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**MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF
NITRATE/NITRITE TRANSPORT IN DENITRIFYING**

***Pseudomonas aeruginosa* PAO1**

Dissertation

Submitted to

The College of Arts and Sciences of the

UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Doctor of Philosophy in Biology

by

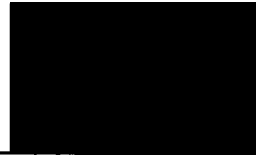
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Dayton, Ohio

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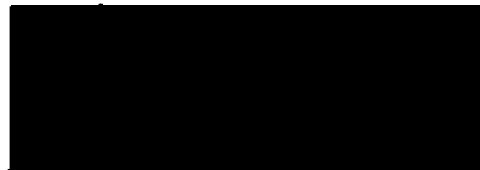
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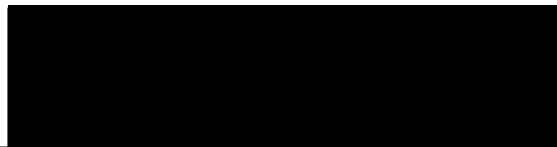
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ABSTRACT

MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF NITRATE/NITRITE TRANSPORT IN DENITRIFYING *Pseudomonas aeruginosa* PAO1

Sharma, Vandana
University of Dayton, 2006

Advisor: Dr. J. J. Rowe

Two transmembrane proteins were tentatively classified as NarK1 and NarK2 in the *Pseudomonas* genome project and hypothesized to play an important physiological role in nitrate/nitrite transport in *Pseudomonas aeruginosa*. The *narK1* and *narK2* genes are located in a cluster along with the structural genes for the nitrate reductase complex. Our studies indicate that the transcription of all of these genes is initiated from a single promoter and the gene complex *narK1K2GHJI* constitutes an operon. Utilizing isogenic mutants of *narK1*, *narK2* and a *narK1K2* double mutant, we explored their effect on growth under denitrifying conditions. While the $\Delta narK1::Gm$

mutant was only slightly affected in its ability to grow under denitrifying conditions, both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ were found to be severely restricted in nitrate dependent anaerobic growth. All three strains demonstrated wild-type levels of nitrate reductase activity. Nitrate uptake by whole cell suspensions demonstrated both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants to have very low yet different nitrate uptake rates while the $\Delta narK1::Gm$ mutant exhibited wild-type levels of nitrate uptake. Finally, *E. coli* K-12 *narK* rescued both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants with respect to anaerobic respiratory growth. Our results indicate that only the NarK2 protein is required as a nitrate and or nitrite transporter by *Pseudomonas aeruginosa* under denitrifying conditions.

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INTRODUCTION

Pseudomonas aeruginosa is an ubiquitous environmental bacterium found in soil, water and clinical specimens. In nature, *P. aeruginosa* is metabolically very versatile due to its ability to use a myriad of energy sources and adapt to different microenvironments (63). Of great biological significance is its ability to metabolize nitrate for two physiologically different purposes and by two different pathways. During aerobic or anaerobic conditions, in the absence of any other nitrogen source, the organism will use nitrate for biosynthetic purposes by the process of assimilation (134). Secondly, under strictly anaerobic conditions, the organism will use nitrate as a terminal electron acceptor for anaerobic respiration by a process termed denitrification (111). Denitrification involves four separate nitrogen oxide reductases and ultimately reduces nitrate to dinitrogen (111).

The process of denitrification has important environmental, agricultural and public health implications and has, therefore, become a major research topic in recent years. One of the intermediates namely, nitrous oxide, is a green-house gas and is partially responsible for the destruction of the ozone layer. Denitrification is also responsible for the removal of organic nitrogen in the terrestrial environment and prevents eutrophication in the aquatic environment. The process has important

applications in water treatment plants where it serves to remove nitrate from wastewater (194).

From a public health perspective, denitrifiers like *P. aeruginosa* are a growing concern. This is because opportunistic pathogens like *P. aeruginosa* have the ability to cause infections in immunocompromised individuals such as burn, AIDS and cystic fibrosis patients. Recent studies of this nosocomial pathogen in CF airways indicated that in these patients, the organism utilizes its ability to survive and proliferate in an anaerobic environment by the process of nitrate respiration (69, 190, 193). More recent investigations of the CF airways indicate that a mutant form of *P. aeruginosa*, *mucA*, is actually responsible for the chronic lung infections in CF patients. This mutant form of *P. aeruginosa* harbors low nitrite reductase activity thereby reducing its capability to metabolize nitrite (192). In lieu of these findings, Yoon and coworkers have shown that the use of acidified nitrite results in killing these mucoid *P. aeruginosa* strains and may ultimately aid in eradicating this organism from CF airways (192).

Nitrate transport across the cytoplasmic membrane is the first step of the denitrification pathway (110). It is a key step because the catalytic site of the nitrate reductase enzyme is oriented towards the cytoplasm and the enzyme reduces nitrate to nitrite at the inner face of the cytoplasmic membrane (134). In contrast, the third step of denitrification, the reduction of nitrite to nitric oxide occurs in the periplasm (194). Thus, with the sites of reduction of the two oxyanions at opposite sides of the membrane, transport systems for both nitrate and nitrite are required. It has been

postulated that for the purpose of anaerobic nitrate respiration, bacteria rely solely on secondary transporters (110).

The project described in this dissertation utilized *P. aeruginosa* PAO1 as a model system for the study of the transport of nitrate during denitrification. The main focus of the study was to identify, confirm and characterize two transport systems of *P. aeruginosa* PAO1 encoded by *narK1* and *narK2* and the function of their corresponding proteins. In determining the overall importance and function of these proteins, isogenic mutants of *narK1*, *narK2* and *narK1K2* were created and their phenotype with respect to anaerobic growth and nitrate respiration determined. Subsequently, physiological studies were performed to reveal possible roles for each in anaerobic metabolism. The results of this study will be discussed in detail in Chapter 3 of this dissertation.

In closing, we started this study because the specific proteins involved in nitrate and or nitrite transport in *P. aeruginosa* had not yet been identified. Characterization of these proteins was important both from the aforementioned public health & environmental perspective as well as from a research perspective. For a reasearcher, examination of bacterial transport systems provides an opportunity to gain insight into a wide range of topics in the area of membrane transport such as, the regulation of transport protein synthesis by environmental factors, the localization of transport proteins to their extra-cytoplasmic destinations and also their specific arrangement in the cell membrane. Our preliminary results from peptide sequence alignment and hydropathy profiles had indicated that the NarK1 and NarK2 proteins

were required as nitrate and nitrite transporters, respectively. Thus, we hypothesized that the NarK1 may be a nitrate uptake protein while the NarK2 may be a nitrite extrusion protein. We expected that a strain that is devoid of both proteins would be rendered incapable of growth anaerobically with nitrate as the terminal electron acceptor. Using genetic and biochemical tools, we set out to elucidate the requirement of these two proteins in *P. aeruginosa* denitrification. The results of this study are discussed more extensively in Chapter 3 of this dissertation.

Chapter 1

Part I

Literature Review

NITRATE/NITRITE TRANSPORT IN PROKARYOTES

1. Introduction to membrane transport

All living cells, from unicellular to multicellular organisms, are enclosed by a biological membrane called the cell membrane. Although the composition of cell membranes differs markedly depending on their various functions, and with the type of organism and its environmental conditions, there is a common thread of structural organization that involves protein and lipid components. The primary backbone of the cytoplasmic membrane is a lipid bilayer stabilized by sterols such as cholesterol or sterol-like compounds called hopenoids. The lipid bilayer possesses a hydrophobic core and hydrophilic surfaces and serves as a semi-permeable barrier, maintaining the cytoplasm as a metabolic soup. Associated with or integrated in the lipid bilayer are proteins termed peripheral or integral proteins. These proteins function physiologically in transport of nutrients, surface binding of nutrients, export of toxic substances, respiration, signal transduction, and in the generation of ATP. The general model for the biological membrane is the fluid mosaic model in that it is a mosaic of protein and lipid. In this model the membrane is proposed to be kept physiologically functional by maintaining a constant crystalline/fluid state in various environmental conditions partly through the modification of the fatty acids covalently linked to the glycerol molecule. For example, at temperatures below 15°C a higher percentage of unsaturated fatty acids are found whereas at temperatures above 45°C

more saturation is observed. The cell membrane acts as a regulatory barrier for the exchange of chemical species, or water from the cells external environment to its intracellular milieu.

Most solutes are unable to pass across the cytoplasmic membrane without a protein carrier that is often termed transporter, porter or permease. This is particularly true of any ion or organic compound with a positive or negative charge.

2. General classification of transporters

Transport proteins are embedded in the membrane and catalyze the specific transport of solutes across the membrane. The importance of transport proteins is illustrated by the fact that a relatively large number of genes encoded on chromosomes are predicted to code for transport proteins, e.g. 18.7 % of the *P. aeruginosa* proteome (98), 6% on the genome of *Saccharomyces cerevisiae* (177) and around 10% on the *Escherichia coli* genome (123).

Usually, transport across the membrane requires metabolic energy and transport proteins can be classified on the basis of their energy requirements. In prokaryotes such as the *Eubacteria* and *Archaea*, four classes of transport proteins can be distinguished:

- Channels, which allow selective transport of solutes down a concentration gradient usually without the need of metabolic energy. They essentially function as selective pores that open in response to a chemical or

electrophysiological stimulus. The glycerol facilitator of *E. coli*, a member of the major intrinsic protein (MIP) family, is an example (120).

- Primary transporters, which are energized by hydrolysis of ATP, photon absorption, electron flow, substrate decarboxylation, or methyl transfer. Examples are the linear electron transfer chains (e.g., cytochrome oxidase), the light driven ion pumps (e.g., bacteriorhodopsin), the sodium-ion translocating decarboxylases, and the ATP-driven transporters (e.g., FoF1-ATPase, members of the ABC super family of binding protein dependent transport systems and others) (74, 91).
- Secondary transporters, often use the electrochemical ion gradients usually protons or sodium, to drive solute transport across the membrane. Generally, these systems are composed of a single protein that binds and translocates the solutes, but some secondary transport systems employ substrate-binding proteins similar to that of binding protein-dependent uptake systems that belong to the ABC superfamily (79).
- Group Translocation Systems, couple translocation to chemical modification of the substrate as it crosses the membrane. Best known are the bacterial phosphotransferase systems that use energy derived from the hydrolysis of phosphoenolpyruvate (PEP) for translocation of sugars. The glucose permease of *E. coli*, a member of the glucose PTS (glc) family, is an example (131, 148).

Bacterial solute transporters are classified as primary transporters and secondary transporters, respectively. Primary solute transporters such as the ATP Binding Cassette (ABC) superfamily contain an ATP hydrolyzing protein that directly couples transport to the release of free energy. These proteins generally consist of multimeric complexes containing nucleotide binding domains, integral membrane domains and an extracellular binding protein. This class of proteins will not be discussed in detail here. Secondary transporters are widely distributed in bacteria and are found in all types of prokaryotic and eukaryotic cells. Most secondary transporters consist of a single polypeptide, an integral membrane protein with 11-15 transmembrane helices. In most bacteria, secondary transporters are the most common class of transporters. In *E. coli* and *Bacillus subtilis*, secondary transport proteins are 1.5 to three-fold more prevalent than ABC transporters (123). *Bacillus subtilis* possesses a remarkably large number of secondary transporters relative to other systems, 68% of all transport proteins are of the secondary type (122).

3. Secondary transporters

Secondary transporters are a class of proteins that are found in all living cells ranging from Prokaryotes to Eukaryotes (104). In secondary transport, the energy of an electrochemical gradient of one solute is used to drive the transport of another solute (including ions). Secondary transport systems can be subdivided in three categories: uniport, symport and antiport.

- Uniport: The transport is called uniport when it is driven by the concentration gradient of the substrate or the electrochemical gradient if it is a charged molecule moving against a concentration gradient. No coupling ions participate in the translocation process. An example is the glucose transporter of *Zymomonas mobilis* and nitrite in *P. aeruginosa* (139, 184).
- Symport: Symport occurs when more than one substrate moves in the same direction. In this case, the electrochemical gradient of one solute (usually proton or sodium-ion) is used to drive the uphill transport of another solute. Since the direction of secondary transport is dependent on the direction of the gradients (both charge and concentration) of transported solutes, excretion of intracellularly formed metabolic end-products may in special cases lead to the generation of a electrochemical gradient (107). The *E. coli* lactose permease of the major facilitator superfamily provides an example (84).
- Antiport: Finally, when substrates move in opposing directions, transport is referred to as antiport. Depending on the charge and stoichiometry of the substrates, the total driving force for the different transport reactions may differ considerably (125). A $\text{Na}^+:\text{H}^+$ antiporter in *Agrobacterium tumefaciens* provides an example (85).

Many transport proteins have been discovered in bacteria, archaea, fungi, plants, animals, and mammals but we have a limited understanding about the molecular mechanisms by which the secondary transporters operate (70, 104, 105, 119, 124, 125, 130). A major reason for this is the difficulty in solving the structure of

membrane proteins. Therefore, most studies exploring the structure/function relationships of these proteins have relied on less sensitive yet reliable methods such as circular dichroism spectroscopy, analysis of amino acid sequence, hypothetical modeling and hydropathy profiles.

4. Driving force of secondary transporters

Secondary transporters use the energy derived from the establishment of ion gradients across the membrane. The cytoplasmic membrane is basically impermeable to ions, which allows for the generation of electrochemical gradients of protons or sodium ions across the membrane that can subsequently be used to drive energy requiring processes such as ATP synthesis, solute and ion transport, flagellar motion, etc. The electrochemical gradients of H^+ and Na^+ are directed inward and exert a force on the ions, termed the proton motive force (PMF) and sodium ion motive force (SMF), respectively (89). In aerobic bacteria, metabolic energy is mainly generated by the primary transport systems of the respiratory chain (electron transfer chain). These systems use the redox energy from electron donors to translocate protons across the membrane. Phototrophic bacteria can derive energy from light to generate electrochemical ion gradients (proton pumps powered in response to green sunlight, mediated by bacteriorhodopsin). In fermentative bacteria that depend only on the energy derived from substrate level phosphorylation, the electrochemical gradients are generated by membrane bound ATPases that use the energy derived from ATP hydrolyses to transport protons or sodium ions out of the cell. More recently,

secondary metabolic energy generating systems were identified (90). In these systems, secondary transport systems convert electrochemical gradients of solutes into electrochemical energy of protons or sodium ions.

5. Modes of energy coupling revisited: Uniport, Symport and Antiport

The proton motive force consists of an electrical potential difference ($\Delta\Psi$) and a chemical concentration difference (ΔpH). The first component depends on the distribution across the membrane of all charged solutes and is usually negative inside relative to outside. The gradient of protons also represents a chemical gradient of protons called the ΔpH , which is alkaline inside relative to outside. Secondary transporters can use the $\Delta\Psi$, or the ΔpH , or both components of the PMF as driving force for solute transport. In a similar fashion, other secondary transporters use an electrochemical gradient of sodium ions or other charged molecules as source of free energy. The general reaction catalyzed by secondary transporters is the conversion of the electrochemical gradients of H^+ or Na^+ into gradients of solutes (90). As mentioned above, three basic modes of energy coupling are recognized: uniport, symport and antiport. Uniporters may be involved in facilitated diffusion (**Figure 1A**), which is the transport of a single molecular species down its solute gradient. However, if the solute is charged it may use the $\Delta\Psi$ to drive transport against a concentration gradient of the solute. The direction of transport is dictated by the direction of its electrochemical gradient and, therefore, both uptake as well as efflux can be mediated by uniporters. In case of uncharged solutes the internal solute

concentration will never exceed the external concentration. In case of a charged solute, the $\Delta\Psi$ will contribute to the accumulation of the solute at either side of the membrane, depending on the charge of the solute.

Symporters transport two or more molecular species together in the same direction in a tightly coupled process (**Figure 1B**). One of the transported species is usually either a proton or a sodium ion, or both. The ions move down their electrochemical gradients thus providing the energy for the accumulation of the solute against its electrical or chemical gradient. Symporters can accumulate solutes to the level at which the electrochemical gradient of the accumulated solute is equal but opposite to the driving force supplied by the PMF or SMF.

Antiporters catalyze the transport of a solute or ion into the cell tightly coupled to transport of another solute or ion out of the cell, or *vice versa* (**Figure 1C**). Typical examples are the H^+/Na^+ antiporters that convert a PMF into a SMF. The mode of transport is not necessarily conserved within families, i.e. a family of homologous proteins may contain symporters, uniporters and antiporters. Some transporters can function both as symporters, using the energy of the PMF or SMF to accumulate a solute, or can function as exchangers usually by coupling the secretion of a metabolic end product to the accumulation of a metabolic precursor. In all cases metabolic energy, stored in concentration gradients of solutes across the membrane, is dissipated in order to accumulate a solute. Thus, in summary, secondary transporters use one concentration gradient to power the formation of another.

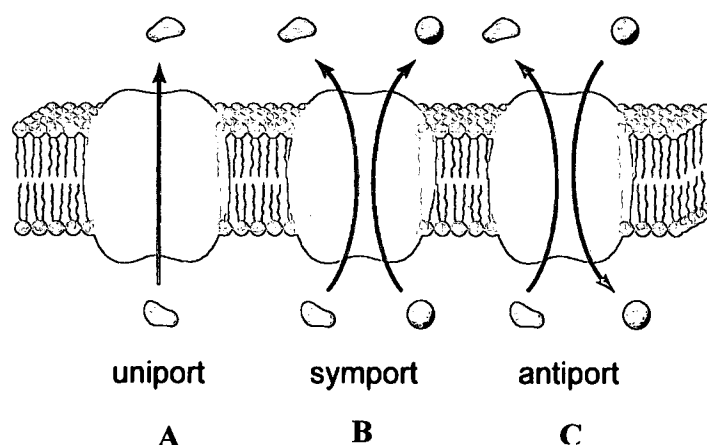


FIGURE 1. Three types of secondary transporters found in bacteria. Any single bacterium uses diverse transport systems to take up sugars, amino acids, ions, vitamins, organic acids, etc. Many of these systems are powered by electrochemical energy (ion gradients). They catalyze uniport (A), symport (B) and antiport (C).

In fermentative bacteria, special secondary transporters have been discovered that generate rather than consume metabolic energy. The general mode of metabolic energy generation is as follows. Exchange of a divalent negatively charged precursor and a monovalent negatively charged product results in the net transport of negative charge to the inside of the cell; a $\Delta\Psi$ of physiological orientation is generated. The cytoplasmic conversion of precursor into product results in proton consumption and a ΔpH of physiological orientation is generated (101). The intracellular accumulation of the product and the internal cytoplasmic conversion of the precursor drives the uptake of the precursor. The resulting PMF is often large enough to drive energy requiring processes such as ATP synthesis by ATPases. For instance, *Leuconostoc mesenteroides* subsp. *mesenteroides* 19D catalyzes citrate uptake by a secondary transporter (CitP). Kinetic studies indicate that CitP catalyzes Hcit²⁻ (dianionic form

of citrate)/H⁺ symport. Translocation of negative charge into the cell during citrate metabolism results in the generation of a membrane potential that contributes to the PMF across the cytoplasmic membrane, i.e. citrate metabolism in *L. mesenteroides* generates metabolic energy. Efficient exchange of citrate and D-lactate, a product of citrate/carbohydrate co-metabolism, is observed, suggesting that under physiological conditions, CitP functions as an electrogenic precursor/product exchanger rather than a symporter (106).

6. Functions of transporters

Transport systems serve the cells in seven ways (141-143, 145-147). First, they allow entry of all essential nutrients into the cell, thereby allowing metabolism of exogenous sources of carbon, nitrogen, sulfur, and phosphorus. Second, they provide a means for the regulation of metabolite concentrations by catalyzing the excretion of end products of metabolic pathways from cells. Third, they mediate the active extrusion of drugs and other toxic substances from either the cytoplasm or the plasma membrane. Fourth, they mediate uptake and efflux of ionic species that must be maintained at concentrations that differ drastically from those in the external milieu. Fifth, transporters participate in the secretion of proteins, complex carbohydrates, and lipids into and beyond the cytoplasmic membrane, and these macromolecules serve a variety of biologically important roles in protection against environmental insult and predation, in communication with members of the same and different species, and in pathogenesis. Sixth, transport systems include the transfer of nucleic acids across cell

membranes, allowing genetic exchange between organisms and thereby promoting species diversification. Seventh, transporters facilitate the uptake and of excretion of a variety of signaling molecules that allow a cell to participate in the biological experience of multicellularity (Quorum sensing). Finally, transport proteins allow living organisms to conduct biological warfare, secreting, for example, antibiotics, antiviral agents, antifungal agents, and toxins of humans and other animals that may confer upon the organism producing such an agent a survival advantage. Many of these toxins are themselves channel-forming proteins or peptides that serve a cell-disruptive transport function. Thus, from a functional standpoint, the importance of molecular transport to a cell cannot be overestimated.

7. Topology of nitrate and nitrite reduction and the relationship to nitrate transport and nitrite detoxification

Three distinct enzymes are synthesized in bacteria for the reduction of nitrate within different cellular compartments (134). The periplasmic nitrate reductase or the Nap has its active site outside the cytoplasm, and therefore no specific biological mechanism is required for transport of the nitrogenous substrate to its site of reduction. On the other hand, two other types of nitrate reductase have their active sites within the cytoplasm: the membrane-bound nitrate reductase (Nar), which is operative under anaerobic conditions as a respiratory enzyme, and the cytoplasmic assimilatory nitrate reductase (Nas). To reach the active sites of these enzymes, nitrate must cross the cytoplasmic membrane barrier. The product of nitrate

reduction, nitrite, is usually reduced via nitrite reductases, which reside in the periplasm or in the cytoplasm. Hence, in many cases the product of nitrate reduction i.e., nitrite, must cross a membrane in order to reach the active site of its reductase, or alternatively the nitrite may be expelled from the cytoplasm into the extracellular milieu. Since nitrite is a highly toxic ion, this export process must not let nitrite accumulate in the cytoplasm.

It has been demonstrated in *P. aeruginosa* (48), *P. stutzeri* (73) and *E. coli* (139) that the product of nitrate respiration, i.e. nitrite, is immediately excreted to the external environment presumably protecting the organism from potential toxic effects. These toxic effects are due to the ability of this anion to bind to the heme groups in electron carriers thereby inhibiting the flow of electrons. Genetic and physiological data suggest that nitrate transport in some bacteria occurs through two different uptake systems. Thus, for the process of nitrate assimilation, ABC transporters as well as secondary transporters are postulated to be used. On the other hand, anaerobically, for the purpose of nitrate respiration it is postulated that bacteria rely solely on secondary transporters.

Most of the data that currently exist in the literature on nitrate transport is based on the physiological and biochemical characteristics of mutants presumed to play a role in the transport of nitrate, and predictions made by analysis of extrapolated protein sequences. The direct examination of the transport systems for nitrate and nitrite have been stymied by the lack of a long-lived radioactive isotope of nitrogen or oxygen. Therefore nitrate movement has been indirectly measured and most such

studies are limited to whole cells where metabolism is ongoing. One approach to a more accurate assessment of the mechanism of transport requires the use of proteoliposomes and radiotracers (139). However, due to the difficulty in purifying transport proteins, the use of proteoliposomes remains limited.

Originally, John (81) demonstrated that membrane permeabilization of the cells significantly enhanced nitrate uptake suggesting the need for a transport protein specific for nitrate. This was corroborated by several other studies which also demonstrated that external nitrate uptake in whole cells was restricted by a permeability barrier. It was also observed that nitrate reduction and nitrate uptake were closely coupled as *narG* deficient mutants did not take up nitrate (139). Others demonstrated that nitrate uptake and reduction resulted in the immediate excretion of nitrite.

Previous studies have proposed that an antiport exchange would most logically allow the movements of nitrate and nitrite across the membrane during nitrate respiration (46). However, experimental evidence for this has been unsatisfactory. Since no change in net charge affecting the membrane potential is associated with the movement of these anions, an antiporter is energetically the most favorable proposition. A nitrate/proton symport, driven by the energized membrane, has also been proposed, both as the principal uptake process and to initiate nitrate uptake prior to the functioning of the antiport system (22, 92). There is some data in the literature that confirms an energy requirement for nitrate uptake that is sensitive to protonophores and may favor a symport mechanism (139). However, there are also

contradictory findings (71), and a passive nitrate-specific pore has been postulated to account for the lack of energy requirement and missing unequivocal evidence for an antiporter. Perhaps the most efficient nitrate uptake system of a denitrifier has been found in the vacuolated sulfide- and elemental sulfur-oxidizing bacterium *Thioploca* sp., where nitrate is concentrated from 25 mM in seawater to 0.5 M inside the cell (57).

Transport systems for nitrate and nitrite are better known outside the denitrifying bacteria. The first genetic locus identified as playing a role in nitrate uptake or nitrite excretion was *narK* of *E. coli* K-12 (34, 46, 114, 139, 170). The current state of knowledge is primarily based on studies in this organism which possesses two nitrate/nitrite transport proteins, NarK and NarU (21, 34, 80). The 12-times membrane-spanning NarK protein of *E. coli* was initially thought to be a $\text{NO}_3^-/\text{NO}_2^-$ antiporter (46). A later, more thorough investigation which made use of membrane vesicles and proteoliposomes (rather than intact cells) and of sensitive techniques (the use of ^{13}N nitrate and nitrite-sensitive fluorophore: *N* - (ethoxycarbonylmethyl) - 6 - methoxyquinilinium bromide) suggested that the physiological role of NarK was that of a nitrite exporter (139). More recent non-radioactive, whole cell studies utilizing a nitrate ionophore and a NarK-NarU double mutant have concluded that both NarK and NarU proteins are required in both nitrate and nitrite import as well as nitrite export (34). The latest study on *E. coli* NarK also suggests antiporter capabilities for the proteins (80). It must be mentioned here that the studies carried out by Clegg and coworkers (34), and Jia and coworkers (80), were

in an actively metabolizing cell and thus may account for the disparate data from Rowe and coworkers (139), that specifically made use of vesicles and proteoliposomes, making the system more sensitive. In summary, based on the studies by Demoss and coworkers (46) and Rowe and coworkers (139), two possibilities have been proposed for the *Escherichia coli* NarK (Figure 2)

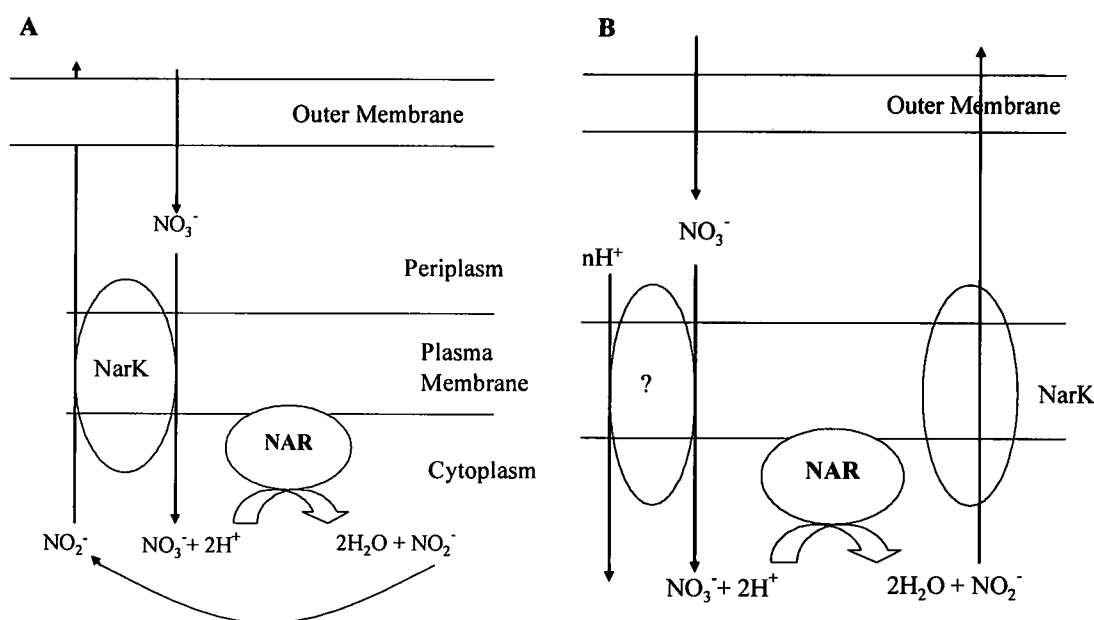


FIGURE 2: Proposed mechanisms of transmembrane fluxes of nitrate and nitrite. (A) Classical antiport model showing NarK mediating nitrate uptake and nitrite extrusion. (B) Uniport model of NarK showing nitrite extrusion.

8. Bioenergetics and putative mechanisms of nitrate/nitrite transport

The uptake of nitrate into an actively metabolizing cell, the molecule requires that it must be moved against a proton electrochemical gradient ($\Delta\mu$), in the order of 180 mV. Given that isolated nitrate reductase has a K_m of approximately 10 μM (121), a passive mechanism of nitrate uptake would yield a K_s for nitrate of around 10

mM. Interestingly, the affinity of nitrate reductase for nitrate appears to be similar to that of the isolated enzyme. However, these measurements have been made in artificial systems thus the physiological verity is in question. If these calculations are correct then it makes a nitrate/nitrite antiporter system very attractive since the net effect of such a system would be electroneutral. However, it is also in keeping with a nitrate/ $n\text{H}^+$ symport mechanism of transport (where $n \geq 1$).

An alternative mechanism for nitrate uptake would involve an ATP-driven nitrate uptake system (like those postulated based on sequence data for nitrate assimilation). Experimental determinations of the $\text{P}/2\text{e}^-$ ratio calculated from medium acidification following pulses of nitrate, nitrite or nitrous oxide, or from the specific rate of ATP synthesis in chemostats of *P. denitrificans* growing with nitrate or nitrite suggest that the transmembrane charge displacement is the same (per electron) for nitrate reduction, nitrite reduction and nitrous oxide reduction (22, 23). These values were in agreement with the theoretical calculations for these reductases based on the known properties of their electron transport chains, and indicate that the nitrate uptake process does not result in a significant loss of Δp , i.e. nitrate uptake is not driven by ATP hydrolysis or the use of >1 proton during the mechanism of a putative nitrate/proton symporters.

9. *NarK* homologues

Multiple homologues of NarK have been found amongst phylogenetically diverse eubacteria capable of respiratory nitrate reduction. Organisms containing NarK homologues include the deeply branched eubacteria *Aquifex aeolicus* (44) and *Thermus thermophilus* (128). NarK homologues have yet to be discovered in the Archaea. There is at least one copy of a *narK* homologue found upstream from *narGHJI* in most nitrate respirers that have been studied genetically so far. For instance the denitrifying bacteria *Pseudomonas stutzeri* (67) contains two different *narK* like genes, namely NarK and NarC which are separate from but close to the *narGHJI* operon. However in another denitrifier *Pseudomonas aeruginosa*, there are two NarK genes namely, NarK1 and NarK2 which are present together in an operon with the *narGHJI* genes (158) (**Table 1**). In *Paracoccus pantotrophus*, a single fusion protein has evolved which contains two domains, a NarK1 and NarK2 (189). In a report of nitrate transport in an extreme thermophile, *Thermus thermophilus*, it was the first study that revealed an organism possessing two forms of *narK* namely, *narK1* and *narK2*, separate from but just downstream of the *narGHJI* operon (128). The NarK2 of that organism was most similar to the NarK of *E. coli*. It was hypothesized that both NarK1 and NarK2 serve to import nitrate as well as export nitrite during anaerobic growth by this organism. However, physiological studies of the mechanism were limited in this report.

TABLE 1. NarK1 and NarK2 homologues of *P. aeruginosa* as determined through BLAST.

Sequence Identity to NarK1 of <i>Pseudomonas aeruginosa</i>		
Organism	Gene Name	Sequence Identity (%)
<i>Pseudomonas fluorescense</i>	<i>narD</i>	53
<i>Paracoccus pantotrophus</i>	<i>narK</i>	48
<i>Thermus thermophilus</i>	<i>narK1</i>	41
<i>Bacillus subtilis</i>	<i>narK</i>	28
<i>Staphylococcus carnosus</i>	<i>NarT</i>	29
Sequence Identity to NarK2 of <i>Pseudomonas aeruginosa</i>		
Organism	Gene Name	Sequence Identity (%)
<i>Pseudomonas fluorescense</i>	<i>narK</i>	72
<i>Paracoccus pantotrophus</i>	<i>narK</i>	62
<i>Thermus thermophilus</i>	<i>narK2</i>	38
<i>Escherichia coli</i>	<i>narK</i>	60
<i>Escherichia coli</i>	<i>narU</i>	58
<i>Mycobacterium tuberculosis</i>	<i>narK2</i>	23

The genome sequence of *Mycobacterium tuberculosis* has revealed the presence of four *narK* homologues, designated *narK1*, *narK2*, *narK3* and *narU* (35). In this organism none of the NarK homologues is found upstream from *narGHJI* operon although *narK2* is located upstream from a gene designated *narX* which may encode a single subunit ('fused') nitrate reductase (this is entirely distinct from nitrate:nitrite sensory gene *narX* of *E. coli*). *NarK3* and *narU* are found in the vicinity of genes required for synthesis of a cytoplasmic assimilatory nitrite reductase. There are no genes known to be involved in nitrogen metabolism in the vicinity of the *narK1* homologues in this organism. The reason(s) for the multiple copies of *narK*

homologues and the absence of a *narK* homologue upstream from *narGHJI* are unclear, and work is hampered by our lack of understanding of the physiology of this organism. However, a recent study carried out in this organism concluded that its NarK2 is functionally identical to the *E. coli* NarK (163). This conclusion was reached due to the ability of the NarK2 of *M. tuberculosis* to complement the *E. coli* NarK-NarU double mutant. However, another interpretation of this finding is that it is involved in nitrate or nitrite transport and can therefore substituted for the *E. coli* deficiency even though it might not be identical in function. Other Gram-positive organisms that contain NarK homologues include *Bacillus subtilis*, which contains two homologues, designated NarK and NasA (96), and *Streptomyces coelicolor* (accession no. CAB53437). The gram-positive nitrate reducer *Staphylococcus carnosus* also contains a *narK* homologue called narT (53). A *narT* mutant grows poorly on nitrate, but uptake of nitrate can be enhanced by treatment with benzyl viologen, which can act as an ionophore. The dependence of nitrate uptake on NarT suggests that NarT may be a nitrate uptake protein (53). Alternatively, NarT may be a nitrite effluxer, and intracellular accumulated nitrite in a *narT* mutant may prevent biological nitrate uptake via a separate nitrate transporter protein (53). Most recently, isogenic mutants of *narK1* and *narK2* genes of *Pseudomonas aeruginosa* have been characterized (158). While a NarK1 mutant is found to be only slightly affected in its ability to grow under denitrification conditions, both NarK2 and NarK1K2 mutants are found to be severely restricted in nitrate dependent anaerobic growth. Additionally, an *E.coli* NarK is able to rescue both the NarK2 and NarK1K2

mutants indicating that functionally the NarK2 of *P. aeruginosa* is similar to the NarK of *E. coli* (158).

10. The Major Facilitator Superfamily

The MFS is a superfamily that includes 28 currently recognized families, each of which shares common descent with the 27 other families of the MFS but is only distantly related to them (144). Sixteen of these families were recognized in 1998 (119), but the superfamily has expanded considerably since then. Most of the well-characterized proteins of the MFS exhibit characteristic sequence motifs localized to specific portions in the proteins. Their topology characteristically exhibits 12 transmembrane spanners (TMSs), of which the first six exhibit sequence similarity with the last six. It is believed that these proteins arose by one (or more) internal tandem gene duplication event(s). Three of the established 28 families of the MFS exhibit 14 instead of 12 TMS, as discussed previously (119).

The MFS is not only one of the largest superfamilies of secondary transporters but it is also one of the most diverse. Its members can transport substrates with either inwardly or outwardly directed polarity, or they can equilibrate their substrates across the membrane without directionality. For a neutral (non-charged) substrate, if mere equilibration is observed, then the transport mechanism usually involves uniport. That is, the substrate is transported across the membrane without the obligatory movement of any other molecular species. If, on the other hand, a neutral solute is accumulated inside the cell against a concentration gradient, its transport is normally coupled to the co-transport (symport) of a monovalent cation, either H^+ or Na^+ (185). More than one

such ion can be symported with the substrate, and substrate accumulation is dependent on the electrochemical gradient of the co-transported cation. When a substrate is pumped out of the cell against a concentration gradient, the energy-coupling mechanism usually involves substrate:H⁺ countertransport (antiport) (180). Influx of H⁺ down its electrochemical gradient drives efflux of the solute. Finally, some carriers preferentially or obligatorily catalyze solute:solute antiport, a process in which one substrate can be taken up in exchange for another substrate of similar structure (129). All these mechanisms are observed for proteins of the MFS. Moreover, a very few MFS members function as receptors, transmitting information from the cell surface to the genetic material of the cell in order to influence rates of gene expression (82, 93). Although MFS homologues serving receptor functions are rare, they have been observed in both prokaryotes (*E. coli*) and eukaryotes (*S. cerevisiae*) (78, 93). It is therefore clear that the MFS is a functionally diverse superfamily.

(10A) Phylogeny: NarK belongs to the Major Facilitator Superfamily

NarK belongs to the Major Facilitator Superfamily (MFS) of transmembrane transporters (105). On the basis of phylogenetic analysis the superfamily has been divided into six clusters; the NarK homologues belong to cluster 6 of the MFS (176). The other members of this cluster are the so-called high-affinity nitrate transporters of plants and fungi (59). This latter group of proteins consists of Δp -driven transporters which import nitrate and/or nitrite into the cell (60). The bacterial and the eukaryotic

proteins clearly have a common ancestor distinct from members of the other clusters of the MFS (176). The MFS cluster most closely related to that containing NarK is cluster 4, which contains phosphate/ester-phosphate antiporters (105), i.e. the most closely related group of transporters also involved in anion transport.

A phylogenetic analysis of the 6 of the MFS transporters shows that the plant and fungal transporters cluster separately from the bacterial proteins (110). Within the bacterial NarK-like proteins there are two distinct subgroups. Type I includes those NarK homologues identified in *S. carnosus*, *B. subtilis* and copies of NarK1 from *Pseudomonas aeruginosa*, *T. thermophilus* and *P. pantotrophus* and NasA homologues from *B. subtilis* and *A. aeolicus* (110). Type II contains NarK and NarU from *E. coli*, copies of NarK2 from *P. aeruginosa*, *T. thermophilus* and *P. pantotrophus* and three of the NarK homologues from *M. tuberculosis* (110).

The distinct types of NarK seem to have diverged from a common ancestor in the distant evolutionary past, but in several cases members of each type are found, in series, in the same organism. For example, both types of NarK are found in the distantly related organisms *P. aeruginosa* and *T. thermophilus* (110, 128). The divergent sequences of each NarK type demonstrate that two copies in series in an organism did not arise by a recent gene duplication event. The high degree of similarity between NarK1 from *P. aeruginosa* and *T. thermophilus* is suggestive of horizontal gene transfer between phylogenetically distant organisms (128).

The two types of NarK seem to be involved in nitrogen oxyanion transport but may have different substrate specificities, i.e. Type I and Type II NarK are

functionally divergent (110). It has been hypothesized that type I NarK's are responsible for nitrate uptake, whereas the type II NarK proteins are nitrite exporters. However, in light of the recent findings that *E. coli* NarK (a member of the type II group) could be involved in both the uptake of nitrate and nitrite and the export of nitrite. If this turn out to be correct then the definition of Type I and Type II NarKs will have to be revised. The arrangements of NarK-like genes and the enzymes they may service are presented (**Figure 3**).

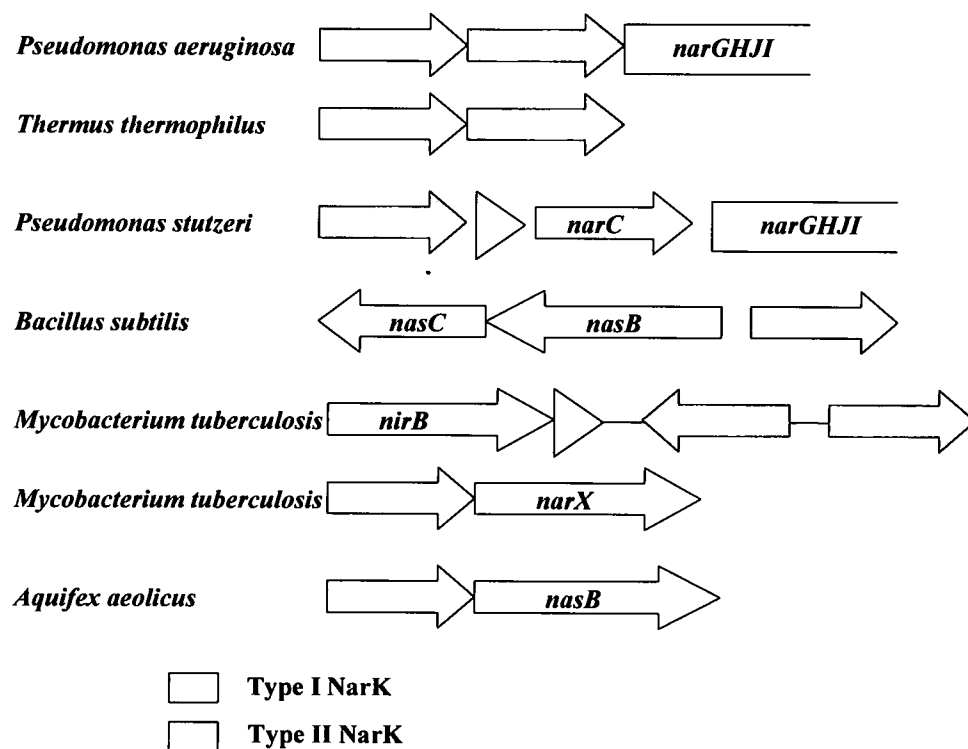


FIGURE 3. Genetic organization of NarK. Partial gene clusters containing *narK* homologues are shown. (Figure adopted from (110)).

11. Structure and function of NarK proteins

Consistent with a role in transmembrane transport, members of the NarK family are predicted to have 12 transmembrane helices. Hydrophobicity plots of members of the NarK family consistently show predicted helices 2 and 8 to be the least hydrophobic (110). This is largely due to the conserved arginine residues located within this predicted membrane spanning region, amongst all members of cluster 6 of the MFS (i.e. all NarK and the plant:fungal nitrate transporters). Both their location as well as the conserved nature of these arginine residues indicates that they may be crucial in anion transport. There are 15 totally conserved residues amongst the members of cluster 6 of the MFS, the two Arg residues, nine Gly residues, seven of which are found within predicted membrane spans, Pro, Phe and Tyr residues within membrane spans and a conserved Asp residue located in the loop predicted between helices 8 and 9. Topology is predicted such that the N- and C-termini are cytoplasmic. Thus, the conserved Asp is predicted to be cytoplasmic and may control access to (or egress from) a network of residues, including the Arg residues, within and crossing the membrane that allow transmembrane transport of anions. Bulky conserved residues Phe and Tyr may also help control substrate access to the transporter. Gly and Pro residues are associated with helix breaking, and may lend flexibility to the membrane protein structure important for substrate movement. Members of the MFS generally possess 12 transmembrane-spanning helices, which are considered to have arisen from ancient duplications of proteins containing 6 transmembrane helices (105). Pair-wise comparisons of the sequences of N-terminal and C-terminal halves of

the members of cluster 6 of the MFS show them to be significantly similar to one another compared with members of other clusters of the MFS (110), thus indicating that the duplication giving rise to cluster 6 occurred after evolutionary divergence from the other clusters of the superfamily. Interestingly, when the two halves of the proteins in the family are aligned, the conserved arginines from helix 2 and helix 8 align together, indicating that the arginine was present in the six-helix ancestor. Perhaps a prototypical nitrogen oxyanion transporter consisted of a dimer of identical six-helical proteins (110).

12. Regulation of nitrate transport

In addition to oxygen regulating nitrate reduction at the level of gene expression, respiratory nitrate reduction is also inhibited in intact cells in the presence of oxygen (72, 81). This effect can be mimicked using ferricyanide, indicating that the nitrate reduction pathway is sensitive to oxidizing conditions, rather than molecular oxygen itself (3, 95). Early studies came from John (81) who investigated the levels of oxygen and nitrate during bacterial respiration. He found that both *Paracoccus denitrificans* and *E. coli* cells cease to reduce nitrate when oxygen is available, and equally rapidly commence nitrate reduction when all the oxygen has been consumed. Conversely, inverted vesicles of *P. denitrificans* are capable of reducing nitrate and oxygen simultaneously. Furthermore, treatment of anaerobically grown cultures of *P. denitrificans* with low concentrations of detergents such as Triton X-100 relieves the inhibitory effect of oxygen on nitrate reduction, whereas

identical concentrations of the detergent allowed the intact cells to reduce chlorate (1). These findings indicate that the oxygen inhibitory effect on nitrate reduction is related to the transport of nitrate and/or nitrite across the cytoplasmic membrane. This supposition is buttressed by the finding that treatment with Triton X-100 leads to a slight increase in oxygen reduction (1).

In a study by Hernandez and coworkers (73), nitrate reduction was found to be inhibited in whole cell suspensions of *P. aeruginosa* exposed to oxygen. Cell-free extracts on the other hand were not found to exhibit this inhibitory effect making the authors conclude that the regulation of nitrate respiration at the level of transport by oxygen was a major mechanism by which the entire denitrification pathway was regulated in *P. aeruginosa* (73). They also found that nitrite uptake was not subject to the same kind of regulation as it commenced at low levels of oxygen. In a follow up study by the same group (72), a wide variety of nitrate-respiring bacteria were analyzed in an attempt to see if inhibition of nitrate transport by oxygen was a universal mechanism. They found that indeed whole cell suspensions of 11 out of the 12 species tested demonstrated the same inhibition as *P. aeruginosa*. The only exception to this was the organism *E. aerogenes* which was able to utilize nitrate both aerobically and anaerobically. From this, they concluded that oxygen inhibition of nitrate uptake was a general regulatory phenomenon (72).

Another study by Dias and coworkers (48) analyzed wild type and nitrite reductase mutant of a halophile, *Pseudomonas stutzeri*. Similar to previous studies, they also found that oxygen exerted an inhibitory effect on nitrate uptake which was

reversible and more pronounced in cells grown on low concentrations of nitrate compared to cells grown at high concentrations of nitrate. They concluded that the high initial nitrate concentrations in culture changed the physiological response of this halophilic organism to both oxygen and nitrite dissimilation. Furthermore, they found that this effect was more pronounced in the nitrite reductase mutant compared to the wild type (48).

In yet another study by Hernandez and coworkers (71), the effects of a metabolic inhibitor on oxygen regulation of nitrate uptake was investigated. This group hypothesized that oxygen regulation of nitrate uptake was in part through redox-sensitive thiol groups since N-ethylmaleimide at high concentrations decreased the rate of nitrate transport. Further they found that oxygen regulation of nitrate transport was relieved by the cytochrome oxygen reductase inhibitors carbon monoxide and cyanide (71).

To further elucidate the mechanism of oxygen inhibition of transport, studies have been carried out in *Paracoccus denitrificans*. In *P. denitrificans*, nitrous oxide (N_2O), like oxygen, inhibits the reduction of nitrate in intact cells. The inhibitory effects of oxygen and nitrous oxide on nitrate reduction are diminished by antimycin, which inhibits electron flow through the cytochrome *bcl* complex (resulting in a more highly reduced quinone pool). This indicates that transport of nitrogen oxyanions may be dependent upon the redox state of the respiratory chain, possibly the redox state of the quinone pool being sensed by the transport process (2), i.e. nitrate reduction occurs only when the respiratory chain is sufficiently reduced.

Denis and co-workers (47) found that inhibition of nitrate reduction by oxygen was greatly lessened in strains of *E. coli* unable to synthesize the respiratory oxidases (*cytochromes bo* and *bd*), supporting the idea that the inhibition of nitrate reduction under aerobic conditions is partially brought about by diversion of electrons to oxygen and the inhibition of transport when the respiratory chain becomes oxidized. Oxygen inhibition of nitrate transport seems to be caused by an indirect mechanism as well as the diversion of electrons away from the catalytic protein NarG (47).

Kucera and co-workers (94) measured internally accumulated nitrate in whole cells of *P. denitrificans*. Provision of oxygen (via the catalytic action of catalase on hydrogen peroxide) actually led to an increase in the steady state nitrate pool inside the cell. The conclusion of this work was that the control of nitrate reduction by oxygen could not be explained by inhibition of nitrate transport. Rather, regulation of nitrate reduction by oxygen may be controlled by both competition for electrons by the oxidases, and control at the level of nitrite export. Finally, it is also very likely that nitrite export may be the site of control by oxygen.

Finally, at the protein level, there are few completely conserved residues amongst the entire NarK family and none of these are residues that might be expected to play a role in oxygen control (110). However, Cys residues, found within predicted transmembrane helices 4 and 10, are conserved between NarK and NarU from *E. coli* and NarK2 from both *Pseudomonas aeruginosa* and *Paracoccus pantotrophus*. A redox-dependent disulfide bridge formation between these two cysteines, causing

reversible inactivation of NarK under oxidising conditions, may be the cause of oxygen inhibition of nitrate:nitrite transport. Some support for the importance of redox-sensitive thiol groups in the transport process comes from findings that in *Pseudomonas aeruginosa*, nitrate uptake is inhibited by *N*-ethylmaleimide (71). It is possible that control of nitrogen oxyanion transport by oxygen is limited to the nitrate-reducing Proteobacteria (e.g. *E. coli*, *Paracoccus* species, *Pseudomonads*).

A working model for nitrate and nitrite transport via type I and type II NarK and the impact of oxidizing conditions on nitrite accumulation and hence inhibition of nitrate uptake via the type I transporter is presented in **Figure 4**.

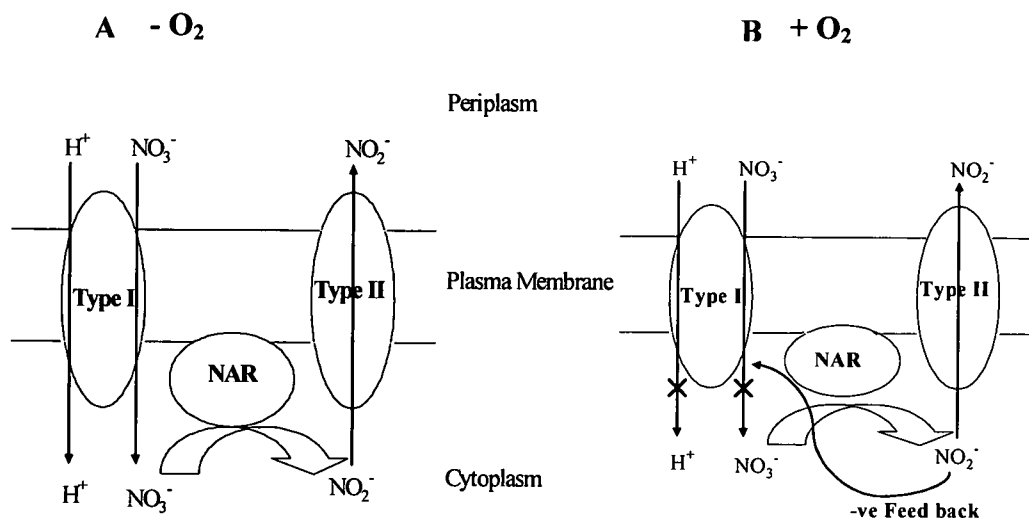


FIGURE 4. Type I and Type II NarK: A proposed mechanism of oxygen inhibition. (A) Type I NarK is hypothesized to import nitrate into the cytoplasm, whereas Type II NarK exports nitrite into the periplasm (B) The proposed role of two cysteine residues found amongst type II NarK is to form a disulfide bridge under oxidising conditions, which prevents nitrite export and hence nitrate uptake by a feedback mechanism.

The effect of *narXL* and *narQP* regulatory proteins on nitrate transport has been discussed in Chapter 1 of this dissertation.

13. Nitrate/Nitrite transport in *P. aeruginosa*

A close look at the loci of the *nar* operon of *P. aeruginosa* identifies two potential nitrate and/or nitrite transporters: NarK1 (PA3877) and NarK2 (PA3876) (175). The genes for these proteins are located within the nitrate reductase operon, *narK1K2GHJI* operon. Our studies have shown that both Δ narK2 and Δ narK1K2 mutants are unable to grow anaerobically with nitrate as the terminal electron acceptor. On the other hand, a Δ narK1 mutant seems to be unaffected in this aspect and demonstrates wild-type levels of growth. Subsequent experiments making use of nitrate electrode and complementation of the Δ narK2 and Δ narK1K2 mutant with *E. coli narK* have shown that the NarK2 protein is very vital in *P. aeruginosa* denitrification. The role of the NarK1 protein however, still remains enigmatic. The details of this study have been included in Chapter 3 of this dissertation.

14. Conclusions

A picture of the mechanisms by which nitrate and nitrite are transported in bacteria is beginning to emerge. Nitrate and nitrite uptake via ATP-driven systems for assimilation is fairly well understood, but the precise functions of NarK homologues in both dissimilatory and assimilatory transport is still somewhat obscure. Combining genetic and physiological analysis and by measuring accumulation of intracellular

nitrate and nitrite with more sensitive techniques, it may be possible to identify the roles of NarK homologues and identify whether they are very specific for certain substrates, or whether they are more promiscuous, being capable of, say, both uptake and excretion of nitrite (type II NarK), or both nitrate:proton symport and nitrate:nitrite antiport (type I NarK).

CHAPTER 2

Literature Review

Part II

NITRATE RESPIRATION IN PROKARYOTES

1. General nitrate metabolism

Nitrogen is a fundamental element for life because it is a component of important biological macromolecules such as nucleic acids, proteins, vitamins, and amino-carbohydrates. Nitrogen in the environment exists in several oxidation states, interconversion of which constitutes the global nitrogen cycle, a process where bacteria play a predominant role (**Figure 1**) (136). The nitrogen cycle can be represented by stages that include nitrogen fixation, ammonification, nitrification, denitrification and assimilatory nitrogen metabolism (194). The first stage, nitrogen fixation, is performed only by bacteria and requires significant amounts of energy. Symbiotic nitrogen fixation is of greatest significance and is an association of leguminous plants with bacteria of the genus *Bradyrhizobium*. However, there also are free-living nitrogen fixers, all bacteria, which are critical in certain ecosystems. Once fixed into biomaterial, microbial decay (ammonification) and nitrification play critical roles in the recycling of fixed nitrogen as well as in the ultimate return of dinitrogen to our atmosphere. Ammonification is the microbial breakdown of proteins, nucleic acids and other plant material releasing ammonia into the environment. The main biochemical process is via the action of proteases that break the proteins into amino acids followed by deamination and the release of ammonium into the ecosystem.

After the release of ammonia, chemolithotrophic autotrophs can oxidize ammonia first to nitrite and subsequently to nitrate. These organisms utilize the ammonia and nitrite as energy sources and fix carbon dioxide. Thus, they are considered non-photosynthetic primary producers. Ammonia can also be biologically recycled to biomass.

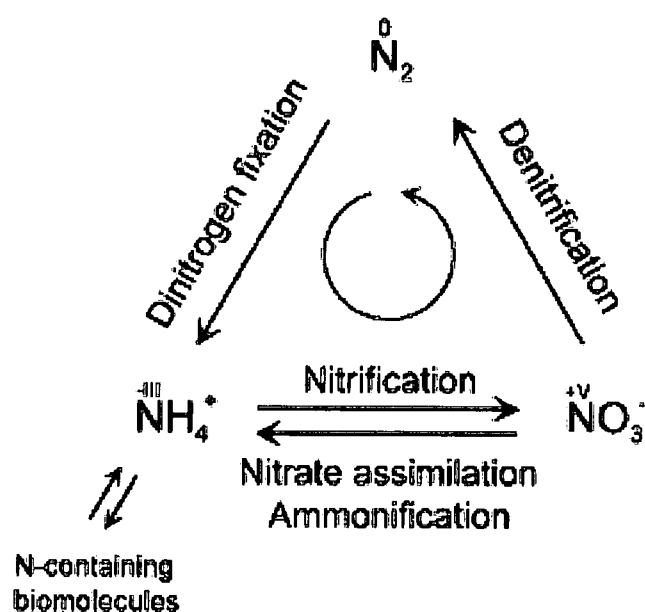


FIGURE 1. Biogeochemical nitrogen cycle. The oxidation states of the various nitrogen species is indicated by the roman numerals. The process of nitrogen fixation is carried out by the enzyme nitrogenase with no reaction intermediates being produced. The process of denitrification involves four different N-oxygen reductases leading to the production of nitrite, nitric oxide and nitrous oxide as reaction intermediates. The only oxidative segment of the nitrogen cycle is nitrification in which hydroxylamine and nitrite are produced as reaction intermediates. Finally, both the process of nitrate assimilation and ammonification involve only ammonia and nitrite as the reaction intermediate. (Figure adopted from (194)).

The fate of the nitrate formed from nitrification is complex but ultimately it will either be incorporated into more biomass (assimilation) or processed anaerobically to return dinitrogen to the atmosphere (respiration and dissimilation). The process of nitrate reduction plays a key role in the biogeochemical nitrogen cycle and has important agricultural, environmental, and public health implications. Assimilatory nitrate reduction, performed by fungi, algae, higher plants and bacteria is one of the most fundamental biological processes and accounts for more than 10^4 megatons of inorganic nitrogen transformed each year (65). This pales in the face of the amount of nitrate converted to dinitrogen by anaerobic denitrification. In modern times, because of the gargantuan amounts of atmospheric nitrogen industrially fixed each year, there is now a global concern over the indiscriminate use of fertilizers for agriculture, leading to the accumulation of nitrates in ground water. It is feared that the natural balance of biogeochemical cycling of nitrogen is being disrupted and the biological activity cannot keep up. Additionally, consumption of nitrate/nitrite contaminated drinking water has been associated with methemoglobinemia and gastric cancer due to endogenous formation of genotoxic *N*-nitroso compounds by bacteria in the gastrointestinal tract, making it a major public health concern (179). Nitrogen oxides generated by denitrification are also associated with the greenhouse effect and ozone depletion (194). Finally, denitrification seems to prevent eutrophication by removing nitrogen from water bodies. Thus, nitrate reduction has become an important focus for research in the last several years, generating a vast literature.

Many bacteria can express multiple, functionally and biochemically distinct, nitrate reductases, enzymes that catalyze the reduction of nitrate to nitrite (111). The process of nitrate reduction can be performed for three different physiological purposes:

- Nitrate Assimilation: the utilization of nitrate as a nitrogen source for generating cellular biomass
- Nitrate Respiration: the generation of metabolic energy, by using nitrate as a terminal electron acceptor
- Nitrate Dissimilation: the dissipation of excess reducing power for redox balancing

Three distinct types of nitrate reductases catalyze the two-electron reduction of nitrate to nitrite in bacteria namely, the cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and the periplasmic dissimilatory (Nap) nitrate reductases (111). In contrast to the assimilatory nitrate reductases, there is a vast amount of biochemical, physiological and genetic data available in the literature for the respiratory nitrate reductases of bacteria. Recent studies on the periplasmic nitrate reductase of *Desulfovibrio desulfuromonas* include the crystal structure (49).

The aim of this chapter is to summarize recent advances in the physiology, biochemistry, and genetics of prokaryotic nitrate reduction, emphasizing the different molecular characteristics of the two respiratory nitrate reductases i.e., Nar and Nap (**Table 1**).

TABLE 1. Characteristic features of respiratory and periplasmic nitrate reductases.

Prokaryote Dissimilatory Nitrate Reduction		
Characteristic	NO ³⁻ respiration	NO ³⁻ reduction
Nitrate reductase Location	Respiratory Nar Membrane	Dissimilatory Nap Periplasm
Reaction catalyzed	NO ₃ ⁻ ⇒ NO ₂ ⁻	NO ₃ ⁻ ⇒ NO ₂ ⁻
Structural genes	<i>narGHI</i>	<i>napAB</i>
Prosthetic groups	cyt ^b ^a , FeS, MGD	cytc, FeS, MGD
Nitrate transport	Yes	No
Function	PMF (nitrate respiration and denitrification)	2H ^{↓b} and denitrification
Regulation ^c		
O ₂	Yes	No/yes
NH ₄ ⁺	No	No
NO ₃ ⁻ /NO ₂ ⁻	Yes	No/yes

^acyt^b, cytochrome *b*.

^b2H[↓], dissipation of reducing power. A PMF can be generated if a proton-translocating complex is involved in the electron transfer, but in most cases, this seems to be insufficient to support ATP synthesis coupled to nitrate reduction.

^cSome differences in regulation in prokaryotic organisms have been reported.

2. Respiratory nitrate reduction

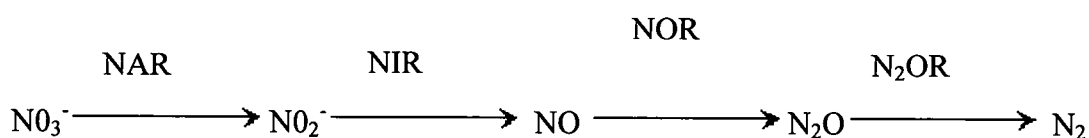
A variety of microbes are capable of generating energy in anaerobic environments via anaerobic respiration (proton)-mediated phosphorylation using nitrate as the terminal electron acceptor. The electron transport system is conceptually analogous to aerobic respiration, but the terminal electron acceptor donates electrons to nitrate instead of oxygen and the specific cytochromes are different from those

involved in aerobic respiration. Anaerobic respiration with nitrate requires the oxidation of a suitable physiological electron donor such as formate, lactate, NADH, or succinate at the front end of the respiratory chain which is then ultimately coupled to the reduction of nitrate to nitrite. This alternate pathway of nitrate respiration allows the organism to carry out relatively efficient energy production in anaerobic environments.

Respiratory nitrate reduction, catalyzed by the proteins encoded by the *Nar* loci, can be divided into two broad classes: the first type is characterized by nitrate reduction in the enterics and has been well studied in *Escherichia coli* (167). In the presence of an appropriate donor, nitrate is reduced to nitrite under anaerobic conditions creating an electrical and chemical potential across the cytoplasmic membrane designated as the proton motive force (PMF). The PMF generated at the expense of nitrate is 67-71% of that generated by oxygen respiration, depending on the initial electron donor to the respiratory chain (24, 167). In some cases, the nitrite generated is further reduced to ammonia. In *E. coli*, nitrite reduction to ammonium is not considered a respiratory step but rather a detoxification reaction that prevents inhibition of respiration by nitrite (140).

The second type of nitrate respiration involves the sequential reduction of nitrate to dinitrogen gas through a process called denitrification (194). The property is characteristic of bacteria living in an anaerobic or micro-aerophilic environment. Some well studied examples of denitrifiers are: *Paracoccus denitrificans* (12, 42, 43), *P. stutzeri* (27, 83), *P. aeruginosa* (4, 29), and *Rhodobacter sphaeroides* (108, 111,

113). The denitrifier very tightly regulates the process of denitrification since some of the products of the reduction of nitrate to nitrogen gas are extremely toxic to cellular metabolism. This may explain the large number of genes involved in the process and the limited number of bacteria that are capable of it. Evidence of four separate enzymatic activities and isolation of the various oxidation states of the substrates involved revealed the following metabolic pathway:



The most common end product of denitrification is elemental nitrogen (N_2) although there are instances where nitrous oxide (N_2O) or nitric oxides (NO) have been reported as the primary end products of nitrate reduction by denitrifiers (194).

As with aerobic respiration, the anaerobic electron transport chain serves to recycle NAD (oxidize NADH) and at the same time generates a proton gradient across the cytoplasmic membrane. However, in contrast to oxygen reduction that consumes two electrons and two protons per reduction, denitrification requires eight electrons in order to reduce nitrate to N_2 . This takes place in separate two electron transfers (for each of the 4 nitrogen oxide substrates). Theoretically, each of these two electron transfers could be linked to extrusion of protons to the outside of the membrane.

In addition to the respiratory nitrate reduction discussed above, both *E. coli* (and many other enterics) and the denitrifiers are capable of producing a second specifically anaerobic nitrate reductase called the periplasmic nitrate reductase. The

genes involved in the periplasmic nitrate assembly are designated NAP. The periplasmic nitrate reductase is a two-subunit complex, located in the periplasmic compartment and is coupled to quinol oxidation via a membrane-anchored tetraheme cytochrome. The Nap seems to be a dissimilatory enzyme in the strict sense because quinol oxidation by Nap is not directly coupled to the generation of a PMF and also because it is independent of the cytochrome *bc*₁ complex. Although it has yet to be experimentally demonstrated, theoretically Nap could generate a PMF if a proton-translocating NADH dehydrogenase is available to reduce the quinone pool. Current evidence does not support that Nap activity can lead to ATP synthesis (112). However, depending on the metabolic fate of nitrite, NAP nitrate reductase can fortuitously and indirectly support anaerobic respiration.

3. Bioenergetics of nitrate respiration

Anaerobic respiration results in the net translocation of protons from the cytoplasmic face of the membrane to the periplasmic space. This generates an electrochemical proton gradient across the membrane, which is called the proton motive force (PMF), measured in millivolts (ΔP). The PMF has two components:

- the electrical or charge gradient/membrane potential ($\Delta\Psi$)
- the chemical gradient i.e., the concentration difference of protons/proton gradient (ΔpH) across the membrane, such that

$$\Delta P = \Delta\Psi - 60 \Delta\text{pH} \quad \text{at } 30^\circ\text{C}$$

The cell uses the PMF generated to drive both the synthesis of ATP (adenosine 5'-triphosphate) from ADP (adenosine 5'-diphosphate) and substrate transport.

Although translocation of protons occurs during both aerobic as well as during anaerobic respiration, it is generally believed that higher energy yields are obtained from oxygen respiration than via anaerobic respiration. Theoretically, the difference between the redox potential of the NAD^+/NADH couple and the O_2 (1 atm)/ H_2O couple is +1.13 V, while that between the NAD^+/NADH couple and the $\text{NO}_3^-/\text{NO}_2^-$ couple is +0.72 V (77). Thus, it might be expected that circumstances where an organism possesses both systems, a terminal electron couple with a more positive redox potential such as the O_2 (1 atm)/ H_2O couple, might override systems designed to function with lower redox couple such as, NAD^+/NADH or $\text{NO}_3^-/\text{NO}_2^-$ (47). True energy yield and differences between aerobic and anaerobic respiratory systems are practically difficult to measure. There have been few investigations where the PMF has been measured in the same organism under both aerobic and anaerobic conditions. In one such study done in *E. coli*, about half the value was observed during anaerobic respiration than during aerobic respiration (24). It should be mentioned that in *E. coli*, electrons enter the anaerobic respiratory chain from formate and the formate/ CO_2 couple is more electro-negative than the NAD^+/NADH couple.

4. Membrane-bound Respiratory Nitrate Reductase (Nar)

(A) Structure and biochemistry

Membrane-bound nitrate reductases are responsible for anaerobic nitrate respiration and denitrification. Although the most comprehensive biochemical and genetic studies have been performed in *E. coli* and *Paracoccus denitrificans*, Nar enzyme complexes have been purified from several denitrifying and nitrate-respiring bacteria (194). In *E. coli*, there are two different membrane-bound isoenzyme complexes (18, 20):

- NRA: which is expressed under anaerobic conditions in the presence of nitrate and represents 90% of the total activity
- NRZ: which is expressed constitutively

The membrane-bound nitrate reductase enzyme is a complex, three-subunit quinol dehydrogenase (**Figure 2**) (134). It contains a catalytic α -subunit (NarG) of 112 to 140 kDa with a MGD cofactor, a cytoplasmic β -subunit (NarH) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-4S] centers, and a 19- to 25-kDa membrane bi-heme cytochrome *b* quinol-oxidizing γ -subunit (NarI). The soluble α - and β -subunits are anchored to the cytoplasmic membrane by the γ -subunit and can be solubilized by detergents or heat. In contrast, NarI is heat-sensitive and, thus, can be lost during the purification procedure, leading to the isolation of a soluble $\alpha\beta$ complex that can reduce nitrate using reduced viologens as electron donors. Finally, a δ polypeptide (NarJ), which is not part of the final enzyme complex, seems to participate in the assembly or stability of the $\alpha\beta$ complex prior to its membrane attachment (19, 51).

Specifically this protein has been proposed to be a chaperone-type protein that allows for the insertion of the molybdenum cofactor in the $\alpha\beta$ - subunits of the respiratory nitrate reductase enzyme.

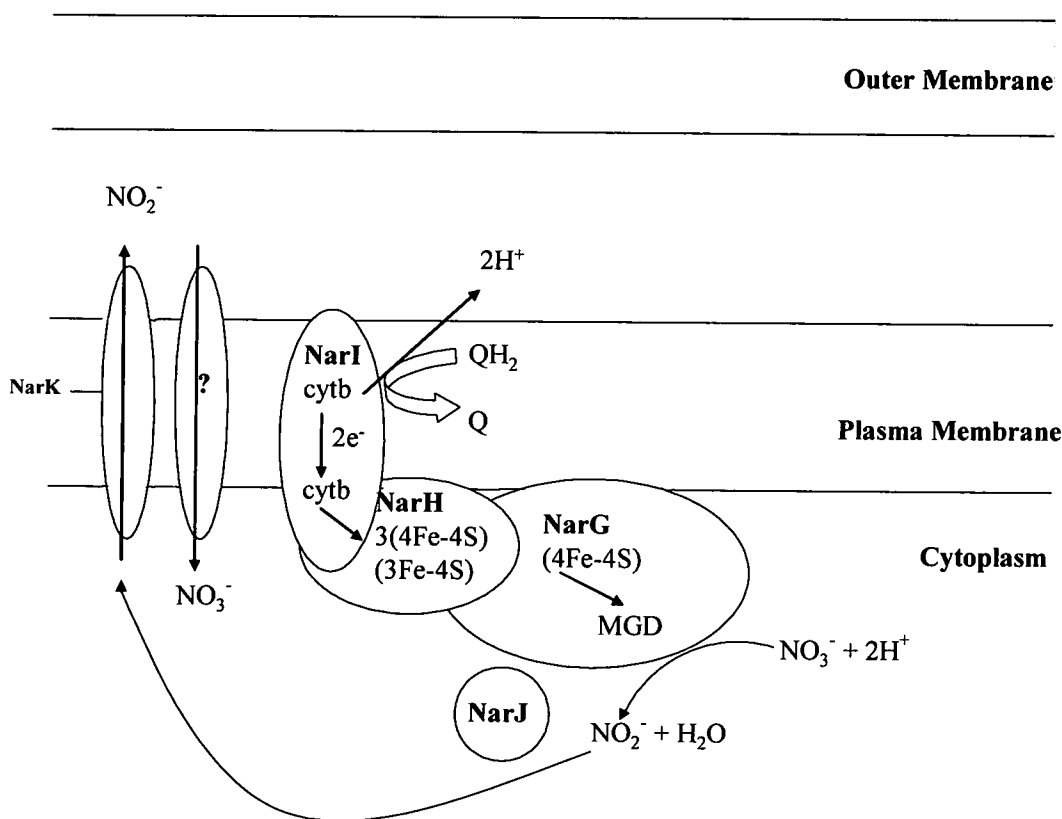


FIGURE 2. Model of membrane-bound respiratory nitrate reductase complex (*nar*). The figure above presents a schematic of the spatial arrangement of the respiratory nitrate reductase enzyme in the membrane. First, the electrons are transferred from the quinone pool to NarI (*cytochrome b*), and then to NarH (electron transfer subunit) and finally to narG (respiratory nitrate reductase, MoCo subunit), where the reduction of nitrate to nitrite occurs. NarJ is a chaperone protein that is responsible for the activation of NarG by inserting MoCo. The NarK protein carries out nitrite export. (Figure modified from (134)).

In general, all Nar enzymes are able to reduce chlorate and are inhibited by azide, chlorate, cyanide, and thiocyanate (76). In addition, intestinal tract bacteria have a Nar system that is involved in nitrosation of aromatic and alkyl amines by nitrite due to a weak NO-producing nitrite reductase activity associated with this enzyme (109). Thus, consumption of drinking water, especially well water, with high nitrate levels can imply a genotoxic risk for humans as indicated by an increase in HPRT variant frequencies and by the endogenous formation of carcinogenic N-nitroso compounds from nitrate-derived nitrite (179). Oddly, although subunits of the *E. coli* NRA and NRZ enzymes are very similar and can form an active hybrid complex (19), only the NRA is implicated in the nitrosation activity, whereas neither the second membrane-bound enzyme NRZ nor the periplasmic nitrate reductase contributes to the nitrosation reaction (109).

Nar I utilizes the quinol pool as the primary physiological electron donor ultimately generating a PMF by the conventional redox loop mechanism (11, 136). All the four proteins work synchronously and are involved in the electron transfer, activation, attachment and catalysis. Thus, NarI oxidizes quinols at the periplasmic face of the membrane and in the process releases two protons into the periplasm. Electrons are then passed on to NarG, via the Fe-S centers of NarH, to reduce nitrate with the consumption of two cytoplasmic protons. The low- and high-potential heme *b* groups of NarI located at opposite sides of the membrane allow an effective transmembrane electron transfer. Studies making use of electron paramagnetic resonance (EPR) spectroscopy and general biochemical characterization of the wild-

type and site-specific NarH mutants have revealed the presence of two pairs of Fe-S clusters in the β -subunit (66). Additionally, it has been proposed that a His-Cys₃ motif in the N-terminal end of NarG could bind a [4Fe-4S] center participating in the electron transfer from the Fe-S centers of NarH to the MGD cofactor (136). However, EPR spectroscopy studies could not detect this center and all the Fe-S clusters of the enzyme were found to be ligated by the Cys groups of the NarH subunit (103).

(B) Location

A variety of experimental approaches have been employed to determine the location of the respiratory nitrate reductase complex. Initially, immunoelectrophoretic studies indicated the enzyme to span the inner membrane (178). Subsequently, isotope-labeling experiments in combination with immunological techniques revealed that in *E. coli*, part of the α -subunit of the nitrate reductase faces the cytoplasmic side of the membrane (25). In contrast, the β -subunit seemed to be incorporated into the membrane (25, 102) while the γ -subunit (cytochrome *b*) was found to be periplasmic (25). The combined evidence now suggests that one facet of the γ -subunit is exposed to the periplasmic side and that the γ -subunit - nitrate reductase complex has a transmembrane orientation. This is in complete agreement with the hydrophilicity profile for the extrapolated proteins by the ORF3 region of the *narI* nucleotide sequence (162). The original *narI* mutation allowed for the accumulation of the α -, β -subunits in the cytoplasm (117, 162). Studies of the *narI* region indicated the presence of two open reading frames, which were denoted ORF3A and ORF3B. It is

now known that ORF3B codes for the narI (γ -subunit) protein and has significant hydrophobicity, while the ORF3A codes for the NarJ protein and contains only a few hydrophobic residues. Early studies proposed NarJ to be involved in attaching the NarG-NarH complex to the membrane-bound, NarI (162). This was in disagreement with the deduced amino acid sequence of the NarG polypeptide which neither showed the presence of hydrophobic domains, nor showed the presence of an N-terminal end signal sequence, suggesting a cytoplasmic location for the α -subunit (17). However, genetic (17), immunological (178), and biochemical (102) evidence indicates that the α -subunit is membrane-bound, so some form of modification must take place. It is now known that NarJ is a chaperone involved in the attachment of MoCo to NarG (16) and is not involved in membrane attachment to NarI (**Figure 2**).

An indirect approach also established the location of the catalytic site of the respiratory nitrate reductase on the cytoplasmic side of the membrane. Chlorate, an analog of nitrate, was found to be impermeable to the cytoplasmic membrane (115). Chlorate reduction was not seen in whole cells. However, chlorate was reduced in octylglucoside permeabilized cells where the transport barrier was destroyed suggesting that the catalytic site of the nitrate reductase is situated on the inner membrane (115).

(C) Genetic organization

As mentioned above, the respiratory nitrate reductase exhibits chlorate reductase activity, a property that was exploited to isolate chlorate resistant mutants also blocked in nitrate respiration. The reduction of chlorate to chlorite creates cellular toxicity and thus was used to identify nitrate reductase deficient mutants in many bacteria namely, *Proteus mirabilis* (45), *Enterobacter aerogenes* (174), *E. coli* (170) and *P. aeruginosa* (181). The early studies did not allow for the identification of regulatory genes or genes associated with the structural components of the enzyme complex.

The latest studies in *E. coli* have shown the presence of two different membrane-bound isoenzymes: NRA and NRZ. The *E. coli* NRA is encoded by the *narGHJI* operon located at 27 min on the chromosome, and NRZ is encoded by the *narZYWV* operon at 32.5 min (20). Many other homologues of *narGHJI* have been reported for other bacteria. In *E. coli*, NRA and NRZ show a high similarity: 76% identity for the catalytic subunits (NarG and NarZ), 75% identity for the β subunits (NarH and NarY), and 87% similarity for the NarI and NarV proteins (18). The *E. coli* also possesses the *narK* gene encoding a nitrite efflux porter (139) and the *narXL* genes encode a nitrate response two-component regulatory system, in which NarX is the nitrate sensor and NarL is the DNA-binding regulator (165). A *narK*-homologous gene (*narU*) is located upstream of *narZYWV* operon. The *narQP* genes coding for a second nitrate sensor (NarQ) and a second nitrate response regulator (NarP) are

located at 53 and 46 min on the *E. coli* genetic map, respectively (**Figure 3**) (20, 168).

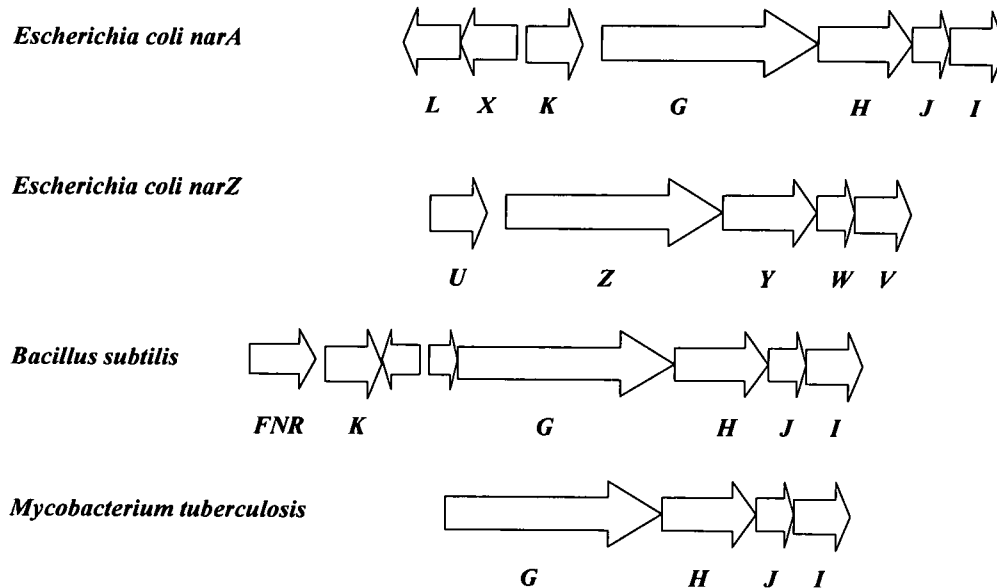


FIGURE 3. The organization of the membrane-bound nitrate reductase complex gene clusters in a number of different bacteria. The *narGHI* encode for the membrane-bound nitrate reductase enzyme complex and *narJ* encodes for a chaperone involved in the activation of the complex. Depending on the organism, the upstream region of *narGHJI* can vary. (Figure adopted from (134)).

(D) Regulation of the membrane-bound nitrate reductase

In *E. coli*, Nar proteins are synthesized only during anaerobic growth. Transcriptional regulation is mediated by FNR (Fumarate and Nitrate Reductase) protein anaerobically and by the two-component signal transduction systems of NarX (sensor)/NarL (response regulator) and NarQ (sensor)/NarP (response regulator) in the presence of nitrate/nitrite (33, 127, 169, 171). It should be

emphasized that the Nar system controls nitrate and nitrite metabolism strictly in response to the environmental need to perform anaerobic respiration and has nothing to do with the use of nitrate or nitrite as nitrogen sources for biosynthesis. Therefore, synthesis of Nar enzymes is unaffected by ammonium (11, 76, 168, 194). Although both NRA and NRZ show a high identity, the *narZ* operon is not regulated either by O₂ (Fnr) or by nitrate (18, 20). Constitutive NRZ could play a role, as proposed for the Nap system, in adaptation to anaerobic metabolism after the transition from aerobic conditions to anoxia or to prime the NRA system.

All the nitrate reduction regulatory mechanisms discussed so far occur at the level of transcription. In addition to these modes of regulation, the enzyme activity can also be regulated at the post-transcriptional level by oxygen. More specifically, oxygen interferes with nitrate transport and also causes a diversion of flow of electrons in the electron transport chain.

(1D) Response to oxygen: The FNR regulatory system

FNR-like factors have been recognized as important elements of the regulatory network of denitrification (164, 169). Mechanistically, FNR binds to a consensus sequence upstream of the FNR-regulated promoters acting as either an activator or repressor, depending on its location (6). Thus, the FNR protein acts as a positive regulator for the *narGHJI* operon (100, 168, 171), *frdABCD* operon (31), *dmsABC* (dimethylsulfoxide/dimethylamine sulfoxide reductase) operon (37, 164) and the *narK* gene (88), in *E. coli* (Figure 4). It negatively regulates the expression

of the genes that encode the terminal oxidases utilized in aerobic respiration namely, *cyoABCDE* (cytochrome *o* oxidase) and *cydAB* (cytochrome *d* oxidase) (36) (**Figure 4**).

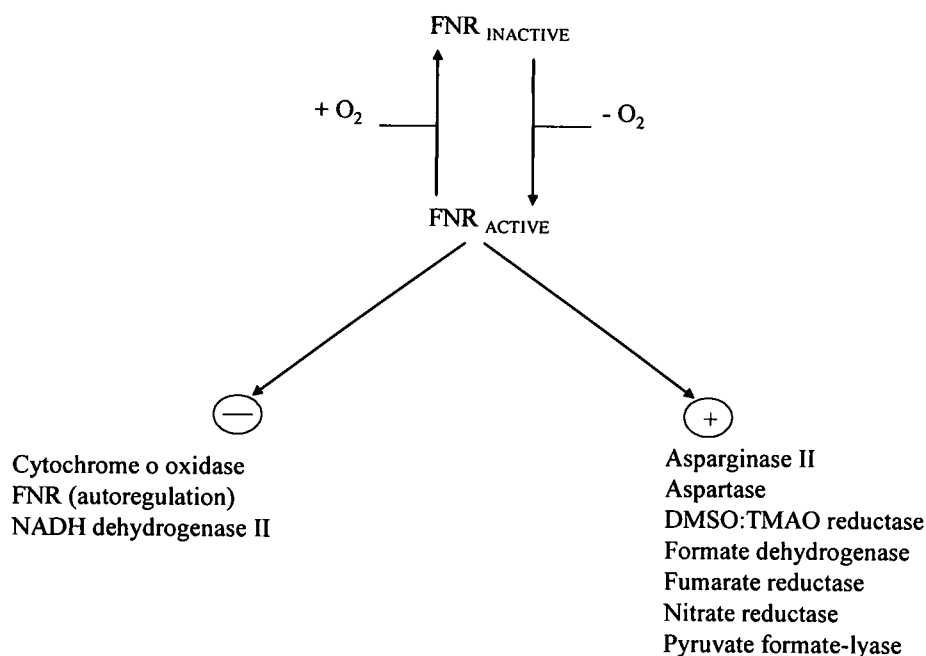


FIGURE 4. Global regulation of various genes by FNR. In the absence of oxygen FNR becomes activated to become an inducer for many anaerobically expressed genes and a repressor for certain aerobically expressed genes. Activation and repression of target operon expression are indicated by + and -, respectively. (Figure adopted from (186)).

To define the oxidation state and type of Fe-S cluster present in the active form of FNR, studies were carried out in anaerobically purified FNR by using Mössbauer spectroscopy. From these studies, reassembly or disassembly of a labile Fe-S center has been proposed as a model for the O₂-dependent FNR inactivation (87). Under anoxia, dimeric Fnr binds to DNA and activates transcription of *nar* and other anaerobic metabolism genes. Under aerobic conditions, the [4Fe-4S]²⁺ clusters are converted to [3Fe-4S]²⁺ or [2Fe-2S]²⁺ centers, resulting in FNR inactivation (87).

Stated another way, active FNR is a homodimer of an iron-sulfur protein with an oxygen-labile $[4\text{Fe-4S}]^{2+}$ cluster in each monomer. Upon exposure to oxygen, the $[4\text{Fe-4S}]^{2+}$ cluster is oxidized. When this happens, a fraction of the FNR loses its iron clusters and becomes an apoprotein. Both the protein bearing an oxidized cluster and the apoprotein are inactive. They bind with very low affinity to the DNA and do not stimulate transcription. The oxidation of the iron-sulfur clusters as well as their loss is reversible and under anaerobic conditions, the Fe-S clusters are restored. Thus, FNR represents a 'one-component' sensor/regulator utilizing Fe for signal perception (87).

(2D) Response to nitrate and nitrite: The Nar regulatory system

NarX and NarL function in *E. coli* as the sensor and transcriptional regulator, respectively, responding to the external nitrate and nitrite levels under conditions of nitrate respiration (33, 171, 172). Nitrate induces the *narGHJI* operon (168), *narK* gene (nitrite export) (88), and *fdnGHI* operon (formate dehydrogenase-N) (99) but represses the operons *frdABCD* (fumarate reductase) (99) and *dmsABC* (dimethyl sulfoxide reductase) (37) and the *adhE* gene (alcohol dehydrogenase) (166). NarX and NarL are duplicated in the homologous factors NarQ (33) and NarP (41) in *E. coli*. However, NarL and NarP are thought to primarily be response regulators for nitrate and nitrite, respectively (126). The *napF* promoter of the operon for periplasmic nitrate reductase transcription is regulated equally by nitrate and nitrite through the NarX and the NarQ sensors. It is under the positive control of NarP and is inhibited by NarL via competition for the NarL DNA-binding site (168).

Sequence comparisons strongly suggest that the NarX/NarQ and NarL/NarP proteins function in phosphoryl transfer reactions (**Figure 5**). Studies with purified proteins have examined some of these interactions in vitro.

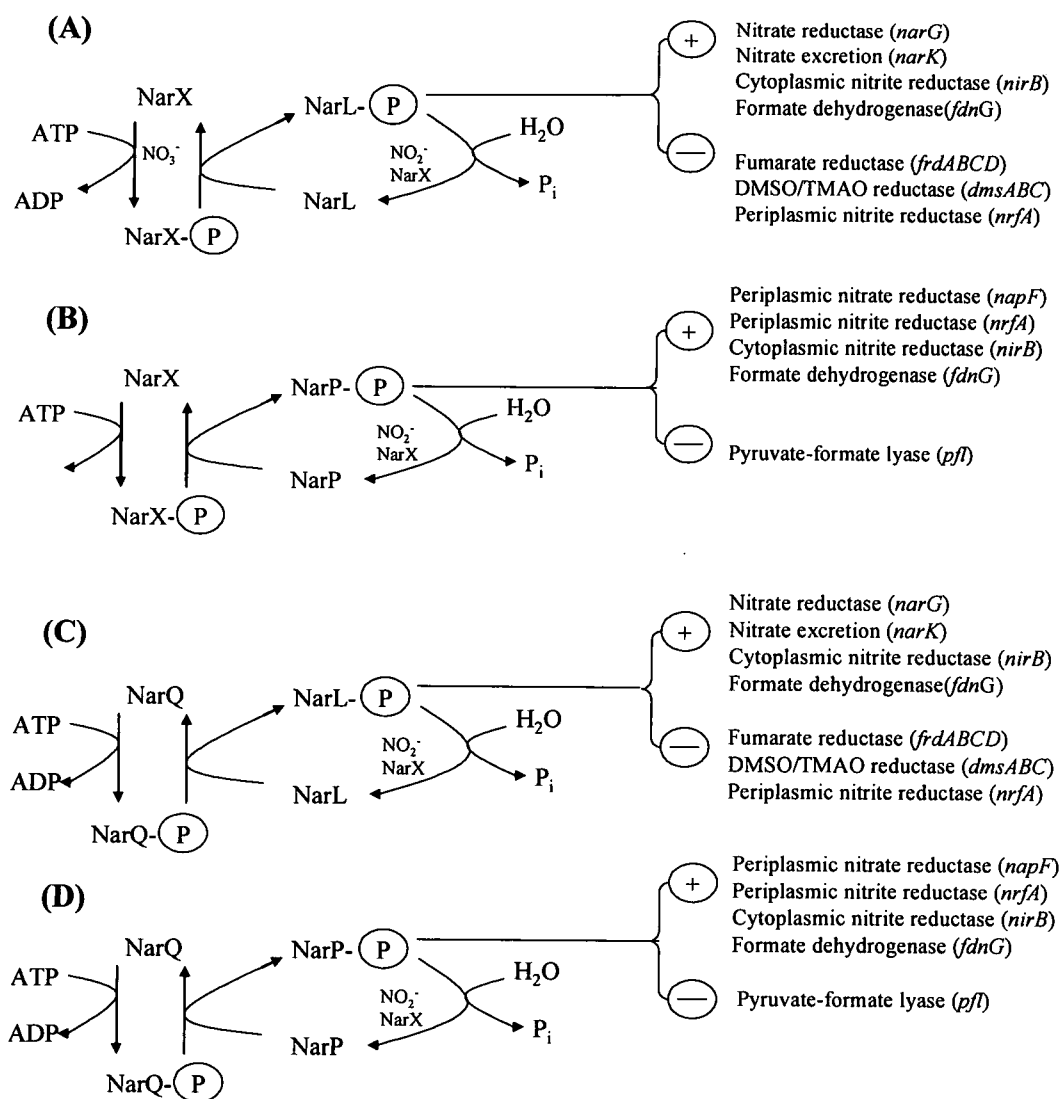


FIGURE 5. Model for nitrate- and nitrite-regulated gene expression. The membrane-bound histidine kinase sensor proteins are NarQ and NarX. The cytoplasmic response regulator proteins are NarL and NarP. Activation and repression of target operon expression are indicated by + and -, respectively. (A) Sensor/regulator pair *narX/narL*, (B) Sensor/regulator pair *narX/narP*, (C) Sensor/regulator pair *narQ/narL*, (D) Sensor/regulator pair *narQ/narP*

The NarX protein (in both full-length and amino-terminal truncated versions) autophosphorylates when incubated with ATP, and NarX-P serves as an efficient substrate for NarL protein phosphorylation (kinase activity) (153). The resultant NarL-P is relatively long-lived, but addition of NarX protein accelerates NarL-P dephosphorylation. This supports previous genetic evidence suggesting that the NarX protein additionally has a NarL-P phosphatase activity as well as NarQ. An amino-terminal truncated version of the NarQ protein has little effect on NarL-P dephosphorylation (32). The stability of NarL-P and NarX-P is affected by acidic and alkaline conditions in a manner consistent with the formation of phosphoaspartyl and phosphohistidyl residues, respectively (182). Finally, inclusion of nitrate in the mix has no influence on the rates of full-length NarX protein autophosphorylation, phosphoryl transfer to the NarL protein, or NarL-P dephosphorylation in the absence or presence of the NarX protein, suggesting that the solubilized NarX protein has lost the ability for ligand-dependant signaling (182).

(3D) Regulation by oxygen at the level of nitrogen oxyanion transport

Nitrate transport has been demonstrated to be a first step regulatory site for the modulation of nitrate reduction by oxygen in a number of organisms, including *E. coli* and *P. denitrificans*, *P. aeruginosa* (6, 17). This regulation has been extensively discussed in Chapter 2 of this thesis.

5. Dissimilatory Periplasmic Nitrate Reductases (Nap)

(A) Structure and biochemistry

Periplasmic nitrate reductases were first reported for phototrophic and denitrifying bacteria, but they are widespread among gram-negative bacteria. The enzyme has been linked with a variety of physiological functions. To begin with, the Nap activity does not appear to be primarily involved in nitrate assimilation or in anaerobic respiration although depending on the organism, the nitrite generated by Nap can be used as a nitrogen source or as a substrate for anaerobic respiration. The Nap enzyme is located in the periplasm and thus doesn't directly contribute to the generation of a PMF. In addition, the Nap system is independent of the energy-conserving cytochrome *bc*₁ complex, but it may be linked to the generation of a PMF when the electrons from NADH are passed through the proton-translocating NADH dehydrogenase by the quinone loop mechanism (13, 136). Nonetheless, even at that, the ΔP is insufficient to support anaerobic nitrate dependent growth in *Rhodobacter sphaeroides* in the dark (86, 112). Moreover, a *T. pantotropha* NarH mutant capable of overexpressing Nap was found to have a threefold decrease in nitrate dependent anaerobic growth, relative to Nar under denitrifying conditions (8). Conversely, in *Pseudomonas* sp. strain G-179, the Nap enzyme catalyzes the first step of denitrification in an energy-generating process, although the mechanism by which this was accomplished is unclear (7). Thus, the physiological role of the Nap system may vary in different organisms or even in the same bacterium under different metabolic conditions. For instance, there are data in support of the fact that the Nap

enzyme is utilized for redox balancing (13, 112, 135, 137, 157). Maintenance of an appropriate redox balance is necessary for optimal bacterial growth under some physiological conditions such as during fermentation in enteric bacteria, oxidative metabolism of highly reduced carbon substrates in aerobic heterotrophs, or anaerobic photoheterotrophic growth in photosynthetic bacteria. Additionally, in the case of *Thiosphera pantotropha*, since oxygen primarily inhibits denitrification at the level of nitrate transport (47) and since the Nap system does not require this step, this particular organism can perform aerobic denitrification, coupling the Nap enzyme to the nitrite and *N*-oxide reductases (9, 13). This aerobic denitrification step could be a valuable attribute for organisms such as *Thiosphera* growing under microaerobic conditions or in environments where there can be a sudden transition from aerobic conditions to anoxia. Other proposed roles for Nap are the adaptation to anaerobic metabolism after transition from aerobic conditions, the utilization of alternate reductants (160), or even a self-defense mechanism forming high nitrite levels to inhibit the growth of potential competing bacteria (86).

Nap systems have been studied at the biochemical and/or genetic level in a variety of bacteria namely, *Alcaligenes eutrophus* (*Ralstonia eutropha*), *T. pantotropha* (*P. denitrificans*), *E. coli*, and *Rhodobacter* species. The enzyme is a heterodimer containing a 90-kDa catalytic subunit (NapA) with MGD cofactor and an N-terminal [4Fe-4S] center and a 15-kDa biheme *c* cytochrome (NapB), which receives electrons from NapC, a membrane-bound tetraheme cytochrome *c* of 25 kDa (13, 14, 132, 133). Enzyme activity utilizing the non-physiological reduced viologen

as electron donors decrease when the NapB subunit is lost during purification of the *R. capsulatus* enzyme (108). The role of NapC in the electron transfer to the periplasmic enzyme complex has been supported by the results of mutational analysis in *R. sphaeroides* (132, 133). NapC homologues are by extrapolation, thought to be involved in electron transfer between the membrane quinol pool and several soluble periplasmic reductases. Electron transfer by the NapC family appears to be cytochrome *bc*₁ independent and is not coupled to proton translocation. The spectroscopic characterization of a soluble form of NapC expressed as a periplasmic protein has been recently reported (**Figure 6**) (138).

The NapA enzyme complex crystallized from *Desulfovibrio desulfuricans* comprises a single-subunit form (NapA) and lacks the biheme cytochrome *c* subunit (NapB) (49). However, it cannot be excluded that the NapB subunit might be lost during purification as the two subunits can be separated in the *Rhodobacter capsulatus* enzyme. The Nap protein is folded into four domains, all of them involved in MGD cofactor binding, and its structure is more similar to that of formate dehydrogenase than to dimethylsulfoxide reductase. The structure of NAP reveals the details of the catalytic molybdenum site, which is coordinated to two MGD cofactors, a Cys residue, and a water/hydroxo ligand. A facile electron-transfer pathway through bonds connects the molybdenum and the [4Fe-4S] cluster (49). This structure suggests a mono-oxo/desoxo catalytic cycle, although a di-oxo/mono-oxo cycle has also been proposed for the *T. pantotropha* enzyme (10).

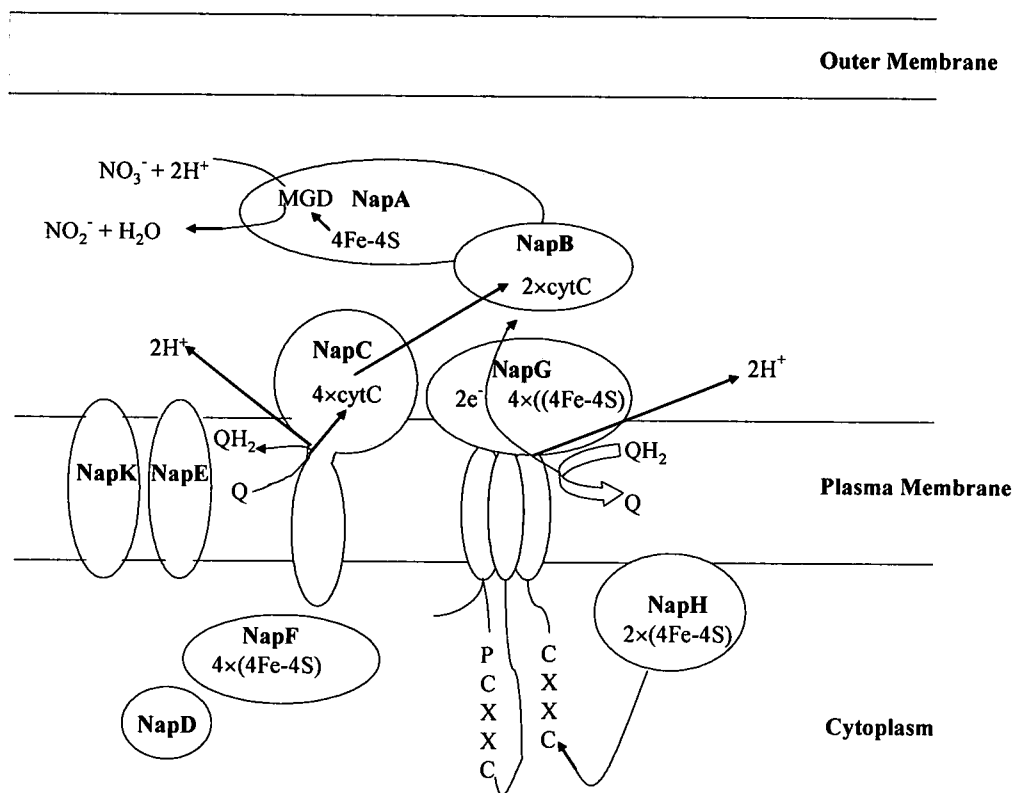


FIGURE 6. Model of dissimilatory periplasmic nitrate reductase (*nap*). Schematic of the spatial arrangement of the periplasmic nitrate reductase enzyme in the periplasm. In most proteobacteria, the *napDABC* cluster is highly conserved where NapA is the terminal bis-MGD-containing reductase, NapB is the diheme, NapC is the tetraheme and NapD is a private chaperone involved in the maturation of NapA prior to its export to the periplasm (Figure adopted from (134)).

The results of electron paramagnetic resonance and X-ray absorption spectroscopy analyses suggest that the Mo environments in the soluble Nas and Nap enzymes are similar to each other but distinct from that in the membrane-bound Nar (10, 28, 62). Nap and Nar are also catalytically distinct: for instance in *Thiosphera pantotropha*, Nap is less sensitive to inhibition by cyanide, does not use chlorate as substrate, and is slightly stimulated by thiocyanate and azide (13, 76). However, in *R.*

sphaeroides, Nap activity is competitively inhibited by chlorate. Both nitrate and chlorate stimulate the phototrophic growth in the wild-type strain, but not in a *napA* mutant. This Nap-dependent chlorate or nitrate stimulation of bacterial growth has been explained in terms of redox balancing; the dissipation of excess photosynthetic reducing power allows optimal growth (113, 137).

(B) Genetic organization

Since the initial identification of *napAB* structural genes of *Alcaligenes eutrophus* (160), several other *nap* genes have been sequenced from various organisms (7, 13, 56, 64, 132, 133). Of significant mention are the organisms *Rhodobacter capsulatus* (188), *Rhodobacter sphaeroides* (30), *Alcaligenes eutrophus* (160), and *Paracoccus denitrificans* (13). In these organisms, the *nap* genes are present endogenously on plasmids and their expression is unaffected by O₂. On the other hand, the *E. coli* *nap* genes are clustered on the chromosome (*aeg-46.5* locus) and are induced only anaerobically by Fnr (40, 64). Thus, the plasmid location of most *nap* genes, the heterologous expression of *nap* genes (133), and the fact that the ability to reduce nitrate is present in only a few wild-type strains of purple bacteria suggests the possibility of horizontal transfer of *nap* genes within the bacterial community.

Rhodobacter sphaeroides contains a seven gene operon *napKEFDABC* involved in periplasmic nitrate reduction (132, 133). Of the seven genes, the *napABC* are the structural genes of the enzyme complex and are, therefore, required for *in vivo*

activity. The NapE and NapK are small transmembrane proteins of unknown function (134). NapF, a soluble protein of 16 kDa with four Cys clusters, probably binds four [4Fe-4S] centers, which are proposed to be involved in the assembly of the iron-sulfur center of NapA (111). Finally, NapD is a 9-kDa cytoplasmic protein that could participate in the maturation or processing of NapA (132). Similar *napEFDABC* and *napEDABC* gene clusters are found in *Pseudomonas* sp. strain G-179 and *T. pantotropha*, respectively (7, 13). Interestingly, in *Pseudomonas* sp. strain G-179, the *nap* genes are linked to *nir* and *nor* genes which are involved in nitrite and nitric oxide reduction, respectively (7). In *E. coli*, seven *nap* genes and eight cytochrome *c* biogenesis genes are clustered in the anaerobically regulated *aeg-46.5* locus (64). This locus lacks a *napE*-homologue but contains two additional *napGH* genes. NapG is a 20-kDa soluble protein with four putative [4Fe-4S]²⁺ centers, and NapH is a 32-kDa membrane protein that probably binds two [4Fe-4S]²⁺ centers. It has been proposed that a putative NapGH complex could act as a redox sensor controlling the electron flow to NapA (13). Sequencing of the *Haemophilus influenzae* genome (56) has shown the presence of a *nap* locus organized identically to that in *E. coli* but unlinked to cytochrome *c* biogenesis genes. (Figure 7)

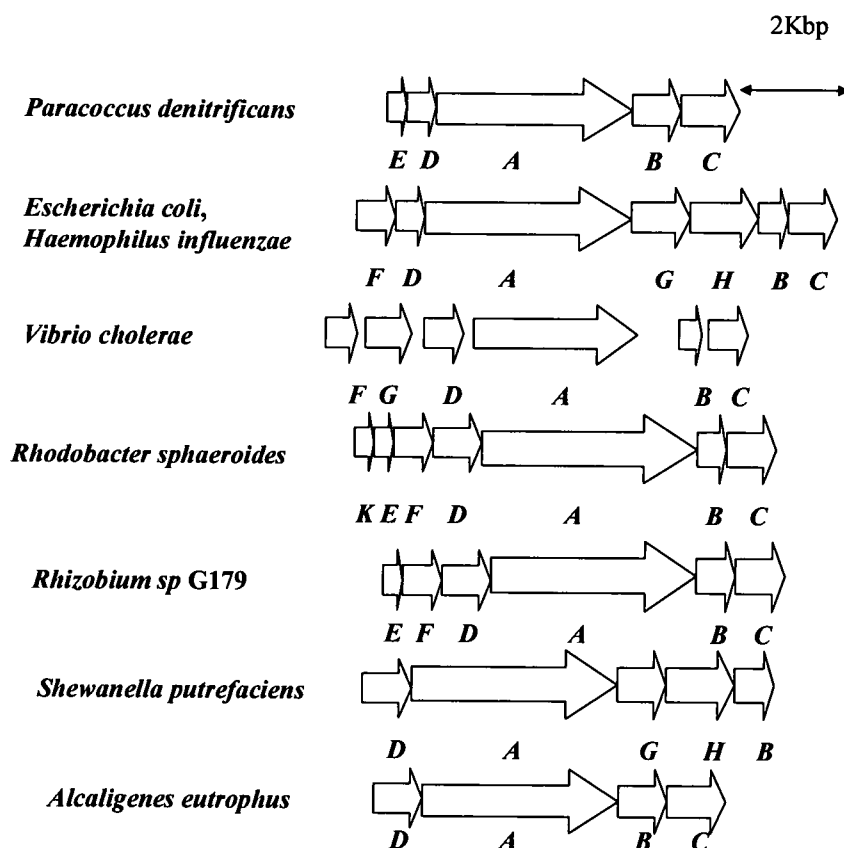


FIGURE 7. The organization of the periplasmic nitrate reductase gene clusters in a number of different bacteria. The *nap* gene clusters amongst different bacteria reveals a considerable amount of heterogeneity in their composition. With the exception of the organism *S. putrefaciens*, the cluster *napDABC* seems to be conserved in all the organisms (Figure adopted from (134)).

(C) Export of Nap to the periplasmic space

The periplasmic location of the Nap enzyme raises important questions about its translocation process. Analysis of NapB, the cytochrome *c* subunit, reveals the presence of a signature N-terminal signal sequence which is required for a Sec-dependent translocation. Thus, the heme binding to the NapB apoprotein can take

place in the cytoplasm, which is consistent with observations made for other cytochrome *c* homologues (118). On the other hand, several observations suggest that the catalytic NapA subunit could be assembled in the cytoplasm and then exported into the periplasm in a folded state by a Sec-independent pathway (111). Firstly, NapA and other periplasmic molybdoenzymes contain N-terminal signal sequences that are unusually long and contain a twin-Arg motif. This pre-sequence can be involved in an alternative translocation system similar to the PMF-dependent thylakoid import pathway (54). Secondly, the MGD cofactor is synthesized in the cytoplasm by the action of five different loci (*moa*, *mob*, *mod*, *moe*, and *mog*), and no cofactor export system has been reported for any bacteria analyzed so far. Finally, the size of the cofactor and the fact that it is almost buried within the protein (49) suggests that the MGD cofactor should be assembled and inserted into the apoprotein in the cytoplasm prior to protein translocation. It has been demonstrated that cofactor insertion into the apoprotein is a prerequisite for the translocation of the *E. coli* trimethylamine *N*-oxide reductase by a Sec-independent pathway (150). Recently, the *E. coli* genes required for the Sec-independent export of cofactor-containing periplasmic proteins have been identified (151, 183).

(D) Regulation of Dissimilatory Periplasmic Nitrate Reductases

Although there are some differences reported in the *nap* gene expression depending on the organisms, the Nap systems are normally unaffected by ammonium or oxygen. In phototrophic bacteria, the Nap activity is present under aerobic and

anaerobic conditions and is unaffected by ammonium or by the intracellular C and N balance. In addition, Nap activity is stimulated by nitrate, although a basal activity is also observed in the absence of nitrate (50, 133). For instance, in *P. denitrificans* the Nap activity is observed in aerobically grown cells even in the absence of nitrate. Activity is maximally expressed during growth on highly reduced carbon sources, such as butyrate, suggesting a Nap regulation in response to the redox state of the bacterium (157). Similarly, the Nap system is not induced by nitrate in *A. eutrophus*, and maximal expression is observed under aerobic conditions at the stationary phase (160). On the other hand, expression of the *E. coli nap* operon (*aeg-46.5* locus) is induced under anaerobic conditions, via the Fnr regulator, and by nitrate or to a lesser extent by nitrite, via the homologous regulators NarL and NarP. Both proteins compete *in vivo* for a common binding site in the *aeg-46.5* promoter region, but only phosphorylated NarP activates gene expression. Thus, NarL has a negative effect on expression of the *aeg-46.5* operon because it antagonizes NarP-dependent activation (39). The *nap* gene cluster of *Pseudomonas* sp. strain G-179 could also be regulated by an Fnr-like protein under anaerobic conditions (7).

6. Denitrification: The *Pseudomonas aeruginosa* model

Denitrification by prokaryotes represents the final step of the nitrogen cycle. As a result of this process, nitrogen in the form of nitrate is utilized for anaerobic respiration where it is ultimately reduced to dinitrogen gas and returned to the biosphere. The best-studied denitrifiers include *P. aeruginosa*, *P. stutzeri*, *Paracoccus*

denitrificans, and *Rhodobacter sphaeroides*. Denitrification is of greater consequence both physiologically and in the grander scheme than the dissimilation of nitrate by enterics because of its many important ramifications to our ecosystem (111), public health (111), as well as in biofilm formation (52). Important negative effects of denitrification include the removal of fixed nitrogen from agricultural soils thus, limiting plant productivity and the generation of nitrous oxide, which is a green house gas and is responsible for depletion of the ozone layer. Additionally, denitrification is performed by the opportunistic pathogen *P. aeruginosa* during biofilm formation and lung infections of patients with CF (52). Denitrification is beneficial in waste-water treatment plants, enabling the removal of excess nitrate and nitrite from waste water.

Genetic and molecular studies of the denitrification pathway of *P. aeruginosa* are limited, but some early genetic and biochemical studies provide basic information. Mutants of the respiratory nitrate reductase of *P. aeruginosa* were isolated and partially characterized, on the basis of chlorate resistance (26, 154). Phenotypic analysis allowed for the characterization of five phenotypes, few of which have been concretely identified in terms of biochemical function. Only the *narG* and the *mod* gene loci could be identified from the early *P. aeruginosa* mutant phenotypes reported. The nitrate reductase from *P. aeruginosa* was partially purified and characterized by two different groups (26, 49). In one case, the enzyme was purified to homogeneity and consisted of two heterogenous subunits of 64 kDa (NarH and 118 kDa (NarG). No cytochrome component was identified, although the techniques used at the time typically resulted in the loss of this component during purification.

Fewson and Nicholas partially purified the nitrate reductase from *P. aeruginosa* but did not characterize the subunits by molecular weight (55). The unusual results from this investigation included the report that NADH served as the electron donor for the reduction of the purified enzyme and a cytochrome *c* was found to be associated with the nitrate reductase complex.

Further genetic and biochemical studies on the nitrate reduction system of *P. aeruginosa* have not been forthcoming. This represents an area of great need in order to elucidate a nitrate reduction system that is significantly different from the non-denitrifying *E. coli*. Much of what we now know about the nitrate reduction systems of *P. aeruginosa* is based strictly on gene and protein homology with experimentally verified components from other organisms such as *E. coli*, *B. subtilis*, *P. stutzeri* and *P. denitrificans*. The completion of the *Pseudomonas* genome project has allowed us to piece together a model for nitrate reduction in this organism but experimental verification is now essential.

The remainder of this chapter will attempt to provide a hypothetical model for the denitrification pathway of *P. aeruginosa*, based on data from the *Pseudomonas* genome project. The only experimentally verified components of the nitrate reduction system are currently the NarG and NarH subunits of the nitrate reductase complex; the ANR, DNR regulation system and the NarK1 and NarK2 transport proteins (Table 2).

TABLE 2. Respiratory and periplasmic nitrate reduction genes of *P. aeruginosa* and their functions.

Respiratory or Periplasmic	Loci	Gene Name	Function
Respiratory	PA3872	narI	Nitrate reductase (<i>cyt b</i>)
	PA3873	narJ	Chaperone for MoCo
	PA3874	narH	Nitrate reductase e- transfer
	PA3875	narG	Nitrate reductase (MoCo)
	PA3878	narX	Two-component sensor
	PA3879	narL	Two-component response regulator
	PA2376	narLII	Two-component response regulator
Periplasmic	PA1174	napA	Nitrate reductase (MGD)
	PA1173	napB	Cytochrome <i>c</i>
	PA1172	napC	Cytochrome <i>b</i>
	PA1175	napD	maturation of NapA
	PA1176	napF	Ferredoxin protein
	PA1177	napE	Transmembrane/unknown

7. Membrane-bound respiratory nitrate reductase of *Pseudomonas aeruginosa*

(A) Structure

In *P. aeruginosa*, the *narG* gene encodes for a 141 kDa protein and is believed to be the α -subunit of the *P. aeruginosa* respiratory nitrate reductase. A *narG::lacZGm* mutant is unable to utilize nitrate as a terminal electron acceptor under anaerobic conditions (116, 173). Adjacent to the *narG* gene is the *narH* gene which encodes for a 58.1 kDa protein and contains 15 conserved cysteine residues which are involved in Fe-S binding suggesting that the NarH of *P. aeruginosa* participates as the electron transfer subunit. Finally, both *narJ* and *narI* are also present in *P. aeruginosa* encoding for 27.3 and 25 kDa proteins, respectively. NarI is the γ -subunit

of the nitrate reductase and is anchored to the cytoplasmic membrane, where it acts as an electron donor for the reduction system.

(B) Genetic organization

In *Pseudomonas aeruginosa*, the *narK1* and *narK2* genes are present in an operon together with the *narGHJI* genes (158). The *narK1* and *narK2* genes are involved in nitrite export and perhaps nitrate uptake and are present upstream of the structural genes of nitrate reductase. In addition, the *narX* and *narL* genes are located adjacent to the *narK1K2GHJI* cluster, but are transcribed in the opposite direction. These genes are part of a two-component signal transduction mechanism that regulate the denitrification pathway. The *nar* genes are located between 4,335,275- and 4,347,533 bp on the chromosomal map of *P. aeruginosa* PAO1.

(C) Regulation of nitrate reduction

In *P. aeruginosa*, Nar proteins are synthesized during anaerobic growth, via the ANR transcriptional regulation (FNR analogue) and DNR proteins. Nitrate or nitrite probably activates the two-component regulatory systems of sensor protein (NarX; a Nar Q has not yet been identified) and DNA-binding regulators (NarL and PA2376- NarLII; NarP does not seem to be required). In addition, synthesis of Nar enzymes seem to be unaffected by ammonium. Several factors of this regulatory network combining the initiator reaction with the main process are just being

recognized (Table 3), and a full experimental dissection of this network is anticipated to be a long-term task.

TABLE 3. Regulatory genes in *P. aeruginosa* denitrification.

Gene Name	Loci	Function
DNR	PA0527	Transcriptional regulator
ANR	PA1544	Transcriptional regulator
narX	PA3878	Two-component sensor
narL	PA3879	Two-component response regulator
narLII	PA2376	Two-component response regulator
narP	PA1397	Two-component response regulator

(1C) The FNR homologue in *P. aeruginosa* is the ANR

A promoter probe vector carrying *lacZ* under the control of an FNR recognition motif first indicated the existence of FNR-like elements in *P. aeruginosa* (58). In the absence of nitrate and oxygen, arginine degradation allows *P. aeruginosa* an anaerobic way of life to sustain motility and growth by substrate level phosphorylation (58). In searching for the regulator for the system of arginine degradation encoded by the *arcDABC* genes, the *nar* mutant S1239 was found to be pleiotropic with respect to nitrate and arginine utilization (58). Investigation of the affected locus led to the discovery of *anr* (58). The *anr* mutation was mapped in the 59-min region of the *P. aeruginosa* chromosome. ANR is a homolog of FNR (51% sequence identity) that can functionally replace FNR in *E. coli*, in activating the *pfl* genes for the pyruvate formate-lyase complex (152). With its N-terminal and central cysteine residues, a DNA-binding motif, and the glycine-rich β -roll structure, ANR conserves essential structural features of an FNR protein (58). It was named ANR to

reflect its role in arginine catabolism and nitrate reduction, because *P. aeruginosa* has no fumarate respiration system.

The *anr* gene encodes for a protein of 244 amino acids (152). Expression of the *anr* gene in a T7 promoter/polymerase system identified ANR as a 31-kDa protein (152). Transcriptional analysis of the *anr* gene has showed that it is monocistronic but apparently lacks the equivalent sites for negative auto-regulation that have been shown to be present in the promoter region of the *E. coli fnr* gene.

The promoter region of the *arcDABC* operon carries at position -39.5 the nucleotide sequence TTGAC-N₄-ATCAG, resembling the FNR box (61). Manipulating this sequence decreases or abolishes the expression of the *arc*-encoded enzymes (264). Most importantly, deletion of *anr* affects all enzyme activities of the four steps of the denitrification pathway (191). ANR, thus, is as a global regulator in *P. aeruginosa* for anaerobic metabolism that, in addition to controlling the *arc* operon and other metabolic activities like cyanogenesis, regulates the entire denitrification process. The details of how this control is achieved remain unknown.

FNR of *E. coli* complements an *anr* mutant of *P. aeruginosa* in activating anaerobic nitrate utilization but not arginine catabolism (58). The promoter of the ANR-dependent *arc* genes is not active in wild-type *E. coli*; i.e., FNR does not recognize the *arcD* promoter. ANR appears to be less stringent in its DNA-binding specificity than FNR. While this provides a rationale for differential activity between the two, it cannot explain how cognate genes are discriminated in the simultaneous presence of two or more FNR factors within the same cell. Recognition helices and

recognition motifs reveal only subtle differences to explain the binding of an FNR factor to a distinct set of target promoters. Future answers may be found in the participation of neighboring nucleotides in the recognition process, as shown for FNR of *E. coli*; the exertion of hierarchical control over the FixK-like factors; and/or further protein requirements for transcriptional activation.

(2C) *P. aeruginosa* requires an additional transcriptional regulator DNR

The gene *dnr* is located in the vicinity of the structural genes for nitrite reductase (*nirS*) and nitric oxide reductase (*norCB*), and the gene for activation of the reductases (*nirQ*) (4). The gene encodes for a protein of 227 amino acids, homologous with the CRP/FNR-family of transcriptional regulators (5). The promoter activities for *nirS*, *nirQ* and *norCB* were considerably reduced in the *dnr* mutant as well as in the mutant of *anr*, which is indicative that both ANR and DNR were required as transcriptional regulators in *P. aeruginosa* (4, 68).

Recent studies have found a cascade form of regulation in effect by ANR and DNR in *P. aeruginosa* (**Figure 8**) (68). Both ANR and DNR had previously shown to be CRP/FNR-related transcriptional regulators necessary for denitrification (5). Specifically, it was found that the transcription of the *dnr* gene was under the control of ANR (5). When the *dnr* gene was expressed by *tac* promoter in an *anr* mutant, the strain recovered the ability to grow under anaerobic conditions by denitrification. Nitrate, nitrite, nitric oxide and nitrous oxide reducing activities were expressed, and the structural genes for nitrite and nitric oxide reductases were transcribed under

anaerobic conditions in the *anr* mutant strain transformed with the *dnr* gene. These findings suggested that the expression of the denitrification system was controlled not by DNR. However, the arginine deiminase activity and cyanide production were not regulated by DNR. This was because it was found that the activation of a consensus FNR-dependent promoter by DNR occurred in response to the presence of nitrite whereas activation by ANR did not, suggesting that only DNR was involved in sensing nitrogen oxides (4).

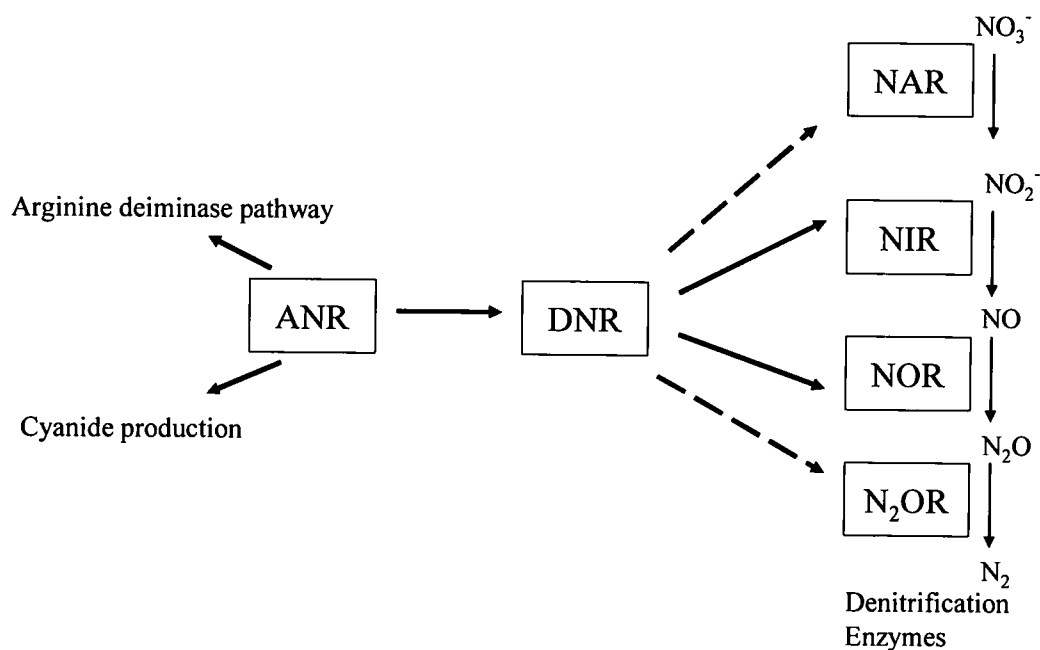


FIGURE 8. A schematic representation of the regulatory cascade involving two CRP/FNR-related transcriptional regulators, ANR and DNR in *P. aeruginosa*. ANR regulates the expression of enzymes for denitrification and the anaerobic arginine deiminase pathway, and the production of cyanide. DNR mediates the ANR-dependent expression of the denitrification enzymes. The dotted arrows represent the enzymes whose activation by DNR has not yet been identified. (Figure adopted from (5)).

(3C) Bacterial response to nitrate/nitrite: Nar regulation

In the *Pseudomonas* genome database, the *narX* and *narL* genes are located adjacent to the *narK1K2GHJI* operon but are transcribed in the opposite direction. The *narX* gene encodes for a 69.3 kDa protein with 48% similarity to NarX of *E. coli*. The NarX of *P. aeruginosa* contains the conserved 15 amino acid "P-box" in the N-terminal region of the protein and the C-terminal region shares similarity to autokinases. The *narL* gene encodes for a 23.7 kDa protein with a 74% similarity to NarL of *E. coli*. Similar to *E. coli* NarL, the *Pseudomonas* NarL also contains the helix-turn-helix DNA-binding domain found in the C-terminal end and the response-type-signaling motif located in the N-terminal region. Interestingly, apart from these two proteins, *P. aeruginosa* contains a second NarL-like protein (Table 2), NarL-II. Mutations in all three proteins i.e., NarX, NarL and NarL-II, renders *P. aeruginosa* incapable of respiring with nitrate as the terminal electron acceptor (unpublished data). Currently, studies that will enable us to functionally characterize these proteins are in progress. It remains to be seen how different the *nar* regulatory system of *Pseudomonas* is compared to *E. coli*.

8. Dissimilatory Periplasmic Nitrate Reductases (NAP) of *P. aeruginosa*

Finally, there is a periplasmic nitrate reductase in *P. aeruginosa*. Unlike that of *E. coli*, there is no homologue of a constitutively expressed *narZ* operon in *P. aeruginosa*. There is, however, a periplasmic nitrate reductase located between 1,272,200 bp and 1,276,784 bp of the *P. aeruginosa* chromosome (175). The

napEFDABC operon is organized in a similar fashion to that of *R. sphaeroides*. The hypothetical *napA* gene encodes for the MGD containing periplasmic nitrate reductase which is 84% similar to that of *E. coli*. The rest of the *nap* cluster is similar to the genes and gene products previously described in *E. coli*. The exceptions are the lack of the *napG* and *napH* gene products, which are presumed to be a redox sensor system to control the electron flow to NapA, and the addition of the *napE* gene product. NapE is a small (16.8 kDa) transmembrane protein with an unknown function. There are two functional possibilities for the periplasmic nitrate reductase. The first is represented in the denitrifier, *P. denitrificans*, in which the membrane-bound nitrate reductase is the primary enzyme for nitrate reduction, while the periplasmic enzyme only has secondary functions, including intermittent aerobic denitrification, transition to anaerobic respiration or dissipation of excess reducing equivalents. The second possibility is represented by the *Pseudomonas* sp. Strain G-179 that may use the periplasmic nitrate reductase for the first step of denitrification. Mutations in the *nap* genes abolish all denitrification activity in this strain of *Pseudomonas*. It remains to be seen however, what role the periplasmic nitrate reductase has in *P. aeruginosa* until, further molecular and biochemical testing is performed.

9. Conclusions

Nitrate respiration is observed in a wide variety of bacteria including *E. coli* and *P. aeruginosa*. Although the net energy gain by this process is not as efficient as aerobic respiration, it still allows cellular metabolism to continue more efficiently than during fermentation. Biochemical and genetic studies of nitrate reduction have revealed a complex process. Denitrification provides the only natural mechanism of returning fixed nitrogen back to the atmosphere in the form of nitrogen gas. Regulation of nitrate respiration is also complex, being induced by nitrate and repressed by oxygen in a number of different ways. It is really interesting to see that bacteria have evolved to possess such a complex regulatory circuit for response to two small inorganic molecules namely, nitrate and nitrite. With advances in the biotechnology and sequencing of bacterial genomes still forthcoming, many of the questions concerning prokaryotic nitrate respiration will be answered.

Chapter 3

INVOLVEMENT OF NARK1 AND NARK2 PROTEINS IN TRANSPORT OF NITRATE/NITRITE IN THE DENITRIFYING BACTERIUM *Pseudomonas aeruginosa* PAO1

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Involvement of NarK1 and NarK2 proteins in transport of nitrate/nitrite
in the denitrifying bacterium *Pseudomonas aeruginosa* PAO1

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Running Title: NarK and Nitrate/Nitrite transport in *P. aeruginosa*

Abstract

Two transmembrane proteins were tentatively classified as NarK1 and NarK2 in the *Pseudomonas* genome project and hypothesized to play an important physiological role in nitrate/nitrite transport in *Pseudomonas aeruginosa*. The *narK1* and *narK2* genes are located in a cluster along with the structural genes for the nitrate reductase complex. Our studies indicate that the transcription of all these genes are initiated from a single promoter and the gene complex *narK1K2GHJI* constitutes an operon. Utilizing isogenic mutants of *narK1*, *narK2* and a *narK1K2* double mutant, we explored their effect on growth under denitrifying conditions. While the $\Delta narK1::Gm$ mutant was only slightly affected in its ability to grow under denitrification conditions, both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ were found to be severely restricted in nitrate dependent anaerobic growth. All three strains demonstrated wild-type levels of nitrate reductase activity. Nitrate uptake by whole cell suspensions demonstrated both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants to have very low yet different nitrate uptakes while the $\Delta narK1::Gm$ mutant exhibited wild-type levels of nitrate uptake. Finally, *E. coli narK* rescued both $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants with respect to anaerobic respiratory growth. Our results indicate that only

the NarK2 protein is required as a nitrate/nitrite transporter by *Pseudomonas aeruginosa* under denitrifying conditions.

Introduction

Denitrification involves four separate nitrogen oxide reductases and ultimately reduces nitrate to dinitrogen (194). Respiratory nitrate reductase, which is the first enzyme in this denitrification pathway, has its active site on the cytoplasmic side of the membrane (134). The enzyme substrate, nitrate, is an ion and cannot be taken up by the simple process of passive diffusion (110). Both of these factors require the bacterium to synthesize transport protein(s) to carry nitrate into the cytoplasm, where reduction of nitrate to nitrite takes place. It has been demonstrated in *P. aeruginosa*, *P. stutzeri* and *E. coli* (48, 73, 139) that the product of nitrate respiration, i.e. nitrite, is immediately excreted to the external environment presumably protecting the organism from potential toxic effects. These toxic effects are due to the ability of this anion to bind to the heme groups in electron carriers thereby inhibiting the flow of electrons (140). Genetic and physiological data suggest that nitrate transport in some bacteria occurs through two different uptake systems. Thus, for the process of nitrate assimilation, ABC transporters as well as secondary transporters are postulated to be used. On the other hand, anaerobically, for the purpose of nitrate respiration it is postulated that bacteria rely solely on secondary transporters (110).

Originally, John (81) demonstrated that membrane permeabilization of the cells significantly enhanced nitrate uptake suggesting the need for a transport protein specific for nitrate. This was corroborated by several other studies which also demonstrated that external nitrate uptake in whole cells was restricted by a permeability barrier (72, 114). It was also observed that nitrate reduction and nitrate

uptake were closely coupled as *narG*-deficient mutants did not take up nitrate (139). Others demonstrated that nitrate uptake and reduction resulted in the immediate excretion of nitrite (7).

The first genetic locus identified as playing a role in nitrate uptake or nitrite excretion was *narK* of *E. coli* K-12 (46, 139, 170). Subsequently other NarK-like proteins were identified by homology and by phenotype. NarK families of proteins belong to the Major Facilitator Superfamily (MFS) of transmembrane transporters, and are categorized as secondary transporters requiring the generation of a proton motive force (PMF) (105). Homologues of NarK seem to be present in a multitude of organisms where they may serve as either nitrate/proton symporters or as nitrate/nitrite antiporters.

E. coli is the paradigm for respiratory nitrate metabolism in bacteria. The current state of knowledge is primarily based on studies in this organism which possesses two nitrate/nitrite transport proteins, NarK and NarU (21). These porters are separate from the *narG* operon which contains the genetic information for the nitrate reductase enzyme complex. Since the first studies in *E. coli*, NarK homologues have been identified in a number of different organisms such as *Bacillus subtilis* (38), *Staphylococcus carnosus* (53), *Thermus thermophilus* (128), *Paracoccus pantotrophus* (189), and *Mycobacterium tuberculosis* (163). Although these studies have enhanced the knowledge about nitrate/nitrite transport in bacteria, the actual mechanism(s) for nitrate transport remain controversial. The studies described here have identified the presence of a unique operon within an organism capable of

denitrification. The system is novel among the *Proteobacteria* as two genes, *narK1* and *narK2* cluster with the *narGHJI* genes in a single operon.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The PAO1 *narG* mutant was previously isolated (75, 116, 155, 156, 161). All bacteria were grown at 37°C from single-colony isolates or overnight cultures in Luria-Bertani (LB) broth (Fisher Scientific). The medium was supplemented with nitrate at a final concentration of 1%. The cultures were also plated on LB medium, 1.5% agar (Difco, Detroit, Mich.). Plasmid integration during mutant construction was checked using Pseudomonas Isolation Agar (PIA) (Difco, Detroit, Mich.)

Aerobic overnight cultures were incubated with shaking at 250 rpm unless otherwise noted. For anaerobic growth cultures, conditions included magnetic stirring in 125 ml Erlenmeyer flasks with rubber stoppers equipped with ports for sample withdrawal and one-way gas release valves. To ensure complete anaerobiosis of the system, the medium was supplemented with 2% (w/v) Oxyrase (Oxyrase, Inc., Mansfield, Ohio) and flushed with argon. For the nitrate reductase assay and the whole cell uptake study, the cultures were grown aerobically to an OD_{660nm} of 0.5-0.6, after which time they were shifted to complete anaerobic conditions.

Antibiotics were used for *E. coli* at the following concentrations (µg/ml): ampicillin, 100; gentamicin, 15. For *P. aeruginosa*, gentamicin and carbenicillin were used at 300 and 500 µg/ml, respectively.

Bioinformatics analyses

Gene, protein and primer sequences for *P. aeruginosa* PAO1 and *E. coli* K-12 were obtained using the *Pseudomonas* genome database site (<http://www.pseudomonas.com/>) and *E. coli* K-12 genome database site (<http://www.ecocyc.com/>), respectively. Prediction of the molecular weight of the proteins, based on amino acid data, was performed with individual proteomics tools available at the ExPASy mirror site (<http://www.au.expasy.org/>) of the Swiss Institute of Bioinformatics. Promoter search was carried out using the promoter prediction software site (<http://www.fruitfly.org/>). Sequence similarity comparisons between PAO1 NarK2 and *E. coli* K-12 NarK were carried out using the Multalin software (<http://www.prodes.toulouse.inra.fr/multalin/multalin.html>). Hydropathy profiles were generated as described (97) with a window size of 23 (<http://www.bio.davidson.edu/courses/compbio/flc/home.html>).

Manipulation of recombinant DNA and genetic techniques

All plasmid and chromosomal nucleic acid manipulations were by standard techniques (149). Plasmid DNA was transformed into *E. coli* DH5 α -MCR (Gibco-BRL), SM10 (75, 116, 155, 156, 161), or *P. aeruginosa* PAO1. Restriction endonucleases, Klenow fragment and T4 DNA ligase were used as specified by the supplier (New England Biolabs). Plasmid DNA was isolated using the QIA prep[®] Spin kit (Qiagen). DNA fragments were isolated from agarose gels using the Gene Clean kit (QBiogene). PCR reactions were performed using *Taq* DNA polymerase,

PCR buffer, dNTPs (Sigma Chemical Co.) in a Peltier Thermal Cycler. All the oligonucleotide primers used in this study are listed in Table 2 (Sigma-Genosys).

Construction of isogenic mutants

The ORFs putatively responsible for the formation of NarK1 and NarK2 were identified by homology as PA3877 and PA3876, respectively, through the *Pseudomonas* Genome Project (175). The genes were amplified from PAO1 using primers based on sequence data from the *Pseudomonas* Genome Database (Table 2). All the strain constructions and manipulations are described in detail in Table 1. PCR fragments were initially cloned into pGEM-T Easy (Promega) or pCR2.1 (Invitrogen) vectors. The genes were inserted into pEX18Ap (75). Isogenic mutants of *narK1*, *narK2* and *narK1K2* were created by deletion of most of the ORFs, followed by insertion of *aacC1*, a gentamicin resistance marker from pUCGM (75, 116, 155, 156, 161). Single-copy chromosomal gene disruptions were created using a gene replacement technique previously described (154). Mutants were confirmed by PCR using primers specified in Table 2 (data not shown). All mutants were complemented by the use of pUCP18 plasmid vector (75, 116, 155, 156, 161) with the gene(s) of interest (Table 1).

Preparation of cell extracts to analyze nitrate reductase activity

To analyze cell-free extracts for enzyme activity, cultures were centrifuged and the cells washed five times with an equal volume of 0.1 M potassium phosphate buffer

(pH 7.2). The cell suspensions were then sonicated five times at 4°C, with 15 second bursts and a rest interval of 1 min, in an ice bath using the Branson 150 sonicator, followed by centrifugation at $10,000 \times g$ for 10 min to remove cell debris.

Determination of nitrate reductase activity

For the assay, 100 μ l of cell extract was added to a 1.5ml eppendorf tube containing 700 μ l 0.1 M potassium phosphate buffer (pH 7.2) followed by 50 μ l of 1 M KNO₃. To start the reaction, 50 μ l of freshly made 0.08% sodium hydrosulfite (dithionite) was added to 100 μ l of methyl viologen, gently mixed, and added to cell extracts. The reaction proceeded for 2 min, after which time all contents were vigorously vortexed and the nitrite concentration was determined by the Griess reaction (102). Enzyme activity is defined as that amount of nitrate reductase required to produce 1 nmol nitrite min⁻¹mg⁻¹ protein. All the assays were performed in triplicate and repeated at least twice with independent cultures.

Uptake of nitrate monitored by nitrate-ion selective electrode

Whole cells were analyzed for rates of nitrate uptake using the Orion 9707 Ionplus® nitrate electrode (Thermo Electron Co) by a method previously described (73). 1 M glucose was used as an energy source and the cells were spiked with 200 – 600 μ M of KNO₃ in an argon-generated anaerobic environment. All the assays were performed in triplicate and repeated at least twice with independent cultures.

Determination of the concentrations of extracellular nitrite

Extracellular nitrite was determined in whole cell suspensions using the Griess reaction as previously described (34). All assays were performed in triplicate and repeated at least twice with independent cultures.

Determination of protein concentrations in whole cell suspensions and cell extracts

The Bradford reagent (Sigma-Aldrich, St. Louis, Mo.) was utilized to determine the protein concentrations for both sonicated as well as for whole cell suspensions (26).

Results

NarK1 and NarK2 as candidates for nitrate import or nitrite export

In the organism *P. aeruginosa* PAO1, the *narK1* and *narK2* genes are found in a cluster of genes which include structural genes for nitrate reductase enzyme complex (*narGHJI*) (Fig.1) and these together appeared to comprise the *narK1K2GHJI* operon (<http://www.pseudomonas.com>). By extrapolation, the *narK1* gene encodes a protein of 431 amino acids with a molecular weight of 47.3 while the *narK2* gene encodes a protein of 468 amino acids with a molecular weight of 50.6 (http://au.expasy.org/tools/pi_tool.html).

A comparison of peptide sequence between the NarK1 and NarK2 proteins yielded a similarity of 28% (<http://prodes.toulouse.inra.fr/multialin.html>). In contrast, a similarity of 74% was observed between NarK2 and NarK of *Escherichia coli* K-12. The NarK1 was found to be 59% similar to the NarK1 protein of *Thermus thermophilus*. Further, a hydrophobicity profile (<http://www.bio.davidson.edu/courses/compbio/flc/home.html>) indicates that both proteins contain 11-12 transmembrane helices. Such a helix profile is in complete agreement with the proposed roles for transporters.

The *narK1K2GHJI* operon

A promoter predictor program (<http://www.fruitfly.org>) indicated the presence of only one promoter for *narK1*, *narK2* and *narGHJI* genes, further suggesting that these genes might form one operon. This was verified by growing the $\Delta narK1K2::Gm$

mutant aerobically in LB supplemented with nitrate and gentamicin. In *P. aeruginosa* PAO1, respiratory nitrate reductase is normally induced only anaerobically in the presence of nitrate (111, 159). Because the $\Delta narK1K2::Gm$ mutant contains a gentamicin cassette insertion (Fig. 1) and consequently contains the gentamicin promoter, the respiratory nitrate reductase genes could be induced even aerobically in LB nitrate through this promoter. Thus, under aerobic conditions, the $\Delta narK1K2::Gm$ mutant yielded normal amounts (330 ± 5 nmol nitrite min⁻¹mg⁻¹ protein) of respiratory nitrate reductase activity while no nitrate reductase activity was detected in the wild type strain further supporting that all of these genes are contained in a single operon.

Effect of *narK1* and *narK2* mutants on anaerobic respiratory growth

All mutants and the respective complemented strains were grown anaerobically in LB supplemented with nitrate (Fig. 2). The $\Delta narK1::Gm$ mutant grew almost as rapidly as the wild type each yielding a generation time of 2.6 ± 0.08 and 2 ± 0.4 h, respectively (Fig. 2A). In contrast, the $\Delta narK2::Gm$ mutant (Fig. 2B) was found to be severely impaired in nitrate-dependent anaerobic growth and yielded a generation time of 8.5 ± 0.6 h. Finally, the $\Delta narK1K2::Gm$ double mutant demonstrated almost no growth (Fig. 2C). A complementation of the $\Delta narK2::Gm$ mutant with *pnarK2* completely rescued the mutant. However, the $\Delta narK1K2::Gm$ mutant was not fully complemented with *pnarK1K2*, demonstrating only a slightly higher growth rate than the mutant (Fig 2C). This was attributed to the overproduction of two membrane

proteins due to the use of a high copy plasmid (53). We have confirmed this inhibitory effect by transforming wild type *P. aeruginosa* with *pnarK1K2*. This strain grew slower than the wild type yielding a generation time of 3.2 ± 0.6 h. As expected, the $\Delta narK1K2::Gm/pnarK1$ mutant was also unable to grow (data not shown), implying a requirement for a functional NarK2 for respiratory nitrate reduction by *P. aeruginosa*.

Nitrate reductase activities in *narK1*, *narK2* and *narK1K2* mutants

The nitrate reductase activity was analyzed in cell-free extracts of all the strains using a non-physiological electron donor, i.e. methyl viologen. For this purpose, strains were grown to an OD_{660nm} of 0.5 – 0.6 aerobically in LB supplemented with nitrate and gentamicin and then switched to anaerobic conditions for 3 h. The cell extracts were subsequently analyzed for methyl viologen-linked nitrate reductase activity (Table 3). Similar nitrate reductase activities were observed in all the strains, as was expected. This confirmed that the deletion-insertion mutagenesis of the genes did not affect the expression of the nitrate reductase genes and the phenotypes observed were due to a defect in nitrate and/or nitrite transport.

Nitrate uptake

To investigate the role of *P. aeruginosa narK1* and *narK2* gene products in nitrate transport, anaerobically grown whole cell suspensions were monitored for external nitrate using a nitrate electrode (Table 4). The $\Delta narK1::Gm$ mutant exhibited uptake

rates similar to the wild type, consistent with the anaerobic growth rates observed. On the other hand, both the $\Delta narK2::Gm$ and the $\Delta narK1K2::Gm$ mutants were found to be severely impaired in their nitrate uptake ability exhibiting uptake rates of 8.4 ± 0.3 nmol nitrate $\text{min}^{-1}\text{mg}^{-1}$ protein and <1.5 nmol nitrate $\text{min}^{-1}\text{mg}^{-1}$ protein, respectively. Complementation with *pnarK2* and *pnarK1K2* was found to rescue this phenotype. Furthermore, similar to the observation made for an *E. coli* nitrate reductase mutant, no nitrate uptake was observed in a *P. aeruginosa* PAO1 *narG* mutant (Table 4).

Nitrite accumulation by *narK1*, *narK2* and *narK1K2* mutants

Next, we wanted to see if there were any differences in nitrite extrusion between the wild type and the mutant strains. Samples were withdrawn during anaerobic nitrate-dependent growth, cells were removed and the amount of nitrite was analyzed (data not shown). Given that the $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants are unable to grow in these conditions and since cytoplasmic nitrite is a result of nitrate reduction, both the mutants were expected to demonstrate limited nitrite excretion, which indeed was the case (data not shown). In contrast, although the $\Delta narK1::Gm$ mutant excreted visibly reduced amounts of nitrite compared to wild type, normalization of the data in terms of protein amounts abolished this difference, giving values of 60.5 ± 0.68 and 60.5 ± 0.7 μmol extracellular nitrite mg^{-1} protein for wild type and $\Delta narK1::Gm$ mutant, respectively.

Isogenic mutant of *narK2* was complemented by the *narK* gene of *Escherichia coli* K-12

Previous studies of nitrate/nitrite transport have been most extensively carried out on the NarK of *Escherichia coli* (34, 46, 80, 139, 170). Thus, to establish the role of the *narK2* in PAO1, we cloned the *narK* gene of *E. coli* into a pUCP18 plasmid vector. The resulting strain was used to complement both the $\Delta narK2::Gm$ strain and the $\Delta narK1K2::Gm$ strain (Fig. 3). The results demonstrate that the *narK* gene of *E. coli* is capable of restoring anaerobic growth in PAO1 deficient in *narK2* and *narK1K2*. The growth rates of these complemented strains were not completely restored to wild-type levels, but that can be attributed to (i) a high copy number of the membrane proteins being produced (53) and (ii) a non-identical protein used for complementation.

Discussion

The goal of the present study was to elucidate the involvement of the *narK1* and *narK2* genes in *P. aeruginosa* denitrification. In this regard, isogenic mutants of *narK1*, *narK2* and *narK1K2* were created and verified. These studies confirmed that the *narK1* and *narK2* genes are in an operon with the *narGHJI* genes. The literature suggests that this is unusual since the *narG* operon of *E. coli* is distinctly separate from *narK* and *narU* (21, 170) as is *narK1* and *narK2* of *Thermus thermophilus* (128) and the *nark* of *P. stutzeri* (67). Only *Paracoccus pantotrophus* (189) has a nitrate/nitrite transporter in the same operon as the genes for the nitrate reductase complex.

Anaerobic nitrate-dependent growth studies showed the $\Delta narK1::Gm$ mutant to be only slightly affected in growth while both the $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants were severely compromised compared to wild type (Fig. 2). This suggests that these proteins serve different roles in nitrate-dependent anaerobic growth. To make sure that these growth phenotypes were not due to an inactive nitrate reductase, all mutants were checked and confirmed for the presence of nitrate reductase activity (Table 3). These results are in contrast to the study of *narK1* and *narK2* of *Thermus thermophilus*. In that study a single mutation of *narK1* or *narK2* did not severely restrict anaerobic growth. Only when both of these genes were mutated was the organism severely restricted in anaerobic growth at the expense of nitrate. Furthermore, in *T. thermophilus*, complementation of the double mutant with either *narK1* or *narK2* restored the ability of the organism to grow anaerobically.

Nitrate uptake studies utilizing a nitrate specific electrode yielded some interesting insights into the NarK1 and NarK2 protein function (Table 4). The *ΔnarK1::Gm* mutant demonstrated nitrate uptake rates similar to wild type. In contrast, both the *ΔnarK2::Gm* and the *ΔnarK1K2::Gm* mutants had very low yet different rates of nitrate uptake. This difference in nitrate uptake rate between the *ΔnarK2::Gm* mutant and the double mutant was more than five-fold, indicating that both the proteins may be involved with nitrate uptake. In addition, we observed no uptake of nitrate in a PAO1 *narG* mutant thus connecting intracellular nitrite generation with nitrate uptake. This is consistent with the observation made for an *E. coli narG* mutant (139) and that of Ramirez *et al* in *T. thermophilus* (128).

It is well established that during denitrifying growth of *P. aeruginosa* in batch culture, there is a sequential reduction of nitrogen oxides (187). Similar results have been observed for other denitrifiers (15). The first product of denitrification, nitrite is very toxic to the cells and thus excreted immediately upon reduction. This extracellular accumulation of nitrite continues to occur until the nitrate supply is exhausted. Moreover, previous studies have shown that nitrite reductase is located in the periplasm but does not participate in nitrite reduction until nitrate disappears from the external medium (159). Thus, we wanted to see if any of our mutants differentially accumulated nitrite in comparison to wild type. The results indicate that both the *ΔnarK2::Gm* and *ΔnarK1K2::Gm* mutants demonstrated limited nitrite excretion while the *ΔnarK1::Gm* mutant excreted amounts of nitrite equivalent to wild type. It is to be expected that a restriction in nitrate uptake would also limit

nitrite production and thus the results obtained for the $\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants may be explained in this manner. The *narK1* mutation did not seem to affect external nitrite accumulation when the wild type and mutant were normalized to protein content.

In a separate experiment we used the *narK* gene of *E. coli* to complement our *narK2* mutant. This experiment was conducted because previous studies on nitrate/nitrite transport had been most extensively carried out in *E. coli* (21, 46, 114, 170), and recent studies concluded that the protein may operate as a nitrate/nitrite antiporter (34, 80). These conclusions were in contrast to the results reported in vesicle and proteoliposomes using ^{13}N nitrate (139) which did not support the antiporter mechanism. In the current study, the NarK protein of *E. coli* complemented both the $\Delta narK2::Gm$ mutant as well as the $\Delta narK1K2::Gm$ mutant of *P. aeruginosa* with respect to anaerobic nitrate-dependent growth. This suggests that functionally the NarK2 of *P. aeruginosa* is similar to the NarK of *E. coli*. However, the issue of antiport vs. uniport remains to be conclusively experimentally proven.

To summarize, in contrast to studies in other denitrifiers such as *T. thermophilus* and *P. pantotrophus* (128, 189), the NarK1 protein is not as important for the anaerobic nitrate-dependent growth and survival of *P. aeruginosa*. However, both the anaerobic growth studies and nitrate uptake studies indicate some involvement of the NarK1 protein in *Pseudomonas* denitrification. For now, its role still remains enigmatic. One possibility is that the NarK1 protein is capable of taking up very low amounts of nitrate. Given that in a $\Delta narK1::Gm$ mutant the *narK2* is

functioning at normal levels, a slight deficiency created by the lack of NarK1 is “masked” by the presence of NarK2. Therefore, no differences in nitrate uptake are observed between the wild type and the $\Delta narK1::Gm$ mutant. However, these differences become apparent on comparison of the $\Delta narK2::Gm$ and the $\Delta narK1K2::Gm$ strains. The 5-fold difference observed between the two strains may be indicative of low amounts of nitrate uptake mediated by the NarK1 protein. Thus, the NarK1 protein may function in *P. aeruginosa* secondarily to NarK2. In the absence of NarK2, the NarK1 would not be able to promote wild-type levels of nitrate dependent anaerobic growth but may provide just enough energy for the organism to sustain itself while it seeks other energy sources. Future studies would be needed to confirm the exact role of this protein.

Finally, in literature, the NarK-like proteins have been divided into two distinct subgroups: Type I and Type II (110). Both *E. coli* NarK as well as the *P. aeruginosa* NarK2 has been classified as members of the Type II group (110). On the other hand, the *P. aeruginosa* NarK1 has been classified as a member of the Type I group (110). Our results agree with the classification scheme for NarK2. However, it is difficult at present time to corroborate the classification of NarK1 since its function is still unknown.

Acknowledgements

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant Genotype and Description	Source or reference
<i>E. coli</i>		
DH5a	<i>recA1 endA1 gyrA96 thi-1 hsdR17supE44 relA1 ΔlacU169 (Φ80lacZΔM15)</i>	Gibco
SM10	Km ^r , Mobilizer strain	157
<i>P. aeruginosa</i>		
PAO1	Wild-type	Al Darzins
Δ <i>narK1</i> ::Gm	Gm ^r , Δ <i>narK1</i> ::Gm	This study
Δ <i>narK2</i> ::Gm	Gm ^r , Δ <i>narK2</i> ::Gm	This study
Δ <i>narK1K2</i> ::Gm	Gm ^r , Δ <i>narK1K2</i> ::Gm	This study
<i>narG</i> :: <i>LacZ</i> Gm	Gm ^r , Φ(PA3875- <i>lacZ</i> Gm)	113
Δ <i>narK1</i> ::Gm/ <i>pnarK1</i>	Gm ^r Cb ^r , Δ <i>narK1</i> ::Gm with PA3877 in pUCP18	This study
Δ <i>narK2</i> ::Gm/ <i>pnarK2</i>	Gm ^r Cb ^r , Δ <i>narK2</i> ::Gm with PA3876 in pUCP18	This study
Δ <i>narK1K2</i> ::Gm/ <i>pnarK1K2</i>	Gm ^r Cb ^r , Δ <i>narK1K2</i> ::Gm with PA3877 and PA3876 in pUCP18	This study
Δ <i>narK2</i> ::Gm/ <i>pnarK</i>	Gm ^r Cb ^r , Δ <i>narK2</i> ::Gm with <i>E. coli narK</i> in pUCP18	This study
Δ <i>narK1K2</i> ::Gm/ <i>pnarK</i>	Gm ^r Cb ^r , Δ <i>narK1K2</i> ::Gm with <i>E. coli narK</i> in pUCP18	This study
PAO1/ <i>pnarK1K2</i>	Cb ^r , wild type PAO1 with PA3877 and PA3876in pUCP18	This study
Plasmids		
pGem:: <i>narK1</i>	Ap ^r , 1.296 kb fragment containing PA3877 in pGEM-T Easy vector (Promega)	This study
pGem:: <i>narK2</i>	Ap ^r , 1.407 kb fragment containing PA3876 in pGEM-T Easy vector (Promega)	This study
pGem:: <i>narK1K2</i>	Ap ^r , 2.703 kb fragment containing PA3876 and PA3877 in pGEM-T Easy vector (Promega)	This study
pCR:: <i>narK E. coli</i>	Ap ^r , 1.392 kb fragment containing <i>E. coli narK</i> in pCR 2.1 vector (Invitrogen)	This study
pEX18Ap	Ap ^r <i>oriT</i> <i>mob sacB</i> gene replacement vector with MCS from pUC18	72
pEX18Ap:: <i>narK1</i>	Ap ^r , ligation of a 1.296 kb <i>EcoRI</i> fragment of PA3877 of pGem:: <i>narK1</i> into <i>EcoRI</i> digest of pEX18Ap	This study
pEX18Ap:: <i>narK2</i>	Ap ^r , ligation of a 1.407 kb <i>EcoRI</i> fragment of PA3876 of pGem:: <i>narK2</i> into <i>EcoRI</i> digest of pEX18Ap	This study
pEX18Ap:: <i>narK1K2</i>	Ap ^r , ligation of a 2.703 kb <i>EcoRI</i> fragment containing PA3876 and PA3877 of pGem:: <i>narK1K2</i> into <i>EcoRI</i> digest of pEX18Ap	This study
pUCGM	Ap ^r Gm ^r <i>aacCI</i>	152
pEX18Ap:Δ <i>narK1</i> ::Gm	Ap ^r Gm ^r , ligation of a 1kb <i>SmaI</i> fragment of pUCGM containing <i>aacCI</i> into a blunt-ended <i>NcoI-SalI</i> deletion of pEX18Ap:: <i>narK1</i>	This study
pEX18Ap:Δ <i>narK2</i> ::Gm	Ap ^r Gm ^r , ligation of a 1kb <i>SmaI</i> fragment of pUCGM containing <i>aacCI</i> into a blunt-ended <i>XhoI-ApaI</i> deletion of pEX18Ap:: <i>narK2</i>	This study
pEX18Ap:Δ <i>narK1K2</i> ::Gm	Ap ^r Gm ^r , ligation of a 1kb <i>SmaI</i> fragment of pUCGM containing <i>aacCI</i> into a blunt-ended <i>NotI-ApaI</i> deletion of pEX18Ap:: <i>narK1K2</i>	This study
pUCP18	Ap ^r , broad-host-range cloning vector	151
<i>pnarK1</i>	Ap ^r , ligation of a 1.296 kb <i>EcoRI</i> fragment of pGem:: <i>narK1</i> into <i>EcoRI</i> site of pUCP18, complementation studies	This study
<i>pnarK2</i>	Ap ^r , ligation of a 1.407 kb <i>EcoRI</i> fragment of pGem:: <i>narK2</i> into <i>EcoRI</i> site of pUCP18, complementation studies	This study
<i>pnarK1K2</i>	Ap ^r , ligation of a 2.703 kb <i>EcoRI</i> fragment of pGem:: <i>narK1K2</i> into <i>EcoRI</i> site of pUCP18, complementation studies	This study
<i>pnarK</i>	Ap ^r , ligation of a 1.392 kb <i>EcoRI</i> fragment of pCR:: <i>narK</i> into <i>EcoRI</i> site of pUCP18, complementation studies	This study

TABLE 2. Oligonucleotide primers used in this study

Primer	Location	Strand ^a	Sequence (5' → 3')
NarK1	<i>narK1</i>	+	CCTGTCACCTCCAAAG
NarK1	<i>narK1</i>	–	AGAAGCTGATATTGGACATG
NarK2	<i>narK2</i>	+	GTGCCTGTTCTTCCTCTC
NarK2	<i>narK2</i>	–	TTGGCGCTGTAGATGTAC
NarK1K2	<i>narK1</i>	+	CCTGTCACCTCCAAAG
NarK1K2	<i>narK2</i>	–	TTGGCGCTGTAGATGTAC
<i>E. coli</i> NarK	<i>narK</i>	+	CTGCTGCTCGAGTCAACTC
<i>E. coli</i> NarK	<i>narK</i>	–	TATAATTCGGTTTACAGGAAGG

^a Forward and reverse primers are indicated by + and -, respectively.

TABLE 3. Reduced methyl viologen-linked nitrate reductase activities of *P. aeruginosa* wild type and mutants grown anaerobically.

Strain ^a	Nitrate reductase activity ^{b, c} in nmol nitrite min ⁻¹ mg ⁻¹ protein
PAO1	334 ± 9
$\Delta narK1 :: Gm$	310 ± 10
$\Delta narK1 :: Gm/pnarK1$	330 ± 12
$\Delta narK2 :: Gm$	278 ± 12
$\Delta narK2 :: Gm/pnarK2$	328 ± 13
$\Delta narK1K2 :: Gm$	229 ± 8
$\Delta narK1K2 :: Gm/pnarK1K2$	283 ± 4

^a All strains were grown aerobically in LB supplemented with 1% nitrate to an OD_{660nm} of 0.5-0.6 and then shifted to anaerobiosis for 3h.

^b Enzyme activities were determined in cell extracts using reduced methyl viologen as electron donor.

^c Mean and standard errors were calculated from 3 independent cell suspensions.

TABLE 4. Effects of mutations in *narK1*, *narK2*, *narK1K2* and *narG* on rates of nitrate uptake.

Strain ^a	Rate of nitrate uptake (nmol nitrate min ⁻¹ mg ⁻¹ protein) ^b
PAO1	175 ± 37
$\Delta narK1$::Gm strain	192 ± 7
$\Delta narK2$::Gm strain	8 ± 0.3
$\Delta narK2$::Gm/ <i>pnarK2</i> strain	254 ± 31
$\Delta narK1K2$::Gm strain	<1.5
$\Delta narK1K2$::Gm/ <i>pnarK1K2</i> strain	186 ± 47
<i>narG</i> :: <i>lacZ</i> Gm strain	0 ^c

^a All strains were grown aerobically in LB medium supplemented with 1% nitrate to an OD₆₆₀ of 0.5 to 0.6 and then shifted to anaerobiosis for 3 h. Washed whole cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) and monitored under argon-generated anaerobic conditions.

^b Glucose (1M) was used as the energy source, and the nitrate concentration was 200 to 600 mM. Means and standard errors were calculated from three independent cell suspensions.

^c Not detected.

Figure Legends

FIG. 1. A map of the *narK1K2GHJI* operon of *Pseudomonas aeruginosa*. The map shows the *narK1* and *narK2* genes to be upstream of the structural genes of nitrate reductase (*narGHJI*). Relevant restriction sites used to create deletions are shown. The endogenous promoter for the operon is shown as P_{nar} . The direction of transcription of both the operon and the gentamicin cassette (*Gm*) are shown with the help of arrows. The orientation of the *Gm* cassette in the gene disruptions was always positive with respect to the gene, as shown in the figure. The figure is not drawn to scale. **(A)** The $\Delta narK1::Gm$ mutant was created by blunt-ending the *Gm* cassette into the *NcoI-SalI* deletion site. **(B)** The $\Delta narK2::Gm$ mutant was created by blunt-ending the *Gm* cassette into the *XhoI-ApaI* deletion site. **(C)** The $\Delta narK1K2::Gm$ mutant was created by blunt-ending the *Gm* cassette into the *NotI-ApaI* deletion site.

FIG. 2. Anaerobic growth of *Pseudomonas aeruginosa* PAO1 in LB medium supplemented with nitrate. All the inocula were prepared by growing the strains overnight in shaker grown starter cultures in LB medium which were then transferred to LB supplemented with 1% nitrate and the appropriate concentrations of gentamicin and/or carbenicillin and switched to anaerobic conditions using oxyrase and argon gas. **(A)** Anaerobic growth of PAO1 (\diamond), $\Delta narK1::Gm$ (\blacksquare), $\Delta narK1::Gm/pnarK1$ (*narK1* complement) (\blacktriangle). **(B)** Anaerobic growth of PAO1 (\diamond), $\Delta narK2::Gm$ (\blacksquare),

ΔnarK2::Gm/pnarK2 (*narK2* complement) (▲). (C) Anaerobic growth of PAO1 (◇), *ΔnarK1K2::Gm* (■), *ΔnarK1K2::Gm/pnarK1K2* (*narK1K2* complement) (▲).

FIG. 3. Complementation of *ΔnarK2::Gm* and *ΔnarK1K2::Gm* with *pnarK*. The *narK* cloned into pUCP18 plasmid vector was obtained from *E. coli* K-12. All strains were grown overnight in LB medium and were then transferred to LB supplemented with 1% nitrate and an appropriate concentration of gentamicin and carbenicillin and switched to anaerobic conditions. Anaerobic Growth: PAO1 (◇), *ΔnarK2::Gm* complemented with *pnarK* (■), *ΔnarK1K2::Gm* complemented with *pnarK* (▲).

FIG. 1.

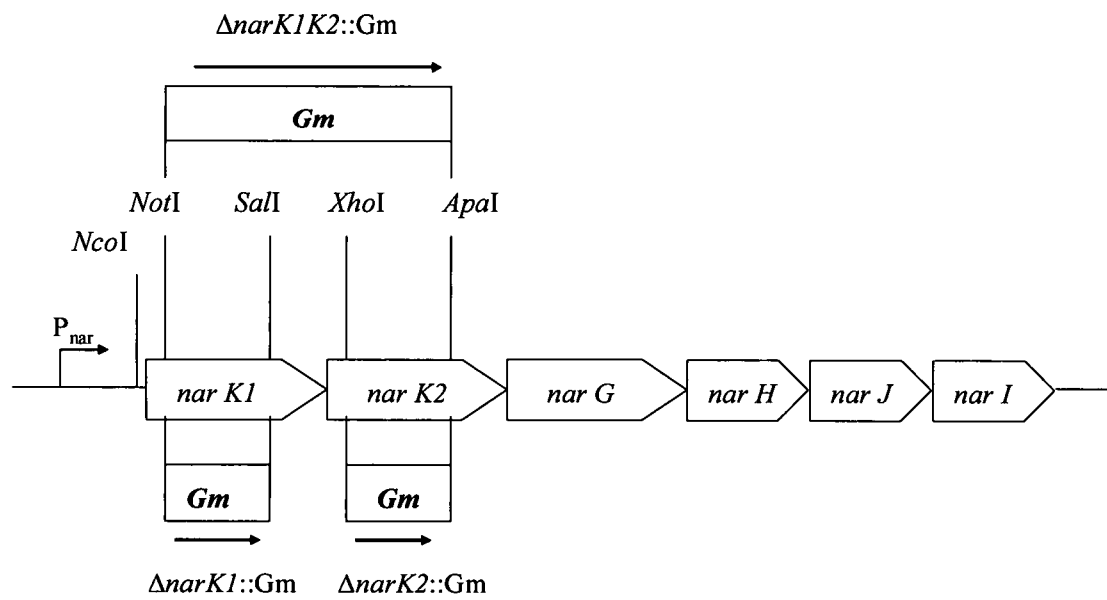
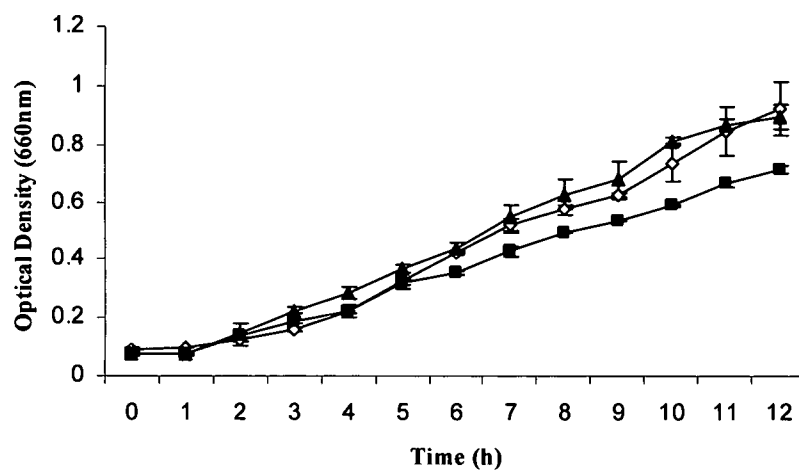


FIG. 2.

2(A) PAO1 $\Delta narK1::Gm$



2 (B) PAO1 $\Delta narK2::Gm$

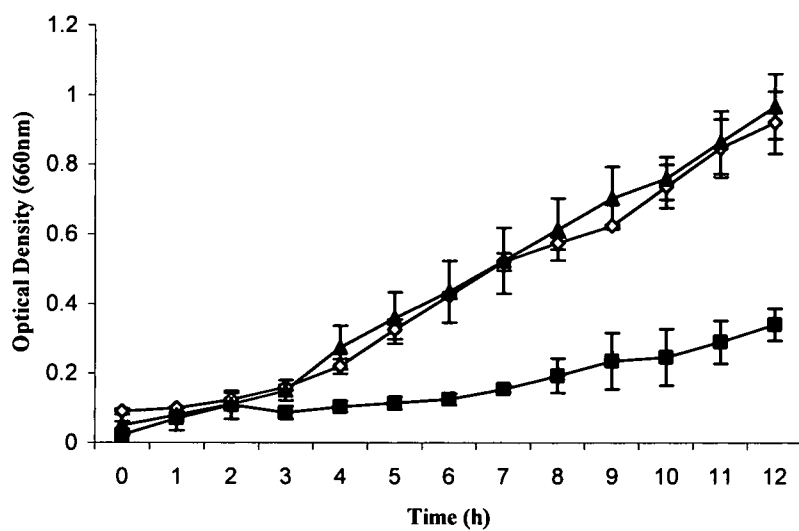


FIG. 2.

2 (C) PAO1 $\Delta narK1K2::Gm$

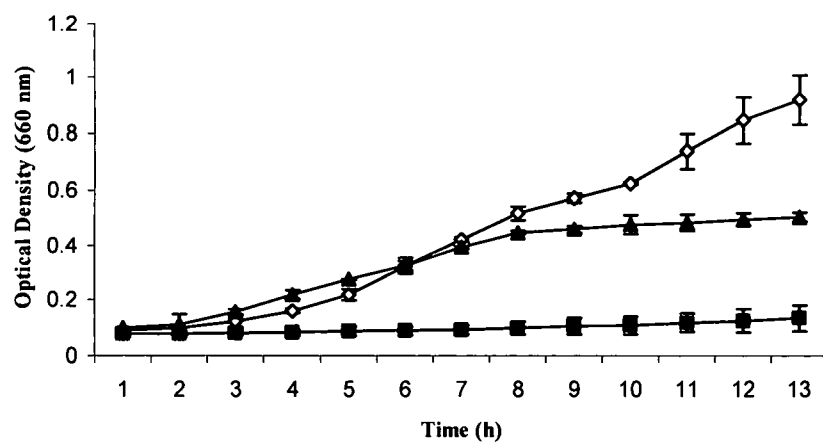
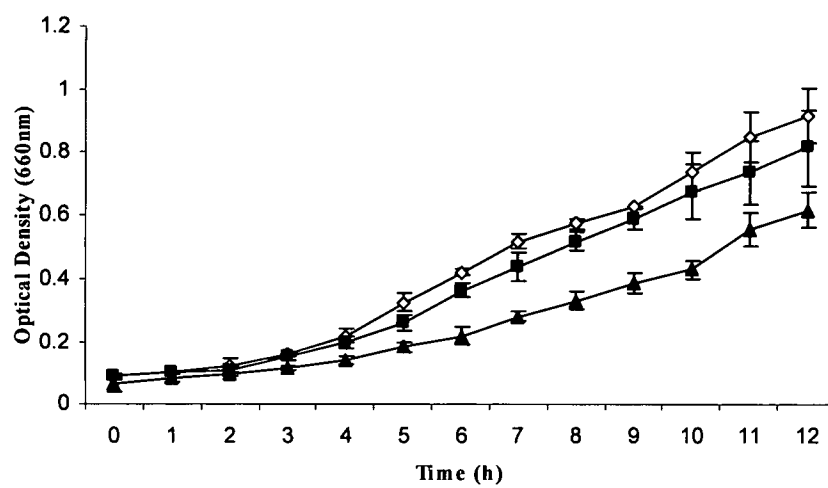


FIG. 3.



Summary and Future Research

Pseudomonas aeruginosa has the ability to reduce nitrate to nitrite through an assimilatory pathway, thereby providing the cell with reduced nitrogen for biosynthesis. *P. aeruginosa* can also reduce nitrate to nitrite and eventually to nitrogen gas by the denitrification pathway that provides the organism with a mode of respiration and ATP generation in the absence of oxygen. The intracellular location of the enzymes that are required to carry out these processes necessitates the synthesis of specific transport proteins. Thus, the basic crux of nitrate metabolism is in the transport of nitrate and nitrite. The focus of the present study was to determine the proteins involved in nitrate/nitrite transport by a denitrifying *P. aeruginosa* and elucidate their respective functional roles. In this regard, the *narK1* and *narK2* genes were identified from the *Pseudomonas* genome database as potential candidates for creating isogenic mutants. To this end, *narK1*, *narK2* and *narK1K2* mutants were created and verified. We have conclusively shown that the *narK1* and *narK2* genes are transcribed by a common promoter together with the *narGHJI* i.e., the *narK1K2GHJI* constitute an operon. Mutational analysis of the single and double mutants gave us very interesting results. We found that while the $\Delta narK1::Gm$ mutant was only slightly affected in its ability to grow under denitrifying conditions, both the

$\Delta narK2::Gm$ and $\Delta narK1K2::Gm$ mutants were severely restricted in nitrate dependent, anaerobic growth. To make sure that these growth phenotypes were not due to an inactive nitrate reductase enzyme, we checked and confirmed all the mutants for the presence of nitrate reductase activity. This indicated to us that a functional NarK2 protein was important in *P. aeruginosa* denitrification, outside of catalysis. This was further corroborated when we monitored whole cell suspensions for nitrate uptake. Again, we found that both the $\Delta narK2::Gm$ and the $\Delta narK1K2::Gm$ mutants had very low yet different rates of nitrate uptake, while the $\Delta narK1::Gm$ mutant exhibited wild-type levels of nitrate uptake. Finally, we were able to rescue both the $\Delta narK2::Gm$ and the $\Delta narK1K2::Gm$ mutants with respect to anaerobic respiratory growth, by complementing with *Escherichia coli narK*, *in trans*. Our results indicate that only the *narK2* protein is required as a nitrate/nitrite transporter by *Pseudomonas aeruginosa* under denitrifying conditions.

In bacteria, three modes of secondary transporters exist namely, uniport, symport and antiport. It is difficult with the present data to speculate on what type of transporter proteins the *narK1* and *narK2* genes encode for. Future research will be required to answer the following questions:

- Does the NarK2 protein function as a nitrite extruder as demonstrated by Rowe *et al.*, (1994) or does it function as a nitrate/nitrite antiporter as proposed by Clegg *et al.*, (2000)?
- If NarK2 is only a nitrite extruder then, which proteins catalyze nitrate uptake in *Pseudomonas aeruginosa*?

- Does the NarK1 protein mediate nitrate uptake together with a yet unidentified protein?
- What are the structures of the NarK1 and NarK2 proteins?

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