nitrate to nitrous oxide, but the contribution of this reduction to cell growth is variable (Usuda, 1995). In prokaryotes and a few filamentous fungi, the reduction of nitrate to gaseous intermediates is a respiratory process. That is, reduction of nitrate is coupled to ATP synthesis via electron transport chains. With one or two exceptions, denitrifiers can also respire with oxygen as the terminal electron acceptor and, because it is usually available at higher concentrations, oxygen is typically the preferred electron acceptor. However, when oxygen becomes limiting, the capacity to utilize nitrate as a terminal oxidant allows denitrifying bacteria to continue respiration using an alternative electron acceptor.

The reduction of nitrate to nitrogen gas is a multi-step process (Fig. 1). The redox couple for each reduction step is greater that 0.35 V, making

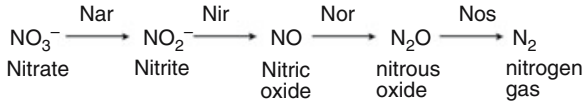

Fig. 1.

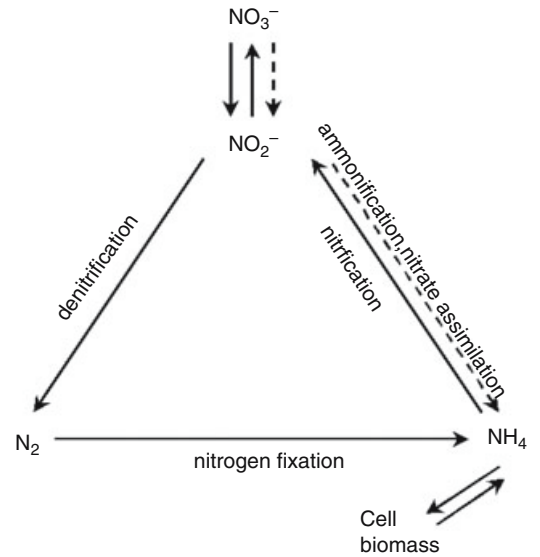

Fig. 2.

denitrification energetically comparable to oxygen reduction. Because each nitrogen oxide reduction has a positive redox couple, every step in denitrification need not be carried out to achieve a net conservation of energy. In fact, it is quite common to isolate bacteria that express only portions of the denitrification electron transport chain. Those prokaryotes that contain partial denitrification chains will be included in this chapter provided these organisms can metabolize the gaseous intermediates in denitrification, nitric oxide and nitrous oxide. So, a bacterium such as *E. coli*, which can reduce nitrate and nitrite, is not considered a denitrifier because it reduces nitrite to ammonia. Conversely, a bacterium such as *Wolinella succinogenes*, even though it also reduces nitrate to ammonia, is included in this review because it also uses nitrous oxide as sole terminal oxidant (Yoshinari, 1980).

Denitrification is a widely dispersed metabolic pathway of prokaryotes. Table 1 shows a list of prokaryotic genera, some of which are denitrifiers. A list of prokaryotic genera suggested to include dentrifying strains is shown in Table 1. This has probably led to an underestimate of the number of denitrifying strains. It is frequently concluded that, if one strain does not denitrify, neither will its close relatives. The essential criterion for inclusion in this list is the metabolism of gaseous nitrogen oxides. In most cases, this means the production of nitrous oxide or nitrogen gas from reduction of nitrate. However, all the strains in this list have not been shown to grow as denitrifiers. Several strains appear to have the capacity to reduce only nitrite or nitric oxide, making them difficult to culture under laboratory conditions.

Archaea

Only a few Archaea capable of denitrification have been isolated. With one exception, all the known archaeal species that have been isolated are also capable of aerobic respiration. Aerobic respiration is relatively frequent among some of the halophilic Archaea, which includes most of the denitrifying Archaea. The denitrification components of these halophilic bacteria have not been characterized extensively. A copper-containing nitrite reductase has been purified from *Haloferax denitrificans* and was shown to be spectroscopically similar to related eubacterial nitrite reductases; but antiserum to the copper-type nitrite reductase from the denitrifier "*Achromobacter cycloclastes*" did not react with the archaeal nitrite reductase (Inatomi, 1996). More recently, other non-halophilic Archaea have been found which are capable of denitrification. One of these is *Pyrobaculum aerophilum*, a hyperthermophile (Volkl, 1993). The genome of this organism is currently being sequenced and information from this effort will be of greatest interest since no genes whose products are required for dentrification have ever been sequenced from an Archaea or a thermophile. The other potential denitrifier from among the Archaea is the strict anaerobe *Ferroglobus placidus* (Hafenbradl, 1996). This bacterium can couple Fe^{++} oxidation to nitrate reduction. Studies on this bacterium have shown cell extracts can reduce nitrite to nitrous oxide, with nitric oxide as an intermediate (Vorholt, 1997). Even though this evidence strongly suggests this organism is a denitrifier, the capacity of whole cells to reduce nitrate to nitrite and small amounts of nitric oxide is puzzling. This result could be due to nitrite toxicity. However, further work should be done to confirm that *F. placidus* is a denitrifier inasmuch as this is one of only two reportedly strictly anaerobic denitrifiers. While denitrification is limited to a few archaeal genera, identification of more archaeal strains capable of denitrification seems likely.

Eubacteria

Denitrification ability is widespread amongst the eubacteria, and almost exclusively in those strains that are capable of aerobic growth. Also,

Table 1. Listing of microbial genera that are suggested to include denitrifiers.

Archaea
Haloarcula
Halobacterium
Haloferax
Ferroglobus
Pyrobaculum

Bacteria
Gram-negative

Aquifex
Flexibacter (formerly *Cytophaga*)
Empedobacter
Flavobacterium
Sphingobacterium
Synechocystis sp. PCC 6803

Purple Bacteria
α subdivision

Agrobacterium
Aquaspirillum
Azospirillum
Blastobacter
Bradyrhizobium
Gluconobacter
Hyphomicrobium
Magnetospirillum
Nitrobacter
Paracoccus
Pseudomonas (G-179)
Rhizobium
Rhodobacter
Rhodoplanes
Rhodopseudomonas
Roseobacter
Sinorhizobium (formerly *Rhizobium*)
Thiobacillus

β subdivision

Achromobacter
Acidovorax
Alcaligenes
Azoarcus
Brachymonas
Burkholderia
Chromobacterium
Comamonas
Eikenella
Hydrogenophage
Janthinobacterium
Kingella
Microvirgula
Neisseria
Nitrosomonas
Ochrobactrum
Oligella
Ralstonia (formerly *Alcaligenes*)
Rubrivivax
Thauera
Thermothrix
Thiobacillus
Vogesella
Zoogloea

γ subdivision

Acinetobacter
Alteromonas
Azomonas
Beggiatoa
Deleya
Halomonas
Marinobacter
Moraxella
Pseudoalteromonas
Pseudomonas
Rugamonas
Shewanella
Thiopioca
Thiomargarita
Xanthomonas

δ subdivision

None

ε subdivision

Wolinella
Campylobacter
Thiomicrospira

Others
Gram-positive

Bacillus
Corynebacterium
Frankia
Dactylosporangium
Dermatophilus
Gemella
Jonesia (formerly *Listeria*)
Kineosporia
Micromonospora
Microtetraspora
Nocardia
Pilimelia
Propionibacterium
Saccharomonospora
Saccharothrix
Spirrilospora
Streptomyces
Streptosporangium

it is found rarely in those bacteria that carry out fermentation. Therefore, genera having strictly respiratory bacteria are likely to have denitrifying strains. Most of the characterized denitrifiers belong to the proteobacteria. However, a number of denitrifiers are in other eubacterial genera.

GRAM-POSITIVE BACTERIA Even though the majority of denitrifiers are Gram-negative, denitrifying bacteria are well represented among Gram-positive bacteria. For example, there have been a number of denitrifying *Bacillus* species described. However, because *Bacillus subtilis*

(the type strain of the most commonly studied strain of the genus) is not a denitrifier, denitrification in *Bacillus* species is often overlooked. It should be pointed out that a strain shown by rDNA analysis to be closely related to *B. subtilis*, *Bacillus azotoformans*, is a denitrifier (Mahne, 1995) and that strains designated as *B. subtilis* have been found to denitrify (Sakai, 1996; Sakai, 1996). However, these *B. subtilis* strain need to be described in more detail. Work done on characterizing denitrification enzymes in *Bacillus* has been limited (Denariaz, 1991). Other Gram-positive bacteria capable of denitrification include strains of *Propionibacterium* (Swartzlander, 1993) and *Jonesia* (originally *Listeria*) *denitrificans* (Rocourt, 1987). These strains are somewhat unusual because they seem to be denitrifying strains in groups of bacteria that are primarily non-denitrifiers. More typical is *Frankia*, in which a screen for denitrification found a number of denitrifying strains (Lensi, 1990). Recently, it has been shown that a number of actinomycetes, including *Streptomyces*, *Dermatophilus* and *Nocardia*, are capable of nitrous oxide evolution from nitrate or nitrite (Shoun, 1998). This work significantly expands the list of denitrifying Gram-positive bacteria and demonstrates that, once denitrification is observed in a group of bacteria, further characterization of its members and close relatives will uncover additional denitrifiers, even if denitrification had never been ascribed previously to any member of the group.

Gram-Negative Bacteria Among the Gram-negative bacteria that are not proteobacteriaare, denitrifying strains are found in the genera *Aquifex* (Huber, 1992), *Flexibacter* (Jones, 1990), and *Flavobacterium* (Coyne, 1989). *Aquifex pyrophilus* is a thermophile that constitutes one of the deepest (earliest) branches of the eubacteria. This may indicate that denitrification represents one of the first forms of respiration. Denitrifying strains of the related *Hydrogenobacter* have not been isolated as yet, but this may be due to incomplete characterization. The gliding bacterium *Flexibacter canadensis* is unique in that its nitrous oxide reductase is apparently insensitive to acetylene, unlike nitrous oxide reductases from other bacteria (Jones, 1990).

Nearly all of the bacteria defined as denitrifiers are identified because of their ability to reduce nitrate or nitrite to gaseous end products. Recently, genomic sequencing efforts indicated that *Synechocystis* sp. strain PCC 6803 is also a denitrifier, but with a truncated electron transport chain (Kaneko, 1996). Analysis of the genome sequence of PCC 6803 showed that it encodes a nitric oxide reductase and a transcriptional regulator that may regulate expression of this enzyme. This strain does not encode a nitrite or nitrous oxide reductase, making it an extreme example of an organism with a truncated denitrification electron transport chain. It will be interesting to see if genomic sequencing finds additional bacteria with truncated denitrification chains.

The majority of currently characterized denitrifiers are found in the group known as the proteobacteria (purple bacteria). The proteobacteria have been subdivided into five subdivisions, α, β, γ, δ and ε. Denitrifiers have been found in four of these. The δ subdivision, which contains a number of strict anaerobes, has not been found to contain any strains that denitrify, as yet. The α subdivision contains a number of well-characterized denitrifiers including *Paracoccus denitrificans*, various *Rhodobacter* strains, and several rhizobia. Even in the α subdivision, in which it is well established, denitrification may be overlooked if the first strains characterized do not denitrify. A good example of this problem is *Rhodobacter sphaeroides*. The original isolates of *R sphaeroides* were not robust denitrifiers, so little effort was made to determine if other strains of *R sphaeroides* were denitrifiers. However, a number of denitrifying isolates were eventually isolated (Michalski, 1988). Further work has indicated that all strains of *Rhodobacter* probably encode nitric oxide and nitrous oxide reductase, but that most have lost the ability to reduce nitrite (Kwiatkowski, 1997).

Denitrifiers of note in the α subdivision include *Hyphomicrobium*, budding bacteria often found in waste-water treatment facilities (Fesefeldt, 1998). *Hyphomicrobium* can be used to help rid water supplies of high nitrate concentrations while growing on inexpensive feedstocks such as methanol. Denitrification among the rhizobia, which are best known for their roles as nitrogen-fixing symbionts, is also noteworthy because these strains have the seemingly contradictory capacity for both nitrogen fixation and denitrification (O'Hara, 1985). This ability to both fix and "unfix" nitrogen is fairly widespread among denitrifiers: *Rhodobacter*, *Hyphomicrobium*, *Frankia*, *Azospirrilum*, *Azoarcus* and some pseudomonad strains can both fix nitrogen and reduce nitrates to nitrogen gas. Some members of the genus *Nitrobacter*, which are nitrifying α-proteobacteria, reportedly produce nitric oxide from nitrite. Even though a putative nitrite reductase has been purified from *Nitrobacter vulgaris* (Ahlers, 1990), other studies have not revealed production of nitric oxide or nitrous oxide in cultures of *Nitrobacter* species (Baumgartner, 1991; Goreau, 1980). Further studies are required to determine if these nitrifying bacteria are also denitrifiers.

Although a number of members of the β-proteobacteria denitrify, they are not as well studied, on the whole, as members of the α or γ subdivisions. However, new developments suggest this situation is likely to be only temporary. One new area of interest is the anaerobic degradation of aromatic organic compounds. In recent years, it has become evident that denitrifying bacteria can metabolize aromatic compounds under denitrifying conditions. A number of aromatic compound-degrading denitrifiers have been isolated, and many belong to the genera *Azoarcus* or *Thauera*, which are both β-proteobacteria (Fries, 1994; Springer, 1998; van Schie, 1998; Anders, 1995). Given the interest in anaerobic degradation of aromatic compounds, there is likely to be more in-depth studies on denitrification in *Azoarcus* or *Thauera*. One strain, *Azoarcus anaerobius*, is a completely anaerobic denitrifier (Gorny, 1992) and is unusual in that it cannot fix nitrogen, a trait common to all other strains of *Azoarcus*.

Another reason to expect that denitrification in members of the β subdivision will receive more attention is that both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are denitrifiers. Even though it has been well documented that several other species of *Neisseria* can denitrify (in fact, one species is named *Neisseria denitrificans*), denitrification in *N. gonorrhoeae* and *N. meningitidis* was originally overlooked because of their apparent sensitivity to nitrite. However, recently it has been shown that the gene *aniA* in *N. gonorrhoeae* encodes a copper-containing nitrite reductase (Mellies, 1997). Ongoing sequence analysis of the genomes of *N. gonorrhoeae* (Roe, 1999) and *N. meningitidis* (Sanger Centre, 1999) has revealed that both organisms encode a nitrite reductase and nitric oxide reductase, but only *N. gonorrhoeae* is known to encode nitrous oxide reductase; whether *N. meningitidis* also has this gene is not determined as yet. Little is known about the regulation of these proteins or what contributions they make to the physiology of these bacteria. Nitrite reductase is apparently not required for pathogenicity (Mellies, 1997). However, because reactive nitrogen oxide is an important part of the host defense response in humans, the role of the denitrification enzymes in these pathogenic *Neisseria* will undoubtedly receive more attention.

Also, among the β subdivision denitrifiers are species of the genus *Nitrosomonas*, a genus defined by the ability to oxidize ammonia to nitrite. It has been known for some time that nitrous oxide is a product of ammonia oxidation (Goreau, 1980; Poth, 1985). Subsequent studies have confirmed these observations and a copper-containing nitrite reductase has been purified from *Nitrosomonas europaea* (Miller, 1985; Dispirito, 1985). This strongly indicates that *Nitrosomonas* species are nitrifying denitrifiers. However, the role of denitrification in *Nitrosomonas*, whether for detoxification or for energy conservation, has not been established as yet.

Denitrification in members of the γ subclass of proteobacteria has been well studied in *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*. Recent characterizations of the pseudomonads has resulted in the reclassification of rRNA group II bacteria to the genus *Burkholderia*, which is in the γ subdivision (Yabuuchi, 1992). Members of the rRNA group I of the pseudomonads are tightly clustered, and *P. stutzeri* and *P. aeruginosa* are in this group.

Some very unusual nitrate respirers are found in the γ group. Both *Beggiatoa* and *Thioploca* species have the unique capacity to accumulate nitrate in internal vacuoles until they contain several thousandtimes the external concentration (McHatton, 1996; Fossing, 1995). The organisms then use this accumulated nitrate as an oxidant and sulfide in the surrounding environment as a reductant. They also make sheaths in which they move between the nitrate-rich waters and sulfide-rich sediments, allowing them to commute from one environment to another depending on their physiological requirements. A unique relative of *Thioploca* and *Beggiatoa*, *Thiomargarita*, has been described recently (Schulz, 1999). *Thiomargarita* accumulates high concentrations of nitrate in a central vacuole. This very large vacuole is the reason *Thiomargarita* is the largest known bacterium. All of these bacteria are presumed to be denitrifiers, however, there is little direct evidence for this. Some evidence suggests *Beggiatoa* species are denitrifiers (Sweerts, 1990), but more work needs to be done to show that these strains contain a nitric-oxide-producing nitrite reductase. The use of reduced sulfur compounds as electron donors is known to occur in several denitrifiers including *Aquifex* (Huber, 1992), *Paracoccus* (Friedrich, 1981), and *Thiobacillus* (Schedel, 1980).

A few denitrifiers have been found in the ε subdivision. Some exhibit truncated denitrification chains. For example, both *Wolinella succinogenes* and some *Campylobacter* species have the capacity to grow with nitrous oxide as sole terminal oxidant (Yoshinari, 1980; Payne, 1982). However, neither bacterium is able to reduce nitrite to nitrous oxide. *Thiomicrospira denitrificans*, which is closely related to the *Campylobacter* group (Muyzer, 1995), can reduce nitrite to gaseous end products (Timmer-ten Hoor, 1975). Also, *T. denitrificans* can use sulfur compounds as reductants.

Enzymology of Denitrification

As shown in Figure 1, complete denitrification is a multi-step process, requiring four separate enzymes for the reduction of nitrate and three intermediate nitrogen oxides, and ending in the evolution of nitrogen gas. The basic arrangement of the nitrogen oxide reductases is shown in Fig. 3. A basic description of the nature of these proteins and ancillary proteins is presented here. Additional information can be obtained from two recent reviews (Berks, 1995; Zumft, 1997).

Nitrate Reductase

The first step in denitrification, the two-electron reduction of nitrate to nitrite, is catalyzed by nitrate reductase. Early studies on nitrate reductase activity in cells demonstrated the existence of at least two types of nitrate reductase: a soluble assimilatory enzyme, used when nitrate is the nitrogen source, and a membrane-associated respiratory enzyme. The situation became more complicated when nitrate reductase activity was found in the periplasm of *R. sphaeroides* IL 106 (Sawada, 1980). Further work has demonstrated that this periplasmic enzyme is found in a wide variety of bacteria, including denitrifiers and *E. coli*.

Most denitrifiers contain more than one type of nitrate reductase. The enzyme typically associated with nitrate respiration is a membrane-bound three-subunit complex, whose membrane-anchoring subunit (γ or NarI) is sometimes lost during purification. A significant amount of the research on the respiratory nitrate reductase has made use of non-denitrifiers, particularly *E. coli* (Berks, 1995). The largest of the three subunits (α or NarG) contains molybdenum, bound by the cofactor molybdopterin guanine dinucleotide and a [4Fe-4S] center. The remaining subunit (β or NarH) contains several [4Fe-4S] centers and a [3Fe-4S] center. NarG and NarH are exposed on the cytoplasmic side of the inner membrane. The membrane anchoring subunit typically contains a b-type heme. The direct electron donor of the respiratory nitrate reductase is quinol. The electrons from quinol are thought to be transferred through the heme in the membrane-anchoring subunit to the [Fe-S] centers in NarH and then to the molybdenum center in NarG where nitrate reduction occurs.

The periplasmic nitrate reductase (Nap) is a heterodimer with prosthetic groups similar to those found in the membrane-bound nitrate reductase. The largest subunit (NapA) binds molybdopterin guanine dinucleotide and a [4Fe-4S] center. The smaller subunit binds heme (NapB) that is required for transfer of electrons to the active site. Electrons are transferred from the membrane-associated quinol pool to the Nap complex by a membrane-bound tetra-heme c-type cytochrome, NapC. Insertional inactivation of NapC in *R. sphaeroides* resulted in the loss of nitrate reductase activity (Reyes, 1996). NapC is related to a larger family of proteins, which apparently bind heme via a bis-His ligation (Roldan, 1998).

Recently the crystal structure of the periplasmic nitrate reductase from the non-denitrifying bacterium *Desulfovibrio desulfuricans* has been reported (Dias, 1999). This enzyme contains a single subunit containing the active site and related cofactors. This subunit has significant similarity to NapA from denitrifiers and *E. coli*. Comparisons with other known structures revealed the enzyme is structurally related to formate dehydrogenase and dimethylsulfoxide reductase, both of which contain molybdenum cofactors. Comparisons of these various struc-

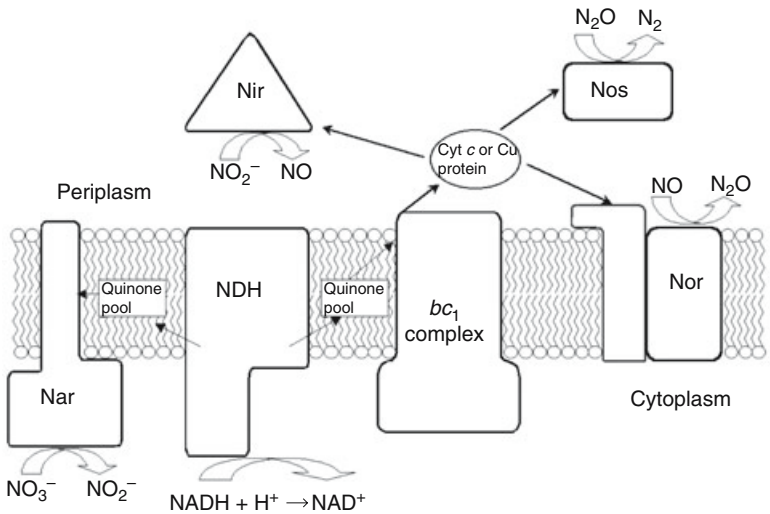

Fig. 3.

tures will be very useful in determining the structural constraints required for nitrate binding and reduction.

The periplasmic and membrane-bound enzymes can be distinguished in several ways. First, the membrane-bound enzyme is sensitive to micromolar levels of azide, whereas the periplasmic form is not (Bell, 1990). Second, the membrane-bound enzyme can reduce chlorate but the periplasmic enzyme is limited to nitrate, a result that led to the development of a useful method to select nitrate reductase mutants. Third, because the active sites of the two enzymes are on different sides of the inner membrane, the differential membrane solubilities of methyl viologen and benzyl viologen can be used to differentiate activities (Carter, 1995). Methyl viologen, which is membrane permeant, can be used as an electron source for both enzymes in whole cell assays. Benzyl viologen, which is membrane impermeant, will act as an electron source only for the periplasmic enzyme in whole cell assays. By comparing the nitrate reductase activity determined with each viologen, the relative levels of activity of each form of nitrate reductase can be estimated. Lastly, the regulation of the two enzymes is different.

While the function of the respiratory nitrate reductase in denitrification is obvious the physiological role of the periplasmic enzyme is unclear. It seems unlikely that the periplasmic enzyme is used by most denitrifiers for energy conservation inasmuch as bacteria such as *R. sphaeroides* 2.4.1 have this enzyme but can not grow anaerobically with nitrate as the sole terminal electron acceptor. Moreover, expression of the periplasmic nitrate reductase is repressed during denitrification in *P. denitrificans* and *R. eutropha* (Warnecke-Eberz, 1993; Sears, 1993). It seems likely that the major role of the periplasmic enzyme is to aid dissipation of excess reductant. This role is supported by the observation that the periplasmic nitrate reductase of *P. denitrificans* GB17 is expressed at a higher level on reduced substrates (that is, those that would likely cause a buildup of reductant) than on more oxidized substrates (Richardson, 1992). However, there are two examples of Nap being the principal nitrate reductase in a denitrifier. Inactivation of the genes encoding Nap in both *Pseudomonas* G-179 (Bedzyk, 1999) and *R. sphaeroides* resulted in a loss of nitrate reductase activity under anaerobic conditions.

The assimilatory nitrate reductase is a single-subunit enzyme. As with the other nitrate reductases the enzyme binds molybdopterin guanine dinucleotide and an [Fe-S] center. The activity of this enzyme is differentiated from the other enzymes, principally because it is only expressed when nitrate is used as a sole source of nitrogen.

Nitrite Reductase

Nitrite reduction is the defining reaction of denitrification—the step that differentiates denitrification from other forms of nitrate metabolism. Nitrite reductase catalyzes the one electron reduction of nitrite to nitric oxide. There are two types of nitrite reductases, but, unlike the different types of nitrate reductase, the nitrite reductases are not structurally related and contain different prosthetic groups. They are, however, both located in the periplasm. They also appear to be functionally redundant (Glockner, 1993). One type of nitrite reductase utilizes copper as a redox active metal (CuNir), and the other utilizes heme-bound iron (cd_1). There is no obvious phylogenetic distribution of the two enzymes. Moreover, both types of enzyme can be found within members of a single genus but have not been found in a single bacterium. In studies assessing the frequency of occurrence of either enzyme in the environment, the cd_1 type was found in a greater number of organisms (Coyne, 1989).

The CuNir has been studied extensively, and much is known about its structure and the nature of the copper centers. Enzymes from several different denitrifiers have been crystallized under different conditions and their high-resolution structures determined (Dodd, 1998; Adman, 1995; Kukimoto, 1994; Godden, 1991). These studies have revealed that the enzyme is a homotrimer with each monomer containing two copper atoms. The copper atom in type-1 centers is attached to Cys, Met and two His residues. Type-1 centers are often referred to as blue copper centers and are found in electron transfer proteins, such as azurin, or in proteins with multiple copper centers, such as laccase (Solomon, 1996). In multi-copper enzymes, including CuNir, the type-1 copper is involved in electron transfer to the active site. The other copper atom in CuNir is bound by three His residues making it a type-2 copper center. Type-2 centers are found in many multi-copper enzymes and are frequently sites of substrate binding. In CuNir, the type-2 center has been shown to be the site of nitrite binding (Adman, 1995). In the CuNir-nitrite cocrystals, the nitrite binds to the type-2 center with its oxygens and upon binding displaces a bound water (Adman, 1995). The copper centers in the cocrystal are in an oxidized state, however, which may influence how the nitrite binds. Studies of synthetic copper centers have suggested that the nitrite nitrogen binds to a reduced copper center (Halfen, 1996).

Less is known about how nitrite reduction proceeds. The redox potential of the type-1 center is higher than that of the type-2 center (Olesen, 1998). This suggests that the type-1 center acts as

a gate, by holding on to an electron until nitrite binds to the type-2 center. Nitrite binding to the type-2 center will raise its potential, permitting electron flow from the type-1 to the type-2 center. Little is known about how nitrite reduction proceeds once the type-2 center is reduced. It has been suggested that the nitrite is reduced and protonated, transiently forming a copper (II)-nitric oxide complex (Murphy, 1997). Because the nitric oxide is bound to oxidized copper, the complex is relatively unstable and nitric oxide can dissociate and diffuse away. It is critical that nitric oxide not bind to the reduced type-2 center, as such a complex would be stable and prevent enzyme function. This may explain how the type-1 center can function as a one-electron gate (Olesen, 1998).

The homodimer cd_1-type nitrite reductase contains a single c-type heme and d_1 heme molecule per monomer. The d_1 heme is a modified tetrapyrrole ring that is partly reduced and has oxo, methyl and acrylate sidegroups (Chang, 1986). The high-resolution structures of the cd_1 type enzymes from *P. denitrificans* GB17 (*Thiosphera pantotropha*) (Fulop, 1995) and *P. aeruginosa* (Nurizzo, 1997) have recently been determined. The c-heme of the oxidized form of the enzyme from GB17 enzyme is bound to two His residues. However, upon reduction, the enzyme refolds causing one of the His ligands to be lost and replaced by a Cys, a more common ligand of c-type cytochromes. This unexpected ligand shuffling does not occur in the *P. aeruginosa* enzyme because both the oxidized and reduced forms have the same His and Cys ligand (Nurizzo, 1998).

The d_1 heme in GB17 enzyme is bound by a Tyr and a His residue. The attachment of the d_1 residue is unusual in that the Tyr residue (identified as the ligand) is not conserved in other cd_1 reductases, nor does a potentially equivalent Tyr in other cd_1 reductases play a role in heme ligation (Cutruzzola, 1997). The high-resolution structure of the *P. aeruginosa* enzyme revealed that the sixth ligand of the d_1 heme is a hydroxide ion. In both the *P. aeruginosa* and GB17 enzymes, the sixth ligand is lost when substrate binds to the d_1 heme. The high-resolution structures of the reduced forms of both enzymes are very similar.

In addition to static structures, a time-resolved structural study of the catalytic cycle of the GB17 enzyme has been carried out (Williams, 1997). This work revealed the large-scale structural changes discussed above. It also indicated that the nitrite nitrogen binds to the reduced enzyme, in contrast with the oxidized CuNir. The ligand shuffling in the GB17 enzyme was postulated to change redox potentials of the heme groups to prevent formation of a Fe(II)-NO complex. The end product (Fe(III)-NO) is ensured by limiting the number of electrons available to the d_1 heme during each nitrite reductase step. Then, Fe(III)-NO can dissociate spontaneously or by the return of the Tyr ligand in the GB17 enzyme.

Both the CuNir and cd_1 enzymes also can reduce oxygen. Early studies often designated the cd_1 enzyme a cytochrome oxidase (Wharton, 1976). The product of oxygen reduction by the cd_1 enzyme is water (Lam, 1969), although it is not clear how four electrons are passed to the oxygen in this process, inasmuch as a one-electron reduction is normally carried out. The oxidase activity of these enzymes is potentially significant because, if activated oxygen species are also produced by CuNir, they can react rapidly with nitric oxide to produce reactive and toxic products such as peroxynitrite (Stamler, 1992).

Nitric Oxide Reductase

The *P. stutzeri* nitric oxide reductase has been the most intensively studied of these enzymes. It is purified as a heterodimer with subunits NorB and NorC being integral membrane proteins (Kastrau, 1994). NorC is c-type cytochrome, proposed to accept an electron from a periplasmic donor and then to pass the electron to NorB. NorB contains two b-type hemes and a non-heme iron. Spectroscopic analysis indicates one b-heme is low spin, whereas the other is high spin and capable of binding carbon monoxide. The low-spin heme is likely to be the direct acceptor of electrons from NorC. Metal analysis revealed that more iron was present than could be accounted for by heme content, indicating non-heme iron. As additional nitric oxide reductases have been purified, similar metal stoichiometry has been observed (Dermastia, 1991; Girsch, 1997). There is no evidence for nitric oxide reductase containing an [Fe-S] center.

The isolation of the genes encoding the nitric oxide reductase in *P. stutzeri* provided significant insight into the structural organization of the enzyme (Zumft, 1994). Examination of the deduced primary sequence indicated that the nitric oxide reductase is related to the heme-copper family of cytochrome oxidases (van der Oost, 1994; Saraste, 1994). Although the overall identity of nitric oxide reductases and cytochrome oxidases is low, a set of six His residues is conserved in pairwise alignments of subunit I of cytochrome oxidase and NorB of nitric oxide reductase. The conservation of these residues is significant because they have been shown to serve as metal center ligands in cytochrome oxidase. In cytochrome oxidase, these His residues bind a six-coordinate heme, five-coordinate heme, and copper—the latter two metal centers

constituting a binuclear center that is the site of oxygen binding and reduction (Iwata, 1995). By comparison, the equivalent His residues in NorB, ligate a six-coordinate heme, a five-coordinate heme and, because there is no copper in nitric oxide reductase, a non-heme iron.

Recent spectroscopic studies on the enzymes purified from *P. denitrificans* (Girsch, 1997) and *P. stutzeri* (Cheesman, 1998) support the structural similarity of nitric oxide reductase and heme-copper oxidases. The active site of nitric oxide reductase is most likely a five-coordinate heme and non-heme iron in close proximity. The two metal centers are close enough to permit interaction of the electron orbitals, as also observed in heme-copper oxidases. NMR, Raman, and FTIR analyses have demonstrated that the local molecular environment of the binuclear center in nitric oxide reductase is distinct from any type of heme-copper oxidase (Moenne-Loccoz, 1998; Mitchell, 1998). The differences in the structure are also manifested in the primary sequences. For example, in the aa_3-type cytochrome oxidases, one of the most highly conserved regions is within membrane spanning helix six, which contains the His residues that bind the five-coordinate heme. Comparison of the aa_3-type consensus sequence of the helix-six region with sequences of the similar region in nitric oxide reductases reveals significant differences in the primary sequences (Fig. 4). Of particular note is the absence of a Glu in nitric oxide reductase that may be involved in proton pumping in cytochrome oxidase and in an overall increase in polarity of the nitric oxide reductase sequence (Verkhovskaya, 1997). The sequence differences also make it possible to differentiate members of this diverse family of proteins. This is important in those bacteria, such as *N. gonorrhoeae* and *N. meningitidis*, where nitric oxide reductase has not been demonstrated, but sequence information indicates the gene encoding this enzyme is present along with other members of the heme-copper oxidase superfamily.

A variant on the *P. stutzeri* prototype structure has recently been identified in the bacterium *Ralstonia eutropha* (*Alcaligenes eutrophus* H16) (Cramm, 1997). This bacterium contains two nitric oxide reductases, one designated NorZ, a product of genes on the chromosome, and the other designated NorB, a product of genes located on a plasmid. The products of these genes have significant identity (>90%) and are functionally redundant. They have significant similarity with NorB of other nitric oxide reductases such as that of *P. stutzeri*, with the exception of an N-terminal extension of about 300 residues. These extra residues of the *R. eutropha* enzymes likely add two additional membrane-spanning regions and a large hydrophilic loop. The primary sequence of the N-terminal extension does not have similarity with other known proteins. Part of its function may relate to the observation that there is no evidence for a gene encoding a NorC equivalent in *R. eutropha*. This suggests that the amino terminal extension is functionally equivalent to NorC, but no direct evidence has been provided in support of this conclusion.

A gene whose product is similar to the NorB and NorZ products has turned up in the genome of *Synechocystis* strain PCC 6803 (Kaneko, 1996). Interestingly, genes encoding nitrite and nitrous oxide reductase were not identified in this bacterium. Because PCC 6803 can not reduce nitrite, it has never been considered as capable of gaseous nitrogen oxide metabolism. This makes the presence of nitric oxide reductase unexpected. However, as discussed above, other strains have recently been characterized which have nitric oxide reductase but lack nitrite reductase, suggesting this mode of truncation of the denitrification electron transport chain may be fairly prevalent. Ongoing genome sequencing efforts in *N. meningitidis* (Sanger Centre, 1999) and *N. gonorrhoeae* (Roe, 1999) have also identified genes whose products are similar to the *R. eutropha* nitric oxide reductases. *Neisseria* and *R. eutropoha* are both in the β-subgroup of the proteobacteria (Table 1), but it is unclear if this class of enzymes will be preferentially found in one taxonomic group. Alignment of nitric oxide reductases also demonstrates that the *R. eutropoha*-type nitric oxide reductases have other unique sequence motifs not found in the *P. stutzeri* type reductases (Fig. 4).

The reduction of nitric oxide to nitrous oxide occurs at the binuclear center. Current models suggest that two nitric oxide molecules bind at the active site, although it is not clear if nitric oxide binds to both metal centers or if a dinitrosyl complex is formed (Ye, 1994; Moenne-Loccoz, 1998). Electrons enter the Nor complex through the cytochrome c in NorC and then flow to the six-coordinate heme in NorB. Electrons are then transferred to the binuclear center. The high affinity of reduced heme for nitric oxide

```
Psaer PENLTRDKFYWWWVVHLWVEGVWELIMGAILAFVLAFITG
Psstu PENLSRDKFYWWWVVHLWVEGVWELIMGAILAFVLIKVTG
Phalo PSNLAVDKLYWWWVVHLWVEGVWELIMASILGYLLIKMTG
Pdeni PANLVLDKQYWWWVIHLWVEGVWELIMAAILAFLMIKLTG
Brjap PANLALDKMYWWWVVHLWVEGVWELIMASVLAYLMIKLNG
Rspha PDNLGLDKMYWWYIVHLWVEGTWELVMAAVLGYLMIKLTG
G-179 PANLALDKMYWWYVVHLWVEGVWELIMASVLSFLMIKLNG
Synec TRISVAEYWRWW-VVHLWVEGFFEVFATVAIAYLCSELGF
Reutr TSITVMEYWRWW-VVHLWVEGFFEVFATVALAFIFSTLGL
Ngono SPIAVMEYWRWW-VVHLWVEGFFEVFATVAFAFVFYNMGF
Nmeni SPIAVMEYWRWW-VVHLWVEGFFEVFATAAFAFVFYNMGF
Sub I GGDPVLYQHLFWFFGHPEVYILILPAFGIISEVISTFSRK
CcoN  VFSGVQDAMVQWWYGHNAVGFFLTAGFLGMMYYFVPKQAE
```

Fig. 4.

makes it unclear if reduction of the catalytic site involves formation of a ferrous heme-nitric oxide species. Irrespective of the exact electron transfer steps, it is clear that proximity of the two nitric oxide molecules is critical in the formation of the N-N bond.

Nitrous Oxide Reductase

The presence of nitrous oxide reductase can be identified independently of the other reductases because many organisms can grow with nitrous oxide as sole terminal oxidant. This has led to the identification of nitrous oxide reductase in several bacteria that are not complete denitrifiers (Yoshinari, 1980; Payne, 1982). The purification and characterization of nitrous oxide reductase was difficult because its activity is lost in cell extracts. Nutritional studies had identified copper as an essential nutrient for nitrous oxide reductase activity (Matsubara, 1982) and further work led to the isolation of a soluble copper protein which, under the proper conditions, had nitrous oxide reductase activity (Zumft, 1982). Additional studies have demonstrated that enzyme purified anaerobically and assayed using reduced viologens as electron donor has the highest specific activity. The latter observation is somewhat puzzling because the natural electron donors to nitrous oxide reductase are likely to have much higher redox potential.

Nitrous oxide reductases from several complete denitrifiers have been extensively characterized (Riester, 1989; SooHoo, 1991; Hulse, 1990; Snyder, 1987). These enzymes are all related and are multi-copper periplasmic proteins. The protein appears to be homodimeric in most preparations, with four coppers per subunit. Current data suggest there are two binuclear copper centers in the active enzyme. These centers undergo spectroscopic shifts depending on their redox state, and this has resulted in the characterization of different colored forms of the enzyme.

One of the copper centers has been structurally defined as a CuA site. The CuA center was described originally in the heme copper oxidases. However, the exact nature of this site was unclear until the related site in nitrous oxide reductase was characterized. Analysis of the deduced primary sequence of the nitrous oxide reductase identified a structural motif found otherwise only in the CuA-containing subunit of cytochrome oxidase (Viebrock, 1988). Additional spectroscopic studies provided further evidence of the similarities between one of the sites in nitrous oxide reductase and the CuA site of cytochrome oxidase (Farrar, 1991; Scott, 1989; Antholine, 1992). A more precise understanding of the structure of the CuA center has been obtained with the determination of the high-resolution structure of two cytochrome oxidases (Tsukihara, 1995; Ostermeier, 1997). In both cytochrome oxidase and nitrous oxide reductase, the CuA site has a role in transferring electrons from external electron donors to the active site. The other copper center in nitrous oxide reductase is, by exclusion, assumed to be the site of nitrous oxide binding and reduction. This site has been designated CuZ. Initial studies suggested both CuZ and CuA sites were bis-thiolate-bridged dinuclear copper sites (Farrar, 1991). Isolation of spectral signals arising from the CuZ center has proved difficult. Spectroscopic analysis of the CuZ signals has relied on poising the enzyme in specific oxidation states to make the CuA center Electron Paramagnetic Resonance (EPR) or optically silent. However, it has been suggested that the signals assigned to the CuZ site are actually different redox states of the CuA center (Farrar, 1998). In this latter work, it was shown that both the CuA site and the putative CuZ site were bis-thiolate-bridged dinuclear copper sites. This would require, if these were separate sites, four conserved Cys residues for formation of the two binuclear centers. Alignment of deduced nitrous oxide reductase sequences does not identify four conserved Cys residues. Instead, there are only two absolutely conserved Cys residues in nitrous oxide reductase. It is possible that the position of a Cys is shifted in one sequence relative to others accounting for the deficiency of conserved residues. However, mutation of one of the Cys residues outside the CuA domain does not lead to a loss of nitrous oxide reductase activity (Dreusch, 1996). Taken together, these data are not consistent with assuming that there are two bis-thiolate-bridged dinuclear copper sites in nitrous oxide reductase.

If conserved residues do not bridge the catalytic center, what then is its structure? Alignment of available nitrous oxide sequences does indicate there are a number of completely conserved His residues. The catalytic center might therefore be a His-ligated structure similar to the Type 3 copper centers found in enzymes such as laccase (Solomon, 1996). However, there is currently no direct evidence for the presence of such a center.

Given the uncertainty in the structure of the catalytic center, it is difficult to develop a useful model of the nitrous oxide reductase catalytic cycle. Nitrous oxide is chemically inert and also a poor ligand, making its reduction an interesting problem in transition metal-ligand chemistry (Kroneck, 1990). Other enzymes, including nitrogenase (Jensen, 1986) and carbon monoxide dehydrogenase (Lu, 1991), also have been found to have nitrous oxide reductase activity, indicat-

ing that it is possible that transition metals other than copper can reduce nitrous oxide.

Only copper-containing nitrous oxide reductases have been purified from complete denitrifiers. However, there appear to be variants of this structure present in other bacteria. The nitrous oxide reductase from *W. succinogenes* has been purified and shown to contain both copper and iron (Teraguchi, 1989; Zhang, 1991). The iron is attributed to an associated cytochrome c, though EPR suggests the presence of a CuA site. Because the primary structure of the *W. succinogenes* enzyme is unknown, the relationship of this enzyme to other nitrous oxide reductases is unknown. Another novel nitrous oxide reductase has been suggested to occur in *Flexibacter canadensis* (Jones, 1990). Most nitrous oxide reductases are inhibited by acetylene (Balderston, 1976; Yoshinari, 1976). However, the nitrous reductase from *F. canadensis* is insensitive to this compound (Jones, 1990). Preliminary characterization of this enzyme suggests it is membrane-associated, further differentiating it from other reductases (Jones, 1992). The nitrous oxide reductase from *R. sphaeroides* IL106 was also thought to be divergent because it was reported to contain Zn and Ni in addition to copper (Michalski, 1986). However, a more rigorous characterization of this enzyme suggests it is similar to typical copper-containing enzymes (Sato, 1998).

Genetics of Denitrification

Gene Organization

Analysis of the structure and organization of denitrification genes has been investigated in denitrifiers that are primarily members of the proteobacteria. The most extensive characterizations have been carried out with genomes of the pseudomonads as well as that of *P. denitrificans* strains. Although no true denitrifier has had its chromosome sequenced, there are several projects to sequence organisms that are partial or complete denitrifiers.

Nitrite and Nitric Oxide Reductase The most extensive examinations of denitrification gene organization have involved *P. aeruginosa* and *P. stutzeri* in which the nitrite reductase and nitric oxide reductase structural genes are about 10 kb apart (Arai, 1995; Braun, 1992). This tight linkage is found also in *P. denitrificans* (Baker, 1998). In general, such tight linkage of the nir and nor gene clusters is not observed in those denitrifiers containing a copper-type nitrite reductase. In *R. sphaeroides* strains IL106 and 2.4.3, the genes for nitrite reductase are not closely linked to the genes for nitric oxide reductase (Schwintner, 1998; Tosques, 1997). This is also the case in *N. gonorrhoeae* (Roe, 1999) and *N. meningitidis* (Sanger Centre, 1999). One exception is *Pseudomonas* G-179 (which is apparently a member of the α proteobacteria subclass) where it has been shown that the gene encoding the copper-type nitrite reductase is in a cluster with the genes for nitric oxide reductase and the periplasmic nitrate reductase (Bedzyk, 1999).

Genes encoding proteins required for assembly of a particular reductase are typically found clustered with the structural gene for that particular reductase. For example, in those bacteria encoding a cd_1-type nitrite reductase, several genes whose products are involved in the synthesis of d_1 heme will be required for the production of an active enzyme. Systematic inactivation of genes closely linked to the nitrite reductase structural gene has identified a number of genes whose products are involved in heme d_1 biosynthesis (Kawasaki, 1997; Palmedo, 1995). Although this has led to the identification of a set of genes uniquely required for heme synthesis during denitrification, the details of the biosynthesis of heme d_1 are not elucidated as yet.

The organization of the genes for assembly of the cd_1 protein is dissimilar in every denitrifier. In *P. aeruginosa*, eleven adjacent genes have been identified whose products are suggested to be involved in nitrite reductase activity (Arai, 1994). These genes are transcribed in the same direction and are postulated to be transcribed in a single transcript (Kawasaki, 1997). In *P. stutzeri*, this cluster of genes has been rearranged so that the genes are no longer adjacent and at least three different transcripts are produced (Palmedo, 1995).

The total number of proteins that is required for expression of an active copper-containing nitrite reductase would be expected to be less than with the heme-type nitrite reductase. Examination of sequence flanking the structural gene encoding the copper-type nitrite reductase in *N. gonorrhoeae* (Roe, 1999), *N. meningitidis* (Sanger Centre, 1999), *R. sphaeroides* 2.4.3 and *Pseudomonas* G-179 (Bedzyk, 1999) revealed only one other conserved gene. In *R. sphaeroides* 2.4.3, this undesignated gene is located about 200 bp downstream of the putative translation stop of nirK (the nitrite reductase structural gene). Preliminary evidence suggests it is transcribed from the nirK transcription start. The role of the product of this gene is unclear. The difference in the amount of DNA required to produce an active copper-containing nitrite reductase versus the heme-containing nitrite reductase is notable. In *R. sphaeroides* 2.4.3, about 2.5 kb are required to encode the copper-

containing nitrite reductase and the accompanying gene of unknown function. In *P. aeruginosa*, about 10 kb appear to be required to code for proteins necessary for assembly of an active nitrite reductase.

The structural genes for the heterodimeric form of nitric oxide reductase, designated norC and norB, have been sequenced from a number of denitrifiers. In every case, the transcription start precedes norC and the gene order is norCB. The complete nor operon consists of norCB and one or two additional genes. For example, in *P. denitrificans* and *R. sphaeroides* norQ and norD follow norB (Bartnikas, 1997; De Boer, 1996). In *P. aeruginosa* and *P. stutzeri*, norD follows norB (Arai, 1995; Zumft, 1994). In both these pseudomonads norQ (designated nirQ) is present, but it is immediately upstream, and divergently transcribed from the structural gene for nitrite reductase, nirS (Arai, 1994; Jungst, 1992). Inactivation of norQ or norD leads to a loss of nitric oxide reductase activity but does not appear to inhibit assembly of nitric oxide reductase (Jungst, 1992; Mitchell, 1998; De Boer, 1996). This suggests the likelihood that both NorQ and NorD are accessory proteins required for the assembly of nitric oxide reductase. One possible role would be insertion of non-heme iron.

Neither norQ nor norD has been found in those denitrifiers encoding the single subunit type of nitric oxide reductase. There are no obvious orthologs of either norQ or norD present in the chromosome of *Synechocystis* sp. strain PCC 6803 (Kaneko, 1996). Nor have norQ or norD orthologs been identified in the ongoing genome sequencing efforts in *N. gonorrhoeae* or *N. meningitidis*. The absence of these proteins in these denitrifiers is somewhat surprising given the sequence similarity of the single subunit and heterodimeric nitric oxide reductases. The sequence of the *R. eutropha* norB is preceded by an open reading frame (ORF) encoding a protein containing a high percentage of His residues (Cramm, 1997). It is possible the product of this gene might play a role in assembly of an active nitric oxide reductase.

R. eutropha is unusual in that it contains two nitric oxide reductase structural genes (Cramm, 1997). One of these, norB, is located on a megaplasmid while the other, norZ, is located on the chromosome. This is the only bacterium described in which functionally redundant terminal N-oxide oxidoreductases have been observed. It is also interesting that in *R. eutropha* norZ does not appear to be tightly linked to the nitrite reductase structural gene. *R. eutropha* contains a cd_1-type nitrite reductase. This genetic organization is different from those bacteria containing a heterodimeric nitrite oxide reductase and a cd_1-type nitrite reductase.

In *P. denitrificans*, there are two additional genes, designated norE and norF, whose products appear to be required for nitric oxide reductase activity (De Boer, 1996). These genes are located immediately downstream of the norCBQD cluster. Insertional inactivation of either norE or norF reduces nitric oxide reductase activity but does not significantly affect its expression. Orthologs of norE have been found in other denitrifiers, for example, the ORF175 protein in *P. stutzeri* (Glockner, 1996) and in *Pseudomonas* sp. G-179 (Bedzyk, 1999). However, no norE ortholog has been identified in *R. sphaeroides* or in those bacteria encoding the single subunit nitric oxide reductase. No obvious norF orthologs have been identified, but genes whose products have some similarity to norF have been described in *P. stutzeri* (ORF82) and in *Pseudomonas* sp. G-179 (Bedzyk, 1999). The role of norE and norF remain undetermined. However, because of its similarity to the subunit III of cytochrome oxidases, it has been suggested NorE is a third subunit of the nitric oxide reductase protein complex (De Boer, 1996). Experimental conformation for NorE as a part of an active nitric oxide reductase complex is lacking.

Nitrous Oxide Reductase While the nir and nor gene clusters are often linked, the location of the nos gene cluster (nos refers to genes related to nitrous oxide reductase and not to genes for nitric oxide synthase) relative to other denitrification genes is more variable. In *P. stutzeri*, the genes encoding nitrous oxide, nitrite and nitric oxide reductase are within a stretch of about 30 kb (Jungst, 1991). In *P. aeruginosa*, the nitrous oxide reductase gene cluster is not as tightly linked to the nitrite and nitric oxide reductase genes (Vollack, 1998). However, the genes for all four nitrogen oxide reductases are located within the 20- to 36-min segment of the *P. aeruginosa* chromosome. In several denitrifiers, including *R. eutropha* (Zumft, 1992), *Sinorhizobium meliloti* (Holloway, 1996) and *R. capsulatus* (Rhodobacter, 1999), nos genes are found on plasmids. By comparison, all denitrifiers characterized in detail have both nir and nor located on the chromosome. The variable location of the nos cluster may reflect nitrous oxide's lack of toxicity, and therefore the accumulation of nitrous oxide that would follow the loss of nitrous oxide reductase activity has only limited consequences.

Gene organization within the nos cluster is much more conserved than is that in the nir or nor clusters. In almost every sequence, nosR is immediately upstream of nosZ, and nosZ is typically followed by nosDFYL. Undefined genes clustered with nosZ are likely to be involved in assembly of an active nitrous oxide reductase,

perhaps in copper incorporation. Inactivation of nosF, nosD or nosY leads to production of an inactive nitrous oxide reductase (Zumft, 1990). Sequence analysis indicates that nosY encodes a membrane-bound protein, nosD a periplasmic protein, and nosF a cytoplasmic nucleotide-binding protein. It has been suggested these proteins form a complex involved in copper processing and insertion into nitrous oxide reductase (Zumft, 1997).

In *S. meliloti*, a gene that has been designated nosX follows nosL. Inactivation of nosX causes a loss of nitrous oxide reductase activity in *S. meliloti* (Chan, 1997). Possible nosX orthologs have been found in other denitrifiers including "*A. cycloclastes*" (McGuirl, 1998) and *B. japonicum* (Genbank accession number {AJ002531}). A gene encoding a product similar to the nosX product has also been identified in *P. denitrificans*, but since it is part of the nir gene cluster it has been designated nirX (Genbank accession number AJ001308). A nosX ortholog has not been identified as yet in the peudomonads. The role of nosX is unclear but current data do not suggest a role in copper processing.

Isolation of mutants of *P. stutzeri* unable to use nitrous oxide as sole terminal oxidant led to the isolation of an additional gene whose product is required for nitrous oxide reductase activity. This gene was designated nosA, and it was shown to encode an outer membrane protein that is required for copper transport into the cell (Lee, 1991; Lee, 1989). Inactivation of nosA results in expression of the nitrous oxide reductase apoprotein. Putative nosA orthologs have been found in other denitrifiers, including *P. aeruginosa* (Yoneyama, 1996), but nosA is not found in the nos cluster of *P. stutzeri*. This organization may be because the nosA product is playing a more general role in cell physiology.

Nitrate Reductase

Of the various nitrate reductases, the respiratory and periplasmic forms have been studied in the most detail in the context of denitrification. Genes encoding the respiratory nitrate reductase, designated nar, have been completely sequenced in *P. aeruginosa* (Genbank accession number {Y15252}) and partially sequenced in *P. denitrificans* (Berks, 1995) and *Pseudomonas fluorescens* (Philippot, 1997). The nar genes are not clustered with other genes required for denitrification in *P. aeruginosa* (Vollack, 1998). The relatively limited interest in the nar genes in denitrifiers is due to the extensive study of nar genes in *E. coli* and other non-denitrifiers.

Genes encoding the periplasmic nitrate reductase have been characterized in several denitrifiers including *P. denitrificans* GB17 (Berks, 1995) and *R. sphaeroides* (Reyes, 1996). The structural genes for this nitrate reductase are napA and napB: napA encodes the molybdopterin cofactor and napB encodes the cytochrome c containing subunit. These two genes are clustered with other genes that have been designated napCDE. The napC gene encodes a membrane-bound cytochrome c, which is the likely electron donor for the periplasmic nitrate reductase; napD encodes a cytoplasmic protein and napE a small membrane protein. The function of these genes' products is unknown. The nap genes of *P. aeruginosa* are present on the chromosome but are not tightly linked to nar or other denitrification-related gene clusters (Vollack, 1998). In *R. eutropha* (Siddiqui, 1993)and *R. sphaeroides* strain 2.4.1 (Schwintner, 1998), the nap genes are localized on plasmids. In *R. sphaeroides* IL106, the nap genes are apparently located on the chromosome (Schwintner, 1998). In *Pseudomonas* G-179, the nap genes are part of a large cluster which includes nir and nor genes (Bedzyk, 1999).

Additional Genes Required for Denitrification

A few other genes frequently associated with nitrogen oxide gene clusters deserve mention. One is hemN, which encodes an oxygen-independent coproporphyrinogen oxidase (Gibson, 1992). *R. sphaeroides* encodes two hemN paralogs, the second of which is designated hemZ (Zeilstra-Ryalls, 1995). The *R. sphaeroides* hemN is clustered with the genes encoding nitric oxide reductase (unpublished). Inactivation of hemN (which was originally designated hemF) inhibits the ability to grow anaerobically under any conditions tested (Gibson, 1992). Aerobic growth is not affected because there is an oxygen-dependent form of this enzyme. The role of the hemZ product is unclear. Interestingly, hemZ is adjacent to fnrL whose product is an important regulator of anaerobically expressed genes (Zeilstra-Ryalls, 1995). These two genes are clustered with the ccoN operon that encodes the cbb_3-type oxidase. The cbb_3-type oxidase is a heme-copper enzyme and is the oxidase with the highest level of similarity to nitric oxide reductase (Saraste, 1994). It is notable then that both hemZ and hemN are clustered with related terminal oxidoreductases and regulatory proteins important in maintaining anaerobic physiology. It is possible that this gene arrangement may have occurred by gene duplication providing further evidence for the evolutionary link between aerobic respiration and denitrification.

The hemN gene is clustered with denitrification genes in *Pseudomonas* sp. G-179 (Bedzyk, 1999) and is clustered with the ccoN genes in both *P. denitrificans* (van Spanning, 1997) and *P. aeruginosa* (Zumft, 1997). A hemN ortholog has not been found in the nor cluster in *P. denitrificans* or *P. aeruginosa*. The regulation of hemN in *P. aeruginosa* (discussed in more detail below) further emphasizes the importance of the activity of the hemN product during denitrification (Rompf, 1998).

Sequencing of the nor cluster in *R. sphaeroides* (Bartnikas, 1997) and nos region in *P. stutzeri* (Glockner, 1996) has revealed a gene present in both regions whose product may be important to the physiology of denitrification. This gene has been designated nnrS in *R. sphaeroides* and orf396 in *P. stutzeri* (Fig. 5). The product of the genes from both bacteria is a membrane protein probably containing twelve membrane-spanning regions. In *R. sphaeroides*, nnrS is expressed only during denitrification and is regulated by NnrR, which also regulates nirK and nor (unpublished). Inactivation of nnrS has no obvious effect on growth under any conditions (unpublished). Though nnrS orthologs have not been found in the denitrification gene clusters of other well studied denitrifiers, examination of ongoing genomic sequencing efforts reveal the presence of nnrS orthologs in every denitrifier. In *R. capsulatus*, a nnrS ortholog is present on a plasmid and is closely linked to the nos genes (Rhodobacter, 1999). There is also an nnrS ortholog located on the chromosome. Copies of genes encoding products similar to nnrS have been identified in the *N. gonorrhoeae* (Roe, 1999), *N. meningitidis* (Sanger Centre, 1999) and *P. aeruginosa* chromosome (Pseudomonas, 1999), although none of these are clustered with denitrification genes. Significantly, nnrS orthologs have not been found in non-denitrifying bacteria. The function of nnrS has not been determined, but it is obviously not essential for denitrification or it would likely have been isolated in mutant screens. However, work in *R. sphaeroides* and its distribution among the bacteria indicates nnrS is a denitrification-associated gene. It seems likely that as more work is done on the genetics of denitrification, many genes that are not essential to, but whose products are physiologically important for, denitrification will be described.

Regulation of Genes Required for Denitrification

This section will focus primarily on the regulation of those genes encoding respiratory nitrogen oxide reductases and genes required for their assembly. As denitrification depends on the presence of nitrogen oxides, it is natural to describe denitrification genes as part of a stimulon, a term that refers to operons responding together to a particular environmental stimulus (Neidhardt, 1990). In general, the regulators of denitrification can be differentiated into the nitrate, nitric oxide, and nitrous oxide stimulons. The organization of denitrification genes roughly reflects the organization of the stimulons. The nitrate and nitrous oxide stimulons are primarily made up of the nar and nos gene clusters, respectively. These two, independently regulated, gene clusters are not linked to each other and are frequently distant from other denitrification-related clusters. The nitric oxide stimulon is made up of both the nir and nor clusters. These two gene clusters are the most strongly linked of any of the denitrification gene clusters.

As denitrification is, in most cases, an anaerobic process, other stimulons and regulons required for anaerobic growth overlap the denitrification-related stimulons. This overlap can make it difficult to differentiate regulatory factors that directly modulate gene expression from those that indirectly affect gene expression. This discussion will focus on those proteins that current data suggest are directly involved in regulation of the nitrogen oxide reductases. It is important to note that a particular stimulon will likely include genes whose products are not directly required for denitrification and, consequently, not covered in this review. However, this does not minimize the usefulness of organizing denitrification genes into stimulons.

One other important consideration is the relationship of denitrification and oxygen respiration. Denitrification is primarily an alternative form of respiration inasmuch as oxygen is a preferred oxidant. However, this does not imply that all denitrifiers have the same set point at which oxygen respiration is switched to nitrogen oxide respiration. Available data suggest that the onset of expression of denitrification genes occurs over a wide range of oxygen concentrations. The best example of an aerobic denitrifier is *P. denitrificans* GB17, which was originally described as an aerobic denitrifier (Robertson, 1984). In addition, other strains have been described as aerobic denitrifiers (Bonin, 1991; Patureau, 1994; van Niel, 1992; Frette, 1997; Robertson, 1995; Ka-Jong, 1997). None of the intensively studied model denitrifiers activate gene expression, even at moderate concentrations of oxygen. Therefore, the molecular mechanisms that permit aerobic denitrification are not currently understood.

Nitrate Stimulon

The presence of nitrate and a reduction in oxygen tension stimulates

```
NmNnrS          ------------------MKFT-KHPVWAMA---FRPFYSLAALYGALSVLLWGFGYT-
NgNnrS          ------------------MKFT-KHPVWAMA---FRPFYSLAALYGALSVLLWGFGYT-
PsORF396        ------------MQUIDRRKALS-IAPIWRLA---FRPFFLAGSLYALLAIPBWVAWWTG
PaNnrS          --------------------MA-IPPIWRLG---FRPFFLGGALFAVLAIALWLAALAG
RsNnrS          ------------MASDRPRTYT-GPALLSYG---FRPFFLLSALFAAGAUPUWLAUWS-
RcNnrS          MAHPHLDSPAAGRHHPDIPAAGAPMTALLRLLSDSFRUFFLLASLWAAAAMALWLWWLWQ
                                            ** *:    *       :*

NmNnrs          ---G-THELSGFY----WHAHEMIWGYAGLUUIAFLLTAUATWTGBGHQPPTRGGULTIF
NGNNRS          ---G-THELSGFY----WHAHEIWGYAGLUUIAFLLTAUATWTGQPPPTRGGULUGLTAF
PsORF396        LWPG-FQPTGGWLA---WHRHEMLFGFAMAIUAGFLLTAUQTWTGQTAPSGNRLUGLAAU
PsNnrS          LWSG-FQPTGGWLA---WHRHEMLFGFGUAIIAGFLLTAUQTWTGUPGLQGRPLALLAGL
RcNnrS          ---G-RIGLAGPFSPIDWHIHEMLFGYTSAUIAGFLFTAIPNWTGRMPRRGLPLAALAAL
RcNnrS          NQIGPGGDLPNALAPSHWHAHELIFGFGMAATAGFFLTAAPNWTGKPUAGPRFIALMAGL
                   *             ** **:::*:  :: :*::**:  ***        ::  :  :

NmNnrs          WLAARIAA-FIPGWGASASGILGTLFFWYGAUCMALPUIRSQNQRNYUAUFALFULGGTH
NGNNRS          WLAARIAA-FIPGWGAAASGILGTLFFWYGAUCMALPUIRSQNRRNYUBAFAIFULGGTH
PsORF396        WLAARLG--WLFGLPAAWLAPLDLLFLUALUWMMAQMLWAUPQKRNYPIUUULSLMLGAD
PsNnrS          WLAARLA--WLFDAPLALLLULQLSFLPLLAWAIGRSLWRURQKRNYPUUGLLLLLTLAD
RcNnrS          WIAGRFAUAGAFGTDPLLULUIDAGFLLAUTLMAUIEIAAGKNWKNLMUUGPUGLYLAAN
RcNnrS          WLAGRGAULLWGSUPPULAAGUULAFPALLTERMARQLIRRPASSEGLYLALLGLITLAE
                *:* *                :   *    -      :           :  : :

NmNnrs          AAFHUQLHNGNLGGLLSGLQSGLUMUSGFIIGLIGTRISFFTSKRLN-----UPQIPSPK
NGNNRS          AAFHUQLHNGNLGGLLSGLQSGLUMUSGFIGLIGMRIISFFTSFFLN-----UPQIPSPK
PsORF396        ULILTGLLQGNDALQRQGULAGLWLUAALMALIGGRUIPFFTQRGLGK----UDAUKPWU
PsNnrS          ALULLGLFEGNDDWQRRASIAALWLIAGMMNLIGGRUIPFFTQRGLGR----QQQUPAIA
RcNnrS          ULFHLEAMQ--QGESDIGRPLGFATUTFLIMLIGGRIIPSFTRNWLAKG--GPGPLPUPF
RcNnrS          ARULLDWLDLPPGDAAAGLRGGLAALUALUAULGGRITPQFTRNALARAGAPPAALPRSF
                : :   :               ::       ::* *: _ **   *         :

NmNnrs          -WUAQASLWLPMLTAMLMAHGU----MPWLSAAFAFAAGUIFTUQUYRWWYKPULKEPML
NGNNRS          -WUAQASLWLPMLTAILMAHGU----MPWLSAAFAFAAUUIFTVQVYRWWYKPVLKEPML
PsORF396        -WLDUALLUGTGUIALLHAFGUAMRPQPLLGLLFU-AIGVGHLLRLMRWYDKGIWKVGLL
PsNnrS          -WLDNGILLGCULUALLTAAGUTTQPTPWLAGLFA-ALGGAQLWRLWRWRDRILWQVPLL
RcNnrS          -GRFDGASLLUAUGALLCUTLA---PDAILTAALLALAAALHUURLURWRGHLUWRSPLL
RcNnrS          PWLDRSVAGCACLAALAAUFPL----SGALAGAAALALGAGQLARMGFWRSRKVLGNPLL
                            : *::           :*  ::: ::       :   *    :    :*

NmNnrs          WILFAGYLFTGLGLIAUGASYFKPAFLN-LGUHLIGUGGIGULTLGMMARTALGHTGNPI
NGNNRS          WILFAGYLFTGLGLIAUGASYFKPAFLN-LGUHLIGUGGIGULTLGMMARTALGHTGNSI
PsORF396        WSLHUAMLWLUUAAFGLALWHFGLLAQSSPSLHALSUGSMSGLILAMIARUTLGHTGRPL
PsNnrS          WSLHLAYFWIAUAPLGMALWSLGLALAPSQSLHALAUGGMGGLILAMLARUTLGHTGRAL
RcNnrS          LMMHUAYGFLPLGLAATAAAAUGWASAP-AGLHLLGIGAIGGMTLAUMMRASLGHTGRPL
RcNnrS          AALHLAMLGTGIGALLQGFAAFGRGEEI-GALHVSAGUGGMILAUMADSRATLAQTGRAL
                : *       :  :      :         :*   :  :  : * :: *  * :**  :

NmNnrs          UPPPKAUPUAFWLMMAATAURMUARFSSGTAYTH---SIRTSSVLFALALLVYAWKYIPW
NGNNRS          YPPPKAUPUAFWLMMAATAURMUAUFSSGTAYTH---SIRTSSULFALALLUYAWKYIPW
PsORF396        QLP-AGIIGAFULFNLGTAAR---UFLSUAWPUG---GLWLAAVCWTLAFALYUWRYAPM
PsNnrS          QPP-AAMPWAFALLNLGCAAR---UFLPSLLPANW--ALPLAGGLWALAFLLFAWFYAPM
RcNnrS          EAG-RALSAGFACIUAAAAAR--TULAATDIGGID--GYSLAAAFWTMGFAIYVGEIGPC
RcNnrS          VAP-RPVVLAYLLLPLAALAR----FAAATWPETYVAAMLSAAALWVLAFALFALSSAPA
                      :   :  : :  ::*    :                   : : :::::    *

NmNnrs          LIRPRSDGRP--G
NGNNRS          LIRPRSDGRP--G
PsORF396        LUAARUDGHP--G
PsNnrS          LCRPRUDGHP--G
RcNnrS          LLRPSLGRTPSKG
RcNnrS          FLGPR--QTP---
                :        *
```

Fig. 5.

expression of the nar genes. Because nitrate respiration leads to the production of the other denitrification intermediates, it can be difficult in wild type cells to demonstrate that nitrate is the effector for only a limited set of genes. Evidence for a nitrate stimulon was then demonstrated by experiments using nitrite reductase-deficient cells. These experiments showed that nitrate alone was not sufficient to activate expression of genes whose products are required for reduction of the other nitrogen oxides (Tosques, 1997; Korner, 1993). Nor does nitrite alone cause significant induction of nitrate reductase. However, there is evidence of cross talk between the nitrate and nitrous oxide stimulons in *P. stutzeri* and *P. denitrificans* (Baumann, 1996; Korner, 1989), although it is difficult to rationalize why this occurs. Experiments monitoring gene expression in relation to oxygen concentration show that the nar genes and nitrate reductase are expressed at higher oxygen levels than the nir and nor genes (Baumann, 1996). Expression of nos occurs at similar oxygen levels.

The molecular mechanisms required for nar activation have not been extensively studied in a denitrifying bacterium. A pair of two component sensor-regulators is responsible for regulating nar in *E. coli*. The function of these proteins has been extensively studied and has been reviewed in Darwin (1996). A nitrate sensor (NarL) and its response regulator (NarX) have been characterized in *P. stutzeri*. However, their deletion did not affect denitrification (Hartig, 1999). This has led to the suggestion that there is a nitrate-responsive system that functions independently of the NarXL system.

Nitric Oxide Stimulon Even before isolation of the proteins regulating the genes encoding the nitrite and nitric oxide reductases, it had been observed that the expression of nitric acid reductase in nitrite reductase mutants was negatively affected. This dependence of nitric oxide reductase expression on the activity of nitrite reductase has been demonstrated in many denitrifiers (Ye, 1992; Tosques, 1997; de Boer, 1994; Zumft, 1994). The decrease in nor gene expression in nitrite reductase mutants appears indirect, as any mutation that leads to a loss of nitrite reductase activity has a negative affect on nor expression (de Boer, 1994; Zumft, 1994). The expression of nir genes is also dependent on nitrite reductase activity (Tosques, 1997). The expression of nir is not directly dependent on nitric oxide reductase, but the accumulation of nitric oxide in nitric oxide reductase mutants probably affects nitrite reductase activity (Kwiatkowski, 1996; Zumft, 1994).

The observation that it is nitrite reductase activity not nitrite reductase per se that is required for the expression of nir and nor genes suggests that a product of nitrite reduction is required for gene expression. An obvious candidate for the likely effector is nitric oxide, a possibility consistent with the observation that addition of nitric oxide generators to nitrite reductase-deficient strains results in expression of both nir and nor genes (Kwiatkowski, 1996). Moreover, trapping of nitric oxide by hemoglobin decreases expression of nir and nor genes (Kwiatkowski, 1996). The accumulated evidence strongly indicates that it is the production of nitric oxide that stimulates expression of those genes in the nitric oxide stimulon. However, it is also possible that a derivative of nitric oxide may be the actual signal.

The role of nitric oxide as a signal molecule in humans is well known (Schmidt, 1994). The first use of nitric oxide as a signal molecule by a living organism, however, was likely by denitrifying bacteria. It is easy to rationalize why nitric oxide or some derivative serves as a signal molecule for denitrifiers. Denitrifying bacteria must keep the steady state levels of nitric oxide low to minimize potential cytotoxic reactions. As nitric oxide acts to regulate expression of the genes whose products are responsible for establishing the steady state levels of nitric oxide, the possibility nitric oxide might accumulate to cytotoxic levels is abated. Also, as it reacts rapidly with oxygen, nitric oxide will accumulate to the levels required to activate gene expression only when oxygen tension is low. This permits a single molecule to be used as an indicator of both oxygen and nitrogen oxide concentrations in the environment. The direct control of nitric oxide levels by oxygen may explain why nitrite reductase is expressed at lower oxygen concentrations than nitrate and nitrous oxide reductase.

In several denitrifiers, a gene has been isolated whose product directly regulates nir and nor expression but not nar or nos genes. The regulation of nir and nor by a single regulatory protein not involved in regulating expression of the other nitrogen oxide reductases is consistent with studies of gene expression indicating three separate stimulons. Not surprisingly, this gene has been given a different name in each bacterium. In *P. denitrificans*, it is designated nnr (van Spanning, 1995), nnrR in *R. sphaeroides* (Tosques, 1996), dnr in *P. aeruginosa* (Arai, 1995) and dnrD in *P. stutzeri* (Hartig, 1999). The family of proteins encoded by these genes will be referred to as the Nnr family in this review. Recent sequencing efforts have also identified likely orthologs in *Synechocystis* strain PCC 6803 (Kaneko, 1996), *Pseudomonas* sp. G-179 (Bedzyk, 1999) and *R. capsulatus* (Rhodobacter, 1999). All of these proteins are members of the

Fnr/CRP family of transcriptional regulators (Spiro, 1994).

Comparison of the sequences of these various proteins reveals little about how they might interact with an effector. Significantly, there are no obvious metal binding motifs in any of these proteins. Fnr and CooA, which are also members of the Fnr/CRP family, have metal centers that are targets for effector interaction. Fnr from *E. coli* binds an iron-sulfur center that apparently undergoes structural changes as the oxygen concentration in the cell changes (Popescu, 1998). CooA from *Rhodospirillum rubrum* binds a heme protein that acts as a carbon monoxide sensor (Shelver, 1997). Current data is not consistent with members of the Nnr family containing any type of metal center.

Phylogenetic analysis of the Nnr family reveals that the relatedness of the proteins does not coincide with relatedness predicted by 16S rRNA analysis. For example, *R. sphaeroides* is closely related to both *R. capsulatus* and *P. denitrificans*. The Nnr from *P. denitrificans* and the putative Nnr ortholog identified in *R. capsulatus* have significant identity but have only limited similarity to NnrR from *R. sphaeroides* (Fig. 6). This suggests species relatedness is not the major factor controlling the degree of relatedness of the members of the Nnr family.

Based on available data, a model can be presented in which nitrite produced by nitrate reductase activity is reduced by nitrite reductase, and some compound (most likely nitric oxide or a nitric oxide derivative) activates the transcriptional regulator resulting in expression of genes in the nitric oxide stimulon. Even though this model may be generally correct, differences appear in the regulation of the expression of various members of the Nnr family. In *R. sphaeroides*, NnrR appears to be constitutively expressed but may be negatively autoregulated (Tosques, 1996). There is no evidence of negative autoregulation of Nnr in *P. denitrificans* (van Spanning, 1995). Expression of Dnr in *P.* may be regulated by another member of the Fnr/Crp family, Anr, an apparent ortholog of Fnr in *E. coli* (Arai, 1995). This suggests a regulatory hierarchy where under low oxygen tension Anr activates expression of dnr, whose product can then activate expression of nir and nor genes under appropriate conditions. This type of regulatory hierarchy is not present in *P. stutzeri* (Hartig, 1999).

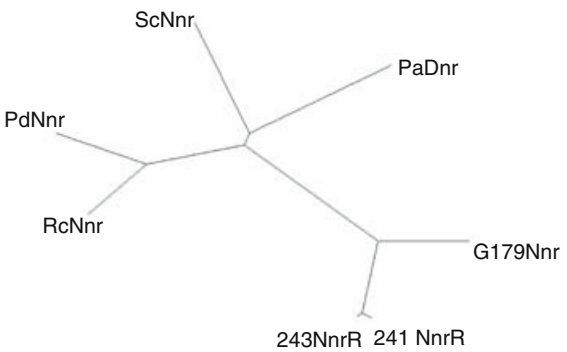

Fig. 6.

In *P. aeruginosa*, Dnr and Anr both regulate hemN, which encodes an oxygen-independent coproporphyrinogen oxidase required for heme synthesis (Rompf, 1998). The hemN gene is also coregulated by NnrR and FnrL in *R. sphaeroides* (Shapleigh, unpublished). Both FnrL and NnrR regulate the gene encoding pseudoazurin in *R. sphaeroides* (Jain and Shapleigh, unpublished). This dual regulation of selective genes by both a global regulator such as Anr/Fnr and a regulator of a limited set of genes such as Dnr/NnrR suggests that transcriptional activation by the global regulator alone is not sufficient for optimal growth under denitrification conditions. Therefore, denitrifiers have developed mechanisms to ensure sufficient levels of expression of genes whose products are in heavy demand during denitrification and other modes of anaerobic growth. Further, in *R. sphaeroides* nitrite accumulation negatively affects expression of FnrL regulated genes (Shapleigh, unpublished). This may provide another explanation as to why FnrL and NnrR are dual regulators of specific genes. It seems likely such dual regulation will be observed in other denitrifiers and will probably encompass a larger set of gene targets.

Nitrous Oxide Stimulon Evidence for a nitrous oxide stimulon was initially provided by the observation that growth on nitrous oxide strongly stimulates nitrous oxide reductase expression, modestly stimulates expression of the nitrate reductase, and does not stimulate expression of nitrite or nitric oxide reductase (Korner, 1989). The observation that inactivation of the regulator of genes in the nitric oxide stimulon did not affect growth at the expense of nitrous oxide provided additional support for existence of a set of genes whose transcription depends solely on the presence of nitrous oxide.

The nature of the genes responsible for regulating nitrous oxide expression is not well defined. One gene suggested to play a role in regulation of nos genes is nosR. Inactivation of nosR in *P. stutzeri* inhibited expression of nosZ, the nitrous-oxide-reductase structural gene (Cuypers, 1992). NosR is a putative membrane protein containing a cytoplasmic C-terminal domain with two motifs that resemble [Fe-S] containing motifs. This unusual combination of a membrane bound, [Fe-S] protein involved

in expression of a copper-containing protein increases interest in this protein. It seems unlikely NosR is involved in copper metabolism, as inactivation of nosR results in the inhibition of nosZ transcription (Cuypers, 1992). In contrast, inactivation of nosFDY, suggested to be involved in copper metabolism, does not cause inhibition of nosZ transcription (Zumft, 1990).

Other Regulatory Proteins Searches for regulatory proteins required for expression of denitrification genes are only just beginning to identify possible regulators and to determine their physiological roles. Although the majority of the genes discussed in the preceding sections have been found in most denitrifiers, other putative regulatory proteins have been identified in only a single bacterium. One example is nirI, a gene implicated in regulation of expression of nirS, the structural gene for nitrite reductase in *P. denitrificans* (Genbank accession number AJ001308). Interestingly, the nirI product is similar to NosR, which is required for expression of nos genes. The involvement of NirI in nitrite reductase expression makes it less likely that NosR is involved in nitrous oxide reductase assembly. Instead, NosR and NirI are likely members of a family of proteins involved in regulation of nitrogen oxide metabolism in denitrifiers. Other regulatory proteins have been found clustered with denitrification genes, but their role in denitrification has not been defined. Clearly, our understanding of the regulation of denitrification genes is very limited. The list of the various proteins involved in regulation will expand, no doubt, as additional denitrifiers are characterized in greater detail.

Metabolism of Related Nitrogen Oxides

Because of its reactivity, nitric oxide will react with many compounds generating a wide range of different nitrogen oxide containing molecules. Some of these derivatives are more toxic than nitric oxide. For example, nitric oxide can react with the thiol of glutathione to generate S-nitrosoglutathione (GSNO), which has been shown to be more toxic for *Salmonella typhimurium* than nitric oxide (De Groote, 1995). Another very toxic nitric oxide derivative, peroxynitrite, is generated from the reaction of nitric oxide and superoxide (Squadrito, 1998). There has been a great deal of interest in the interaction of these types of compounds with pathogenic bacteria inasmuch as they are generated as part of the host defense mechanism during infection. These compounds could possibly be generated during denitrification as well. If so, denitrifiers have probably developed mechanisms to mitigate the potentially cytotoxic effects of such derivatives.

Despite the paucity of work on the metabolism of nitric oxide derivatives by denitrifiers, some data suggest that denitrifiers are useful models for understanding how cells mitigate nitric oxide toxicity. In experiments assessing the sensitivity of *R. sphaeroides* strains to GSNO, those strains with nitrite reductase activity were completely resistant to levels of GSNO to which *S. typhimurium* exhibited sensitivity (Wu, 1998). Comparable assays on a naturally occurring nitrite reductase-deficient strain of *R. sphaeroides* demonstrated that its sensitivity to GSNO was similar to that exhibited by *S. typhimurium*. There was no indication that the resistant strain had any special capacity to degrade GSNO suggesting the likely modification of a GSNO-sensitive target in the resistant strain. The results of this study need to be extended to other denitrifiers to determine whether general resistance to GSNO is intrinsic to denitrifiers. Also, it will be interesting to determine if denitrifying bacteria have any resistance mechanisms to other toxic nitric oxide derivatives such as peroxynitrite. Probing the molecular mechanisms denitrifiers have developed for resistance to nitric oxide derivatives is of broad scientific interest and is further justification for the study of the diverse group of bacteria linked by their shared capacity to reduce nitrate and nitrite to gaseous nitrogen oxides and nitrogen gas.

Acknowledgments. I would like to thank W. J. Payne for critically reading the manuscript. I would also like to thank him for nurturing my interest in microbiology and introducing me to denitrification. I would also like to thank my colleagues working in this field for their contributions. The work in my laboratory is funded by the U.S. Department of Energy.

Literature Cited

Adman, E. T., Godden, J. W., and Turley, S. 1995. The structure of copper-nitrite reductase from Achromobacter cycloclastes at five pH values, with NO_2- bound and with type II copper depleted. J. Biol. Chem. 270:27458–27474.

Ahlers, B., Konig, W., and Bock, E. 1990. Nitrite reductase activity in Nitrobacter vulgaris. FEMS Microbiol. Lett. 67:121–126.

Anders, H. J., Kaetzke, A., Kaempfer, P., Ludwig, W., and Fuchs, G. 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the

genera Thauera, as Thauera aromatica sp. nov., and Azoarcus, as Azoarcus evansii sp. nov., respectively, members of the beta subclass of the Proteobacteria. Int. J. System. Bacteriol. 45:327–333.

Antholine, W. E., Kastrau, D. H. W., Steffens, G. C. M., Buse, G., Zumft, W. G., and Kroneck, P. M. H. 1992. A comparative EPR investigation of the multicopper proeins nitrous-oxide reductase and cytochrome c oxidase. Eur. J. Biochem. 209:875–881.

Arai, H., Igarashi, Y., and Kodama, T. 1995. Expression of the nir and nor genes for denitrification of Pseudomonas aeruginosa requires a novel CRP-FNR-related transcriptional regulator, DNR, in addition to ANR. FEBS Lett. 371:73–76.

Arai, H. Y., Igarashi, Y., and Kodama, T. 1995. The structural genes for nitric oxide reductase from Pseudomonas aeruginosa. Biochim. Biophys. Acta 1261:279–284.

Arai, H., Yasuo, I., and Kodama, T. 1994. Structure and ANR-dependent expression of the nir genes for denitrification from Pseudomonas aeruginosa. Biosci. Biotech. Biochem. 58:1286–1291.

Baker, S. C., Ferguson, S. J., Ludwig, B., Page, M. D., Richter, O. M. H., and van Spanning, R. J. M. 1998. Molecular genetics of the genus Paracoccus: metabolically versatile bacteria with bioenergetic flexibility. Microbiol. Mol. Biol. Rev. 62:1046–1078.

Balderston, W. L., Sherr, B., and Payne, W. J. 1976. Blockage by acetylene of nitrous oxide reduction in Pseudomonas perfectomarinus. Appl. Env. Microbiol. 31:504–508.

Bartnikas, T. B., Tosques, I. E., Laratta, W. P., Shi, J., and Shapleigh, J. P. 1997. Characterization of the region encoding the nitric oxide reductase of Rhodobacter sphaeroides 2.4.3. J. Bacteriol 179:3534–3540.

Baumann, B., Snozzi, M., Zehder, A. J. B., and van der Meer, J. R. 1996. Dynamics of denitrification activity of Paracoccus denitrificans in continuos culture during aerobic-anaerobic changes. J. Bacteriol. 178:4367–4374.

Baumgartner, M., Sameluck, F., Bock, E., and Conrad, R. 1991. Production of nitric oxide by ammonium-oxidizing bacteria colonizing building stones. FEMS Microbiol. Lett. 85:95–100.

Bedzyk, L., Wang, T., and Ye, R. W. 1999. The periplasmic nitrate reductase in Pseudomonas sp. strain G-179 catalyzes the first step in denitrification. J. Bacteriol. 181:2802–2806.

Bell, L. C., Richardson, D. J., and Ferguson, S. J. 1990. Periplasmic and membrane-bound respiratory nitrate reductases in Thiosphaera pantotropha. The periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett. 265:85–87.

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

Berks, B. C., Page, M. D., Richardson, D. J., Reilly, A., Cavill, A., Outen, F., and Ferguson, S. J. 1995. Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron-carrying arm of a redox loop. Mol. Microbiol. 15:319–331.

Berks, B. C., Richardson, D. J., Reilly, A., Willis, A. C., and Ferguson, S. J. 1995. The napEDABC gene cluster encoding the periplasmic nitrate reductase system of Thiosphaera pantotropha. Biochem. J. 309:983–992.

Bonin, P., and Gilewicz, M. 1991. A direct demonstration of co-respiration of oxygen and nitrogen oxides by Pseudomonas nautica: some spectral and kinetic properties of the respiratory components. FEMS Microbiol. Lett. 80:183–188.

Braun, C., and Zumft, W. G. 1992. The structural genes of the nitric oxide reductase complex from Pseudomonas stutzeri are part of a 30-kilobase gene cluster for denitrification. J. Bacteriol. 174:2394–2397.

Carter, J. P., Hsiao, Y. H., Spiro, S., and Richardson, D. J. 1995. Soil and sediment bacteria capable of aerobic nitrate respiration. Appl. Environ. Microbiol. 61:2852–2858.

Chan, Y.-K., McCormick, W. A., and Watson, R. J. 1997. A new nos gene downstream from nosDFY is essential for dissimilatory reduction of nitrous oxide by Rhizobium (sinorhizobium) meliloti. Microbiol. 143:2817–2824.

Chang, C. K., Timkovich, R., and Wu, W. 1986. Evidence that heme d_1 is a 1,3 porphyrindione. Biochemistry 25:8447–8453.

Cheesman, M. R., Zumft, W. G., and Thomson, A. J. 1998. The MCD and EPR of the heme centers of nitric oxide reductase from Pseudomonas stutzeri: evidence that the enzyme is structurally related to the heme-copper oxidase. Biochemistry 37:3994–4000.

Coyne, M. S., Arunakumari, A., Averill, B. A., and Tiedje, J. M. 1989. Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. 55:2924–2931.

Cramm, R., Siddiqui, R. A., and Friedrich, B. 1997. Two isofunctional nitric oxide reductases in Alcaligenes eutrophus H16. J. Bacteriol. 179:6769–6777.

Cutruzzola, F., Arese, M., Grasso, S., Bellelli, A., and Brunori, M. 1997. Mutagenesis of nitrite reductase from Pseudomonas aeruginosa: tyrosine-10 in the c heme domain is not involved in catalysis. FEBS Lett. 412:365–369.

Cuypers, H., Viebrock-Sambale, A., and Zumft, W. G. 1992. NosR, a membrane-bound regulatory component necessary for expression of nitrous oxide reductase in denitrifying Pseudomonas stutzeri. J. Bacteriol. 174: 5332–5339.

Darwin, A. J., and Stewart, V. J. 1996. The NAR modulon systems:nitrate and nitrite regulation of anaerobic gene expression. Lin, E. C. C. and Lynch, A. S. Regulation of gene expression in Escherichia coli. R. G. Landes Co.. Austin, TX.

de Boer, A. P., Reijnders, W. N., Kuenen, J. G., Stouthamer, A. H., and van Spanning, R. J. 1994. Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in Paracoccus denitrificans. Antonie Van Leeuwenhoek. 66:111–127.

De Boer, A. P. N., Reijnders, W. N. M., Van der Oost, J., Stouthamer, A. H., and Van Spanning, R. J. M. 1996. Mutational analysis of the nor gene cluster encoding nitric oxde reductase from Paracoccus denitrificans. Eur. J. Biochem. 242:592–600.

De Groote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R., and Fang, F. C. 1995. Genetic and redox determinants of nitric oxide cytotoxicity in a Salmonella typhimurium model. Proc. Natl. Acad. USA 92:6399–6403.

Denariaz, G., Payne, W. J., and Legall, J. 1991. The denitrifying nitrite reductase of Bacillus halodenitrificans. Biochim. Biophys. Acta 1056:225–232.

Dermastia, M., Turk, T., and Hollocher, T. C. 1991. Nitric oxide reductase: purification from Paracoccus denitrificans with use of a single column and some charcteristics. J. Biol. Chem. 266:10899–10905.

Dias, J. M., Than, M. E., Humm, A., Huber, R., Bourenkov, G. P., Bartunik, H. D., Bursakov, S., Calvete, J., Caldeira, J., Carneiro, C., Moura, J. J., Moura, I., and Romao, M. 1999. Crystal structure of the first dissimilatory nitrate reductase at 1.9 A solved by MAD method. Structure Fold. Des. 7:65–79.

Dispirito, A. A., Taaffe, L. R., Lipscomb J. D., and Hooper, A. B. 1985. A blue copper oxidase from Nitrosomonas europaea. Biochim. Biophys. Acta 827:320–326.

Dodd, F. E., Van Beeumen, J., Eady, R. R., and Hasnain, S. S. 1998. X-ray structure of a blue-copper nitrite reductase in two crystal forms. The nature of the copper sites, mode of substrate binding and recognition by redox partner. J. Mol. Biol. 282:369–382.

Dreusch, A., Riester, J., Kroneck, P. M. H., and Zumft, W. G. 1996. Mutation of the conserved Cys165 outside the CuA domain destabilizes nitrous oxide reductase but maintains its catalytic activity: evidence for disulfide bridges and a putaitve disulfide isomerase gene. Eur. J. Biochem. 237:447–453.

Farrar, J. A., Thompson, A. J., Cheesman, M. R., Dooley, D. M., and Zumft, W. G. 1991. A model of the copper centres of nitrous oxide reductase (Pseudomonas stutzeri) Evidence from optical, EPR and MCD spctroscopy. FEBS Lett. 294:11–15.

Farrar, J. A., Zumft, W. G., and Thomson, A. J. 1998. CuA and CuZ are variants of the electron transfer center in nitrous oxide reductase. Proc. Natl. Acad. USA 95:9891–9896.

Fesefeldt, A., Kloos, K., Bothe, H., Lemmer, H., and Gliesche, C. G. 1998. Distribution of denitrification and nitrogen fixation genes in Hyphomicrobium spp. and other budding bacteria. Can. J. Microbiol. 44:181–186.

Fossing, H., Gallardo, V. A., Jorgensen, B. B., Huttel, M., Nielsen, L. P., Schulz, H., Canfield, D. E., Forster, S., Glud, R. N., Gunderson, J. K., Kuver, J., Ramsing, N. B., Teske, A., Thamdrup, B., and Ulloa, O. 1995. Concentration and transport of nitrate by the mat-forming sulphur bacterium Thioploca. Nature 374:713–715.

Frette, L., Gejlsbjerg, B., and Westermann, P. 1997. Aerobic denitrifiers isolated from an alternating activated sludge system. FEMS Microbiol. Ecol. 24:363–370.

Friedrich, C., and Mitrenga, G. 1981. Oxidation of thiosulfate by Paracoccus denitrificans and other hydrogen bacteria. FEMS Microbiol. Ecol. 10:209–212.

Fries, M., Zhou, J., Chee, S. J., and Tiedje, J. M. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802–2810.

Fülöp, V., Moir, J. W. B., Ferguson, S. J., and Hajdu, J. 1995. The anatomy of a bifunctional enzyme: Structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome cd_1. Cell 81:369–377.

Gayon, U., and Dupetit, G. 1886. Recherches sur la reduction des nitrates par les infinement petits. Mem. Soc. Sci. Phys. Nat. Bordeaux. Ser. 3:201–307.

Gayon, U., and Dupetit, G. 1882. Sur la fermentation des nitrates. C. R. Acad. Sci. 95:644–646.

Gibson, L. C. D., McGlynn, P., Chaudri, M., and Hunter, C. N. 1992. A putative anaerobic coproporphyrinogen III oxidase in Rhodobacter sphaeroides. II. Analysis of a region of the genome encoding hemF and the puc operon. Mol. Microbiol. 6:3171–3186.

Girsch, P., and de Vries, S. 1997. Purification and initial kinetic and spectroscopic characterization of NO reductase from Paracoccus denitrificans. Biochim. Biophys. Acta 1318:202–216.

Glockner, A. B., Juengst, A., and Zumft, W. G. 1993. Copper containing nitrite reductase from Pseudomonas aureofaciens is functional in a mutationally cytochrome Cd-free background nirs- negative of Pseudomonas stutzeri. Arch. Microbiol. 160:18–26.

Glockner, A. B., and Zumft, W. G. 1996. Sequence analysis of an internal 9.72-kb fragment from the 30-kb denitrification gene cluster of Pseudomonas stutzeri. Biochim. Biophys. Acta 1277:6–12.

Godden, J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., Payne, W. J., and Legall, J. 1991. The 2.3 angstrom X-ray structure of nitrite reductase from Achromobacter cycloclastes. Science 253:438–442.

Goreau, T. J., Kaplan, W. A., Wofsy, S. C., Mcelroy, M. B., Valois, F. W., and Watson, S. W. 1980. Production of nitrite and nitrous oxide by nitrifying bacteria at reduced concentrations of oxygen. Appl. Environ. Microbiol. 40:526–532.

Gorny, N., Wahl, G., Brune, A., and Schink, B. 1992. A strictly anaerobic nitrate reducing bacterium growing with resorcinol and other aromatic compounds. Arch. Microbiol. 158:48–53.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Rossnagel, P., Burggraf, S., Huber, H., and Stetter, K. O. 1996. Ferroglobus placidus gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe2+ at neutral pH under anoxic conditions. 166:308–314.

Halfen, J. A., Mahapatra, S., Wilkinson, E. C., Gengenbach, A. J., Young, V. G., Que, L., and Tolman, W. B. 1996. Synthetic modeling of nitrite binding and activation by reduced copper proteins: characterization of copper(I)-nitrite complexes that evolve nitric oxide. J. Amer. Chem. Soc. 118:763–776.

Hartig, E., and Zumft, W. G. 1999. Kinetics of nirS expression (Cytochrome cd_1 nitrite reductase) in Pseudomonas stutzeri during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. J. Bacteriol. 181:161–166.

Holloway, P., McCormick, W., Watson, R. J., and Chan, Y. K. 1996. Identification and analysis of the dissimilatory nitrous oxide reduction genes, nosRZDFY, of Rhizobium meliloti. J. Bacteriol. 178:1505–1514.

Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., Koenig, H., Rachel, R., Rockinger, I., Fricke, H., and Stetter, K. O. 1992. Aquifex pyrophilus, new genus new species, represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Sys. Appl. Microbiol. 15:340–351.

Hulse, C. L., and Averill, B. A. 1990. Isolation of a high specific activity pink, monomeric nitrous oxide reductase from Achromobacter cycloclastes. Biophys. Biochem. Res. Comm. 166:729–735.

Inatomi, K. I., and Hochstein, L. I. 1996. The purification and properties of a copper nitrite reductase from Haloferax denitrificans. Curr. Microbiol. 32:72–76.

Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. 1995. Structure at 2.8Ã resolution of cytochrome c oxidase from Paracoccus denitrificans. Nature 376: 660–669.

Jensen, B. B., and Burris, R. H. 1986. N2O as a substrate and as a competitive inhibitor of nitrogenase. Biochemistry 25:1083–1088.

Jones, A. M., Adkins, A. M., Knowles, R., and Rayat, G. R. 1990. Idenitification of a denitrifying gliding bacterium isolated from soil and able to reduce nitrous oxide in the presence of sulfide and acetylene as Flexibacter canadensis. Can. J. Microbiol. 36:765–770.

Jones, A. M., Hollocher, T. C., and Knowles, R. 1992. Nitrous oxide reductase of Flexibacter canadensis: a unique membrane-bound enzyme. FEMS Microbiol. Lett. 95:205–210.

Jungst, A., Braun, C., and Zumft, W. G. 1991. Close linkage in Pseudomonas stutzeri of the structural genes for respiratory nitrite reductase and nitrous oxide reductase, and other essential genes for denitrification. Mol. Gen. Genetic. 225:241–248.

Jungst, A., and Zumft, W. G. 1992. Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of nirQ, a novel gene in the denitrification gene cluster of Pseudomonas stutzeri. FEBS Lett. 314:308–314.

Ka-Jong, O., Urbance, J., Ye, R. W., Ahn, T. Y., and Tiedje, J. M. 1997. Diversity of oxygen and N-oxide regulation of nitrite reductases in denitrifying bacteria. FEMS Microbiol. Lett. 156:55–60.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). DNA Res. 3:185–209.

Kastrau, D. H. W., Heiss, B., Kroneck, P. M. H., and Zumft, W. G. 1994. Nitric oxide reductase from Pseudomonas stutzeri, a novel cytochrome bc complex. Eur. J. Biochem. 222:293–303.

Kawasaki, S., Arai, H., Kodama, T., and Igarashi, Y. 1997. Gene cluster for dissimilatory nitrite reductase (nir) from Pseudomonas aeruginosa: Sequencing and identification of a locus for heme d_1 biosynthesis. J. Bacteriol. 179:235–242.

Korner, H. 1993. Anaeroic expression of nitric oxide reductase from denitrifiying Pseudomonas stutzeri. Arch. Microbiol. 159:410–416.

Korner, H., and Zumft, W. G. 1989. Expression of denitrification enzymes in response to the dissolved oxygen and respiratory substrate in continuous culture of Pseudomonas stutzeri. Appl. Env. Microbiol. 55:1670–1676.

Kroneck, P. M. H., and Zumft, W. G. 1990. Bio-inorganic aspects of denitrification: structures and reactions of NxOy compounds and their interaction with iron and copper proteins. Revsbech, N. P. and Sfrensen, J. Denitrification in soil and sediment. Plenum Press. New York, NY. 37–55.

Kukimoto, M., Nishiyama, M., Murphy, M. E. P., Turley, S., Adman, E. T., Horinouchi, S., and Beppu, T. 1994. X-ray structure and site-directed mutagenesis of a nitrite reductase from Alcaligenes faecalis S-6: Roles of two copper atoms in nitrite reduction. Biochemistry 33:5246–5252.

Kwiatkowski, A., and Shapleigh, J. P. 1996. Requirement of nitric oxide for induction of genes whose products are involved in nitric oxide metabolism in Rhodobacter sphaeroides 2.4.3. J. Biol. Chem. 271:24382–24388.

Kwiatkowski, A. V., Laratta, W. P., Toffanin, A., and Shapleigh, J. P. 1997. Analysis of the role of the nnrR gene product in the response of Rhodobacter sphaeroides 2.4.1 to exogenous nitric oxide. J. Bacteriol. 179:5618–5620.

Lam, Y., and Nicholas, D. J. D. 1969. A nitrite reductase with cytochrome oxidase activity from Paracoccus denitrificans. Biochim. Biophys. Acta 180:459–472.

Lee, H. S., Abdelal, A. H., Clark, M. A., and Ingraham, J. L. 1991. Molecular characterization of nosA, a Pseudomonas stutzeri gene encoding an outer membrane protein required to make copper-containing N_2O reductase. J. Bacteriol. 173:5406–5413.

Lee, H. S., Hancock, R. E., and Ingraham, J. L. 1989. Properties of a Pseudomonas stutzeri outer membrane channel-forming protein (NosA) required for production of copper-containing N2O reductase. J. Bacteriol. 171:2096–2100.

Lensi, R., Beaupied, H., and Moiroud, A. 1990. Denitrifying activity in the actinorhizae. Acta Oecol. 11:391–398.

Liu, H. P., Takio, S., Satoh, T., and Yamamoto, I. 1999. Involvement in denitrification of the napKEFDABC genes encoding the periplasmic nitrate reductase system in the denitrifying phototrophic bacterium Rhodobacter sphaeroides f. sp. denitrificans. Biosci. Biotechnol. Biochem. 63:530–536.

Lu, W.-P., and Ragsdale, S. W. 1991. Reductive activation of the coenzyme A/acetyl-CoA isotopic exchange reaction catalyzed by carbon monoxide dehydrogenase from Clostridium thermoaceticum and its inhibition by nitrous oxide and carbon monoxide. J. Biol. Chem. 266:3554–3556.

Mahne, I., and Tiedje, J. M. 1995. Criteria and methodology for identifying respiratory denitrifiers. Appl. Env. Microbiol. 61:1110–1115.

Matsubara, T., and Zumft, W. G. 1982. Idenitification of a copper protein as part of the nitrous oxide-reducing system in nitrite respiring (denitrifying) pseudomonads. Arch. Microbiol. 132:322–328.

McGuirl, M. A., Nelson, L. K., Bollinger, J. A., Chan, Y. K., and Dooley, D. M. 1998. The nos (nitrous oxide reductase) gene cluster from the soil bacterium Achromobacter cycloclastes: cloning, sequence analysis, and expression. J. Inorg. Biochem. 70:155–169.

McHatton, S. C., Barry, J. P., Jannasch, H. W., and Nelson, D. C. 1996. High nitrate concentrations in vacuolate, autotrophic marine Beggiatoa spp. Appl. Env. Microbiol. 62:954–958.

Mellies, J., Jose, J., and Meyer, T. F. 1997. The Neisseria gonorrhoeae gene aniA encodes an inducible nitrite reductase. Mol. Gen. Genet. 256:525–532.

Michalski, W., and Nicholas, D. J. D. 1988. Identification of two new denitrifying strains of Rhodobacter sphaeroides. FEMS Microbiol. Lett. 52:239–244.

Michalski, W. P., Hein, D. H., and Nicholas, D. J. D. 1986. Purification and characterization of nitrous oxide reductase from Rhodopseudomonas sphaeroides f. sp. denitrificans. Biochim. Biophys. Acta 872:50–60.

Miller, D. J., and Nicholas, D. J. D. 1985. Characterization of a soluble cytochrome oxidase-nitrite reductase from Nitrosomonas europaea. J. Gen. Microbiol. 131:2851–2854.

Mitchell, D. M., Wang, Y., Alben, J. O., and Shapleigh, J. P. 1998. Fourier transform infrared analysis of membranes of Rhodobacter sphaeroides 2.4.3 grown under microaerobic and denitrifying conditions. Biochim. Biophys. Acta 1409:99–105.

Moenne-Loccoz, P., and de Vries, S. 1998. Structural characterization of the catalytic high-spin heme b of nitric oxide reductase: a resonance Raman study. J. Am. Chem. Soc. 120:5147–5152.

Murphy, M. E., Turley, S., and Adman, E. T. 1997. Structure of nitrite bound to copper-containing nitrite reductase from Alcaligenes faecalis. Mechanistic implications. J. Biol. Chem. 272:28455–28460.

Muyzer, G., Teske, A., Wirsen, C. O., and Jannasch, H. W. 1995. Phylogenetic relationship of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164:165–172.

Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates. Sunderland, MA. 382–383.

Nurizzo, D., Cutruzzola, F., Arese, M., Bourgeois, D., Brunori, M., Cambillau, C., and Tegoni, M. 1998. Conformational changes occurring upon reduction and NO binding in nitrite reductase from Pseudomonas aeruginosa. Biochemistry 37:13987–13996.

Nurizzo, D., Silvestrini, M. C., Mathieu, M., Cutruzzola, F., Bourgeois, D., Fulop, V., Hajdu, J., Brunori, M., Tegoni, M., and Cambillau, C. 1997. N-terminal arm exchange is observed in the 2.15 A crystal structure of oxidized nitrite reductase from Pseudomonas aeruginosa. Structure 15:1157–1171.

O'Hara, G. W., and Daniel, R. M. 1985. Bacterial denitrification: a review. Soil. Biol. Biochem. 17:1–9.

Olesen, K. O., Veselov, A., Zhao, Y., Wang, Y., Danner, B., Scholes, C. P., and Shapleigh, J. P. 1998. Spectroscopic, kinetic and electrochemical characterization of heterologously expressed wild type and mutant forms of copper-containing nitrite reductase from Rhodobacter sphaeroides 2.4.3. Biochem. 37:6086–6094.

Ostermeier, C., Harrenga, A., Ermler, U., and Michel, H. 1997. Structure at 2.7 resolution of the Paracoccus denitrificans two-subunit cytochrome c oxidase complexed with an antibody FV fragment. Proc. Natl. Acad. Sci. USA 94:10547–10553.

Palmedo, G., Seither, P., Korner, H., Matthews, J. C., Burkhalter, R. S., Timkovich, R., and Zumft, W. G. 1995. Resolution of the nirD locus for heme d_1 synthesis of cytochrome cd_1 (respiratory nitrite reductase) from Pseudomonas stutzeri. Eur. J. Biochem. 232:737–746.

Patureau, D., Davison, J., Bernet, N., and Moletta, R. 1994. Dentrification under various aeration conditions in Comomonas sp., strain SGLY2. FEMS Microbiol. Lett. 14:71–78.

Payne, W. J., Grant, M. A., Shapleigh, J. P., and Hoffman, P. 1982. Nitrogen oxide reduction in Wolinella succinogenes and Campylobacter species. J. Bacteriol. 152:915–918.

Philippot, L., Clays-Josserand, A., Lensi, R., Trinsoutreau, I., Normand, P., and Potier, P. 1997. Purification of the dissimilative nitrate reductase of Pseudomonas fluorescens and the cloning and sequencing of its corresponding genes. Biochim. Biophys. Acta 1350:272–276.

Popescu, C. V., Bates, D. M., Beinert, H., Munck, E., and Kiley, P. 1998. Mossbauer spectroscopy as a tool for the study of activation/inactivation of the transcription regulator FNR in whole cells of Escherichia coli. Proc. Natl. Acad. Sci. USA 95:13431–13435.

Poth, M., and Focht, D. D. 1985. 15N kinetic analysis of N2O production by Nitrosomonas europaea: an examination of nitrifier denitrification. Appl. Env. Microbiol. 49:1134–1141.

Reyes, F., Roldan, M. D., Klipp, W., Castillo, F., and Moreno-Vivian, C. 1996. Isolation of periplasmic nitrate reductase genes from Rhodobacter sphaeroides DSM 158: Structural and functional differences among prokaryotic nitrate reductases. Mol. Microbiol. 19:1307–1318.

Richardson, D. J., and Ferguson, S. J. 1992. The influence of carbon substrate on the activity of the periplasmic nitrate reductase in Thiospaera pantotropha. Arch. Microbiol. 157:535–537.

Riester, J., Zumft, W. G., and Kroneck, P. M. H. 1989. Nitrous oxide reductase of Pseudomonas stutzeri, redox properties and spectroscopic characterization of different forms of the multicopper enzyme. Eur. J. Biochem. 178:751–762.

Robertson, L. A., Dalsgaard, T., Revsbech, N. P., and Kuenen, J. G. 1995. Confirmation of "aerobic denitrification" in batch cultures, using gas chromatography and ^{15}N mass spectrometry. FEMS Microbial Eco. 18:113–119.

Robertson, L. A., and Kuenen, J. G. 1984. Aerobic denitrification: a controversy revisited. Arch. Microbiol. 139:351–354.

Roe, B. A., Lin, S. P., Song, L., Yuan, X., Clifton, S., Ducey, T., Lewis, L., and Dyer, D. W. 1999. Gonococcal Genome Sequencing Project The University of Oklahoma.

Rocourt, J., Wehmeyer, U., and Stackebrandt, E. 1987. Transfer of Listeria denitrificans to a new genus, Jonesia gen. nov., as Jonesia denitrificans comb. nov. Int. J. Syst. Bacteriol. 37:266–270.

Roldan, M. D., Sears, H. J., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C., and Richardson, D. J. 1998. Spectroscopic characterization of a novel multiheme c-type cytochrome widely implicated in bacterial electron transport. J. Biol. Chem. 273:28785–28790.

Rompf, A., Hungerer, C., Hoffmann, T., Lindenmeyer, M., Romling, U., Gross, U., Doss, M. O., Arai, H., Igarashi, Y., and Jahn, D. 1998. Regulation of Pseudomonas aeruginosa hemF and hemN by the dual action of the redox response regulators Anr and Dnr. Mol. Microbiol. 29:985–997.

Sakai, K., Ikehata, Y., Ikenaga, Y., Wakayama, M., and Moriguchi, M. 1996. Nitrite oxidation by heterotrophic bacteria under various nutritional and aerobic conditions. J. Fermen. Bioeng. 82:613–617.

Sanger Centre. 1999. These sequence data were produced by the Microbial Genome Sequencing Group at the Sanger Centre (http://www.sanger.ac.uk/Projects/N_meningitidis/).

Saraste, M., and Castresana, J. 1994. Cytochrome oxidase evolved by tinkering with denitrification enzymes. FEBS Lett. 341:1–4.

Sato, K., Okubo, A., and Yamazaki, S. 1998. Characterization of a multi-copper enzyme, nitrous oxide reductase, from Rhodobacter sphaeroides f. sp. denitrificans. J. Biochem. 124:51–54.

Sawada, E., and Satoh, T. 1980. Periplasmic location of dissimilatory nitrate and nitrite reductases in a denitrifying phototrophic bacterium, Rhodopseudomonas sphaeroides forma sp. denitrificans. Plant Cell Physiol. 21:205–210.

Schedel, M., and Truper, H. 1980. Anaerobic oxidation of thiosulfate and elemental sulfur in Thiobacillus denitrificans. Arch. Microbiol. 124:205–210.

Schindelin, H., Kisker, C., Hilton, J., Rajagopalan, K. V., and Rees, D. C. 1996. Crytal structure of DMSO reductase: Reodx-linked changes in molybdopterin coordination. Nature 272:1615–1621.

Schmidt, H. H. H. W., and Walter, U. 1994. NO at work. Cell 78:919–925.

Schulz, H. N., Brinkhoff, T., Ferdelman, T. G., Marinél Hernálndez, M., Teske, A., and Jørgensen, B. B. 1999. Dense populations of a giant sulfur bacterium in Namibian shelf sediments. Science 284:493–495.

Schwintner, C., Sabaty, M., Berna, B., Cahors, S., and Richaud, P. 1998. Plasmid content and localization of the genes encoding the denitrification enzymes in two strains of Rhodobacter sphaeroides. FEMS Microbiol. Lett. 165:313–321.

Scott, R. A., Zumft, W. G., Coyle, C. L., and Dooley, D. M. 1989. Pseudomonas stutzeri N_2O reductase contains Cu_A-type sites. Proc. Natl. Acad. Sci. USA 86:4082–4086.

Sears, H. J., Ferguson, S. J., Richardson, D. J., and Spiro, S. 1993. The idenitfication of a periplasmic nitrate reductase in Paracoccus denitrificans. FEMS Microbiol. Lett. 113:107–112.

Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. 1997. CooA, a CO-sensing transcription factor from Rhodospirillum rubrum, is a CO-binding heme protein. Proc. Natl. Acad. Sci. USA 94:11216–11220.

Shoun, H., Kano, M., Baba, I., Takaya, N., and Matsuo, M. 1998. Denitrification of actinomycetes and purification of dissimilatory nitrite reductase and azurin from Streptomyces thioluteus. J. Bacteriol. 180:4413–4415.

Siddiqui, R. A., Warnecke-Eberz, U., Hengsberger, A., Scneider, B., Kostka, S., and Friedrich, B. 1993. Structure and function of a periplasmic nitrate reductase in Alcaligenes eutrophus H16. J. Bacteriol. 175:5867–5876.

Snyder, S. W., and Hollocher, T. C. 1987. Purification and some characteristics of nitrous oxide reductase from Paracoccus denitrificans. J. Biol. Chem. 262:6515–6525.

Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96:2563–2605.

SooHoo, C. K., and Hollocher, T. C. 1991. Purification and characterization of nitrous oxide reductase from Pseudomonas aeruginosa strain P2. J. Biol. Chem. 266:2203–2209.

Spiro, S. 1994. The FNR family of transcriptional regulators. Antonie von Leeuwenhoek. 62:23–36.

Springer, N., Ludwig, W., Philipp, B., and Schink, B. 1998. Azoarcus anaerobius sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium. Int. J. Sys. Bacteriol. 48:953–956.

Squadrito, G. L., and Pryor, W. A. 1998. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. Free Radic. Biol. Med. 25:392–403.

Stamler, J. S., Singel, D. J., and Loscalzo, J. 1992. Biochemistry of nitric oxide and its redox-activated forms. Science 258:1898–1902.

Swartzlander, J. H., Rehberger, T. G., and Hibberd, C. A. 1993. In vivo denitrification by a selected strain of Propionibacterium acidipropionici in beef cattle. J. Anim. Sci. 71:277–277.

Sweerts, J. P. R. A., DeBeer, D., Nielsen, P., Verduow, H., Heuvel, J. C. V., Cohen, Y., and Cappenberg, T. E. 1990. Denitrification by sulfur oxidizing Beggiatoa spp. mats on freshwater sediments. Nature 344:762–763.

Teraguchi, S., and Hollocher, T. C. 1989. Purification and some characteristics of a cytochrome c-containing nitrous oxide reductase from Wolinella succinogenes. J. Biol. Chem. 264:1972–1979.

Timmer-ten Hoor, A. 1975. A new type of thiosulphate-oxidizing, nitrate reducing microorganism: Thiomicrospira denitrificans sp.nov. Neth. J. Sea Res. 9:344–350.

Tosques, I. E., Kwiatkowski, A. V., Shi, J., and Shapleigh, J. P. 1997. Characterization and regulation of the gene encoding nitrite reductase in Rhodobacter sphaeroides 2.4.3. J. Bacteriol. 179:1090–1095.

Tosques, I. E., Shi, J., and Shapleigh, J. P. 1996. Cloning and characterization of nnrR, whose product is required for the expression of proteins involved in nitric oxide metabolism in Rhodobacter sphaeroides 2.4.3. J. Bacteriol. 178:4958–4964.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. 1995. Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å. Science 269:1069–1074.

Usuda, K., Toritsuka, N., Matsuo, Y., Kim, D. H., and Shoun, H. 1995. Denitrification by the fungus Cylindrocarpon tonkinense: anaerobic cell growth and two isozyme forms of cytochrome P-450nor. Appl. Env. Microbiol. 61:883–889.

van der Oost, J., de Boer, A. P. N., De Gier, J.-W. L., Zumft, W. G., Stouthamer, A. H., and van Spanning, R. J. M. 1994. The heme-copper oxidase family consists of three distinct types of oxidases and is related to nitric oxide reductase. FEMS Microbiol. Lett. 121:1–9.

van Niel, E. W., Braber, K. J., Robertson, L. A., and Kuenen, J. G. 1992. Heterotrophic nitrification and aerobic denitrification in Alcaligenes faecalis strain TU. Antonie von Leeuwenhoek. 62:231–237.

van Schie, P. M., and Young, L. Y. 1998. Isolation and characterization of phenol-degrading denitrifying bacteria. Appl. Env. Microbiol. 64:2432–2438.

van Spanning, R. J. M., De Boer, A. P. N., Reijnders, W. N. M., Westerhoff, H. V., Stouthamer, A. H., and Van Der Oost, J. 1997. FnrP and NNR of Paracoccus denitrificans are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. Mol. Microbiol. 23:893–907.

van Spanning, R. J. M., DeBoer, A. P. N., Reijnders, W. N. M., Spiro, S., Westerhoff, H. V., Stouthamer, A. H., and Van der Oost, J. 1995. Nitrite and nitric oxide reduction in Paracoccus denitrificans is under the control of NnrR, a regulatory protein that belongs to the FNR family of transcriptional activators. FEBS Lett. 360:151–159.

Verkhovskaya, M. L., Garcia-Horsman, A., Puustinen, A., Rigaud, J. L., Morgan, J. E., Verkhovsky, M. I., and Wikstrom, M. 1997. Glutamic acid 286 in subunit I of cytochrome bo3 is involved in proton translocation. Proc Natl Acad Sci USA. 94:10128–10131.

Viebrock, A., and Zumft, W. G. 1988. Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide

reductase from denitrifying Pseudomonas stutzeri. J. Bacteriol. 170:4658–4668.

Volkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A., and Stetter, K. O. 1993. Pyrobaculum aerophilum sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. Appl. Environ. Microbiol. 59:2918–2926.

Vollack, K. U., Xie, J., Hartig, E., Romling, U., and Zumft, W. G. 1998. Localization of denitrification genes on the chromosomal map of Pseudomonas aeruginosa. Microbiol. 144:441–448.

Vorholt, J. A., Hafenbradl, D., Stetter, K. O., and Thauer, R. K. 1997. Pathways of autotrophic CO_2 fixation and of dissimilatory nitrate reduction to N_2O in Ferroglobus placidus. Arch. Microbiol. 167:19–23.

Warnecke-Eberz, U., and Friedrich, B. 1993. Three nitrate reductases activities in Alcaligenes eutrophus. Arch. Microbiol. 159:405–409.

Wharton, D. C., and Gibson, Q. C. 1976. Cytochrome oxidase from Pseudomonas aeruginosa IV. Reaction with oxygen and carbon monoxide. Biochim. Biophys. Acta 292:611–620.

Williams, P. A. Fülöp, V., Garman, E. F., Saunders, N. F., Ferguson, S. J., and Hajdu, J. 1997. Haem-ligand switching during catalysis in crystals of a nitrogen-cycle enzyme. Nature 389:406–412.

Wu, Q., Storrier, G. D., Wu, K. R., Shapleigh, J. P., and Abru__, H. D. 1998. Electrocatalytic reduction of S-nitrosoglutathione at electrodes modified with an electropolymerized film of a pyrrole derived viologen system and their application to cellular S-nitrosoglutathione determinations. Anal. Biochem. 263:102–112.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M. 1992. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36:1251–1257.

Ye, R. W., Arunakumari, A., Averill, B. A., and Tiedje, J. M. 1992. Mutants of Pseudomonas fluorescens deficient in dissimilatory nitrite reduction are also altered in nitric oxide reduction. J. Bacteriol. 174:2560–2564.

Ye, R. W., Averill, B. A., and Tiedje, J. M. 1994. Denitrification: production and consumption of nitric oxide. Appl. Env. Microbiol. 60:1053–1058.

Yoneyama, H., and T. Nakae, T. 1996. Protein C (OprC) of the outer membrane of Pseudomonas aeruginosa is a copper-regulated channel protein. Microbiol. 142:2137–2144.

Yoshinari, T. 1980. N_2O reduction by Vibrio succinogenes. Appl. Env. Microbiol. 39:81–84.

Yoshinari, T., and Knowles, R. 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Comm. 69:705–710.

Zeilstra-Ryalls, J. H., and S. Kaplan, S. 1995. Aerobic and anaerobic regulation in Rhodobacter sphaeroides 2.4.1: the role of the fnrL gene. J. Bacteriol. 177:6422–6431.

Zhang, C.-s., Hollocher, T. C., Kolodziej, A. F., and Orme-Johnson, W. H. 1991. 1991. Electron paramagnetic resonance observations on the cytochrome c-containing nitrous oxide reductase from Wolinella succinogenes. J. Biol. Chem. 266:2199–2202.

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

Zumft, W. G., Braun, C., and Cuypers, H. 1994. Nitric oxide reductase from Pseudomonas stutzeri: Primary structure and gene organization of a novel bacterial cytochrome bc complex. Eur. J. Biochem. 219:481–490.

Zumft, W. G. A., Dreusch, A., Lochelt, S., Cuypers, H., Friedrich, B., and Schneider, B. 1992. Derived amino acid sequence of the nosZ gene (respiratory N2O reductase) from Alcaligenes eutrophus, Pseudomonas aeruginosa and Pseudomonas stutzeri reveal potential coppper-binding sites. Eur. J. Biochem. 208:31–40.

Zumft, W. G., and Matsubara, T. 1982. A novel kind of multicopper protein as terminal oxidoreductase of nitrous oxide respiration in Pseudomonas perfectomarinus. FEBS Lett. 148:107–112.

Zumft, W. G. A., Viebrock-Sambale, A., and Braun, C. 1990. Nitrous oxide reductase from denitrifying Pseudomonas stutzeri: genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. Eur. J. Biochem. 192:591–599.