MOLECULAR EVOLUTION OF THE DISSIMILATORY NITRATE REDUCTASE: A SURVEY TO ASSESS ITS DIVERSITY

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Brent Craig Christner

UNIVERSITY OF DAYTON

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APPROVED BY:

John J. Rowe, Ph.D.
Chairman
Major Advisor

Panagiotis A. Tsonis, Ph.D.
Committee Member

Jayne B. Robinson, Ph.D.
Committee Member

ABSTRACT

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Christner, Brent Craig
University of Dayton, 1995

Advisor: Dr. John J. Rowe

The *narG* gene, which codes for the α subunit of *Escherichia coli*'s dissimilatory nitrate reductase (NR), was utilized to construct two DNA probes which were subsequently used in a survey of nitrate-reducing bacteria. Both of these probes had previously been reported to share homology with the genomic DNA of prominent denitrifiers such as *Pseudomonas aeruginosa* and *P. stutzeri*. Of the 15 species surveyed which contain a respiratory NR (or enzyme analogous to typical dissimilatory NRs), 9 demonstrated homology with the probes (6 different genera). The NR enzyme appears to be genetically diverse like other NO_x reductases that have been characterized. These molecular probes have also identified a second *narG*-like gene in some organisms, several of which are known to contain forms of NR which appear to have no physiological function. The current survey suggests that *narG* gene is distributed over a wide variety of nitrate-reducing bacteria, thus implicating its potential use in conjunction with other genetic probes to assess environmental samples.

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INTRODUCTION

Our global ecosystem depends on the metabolic activity of microorganisms. Bacteria are responsible for the geochemical recycling of important biological constituents like carbon, nitrogen and sulfur. As Carl Woese has said, "In a fundamental sense, the biosphere is the bacterio-sphere" (93). Because of their role in the natural order of things, it is important to understand the metabolic processes carried out by these environmentally significant life forms. In the past, an accurate assessment of microbial diversity was not possible. Often, ecological studies were limited to organisms which could be grown in the laboratory. These enrichment techniques not only disrupt natural community balances, but they also inadequately represent the diversity which exists in a bacterial population. Consequently, much of what is known about the prokaryotes is a result of scientific investigations which have narrowly focused on a small group of culturable organisms.

Molecular biology has permeated every domain of the life sciences, but perhaps no two fields have been impacted more than microbial ecology and evolution. Before the advent of current molecular approaches, prokaryotic classification was based on superficial characteristics such as morphology and physiology. These classification bases presented problems to both the microbial ecologist and evolutionist: no concrete method to establish relationships among microorganisms. Nucleic acid sequence analysis has provided a basis to establish a substantial evolutionary framework. As a result, a correct genealogical perspective concerning these organisms, their evolution and ecological diversity has been made possible. The current molecular techniques also allow bacteria to be detected without laboratory enrichment, thus giving microbial ecologists the potential to identify and study all species in a given ecological niche. Tracking an organism (or group of organisms) is as simple as finding a molecular probe which selectively identifies a particular genotype.

Denitrification is an anaerobic respiratory pathway by which many microorganisms supplement the low energy yields of glycolysis in the absence of oxygen. Oxidized nitrogen, in the form of nitrate or nitrite (NO^-_3/NO^-_2), is utilized as a terminal electron acceptor and is incrementally reduced to nitrogen gas (N_2) in a process that is similar but substantially more complex than aerobic respiration. A second form of nitrate respiration exists where nitrate is reduced to nitrite (NO^-_2) which is subsequently excreted or assimilated into biomass. These interesting bioenergetic pathways have vital environmental consequences in that they are collectively responsible for the recycling of fixed nitrogen to N_2 on the Earth's surface, i.e. the last step of the nitrogen cycle. Techniques that determine the distribution of bacteria capable of nitrate respiration are critical in developing a rudimentary understanding about this important form of metabolism.

The purpose of this study was to develop molecular probes to determine the genetic diversity of nitrate reductases (NR). Gene probes have been constructed from a known nitrate reduction gene and used to determine homology with several NR-containing bacteria (55). This data suggest that this gene sequence is relatively conserved in some organisms, however there may be totally different genetic forms in existence. These NR-specific probes will be subsequently used in combination with existing gene probes derived from known denitrifier genes to better understand the evolution and ecological distribution of microorganisms capable of nitrate respiration.

CHAPTER 1

LITERATURE REVIEW

NITRATE METABOLISM IN PROKARYOTES

Assimilatory and Dissimilatory Nitrate Reduction

The largest reservoir of nitrogen, dinitrogen (N_2), is a gas in the atmosphere. The flux of this element between the atmosphere and the Earth's surface is primarily controlled by bacteria and the activity of man. Fixed (organic) nitrogen is utilized by bacterial cells to form important molecules like proteins, nucleic acids, phospholipids, and structurally significant carbohydrates such as N-acetylmuramic acid and N-acetylglucosamine. The cycle begins with the biological or synthetic fixation of nitrogen and ends with the biological reduction of oxidized nitrogen compounds to dinitrogen (12). Nitrate (NO_3^-) is the most common form of fixed nitrogen on the Earth's surface. Microbial reduction of nitrate can be separated into two categories: assimilatory and dissimilatory.

Most bacteria can utilize the ammonium ion (NH_4^+) as an inorganic nitrogen source. Some have evolved pathways to reduce nitrate to nitrite (NO_2^-) via an assimilatory nitrate reductase (ANR or NR B) and then to the ammonium ion by an assimilatory nitrite reductase (56). This type of nitrate metabolism is not restricted to prokaryotes and can be found in all plants and some fungi. The enzymes of this system are soluble, expressed under aerobic and anaerobic conditions, and inhibited by ammonia (58).

The first anaerobically respiring organisms would have gained a powerful bioenergetic advantage over their anaerobically fermenting counterparts. Cells supplementing the low energy yields of fermentative metabolism with ATP from respiration quickly dominated the ecosystems they evolved in. Recent theories on the evolution of respiration imply that it evolved in an anaerobic environment (46,69). Anaerobic respiration occurs only in bacteria and is much like its aerobic counterpart. Instead of oxygen as the terminal electron acceptor, another inorganic compound is reduced. Nitrate respiration has been studied more than any other form of anaerobic respiration. There are two primary models of dissimilatory nitrate reduction. The first is found in organisms such

as *Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis*. These bacteria reduce nitrate to nitrite via a membrane-bound dissimilatory nitrate reductase (DNR or NR). The nitrite either accumulates or can be reduced further to ammonium by one of the two forms of nitrite reductase (67,79). The second model, termed denitrification, is found in obligate respiratory bacteria such as *Paracoccus denitrificans, Pseudomonas stutzeri,* and *P. aeruginosa* and is physiologically very different from the *E. coli* type of nitrate respiration. In denitrification, four reactions catalyzed by four different membrane-bound enzymes are responsible for the reduction of nitrate to dinitrogen:

$$NO_3^- \text{ ----> } NO_2^- \text{ ----> } NO \text{ ----> } N_2O \text{ ----> } N_2$$

The first enzyme, nitrate reductase (NR), reduces nitrate to nitrite. Next, nitrite is reduced to nitric oxide (NO) by the nitrite reductase (NiR). It is this reaction that distinguishes denitrifiers from nitrate respirers. Nitric oxide reductase (NOR) catalyzes the reduction of nitric oxide to nitrous oxide (N_2O). Finally, nitrous oxide reductase (NOS) converts nitrous oxide to dinitrogen (29).

Denitrification has received much attention because of its agricultural and environmental consequences. It is responsible for the removal of fixed nitrogen from soil and water. In agroecosystems, denitrification diminishes agricultural productivity by nitrogen limitation. Waste-water treatment facilities use these organisms as a nitrogen pollution buffer. This process is also responsible for the generation of nitrous oxide which has been implicated as an ozone depleting compound and is considered an important greenhouse gas. Lastly, denitrification is the final step of the nitrogen cycle (59).

Dissimilatory Nitrate Reductase of *Escherichia coli*

Preliminary genetic and physiological investigations done on both models of nitrate respiration suggest that regardless of the end product, the enzyme which reduces nitrate to nitrite appears to be relatively the same. They both are involved in an anaerobic respiratory

function, contain iron, labile sulfide, and molybdenum in the form of a Mo-cofactor (29,59,79). However, no molecular biological data exists which confirms or refutes this presumed conserved nature among a wide variety of bacteria. As a result, much of what is known about this enzyme is based on information revealed from scientific investigations of *E. coli*'s NR. The NR enzyme complex of *E. coli* is coded by the *narGHJI* operon and has been cloned and sequenced (9,75). This operon is 7 kb and is transcribed with the order *narG-H-J-I*. The four subunit enzyme is oriented such that the active site faces the cytoplasm. The α subunit, encoded by *narG*, contains molybdenum in the form of a Mo-cofactor and is the site for nitrate reduction. Mo-cofactor is noncovalently bound to the protein and its function is that of a prosthetic group. Even though the exact role of the β subunit (*narH*) is not known, it is believed to mediate interactions between the subunits, be involved with membrane association, and may have a role in electron transfer to the catalytic site (9). The γ subunit (*narI*), recognized as *cyt b*, is tightly associated with the other two and appears to act as an immediate physiological donor of electrons to the α subunit. The δ subunits (*narJ*) function is not known, however it is believed to mediate the formation of an active complex by allowing for subunit interactions (75).

The enzymes and membrane proteins involved in nitrate respiration are synthesized at their highest levels in the presence of nitrate, under anaerobic conditions (32,36,61,79). In *E. coli*, the *narGHJI* operon and *narK*, a gene upstream of the operon which encodes a nitrite extrusion system (67), are regulated in different ways by the DNA-binding proteins NarL and FNR (36,79,80). NarL is the response regulator of a two-component system which activates the NR operon in the presence of nitrate and represses other anaerobic respiratory pathways (32,80). Two membrane-associated sensor proteins (histidine kinases), NarX and NarQ, are controlled by different promoters and respond to external concentrations of nitrate (61). Both sensors act separately to carry regulatory functions,

but not to the same degree (36). Oxygen regulates nitrate respiration at two levels: transcriptional (*fnr*) and post-translational. FNR is a positive regulator responsible for the induction of *narGHJI*, *frdABCD* (fumarate reductase), *dmsABC* (dimethyl-[amine]-sulfoxide reductase) and *narK* (20,36,79). Additionally, it down regulates genes which encode aerobic terminal oxidases. The expression of anaerobic respiratory genes in *P. aeruginosa* is under control of a FNR-like mechanism similar to *E. coli*'s called ANR (23). Oxygen is believed to inhibit at the post-translational level by competing for the electrons that are used to reduce nitrate via NR under anaerobic conditions and also by affecting the assembly of NR (19,79). Nitrate uptake and its inhibition by oxygen is still not well understood, however, physiological evidence supports a uniport model which couples transport to the reduction of nitrate (27,67).

The Evolution of Nitrate Metabolism

An understanding of the evolution of nitrogen cycling is critical to developing a correct perspective concerning the origin and evolution of life. The earliest organisms would have required mechanisms to biologically transform fixed nitrogen. Earth's prebiotic reducing atmosphere was probably composed of carbon dioxide, nitrogen, hydrogen, methane, ammonia and water (38,46). Stanley Miller subjected mixtures of these compounds to spark-discharges, which simulated lightning, and analyzed the condensate (52). A variety of organic molecules were generated including α-amino acids and simple organic acids. This classical experiment led to a proposed abiotic mechanism for which primordial biological building blocks could have been formed. Miller hypothesized that hydrogen cyanide (HCN) was formed in a reaction between methane and ammonia. This HCN could then react with hydrocarbons produced as a result of the electric discharge and contribute to the formation of important amino acids such as alanine (38). Nitrosyl hydride (HNO) is another highly reactive product observed in these

prebiotic experiments that mimic the conditions of Earth's early atmosphere (46). HNO is unstable and is eventually reduced to nitrate or nitrite. These reactions are believed to be responsible for the majority of fixed nitrogen which accumulated in the primordial soup. As life evolved and diversified, the environment slowly changed by the chemical transformations made by organisms. An evolutionary sequence has been proposed for the appearance of biological nitrogen transformation reactions: Ammonification - Denitrification - Nitrification - Nitrogen fixation (46).

Aerobic terminal cytochrome oxidases are membrane-bound enzymes which reduce oxygen to water in the terminal step of aerobic respiration. A cytochrome oxidase from *Bradyrhizobium japonicum* (FixN) has been sequenced and identified as the most distant member of the heme-copper cytochrome oxidase family (69). Its high affinity for oxygen suggests that it may be related to a primordial cytochrome oxidase which functioned under microaerobic conditions. Sequence analysis between FixN and the NOR of *P. stutzeri* suggests that the former has diverged from the latter and thus challenges the dogma that denitrification evolved from aerobic respiration. It has been proposed that the enzymes of denitrification arose from those of assimilatory nitrate metabolism (46). In fact, DNA homology has been demonstrated between *E. coli*'s *narG* and the assimilatory NR of the cyanobacterium *Synechococcus* (39). According to the current theory, aerobic respiration evolved from denitrification when oxygen entered the biosphere, from oxygenic photosynthesis, and became the preferred acceptor (46). In the words of Francois Jacob:

> "In contrast to the engineer, evolution does not produce innovations from scratch. It works on what already exists, either transforming a system to give it a new function or combining several systems to produce a more complex one. Natural selection has no analogy with any aspect of human behavior. If one wanted to use a comparison, however, one would have to say that this process resembles not engineering but tinkering, *bricolage* we say in French. While the engineer's work

relies on his having the raw materials and tools that exactly fit his project, the tinkerer manages with odds and ends (33)."

The early evolutionary appearance of nitrate metabolism is demonstrated by its occurrence in a diverse number of prokaryotes (eu- and archaebacteria) from a wide variety of environments (29). The complexity of its genetic organization is strong evidence against a lateral gene transfer theory.

Based on the evolutionary development of nitrate reduction, one would expect to see genetic variations in the enzymes associated with nitrate respiration and denitrification. For example, two different types of denitrifying NiR (nitrite reductase) have been reported (40,74,89). The first contains the *c-d* cytochrome molecule (heme-type). This enzyme was mistakenly identified as an aerobic terminal cytochrome oxidase because it readily reduced oxygen to water. A second type of NiR which contains copper has also been identified (40,74,89).

There is evidence from studies of *Geobacter metallireducens* (55) and *Rhodobacter capsulatus* (63) for a different type of NR (nitrate reductase). Southern blot analysis using probes derived from the *narG* of *E. coli* revealed no homology with the genome of *G. metallireducens* but did hybridize to genomic DNA of the two denitrifiers *P. aeruginosa* and *P. stutzeri*. Antisera to *P. stutzeri*'s NR reacted with the NR of *P. aeruginosa* and *E. coli*, but not with *G. metallireducens*. Additional experiments revealed that the *Geobacter* NR does not possess *cyt b* but does contain a *cyt c* similar to that found in *R. capsulatus* (55). The discovery of novel NRs supports a diverse evolutionary origin. It is logical to presume this enzyme has the genetic variations that would be expected in any ancient biological system. Because of recent data suggesting that the NO_x reductases may be more diverse than previously thought, it is important to establish their degree of variation in natural environments.

MOLECULAR APPROACHES TO MICROBIAL EVOLUTION AND ECOLOGY

Reconstructing the Molecular Evolution of Nitrate Reductase

Determining the evolutionary history of an enzyme requires a large amount of molecular data. Based on existing family trees, two types of genetic development have been observed. The genes for some enzymes appear to have very different histories but have evolved to perform the same function. For instance, there are three classes of alcohol dehydrogenase that have been characterized (34,65,91). Mammals and yeast contain a similar enzyme. The other types, found in prokaryotes and Drosophila, are distinct but have a related three-dimensional structure. Evolution in which two or more groups of organisms develop different mechanisms to perform the same function is termed convergent evolution (91). The second pattern of gene evolution is best illustrated by the highly conserved enzymes of glycolysis. This metabolic pathway predates respiration and was present in the first forms of life. The glycolytic enzymes of prokaryotes are 50% identical with our own (51,91). Their conserved nature implies that this system is firmly rooted in all organisms and any further modification would most probably not offer a selective advantage.

Biochemical studies of NR from a variety of bacteria have suggested a conserved quaternary structure for this protein. The NR from the enterics and other organisms is composed of an $\alpha\beta\gamma$ enzyme complex: the α subunit which averages between 104 to 150 kd and contains the molybdenum cofactor, β subunit (52 to 64 kd), and often a γ subunit (19 to 23 kd) with a *b*-type cytochrome can be purified when detergents are used (29,79). Nitrate reduction is inhibited by oxygen, azide, thiocyanate, toluene-3,4, dithiol, cyanide and reduces chlorate and bromate in addition to nitrate (29). These physiological data strongly suggest that the NR complex is relatively the same in a diverse group of microbes.

However, recently this conclusion has been challenged. Gorby and Lovley (1991) reported that the NR of *G. metallireducens* could use artificial electron donors and was inhibited by oxygen, azide and cyanide (26). Stolz *et al.* later demonstrated that the enzyme has no genetic or immunological relationship with the NR of *E. coli* or *P. stutzeri*, respectively (55). Therefore, physiological and biochemical similarities do not necessarily infer genetic relatedness.

The discovery of different forms of denitrifying enzymes raises questions about the origin of this pathway. Did it emanate from a common ancestor and diverge or have many unrelated forms evolved in parallel? The enzymes of this system were once thought to be highly conserved, but recent molecular evidence has challenged that belief.

The second step in denitrification, the reduction of nitrite to nitric oxide, is catalyzed by the dissimilatory NiR. It is this reaction that distinguishes denitrifiers from nitrate respirers. There are two known forms of NiR: The heme (*c-d*) and copper type. Probes have been designed from the NiR gene sequence of *Alcaligenes eutrophus* (40) and *P. stutzeri* (74,89). Both of these organisms contain the *c-d* cytochrome molecule in their NiR. Strains which contain the copper NiR are only weakly recognized by the heme-type probes. Based on the copper type's rare occurrence, it may have evolved much later than the widely distributed heme enzyme (46). Gene probes have also been designed from *P. stutzeri*'s nitrous oxide reductase (40). The existing probes detect viable denitrifying species with a 75% confidence (40) and have been useful in investigations which detect these organisms in environmental samples.

The discovery of a novel NR suggests that other genetic forms may be distributed among nitrate-reducing bacteria. It therefore becomes important to assess the diversity of this enzyme. One approach might involve constructing probes from a known nitrate-reduction gene to be used in a survey of nitrate-reducing bacteria. To date, *E. coli*'s dissimilatory NR is the only one that has been cloned and sequenced (9,75). *E. coli* contains a second NR, coded by *narZYWV*, which is similar to the anaerobic respiratory

enzyme except that it is expressed under aerobic conditions (10). It is believed that the two enzymes share a common evolutionary origin and are a result of gene duplication. A highly conserved domain of both *narZ* and *narG* contains a base sequence that is believed to code for the protein region which anchors the molybdenum cofactor to the NR complex. Probes derived from *narG* have homology with the assimilatory NR of *Synechococcus* (39) and the dissimilatory NR of *P. stutzeri* and *P. aeruginosa* (55). The β subunit (*narH*) is believed to be the polypeptide involved in electron transfer. When its amino acid sequence was compared to other known electron transfer proteins (dimethyl sulfoxide reductase and formate dehydrogenase), four cysteine clusters characteristic of iron-sulfur ferredoxin centers were located (9). All well characterized NRs isolated thus far contain a β-type subunit (NarH) of analogous size and apparent function. Most contain a similar γ subunit which is a specific NR b-type cytochrome. The exception to this are the *cyt c*-containing NRs of *G. metallireducens* and *R. capsulatus*. In *E. coli*, the NR cytochrome b is encoded by *narI*. Perhaps the *narI* gene is under strong selective pressure and could be used to specifically identify organisms with a *cyt b*-containing NR (75).

Screening a diverse group of bacteria with probes derived from the *E. coli* genes *narGHJI* would not only yield additional information on the genetic diversity of this enzyme, it would also provide preliminary data and rationale for designing other NR-specific probes to be used in environmental analyses. There is a critical need to develop techniques which accurately detect microbial denitrifiers. Much of the existing information about their natural distribution comes from detection methods which rely on selective enrichment. Such investigations have lead to ambiguous and inaccurate conclusions. For example, half the reports studying the effect of root proximity on denitrification conclude that it is enhanced, while the other half show that roots have no effect (82). It would be rewarding to better understand the molecular diversity of NO_x reductases so that

physiological or strain-specific probes can be customized for identifying these bacteria in nature.

Identifying Microorganisms with Molecular Probes

Many techniques have been developed in an effort to detect naturally occurring microbial communities (66). The majority of these techniques depend on the ability of an organism to be cultured. However, no medium exists which can meet the variety of metabolic requirements needed to grow all organisms within a natural bacterial community. For example, a medium designed for autotrophs does not complement heterotrophs and methanogenic bacteria do not grow on a medium designed to count spore-formers (12). It has been estimated that less than 1% of the soil bacteria observed by fluorescence microscopy can be grown in the laboratory using a particular growth medium (84). Therefore, detection methods which rely on culturing bacteria from environmental samples greatly underestimate microbial diversity.

Recent advances in molecular biology make it possible to identify microorganisms and microbial activities with probes that target a gene or protein of interest. These techniques detect specific molecular sequences and may more accurately reflect the diversity which exists in a microbial niche. Molecular probes are now commercially available for the detection of individual or groups of bacteria. They allow rapid identification of organisms which are unculturable, difficult to grow, or highly contagious (25). Hence, microbial ecology finally possesses the necessary tools to develop a clear understanding of the natural relationships between microorganisms (See 93).

It has been demonstrated that common activities within a bacterial community can be assessed by using molecular probes. Chromosomal DNA or proteins from bacterial isolates can be detected with antibodies or radiolabeled DNA sequences. The high specificity of antibodies have made them useful in the detection of a single bacterial species

which exists in a complex habitat such as soil or sea water (12,90). Comparative studies suggest that DNA probes are more generic and thus better candidates for cross-species reactivity (90). The main problem with this approach is constructing a probe which discriminates the target organism (or group of organisms) from others in its environment.

Tracking Microbes in Environmental Samples

The development of colony hybridization techniques allow large populations to be screened for a specific genotype (1,3,21,22,40,70,74,89). These methods complement phenotypic analysis, but still depend on culturing the organism. Smith and Tiedje (74) performed Southern hybridization with the *nir* probe on total DNA. Although no signal was detected in DNA that was directly extracted from soil and wetland sediments, the probes did react with DNA from enriched samples. One conclusion reached from this study was that laboratory culture disrupts the community balance by selecting for portions of the population atypical of natural low-nutrient habitats (66). Analysis of total DNA may be appropriate in some instances (30,83,84), but the study by Smith and Tiedje suggests that this method lacks the sensitivity required to detect the limited gene copies that may be present in a sample. Factors that may influence detection include: the efficiency of cell lysis and DNA extraction, abundance of target sequence, specificity and specific activity of the probe, and the limited amount of DNA that can bind to a filter (78).

An alternate quantitative approach presented by Voordouw *et al.* (88), termed reverse sample genomic probing (RSGP), can be employed to survey microbial communities without culturing. DNA extracted from samples is labeled and used to probe a filter spotted with known bacterial standards. The standards are characterized strains and/or isolates from a sample of interest. RSGP can be a useful tool for fingerprinting a population, but it has obvious limitations. The technical barriers are overshadowed by the

fact that the experimental design of this method relies on some knowledge of the community structure. Furthermore, this procedure does not apply to organisms which are unculturable.

The polymerase chain reaction (PCR) has the capability to generate large quantities of specific DNA sequence from environmental samples. Theoretically, only one copy of the target sequence is required for PCR amplification. Microbial ecologists therefore may be able to identify specific diminutive genotypic aspects of a population through PCR amplified DNA. A case in point involves a study which established what phylogenetic types occupy a particular niche based on rRNA sequence obtained by PCR amplification of a mixed population (4). The sequence-based surveys report unprecedented diversity. PCR analysis is limited by the fact that molecular data are required to design primers; thus rRNAs make excellent molecular chronometers allowing a broad range of phylogenetic relationships to be measured (92). Highly conserved domains make it possible to design degenerate oligonucleotides which amplify sequence from a diverse species of molecules. Ribosomal RNA phylogeny has substantially enhanced our evolutionary perspective in a short period of time.

Amplified DNA segments which encode proteins involved in specific metabolic activity have been used to assess community function and structure (2,5,72,78,86,90). If the gene belongs to a large group of targeted organisms, novel sequences can be cloned, screened with a variety of probes, and sequenced. Obtaining this type of molecular biological data can be time consuming, especially if one wants to study the diversity of a population. A rapid and inexpensive technique called "denaturing or temperature gradient gel electrophoresis" (DGGE or TGGE) allows PCR fragments of the same length to be distinguished by sequence (54). A polyacrylamide gel, which contains a denaturant gradient, separates DNA based on the melting of sequence-specific domains. This technique has been used to estimate the distribution of *Desulfovibrio* species in natural microbial mats and an experimental bioreactor (90). DGGE separated the PCR products

into specific bands and accurately detected the species diversity of this group in four different environmental samples.

The PCR amplification procedures are 10^3-fold more sensitive than dot-blot analysis of community DNA extracted directly from environmental samples. Only 1 cell/gram of sample is necessary to detect a target organism (78). DGGE identifies species rapidly by gene sequence and provides a community fingerprint. Each variation of genotype is distinguishable by its molecular signature. The greatest advantage is that non-culturable organisms are represented. Bej *et al.* (5) questioned the ability of this approach to differentiate between dead or alive bacteria. They found that positive signals were only detected from viable cells; i.e. cells capable of division, metabolism, or gene transcription (66).

In environmental monitoring, particularly in denitrification surveys, activities are more important than simply detecting the presence of an organism (or genotype). Molecular analysis can ascertain the identity of proteins or genes, but it reveals nothing about the physiological role of that component *in situ.* Messenger RNA isolated from bacterial cells can be reverse transcribed into cDNA and PCR amplified (5,44). This technique can be used to measure the expression of a targeted gene. DGGE or Southern blot analysis could detect which species and physiologically types represent a niche of interest. However, native nucleases make mRNA difficult to purify. For this reason, mRNA detection is found to be less sensitive than direct amplification of DNA (1,000 cells needed compared with 1, respectively (44)). Although it has promise, reverse-transcription PCR techniques must find a way to increase the half-life of mRNA.

The main objective of this study was to ascertain the conserved nature of the *E. coli*-type NR and judge the worth of *narG* as a potential molecular probe to be used in environmental analyses. Surveying a wide variety of nitrate-reducing bacteria with probes derived from the cloned genes of *E. coli*'s NR provides preliminary data and rationale to be used in the design of other NR-specific probes. Such a study will also serve to answer

some pedigree questions regarding the origin and evolution of this environmentally essential metabolic pathway.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Strain designations, growth temperatures, culture medium and sources of bacteria are listed in Table 1. Genomic DNA was isolated from organisms that were cultured aerobically (250 ml Erlenmeyer flask containing 50 ml of medium) with agitation (150-300 RPM).

For the nitrate uptake experiments (Table 3), all bacteria, with the exception of *Azotobacter vinelandii*, *P. fluorescens* (ATCC 13525), *Xanthomonas maltophilia* and M91, were grown anaerobically in the presence of 50 mM KNO_3. These cultures were grown in sealed 500 ml Wheaton bottles which contained a stir bar to prevent cell clumping. Culture bottles were flushed with argon for 15 minutes to create anaerobic conditions. The nitrate reducing system of the obligate aerobe *X. maltophilia* was induced under microaerobic conditions (100 RPM) in the presence of 50 mM KNO_3 (1000 ml Erlenmeyer flask containing 500 ml of medium). The NRs of *Rhizobium meliloti* and *Rhodobacter capsulatus* were induced by growing the organisms microaerobically (500 mls of medium in a 1 L screen top flask) until saturation, then 500 mls of fresh media was added and flasks were sealed and flushed with argon to create anaerobic conditions. These cultures were subsequently incubated overnight.

Preparation of Genomic DNA

Genomic DNA was isolated according to the method of Chen and Kuo (17). Cells in late log phase were harvested by centrifugation of 1.5 ml of culture medium at 12,000xg for 3 min. at room temperature. Pellets are resuspended in 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, and 1% SDS). Lysozyme (100 µl of a 2 mg/ml stock) was added to cell suspensions and incubated at 37°C for 30 min. when chemical treatment resulted in poor DNA yields. *Staphylococcus aureus* was lysed

by incubating cells at 37°C for 30 min. in 100 µl of 50 mg/ml lysostaphin (60). After the addition of 66 (105) µl of 5 M NaCl, the viscous mixture is centrifuged at 12,000xg (4°C) until the cell debris is pelleted; 10-15 min. The supernatant is extracted with an equal volume of chloroform followed by centrifugation at 12,000xg for 3 min. Preparations with high protein content required one or two additional phenol-chloroform extraction's. DNA is precipitated at -70°C in two volumes of 100% ethanol followed by centrifugation at 12,000xg for 15 min. at 4°C. Pellets were washed with 70% ethanol, dried in a Speed-vac and amended in TE. Cellular RNA was removed by the addition of 1 U of RNase (Sigma) followed by a 30 min. room temperature incubation. Genomic DNA was fragmented by restriction digestion with BamHI (Promega) and electrophoresed on a 0.7% agarose gel stained with ethidium bromide (0.5 µg/ml final) to estimate concentration. Attempts to quantitate genomic DNA with a spectrophotometer at 260 nm were unsuccessful (even with 1.8-2.0 260/280 nm ratios), possibly due to the presence of interfering polysaccharides (17). This method yielded DNA of high molecular mass, with no significant trace of shearing or degradation. All DNAs were stored at -20°C.

Gene Probe Construction and Southern Hybridization

All transformations, plasmid DNA isolation, agarose gel electrophoresis and DNA restrictions were done as detailed by Sambrook *et al.* (68). Probes from *narG* were designed from the plasmid pFB71 which contains the 3.7 kb gene from *E. coli* (with permission from F. Blasco, kindly provided by J.F. Stolz). A restriction map of this clone may be seen in Figure 1. This plasmid was propagated in *E. coli* DH5α in the presence of chloramphenicol (50 µg/ml). The probes, 2.1 kb HpaI-HpaI and 0.84 kb HindIII-HindIII, were constructed by complete endonuclease digestion of 5-10 µg of plasmid. Fragments were separated on a 1% low melting agarose gel and subsequently purified using Gene-Clean (Bio 101 Inc.). Quantitation of DNA was by the ethidium bromide (0.5 µg/ml final)

agarose plate method (68). DNA was random prime labeled (Boeringer Mannheim Biochemicals) with α-^{32}P dCTP (Amersham). Unincorporated nucleotides were removed with a Sephadex G-50 NICK column (Pharmacia Biotech). Specific activity of labeled DNA was determined using a Beckman liquid scintillation counter.

Southern blot analysis was carried out as described by Southern (68,76). Genomic DNAs (10-15 µg) were restricted with an excess of BamHI overnight at 37°C. Agarose gels (0.7%) used in transfers were run at low voltage (20-30 volts) for 10-15 hours to improve the resolution of larger DNA fragments. The size of hybridized fragments was based on the migration of a molecular weight standard (λ DNA cut with HindIII) separated in conjunction with the BamHI-restricted genomic DNAs. Capillary transfer of single stranded DNA to a supported nitrocellulose membrane (Schleicher and Schuell; BA-S 83, pore size 0.2 µm) was allowed to proceed overnight. DNA was fixed by baking the membrane in a vacuum oven for 1 hour at 80°C. Blots were hybridized overnight at 42°C with at least 10^8 cpm/µg of labeled probe in 10 ml of 6x SSPE, 5x Denhardt's solution, 0.5% SDS, 100 µg/ml salmon sperm DNA and 50% formamide. Membranes were initially washed twice at room temperature in 2x SSPE and 0.1% SDS for 15 minutes. Stringent washes were at 65°C in 0.1-0.5x SSPE and 0.1% SDS for 15 minutes. Autoradiograph exposure times were as long as 4 days at -70°C for blots that received stringent washing.

Measurement of Nitrate Uptake

Changes in the concentration of nitrate were measured with an Orion digital ion analyzer (model 601 A) connected to an Orion nitrate electrode (model 93-07) and a double junction reference electrode (model 90-02). This device was calibrated directly with known concentrations of KNO_3 in 50 mM KPO_4 pH 7.0 and 2% ISA (ionic strength adjuster - Orion #930711). Induced late log cultures (500 ml) were harvested by centrifugation at

10,000xg for 10 minutes at room temperature. Pellets were washed twice in several volumes of 50 mM KPO_4 pH 7.0 supplemented with 50 µg/ml chloramphenicol. Cells were suspended 1 g (wet weight)/5 ml of buffer. The 10 ml reaction vessel contained the following: 9.4 ml 50 mM KPO_4 pH 7.0, 0.4 ml ISA, 0.2 ml of cell stock suspension (1 g/5 ml) and 50 µl of 100 mM KNO_3 (500 µM final). Anaerobic conditions were maintained by continually flushing the vessel with argon. After an initial period of fluctuation, a baseline concentration of nitrate was observed. The uptake of nitrate was initiated by the addition of 500 µl of 1 M formate (0.5 mM final concentration). Nitrate concentrations were monitored for one minute (15 second intervals) after addition of the electron donor. For each organism, uptake measurements were done in triplicate. The protein content (mg/ml) of cell suspensions was determined after whole cells were disrupted by sonication or with 1N NaOH using the coomassie blue method (595 nm) with a standard curve of known bovine serum albumin concentrations (11). Rates of nitrate uptake are expressed in nmole min^{-1} mg $protein^{-1}$.

CHAPTER 3

RESULTS AND DISCUSSION

Development of Probes from *narG* of *Escherichia coli*

narG was selected to construct the probes used in this study because previous data indicated that this gene may be genetically conserved (10,55). When Bonnefoy *et al.* (1987) discovered a second NR (NarZ coded by *narZYWV*) in *E. coli*, they also found that partial but significant homology existed between it and the NR complex coded by *narGHJI* (10). Southern hybridization experiments revealed that an internal 2.1 kb HpaI-HpaI of *narG* shared a high degree of homology with a corresponding 1.05 kb HpaI-HpaI fragment of *narZ*. The *narG* and *narZ* genes encode the α subunit of NR and it is likely they are conserved because of this polypeptides role in anchoring the molybdenum cofactor to the catalytic unit. The restriction map in Figure 1 shows the internal location of the 2.1 kb HpaI-HpaI fragment (Probe B) relative to *narG*. Stolz *et al.* used the latter along with a 0.84 kb HindIII-HindIII fragment (Figure 1) located upstream of Probe B designated Probe A (55). They discovered that both demonstrated homology with genomic DNA of *P. aeruginosa* and *P. stutzeri,* but not with *G. metallireducens.* Based on this molecular evidence, both probes were used in this survey.

Genetic Relatedness and Expression of Nitrate Reductase in Diverse Organisms

The 13 genera of bacteria surveyed in this study reside in two phyla; the purple bacteria and the gram positive eubacteria. The eubacteria are taxonomically organized into ten phyla based on 16S rRNA sequence comparisons (92). This diverse group of organisms share a common ancestor that existed 5-10 x 10^8 years ago (47). The phylogenetic tree for the purple bacteria, or proteobacteria, is separated into 4 subdivisions: α, β, γ and δ. The study presented in this thesis includes organisms from the α, β and γ

subdivisions of the purple bacteria and gram positive species from the low G+C subdivision of the Eubacteriacea (Figure 2). The common ancestor of these two phyla and the related prokaryote, cyanobacteria, was believed to be an anoxygenic phototroph due to the distribution of this phenotype in the eubacterial phylogenetic tree (12,47,92). It is believed that the capability to photosynthesize has since been lost by many organisms and replaced with other energy generating processes (92). This has resulted in the great metabolic diversity observed among the members of this subkingdom.

The microbes chosen for this study represent a phylogenetically and physiologically diverse group of nitrate-reducing bacteria. Of the 15 species surveyed which contain a respiratory NR (or enzyme that resembles a typical dissimilatory NR), 9 demonstrated homology with the *narG* probes. Continuous selection for the same enzymatic function has evidently preserved the α subunit's sequence in these lineages. The molecular analysis done in this study shows that some organisms contain a gene(s) homologous to *narG* of *E. coli*. However, this approach reveals nothing about the expression or physiological function of the respective gene product. Therefore, bacteria were also examined physiologically to confirm their ability to reduce nitrate anaerobically.

A variety of techniques exist which can determine if a given bacterium is capable of dissimilatory nitrate reduction/denitrification (45). A common method used to detect the expression of NR involves monitoring the ability of a cell suspension to remove nitrate from the external milieu under anaerobic conditions. The activity of NR has been shown to be necessary for nitrate uptake to occur (19,20,27,67). The current model of nitrate uptake in *E. coli* proposes that transport is metabolically coupled to nitrate reduction (67). In this study, a nitrate specific electrode was used to measure rates of uptake (change in external nanomolar concentrations of nitrate versus time, Table 3). All cells were grown in the presence of an appropriate nitrogen source, to repress assimilatory pathways, thus they would not contribute to nitrate utilization. Organisms were grown anaerobically with non-fermentable glycerol when it was possible. Those that could not support growth on this

carbon source were grown on substrates that had previously complemented nitrate respiration, as indicated by the literature.

Purple Bacteria

Of the 10 eubacterial phyla identified, the purple bacteria are the largest and most physiologically diverse. Our contemporary perception of microbiology is the result of scientific research which has primarily focused on the members of this phyla. A variety of metabolic processes are observed in this group: the widely distributed phototrophic phenotype (which provides the backbone for classifying these organisms), heterotrophy, autotrophy, lithotrophy and sulfate reduction (12). In addition, many genera are also able to respire anaerobically using nitrate as a terminal electron acceptor.

Rhizobium meliloti belongs to the α subdivision of the purple bacteria. It is an agriculturally important nitrogen-fixing bacterium that lives in symbiosis with legumes. *Rhizobium* and *Bradyrhizobium* are the only nitrogen-fixing genera which contain denitrifying strains (15). These bacteria are also known to contain an assimilatory NR. Some nitrate respirers do not contain all four reductases of the pathway. For example, *R. meliloti* (ATCC 9930) lacks a N_2O reductase and thus performs a truncated kind of denitrification. Remarkably, several *R. meliloti* strains keep some of their denitrification genes on a megaplasmid where genes for nodulation and symbiotic nitrogen fixation are located (16). It is not currently known if genes for NR are also located on this megaplasmid. Chan *et al.* constructed a probe from the gene sequence of *P. stutzeri*'s NOS which hybridized to all *R. meliloti* isolates possessing N_2O reduction activity (see above), one strain of denitrifying *A. eutrophus* and several other denitrifying *Pseudomonads* (16). Total DNA isolated from *R. meliloti* did not react with either *narG* probe (Figure 3). It may share NOS homology with *A. eutrophus* and a number of *Pseudomonads,* but its NR appears to be genetically distinct from the one possessed by the latter organisms. Induction

of nitrate respiration and the uptake experiments (Table 3) demonstrate that this microbe's dissimilatory NR is an expressible, functional complex. Anaerobic growth of *R. meliloti* was not initially successful. To express its NR, cultures were grown micro-aerobically in the presence of nitrate till early log phase, then a volume of fresh medium was added and the conditions were made anaerobic. This procedure did induce nitrate respiration in this microbe as demonstrated by the uptake data (Table 3).

Hyphomicrobium vulgare and *H. zavarzinii* are budding methylotrophic bacteria that form round to oval cells with one or more short hyphae up to three times the length of the mother cell (28). These microbes belong to the α subdivision of the proteobacteria (77). Cell division in these organisms is very interesting because instead of binary fission, new cells form by budding and the mother cell retains its identity. The appendages are used for attachment and increase surface:volume ratios which may offer a competitive advantage in the dilute aquatic environments they commonly inhabit (12). These bacteria are used in some methods of tertiary water treatment to decrease the BOD of industrial effluent. They are able to denitrify at relatively high oxygen tensions with methanol or methylamine as a carbon source (40). This phenomenon was demonstrated in the *Hyphomicrobium* long before "aerobic denitrification" was characterized in *Thiosphaera pantotropha* (6,7,64). Expression of NR and the uptake experiments were performed only under anaerobic conditions in this study (Table 3). *Hyphomicrobium vulgare* and *H. zavarzinii* shared similar autoradiograph patterns; two strong hybridization signals at 22 and 18 kb with both probes (Figure 3). It is very likely that each signal represents a separate gene. This inference is substantiated by the identical autoradiograph patterns exhibited with both probes. If a probe derived from the 5' region of a gene produced two hybridization signals because the homologous region was bisected with a restriction site (BamHI), another probe from sequence further downstream of the latter should hybridize to only one of the large genomic fragments. These conclusions are based on the assumption that

organisms which contain an *E. coli*-like NR (α subunit) also share a similar genetic arrangement. These data suggest that the *Hyphomicrobium*, like many other NR-containing genera, may have members which possess an additional form (or gene copy) of NR in addition to the known respiratory type.

Thiosphaera pantotropha (α subdivision)was originally isolated by Robertson and Kuenen from an industrial effluent-treatment plant on account of its ability to grow aerobically and anaerobically on reduced sulfur compounds and hydrogen, while fixing carbon dioxide (64). Its entire denitrification pathway from nitrate to N_2 is expressed and active under low oxygen tension (6). *Thiosphaera pantotropha* has become a scientifically significant organism because it grows mixotrophically and heterotrophically on a variety of substrates, and thus could offer an inexpensive alternative to traditional microbial treatments that remove nitrate and reduced sulfur compounds from industrial effluent. This organism has a NR which is membrane-bound and resembles the respiratory enzyme found in enteric nitrate respirers both in respect to its catalytic properties and translational/post-transcriptional regulation by oxygen. In addition, *T. pantotropha* has a second periplasmic NR which is expressed under both aerobic and anaerobic growth conditions (7). For the purpose of this investigation, expression of nitrate respiration and the uptake experiments on this organism only were only performed under anaerobic conditions (Table 3). A 9 kb fragment of this microbe's genomic DNA strongly hybridized to the probes (Figure 3). Another band at 20 kb (data not shown) is observed under low stringency. It is possible that the band removed under stringent washing (20 kb) represents the periplasmic enzyme and the stronger signal at 9 kb is the membrane-bound form. This preliminary conclusion is entirely based on the regulatory and physiological properties of these NRs (7).

Paracoccus denitrificans (α-3) is a facultative autotroph that grows aerobically or anaerobically with NO_x as a terminal electron acceptor and is commonly found in soils (37). Under anaerobic conditions, it may obtain energy heterotrophically or by autotrophic

hydrogen-linked denitrification (59). It is a scientifically significant organism because it is believed to be closely related to the endosymbiont from which mitochondria arose (81). This bacterium contains a respiratory NR that is thought to be physiologically and biochemically very similar to the one found in *E. coli* (37). The *Pa. denitrificans* NR complex possesses α, β and γ subunits that are indistinguishable from those of *E. coli* on SDS-PAGE (18). Furthermore, electron paramagnetic resonance spectroscopy revealed that the "ligand environment" of the molybdenum cofactor is identical in NRs from *Pa. denitrificans, P. aeruginosa,* and *E. coli* (85). Based on this evidence, it is very interesting that *Pa. denitrificans* did not hybridized with either *narG* probe (Figure 3). No signal was detected even under low stringency (data not shown). Sequence comparisons of 16S rRNA between *Pa. denitrificans* and *T. pantotropha* revealed an identical primary structure, suggesting these organisms have a close evolutionary link (41). One would expect that these bacteria would also share many other genetically conserved regions. Nevertheless, the NRs they possess appear to be genetically different. Nitrate uptake experiments demonstrate that this strain expressed NR when cultured under anaerobic conditions in the presence of nitrate (Table 3).

Rhodobacter capsulatus (α-3) is a metabolically versatile organism that is abundant in polluted waters and is commonly found in mud and stagnant water that receives exposure to light (12). This microbe can grow photoautotrophically, photoheterotrophically, chemoautotrophically, chemoheterotrophically or fermentatively (43). It also possesses dissimilatory NR activity when the organism is continuously subcultured in nitrate medium under phototrophic conditions (48). It will not grow under heterotrophic/anaerobic conditions in the dark because nitrate respiration in *R. capsulatus* does not appear to provide enough energy for growth and probably only acts to supplement the energy generated through photosynthesis. Attempts to express NR in *R. capsulatus* under photoheterotrophic conditions, as described by McEwan *et al.* (48), were not

successful. The NR complex of this bacterium, however, does appear to be expressed when it is cultured anaerobically under chemoheterotrophic conditions as revealed by the uptake experiments (Table 3). The NR of this organism was expressed in a manner similar to that described previously for *R. meliloti* (See pg. 25). The expression study implies that nitrate respiration in this bacterium, like many others, is regulated by oxygen and nitrate due to NRs derepression under anaerobiosis in the presence of nitrate. The activity and role of this enzyme in energy generation are still not clear. The *narG* probes did not hybridize to genomic DNA from this organism which supports the existing belief that it contains a novel form of NR (63).

Alcaligenes eutrophus belongs to the β subdivision of the purple bacteria (β-2) and is a soil/water bacterium that can grow heterotrophically, autotrophically and use nitrate/nitrite for anaerobic respiration (73). After the *Pseudomonads*, the members of *Alcaligenes* are believed to be the second most abundant group of denitrifiers (24). The rate of nitrate uptake in *A. eutrophus* was greater than any other observed, 244 $\pm$ 46 nmol/min/mg (Table 3). This particular organism has three distinct NR activities. One has an assimilatory function and another is typical of respiratory NRs. The third type, nitrate reductase periplasmic (NAP), is composed of two subunits and has no clear physiological function (73). Sequence data suggests that NAP is a soluble molybdenum-containing protein with two conserved segments which resemble the 4Fe-4S centers of bacterial ferredoxins and a segment of the small subunit that resembles heme-binding sites typical of c-type cytochromes. The role of this enzyme may be that of a specific oxidoreductase for certain reducing equivalents or it could also function to maintain the cellular redox balance when the organism is in transition between aerobic and anaerobic conditions (73). *Alcaligenes eutrophus* has hybridization signals at 23 and 16 kb with both probes indicating that it contains two genes homologous to *narG*. This observation may be direct evidence that the NAP form of NR may have arisen by gene duplication of respiratory NR. However, it is also possible that one of the signals corresponds to the assimilatory NR this

organism is known to contain. It is unlikely this is the case given that other eubacterial assimilatory NRs appear not to have cross-react with these probes. Additionally, sequence comparisons between the periplasmic enzyme and other molybdenum-containing enzymes such as NR (assimilatory and dissimilatory) and formate dehydrogenase have revealed conserved elements (73).

An enrichment survey of environmental samples for denitrifying organisms from around the world indicated that *Pseudomonas fluorescens* (γ-3) strains represented the majority of organisms isolated (24). It is believed that members of the genus *Pseudomonas* are probably the most common and widely distributed denitrifiers (59). Homology to both probes was demonstrated with *P. aeruginosa* (23 kb) as previously shown (55), but surprisingly not the initial marine strain of *P. stutzeri* (Presque-Isle) chosen. It is interesting to observe NR diversity between organisms of the same genus. We therefore obtained the clinical isolate of *P. stutzeri* used in the *Geobacter* study (55) and both probes did hybridize to a 22 kb band of its chromosomal DNA. These organisms were used as positive controls in this study. *Pseudomonas fluorescens* is an environmentally significant organism that is closely related to *P. aeruginosa* and *P. stutzeri.* Two biotypes of *P. fluorescens* were used in this survey. Biotype C denitrifies (ATCC 17400), but biotype A (ATCC 13525) does not and thus served as a negative control. Both probes hybridized to a 16 kb fragment of biotype C (Figure 3, light band in blot A) and no signals were detected from biotype A. Biotype C was grown under nitrate respiring conditions and a significant rate of nitrate uptake was observed (Table 3). Biotype A was not capable of anaerobic growth.

The obligate aerobe *X. maltophilia* is a γ subdivision (γ-3) member of the purple bacteria and is found in soil surrounding the rhizosphere of some plants and can also be an opportunistic human pathogen (94). It converts nitrate to nitrite while growing aerobically. Two NRs have been purified from this organism (35). One contains *cyt b* and

molybdenum while the other is smaller and has not been well characterized biochemically. Furthermore, *X. maltophilia* has no detectable NiR activity. It has been speculated that this organism uses nitrate as an alternative electron acceptor under semi-aerobic conditions thus oxidizing excess NADH and NADPH (94). It is not clear whether energy is generated from nitrate metabolism. This process likely contributes to the cycling of nitrogen in aerobic environments generating nitrite that can be oxidized by nitrifying bacteria (8). Southern hybridization indicated that *Xanthomonas maltophilia* DNA has two signals at 20 and 15 kb that share homology to *narG* (Figure 3). A third band is observed with Probe B at around 4 kb, but was removed during stringent washing (data not shown). Perhaps the two forms of NR it contains have a respiratory origin, but have assumed alternate functions in this obligate aerobic microorganism. This bacterium is a close relative of *Pseudomonas* that along its evolutionary journey has lost the ability to grow anaerobically. Attempts to express the NR of *X. maltophilia* under fully aerobic conditions in the presence of nitrate were not successful. This system is only expressed when this organism is grown microaerobically in the presence of nitrate (Table 3).

Azotobacter vinelandii is a free-living obligate aerobic nitrogen-fixing soil bacterium that assimilates nitrate by ultimately reducing it to ammonia via the type B assimilatory NR and NiR. As previously mentioned, Lightfoot *et al.* demonstrated cross hybridization between *E. coli*'s *narG* and a single chromosomal region of the cyanobacterium *Synechococcus* (39). This segment is believed to encode an assimilatory NR gene for this blue green algae. Therefore, it was important to determine whether this gene for the catalytic activity of NR is highly conserved throughout the microbial world. No signal was detected suggesting that these assimilatory NR genes are genetically distinct from those encoding dissimilatory reductases.

Proteus mirabilis (γ-3) is physiologically like *E. coli* and is classified as an enteric bacteria. This group is of considerable medical importance because many members are pathogenic. *Proteus mirabilis* infections are frequently observed in the urinary tract or

kidneys (12). Its NR contains molybdenum cofactor and the same *cyt b* as *E. coli*'s (79). This study has surprisingly shown that no genetic relationship appears to exist between the two enzymes. The rate of nitrate uptake obtained for *P. mirabilis* confirms that this organism possesses a NR and has the ability to respire anaerobically (Table 3).

M91-3 is a soil isolate that has the ability to mineralize the pesticide atrazine and use it as a sole nitrogen and carbon source. The API-NFT system of classification identified this microbe as *Agrobacterium radiobacter* (α subdivision (92)) and fatty acid profiling identified it as a member of the genus *Xanthobacter* (62). This organism is able to reduce nitrate to nitrite as it slowly degrades atrazine anaerobically, but it is not known for sure if these processes are coupled to dissimilatory nitrate reduction or denitrification. The genomic DNA from this organism did not hybridize with either probe.

Gram Positive Eubacteria

Originally, prokaryotes were classified based on the structure and staining of their cell wall. Gram negative bacteria, many of which share a cell wall that consists of several complex layers (peptidoglycan, phospholipid and lipopolysaccharide (12)), are not a legitimate phylogenetic unit and comprise many eubacterial phyla. However, the gram positive bacteria do form an evolutionarily related group (92). The cell walls of gram positive bacteria are made of one thick layer that mainly consists of peptidoglycan (12). Some members of this phyla contain a NR and are capable of nitrate respiration. The majority of reported thermophilic denitrifiers have been *Bacillus* species, which suggests that these organism are ecologically significant members of thermophilic communities (31). The following is an overview of the two NR-containing gram positive (low G+C species (92)) microorganisms surveyed in this study.

The facultative aerobe *Bacillus licheniformis* is a common spore-forming bacterium that is readily isolated from soil or air (12). This organism is susceptible to lysis when the

ATP-dependent synthesis of cell wall constituents is interrupted (71). Autolysis can be avoided under anaerobic conditions through ATP synthesis via nitrate respiration or fermentation. Most enterics, such as *E. coli* have less than optimum NR activity under anaerobic conditions in the absence of nitrate. In contrast, the transcription of the NR genes in *B. licheniformis* are regulated by the intracellular redox potential and not the presence of nitrate. The NR complex is inactive only when conditions are fully aerobic and in the absence of an electron donor; thus inhibition requires complete withdrawal of electron flow from the enzyme (71). *Bacillus licheniformis* has an 18 kb band that reacted strongly under stringent conditions with probe A, but the signal from probe B was removed under stringent washing (Figure 3). It's NR was expressed under anaerobic conditions and nitrate uptake was observed (Table 3).

Staphylococcus aureus is a common human pathogen associated with such conditions as boils, pimples, pneumonia, osteomyelitis, meningitis and arthritis (12). It contains a membrane-bound respiratory NR (*cyt b*) along with a form which occurs in the cytoplasm (14). The cytoplasmic form accounts for 50% of the total activity. Antiserum to the membrane-bound NR reacts with both forms and thus the two enzymes are immunologically indistinguishable (13). The relationship, genetic and/or physiological, that exists between the two enzymes is still not clear. *Staphylococcus aureus* did not have homology with either probe. Attempts to culture this bacterium anaerobically in the presence of nitrate with the non-fermentable carbon source glycerol were not successful. It did grow anaerobically with glucose and a rate of nitrate uptake was obtained (Table 3).

Comments

This study suggests that the evolutionary history nitrate respiration is not like that of sulfate reduction or lithotrophy, i.e. recently attained by microorganisms to replace the photosynthetic apparatus (12,92). Instead, it implies that nitrate respiration was deeply

embedded in the common ancestor that purple and gram-positive eubacteria diverged from. Alternatively, the appearance of this gene in a heterogeneous group of bacteria could be the result of lateral gene transfer. Some enzymes involved in denitrification are known to be coded on native plasmids (16), nevertheless it is highly improbable that these genes were expansively distributed in this manner due to the genetic complexity of this system (46) and its widespread appearance among a diverse variety of eu- and archaebacteria (extremely halophilic branch) (29). These data support an ancient origin for NR and are consistent with current theories on the early evolutionary appearance of nitrate respiration (46,69).

An important ancestry question is now raised about the origin, evolution and distribution of NR encoding genes (specifically the α subunit) in nitrate-respiring bacteria that did not hybridize to the *narG* probes. These include: *P. stutzeri* (Presque-Isle), *R. meliloti, Pa. denitrificans, P. mirabilis, S. aureus, R. capsulatus* and *G. metallireducens* (55). These organisms appear to possess a NR genetically distinct from the enzyme found in dominant nitrate-respiring genera such as *Pseudomonas* and *Alcaligenes*. No exclusive phylogenetic relationship appears to exist between them. Genetic diversity was further demonstrated between members of the same genus since a marine strain of *P. stutzeri* (Presque-Isle) failed to hybridize with either probe while the clinical isolate of *P. stutzeri* did. These findings rule out the possibility that a single form of NR is distributed throughout the eubacterial tree. The ability to use nitrate as a terminal electron acceptor is an adaptation that provided many organisms with a selective advantage in anaerobic environments. It appears that the NR phenotype may have arisen from more than one evolutionary solution to anaerobic respiration over a long evolutionary time frame. However, such conclusions may be premature in view of the narrow range of this survey and limited data used to establish the ancestral lineages of NR in prokaryotes.

An alternative explanation might be that all forms of the enzyme are distantly related and subsequent divergence resulted from the dynamic evolutionary pressures which shape genes and organisms. When genes of a common ancestor are selected for by the same

pressures, they tend to evolve in parallel. The selective forces often involve an integral component of the gene products structure or function. Conservation of *narG* could be a result of the α subunits role in binding the molybdenum cofactor. The cofactor binding sequence would be strongly selected for in bacteria dependent on nitrate respiration. The acquisition of alternate metabolic pathways provided many organisms with several physiological options and the potential to colonize a variety of ecological niches. It is possible that these evolutionary processes reduced the "importance" of nitrate respiration in some microbes. This would alleviate a great deal of the selective pressure placed on these genes and result in divergence from the common ancestral sequence. As Carl Woese has written (92):

> "If the environment in which a phenotype arises (first stabilizes) persists, that phenotype will persist, fundamentally unchanged." "On the other hand, all drastic (broad-ranging) changes in ancestral phenotype necessarily result from increased mutation rates, which tend to occur under unusual, drastic environmental conditions, when selection is relaxed in ways that allow the mutational rate to rise."

It is quite evident that further molecular biological data regarding NR will need to accumulate before any explicit pedigree questions can be answered. What has been made clear about this enzyme is that like other NO_x reductases, it appears to be genetically diverse contrary to the biochemical and physiological evidence which has implied that it was highly conserved. This study has confirmed that a genetically conserved form of NR is distributed among the eubacteria and found in dominant nitrate-respiring genera like *Pseudomonas* and *Alcaligenes*. This suggests that the *narG* probes may be suitable to detect such organisms in environmental samples. A fundamental understanding concerning the distribution of NR and other NO_x reductases is as essential to the microbial evolutionist as it is to the microbial ecologist. Nitrate respiration is a strong candidate for the first form of respiration to have evolved. Even if not the first, it is an ancient form of respiration and

knowledge of its development would reveal much information about early prokaryotes and their evolution.

Future Experiments

These results indicate that the *narG* sequence may be appropriate to use in conjunction with conserved regions of NiR and NOS genes to develop probes (or degenerate PCR primers) designed to identify denitrifiers and nitrate respirers (2,5,38,72,78,86,89,90). Some molecular data on NR exist which could be used to design degenerate PCR primers without obtaining more sequence data: i.e. the NAP of *A. eutrophus* (73), *narZYWV* of *E. coli* (10) and several other molybdenum-containing proteins of *E. coli*. Recent advances in molecular microbial ecology allow investigators to track microorganisms in environmental samples based on the sequence of a gene or protein. Eventually, these techniques will be able to accurately assess the composition and activity of microbial communities. The dispersal of the *narG* gene in a wide variety of bacteria suggests that other cloned *E. coli* NR genes, *narH* and *narI*, may also be highly conserved and therefore good candidates for the development of other genetic probes to be used in evolutionary and environmental analyses.

The *narG* probes may also be an easy way to detect NR-containing bacteria with dissimilatory genes that are poorly to expressed. Often an organism is thought not to possess a pathway because the particular phenotype can not be expressed in laboratory culture. The gene probe method allows an isolate to be screened for the presence of a certain gene, in this case a gene of its NR. A positive signal does not mean that the organism is a nitrate-respirer, it merely indicates that the microbe contains a dissimilatory NR-like gene found in many nitrate-respiring and denitrifying bacteria.

Finally, *narG* could be used to further study the molecular biology of NR in organisms which hybridized to the probes. Cloning experiments are often designed such

that specific sequences are used as probes to identify a given gene. When little is known about the molecular biology of a system, the protein of interest is purified and amino acid sequence from the N- and C- terminal ends is used to construct oligonucleotides. These oligonucleotides are then used to screen a library and locate a clone that contains the homologous DNA sequence. Because probes derived from *narG* cross-reacted with the NR of many diverse organisms, they could also be used to clone novel NRs from a variety of species.

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Table 1. Organisms, culture medium, growth temperatures and sources of bacteria.

(*) Growth medium as detailed by the American Type Culture Collection

Organism	Culture Medium	Growth Temperatures °C	Source
α-Purple Bacteria			
Rhizobium meliloti	YEM-Mannitol, MSG*	25	ATCC 9930
Azotobacter vinelandii	Burks-15mM NH_4Cl*	25	ATCC 13705
Thiosphaera pantotropha	T. pant media-NaOAc*	37	ATCC 3551
Paracoccus denitrificans	T-soy-Glycerol	30	ATCC 19367
Rhodobacter capsulatus	Van Niel's-Succinate*	30	ATCC 23782
Hyphomicrobium vulgare	Hyph.media-methanol (39)	25	ATCC
Hyphomicrobium zavarzinii	Hyph.media-methanol (39)	25	ATCC
β-Purple Bacteria			
Alcaligenes eutrophus	T-soy-Glycerol	25	ATCC 17699
γ-Purple Bacteria			
Pseudomonas fluorescens Biotype A	T-soy-Glycerol	25	ATCC 13525
Pseudomonas fluorescens Biotype C	T-soy-Glycerol	25	ATCC 17400
Pseudomonas aeruginosa PAO1	T-soy-Glycerol	37	Lab. Stock
Pseudomonas stutzeri Marine strain	T-soy-Glycerol-1% Sea Salt	37	Presque-Isle
Pseudomonas stutzeri Clinical isolate	T-soy-Glycerol	37	J.F. Stolz
Xanthomonas maltophilia	T-soy-Glycerol	30	ATCC 17666
Proteus mirabilis	T-soy-Glycerol	37	ATCC 29906
Escherichia coli RK4353	T-soy-Glycerol	37	Lab. Stock
M91-3	T-soy-Glycerol	25	J.B. Robinson
Low G+C-Gram Positive Bacteria			
Bacillus licheniformis	T-soy-Glycerol	37	ATCC 14580
Staphylococcus aureus	T-soy-Glucose	37	ATCC 33528

Table 2. Summary of the Southern blot survey with probes derived from the *narG* of *Escherichia coli*.

Key: (+) Hybridization under stringent conditions.

(±) Hybridization under low stringency.

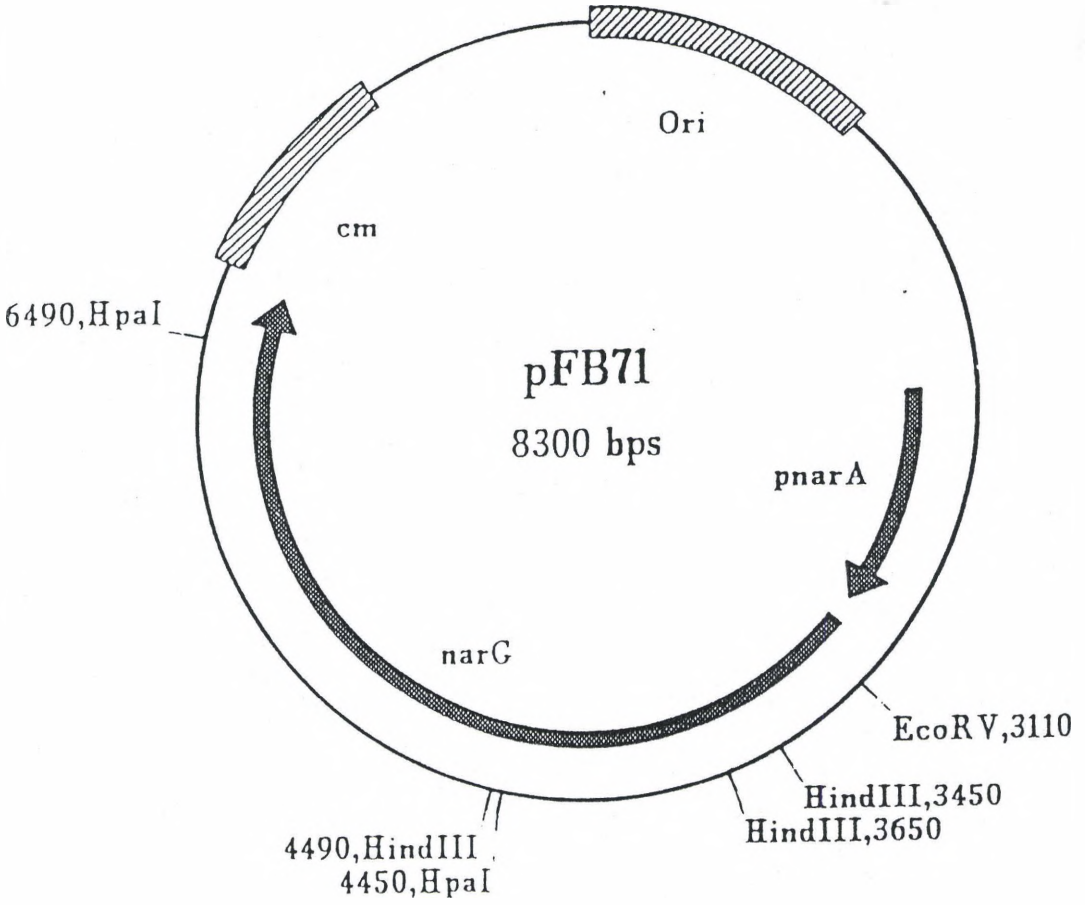
(-) No hybridization.

Organism	Probe A (0.84 kb HindIII) Hybridization	Mol. wt.	Probe B (2.1 kb HpaI) Hybridization	Mol. wt.
Rhizobium meliloti	(-)		(-)	
Azotobacter vinelandii	(-)		(-)	
Thiosphaera pantotropha	(+) (±)	9 kb 20 kb	(+) (±)	9 kb 20 kb
Paracoccus denitrificans	(-)		(-)	
Rhodobacter capsulatus	(-)		(-)	
Hyphomicrobium vulgare	(+)	22,18 kb	(+)	22,18 kb
Hyphomicrobium zavarzinii	(+)	22,18 kb	(+)	22,18 kb
Alcaligenes eutrophus	(+)	23,16 kb	(+)	23,16 kb
Pseudomonas fluorescens Biotype A	(-)		(-)	
Pseudomonas fluorescens Biotype C	(+)	16 kb	(+)	16 kb
Pseudomonas aeruginosa PAO1	(+)	23 kb	(+)	23 kb
Pseudomonas stutzeri Marine strain	(-)		(-)	
Pseudomonas stutzeri Clinical isolate	(+)	22 kb	(+)	22 kb
Xanthomonas maltophilia	(+)	20,15 kb	(+) (±)	20,15 kb 4 kb
Proteus mirabilis	(-)		(-)	
Escherichia coli (not shown) RK4353	(+)	18 kb	(+)	18 kb
M91-3	(-)		(-)	
Bacillus licheniformis	(+)	18 kb	(±)	18 kb
Staphylococcus aureus	(-)		(-)	

Table 3. Rates of nitrate uptake measured in nitrate-respiring bacteria. Carbon sources used in anaerobic growth of organisms selected to be non-fermentable when possible. Uptake was initiated by addition of the electron donor formate (500 μM).

Organism	Rate of NO_3^- Uptake nmole min^{-1} mg^{-1} protein +/- SEM	Carbon Source
Rhizobium meliloti	186 ± 15	Mannitol
Thiosphaera pantotropha	147 ± 44	Acetate
Paracoccus denitrificans	134 ± 21	Glycerol
Rhodobacter capsulatus	243 ± 37	Succinate
Hyphomicrobium vulgare	146 ± 24	Methanol
Hyphomicrobium zavarzinii	203 ± 11	Methanol
Alcaligenes eutrophus	244 ± 46	Glycerol
Pseudomonas fluorescens Biotype C	151 ± 29	Glycerol
Pseudomonas aeruginosa	169 ± 59	Glycerol
Pseudomonas stutzeri	147 ± 36	Glycerol
Pseudomonas stutzeri Presque-Isle	192 ± 28	Glycerol
Xanthomonas maltophilia J.F. Stolz	135 ± 22	Glycerol
Proteus mirabilis	115 ± 19	Glycerol
Escherichia coli	135 ± 5	Glycerol
Bacillus licheniformis	158 ± 18	Glycerol
Staphylococcus aureus	158 ± 57	Glucose

Figure 1. Restriction map of pFB71 (*narG*) and internal position of probe A (0.84 kb HindIII) and probe B (2.1 kb HpaI) relative to *narG*. Kindly provided by J.F. Stolz.


Ori
cm
6490,HpaI
pFB71
8300 bps
pnarA
narG
EcoRV,3110
HindIII,3450
HindIII,3650
4490,HindIII
4450,HpaI

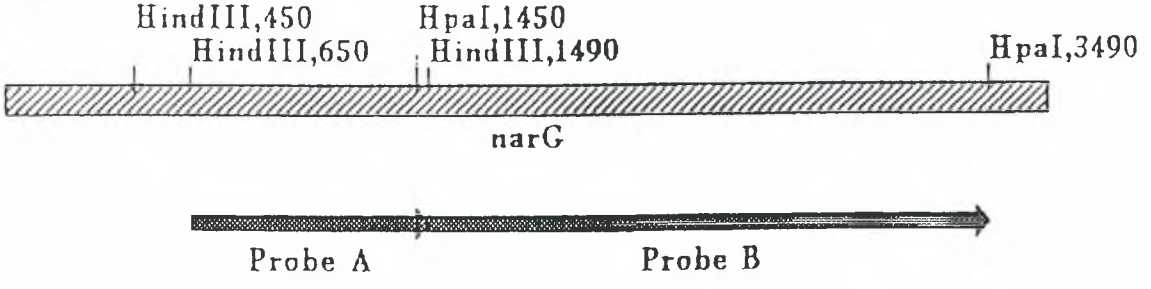

HindIII,450
HindIII,650
HpaI,1450
HindIII,1490
HpaI,3490
narG
Probe A
Probe B

Figure 2. Schematic diagram of phylogenetic relationships among surveyed nitrate-reducing bacteria based on 16S rRNA phylogeny as presented by Woese (92). Evolutionary distances are not drawn to scale.

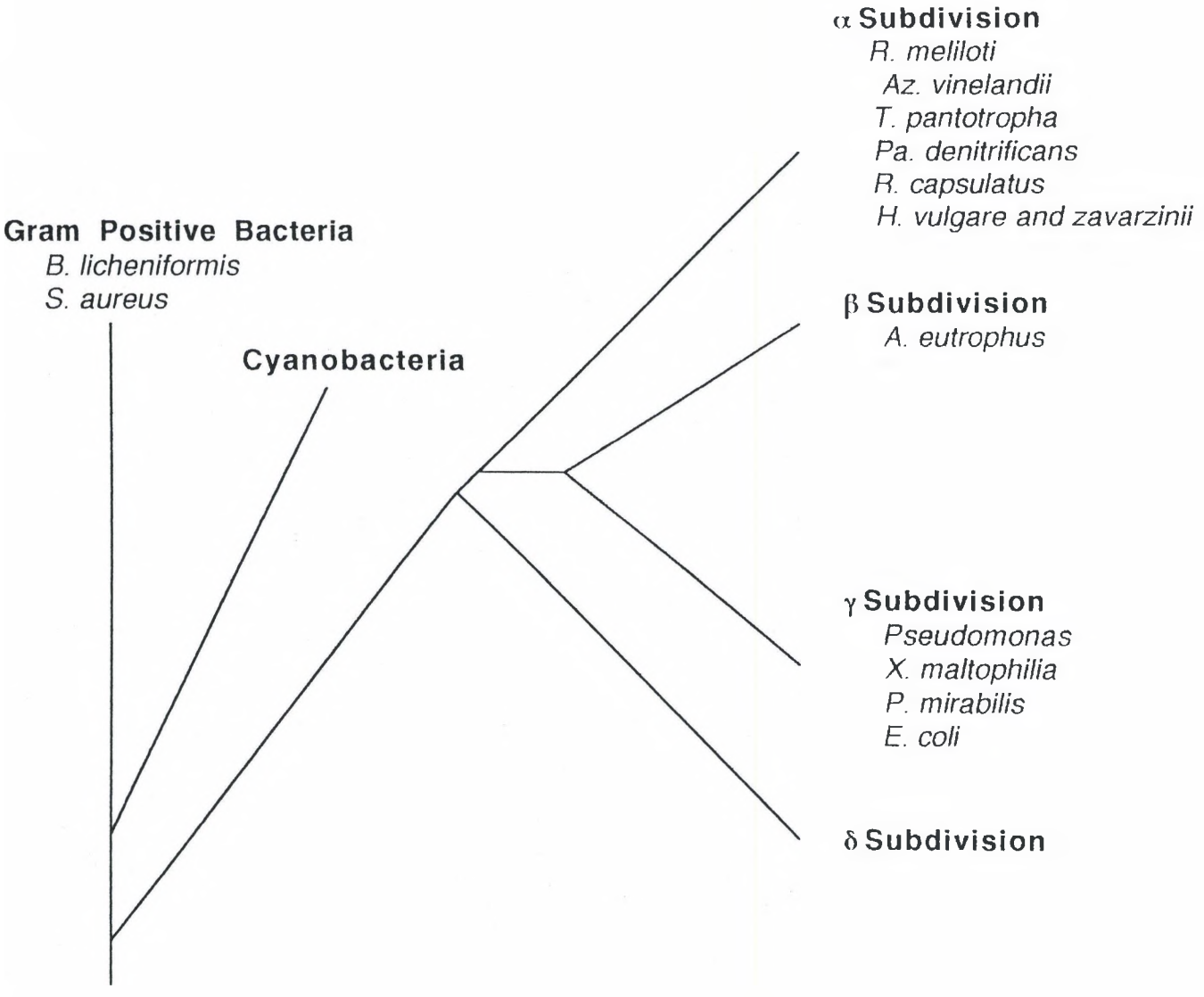

α Subdivision
R. meliloti
Az. vinelandii
T. pantotropha
Pa. denitrificans
R. capsulatus
H. vulgare and zavarzinii
Gram Positive Bacteria
B. licheniformis
S. aureus
β Subdivision
A. eutrophus
Cyanobacteria
γ Subdivision
Pseudomonas
X. maltophilia
P. mirabilis
E. coli
δ Subdivision

Figure 3 A and B. Southern blots of BamHI restricted bacterial genomic DNA with the *narG* probes.

3 A - Probe A (0.84 kb HindIII) 3 B - Probe B (2.1 kb HpaI)

1. pFB71 cut with HpaI 2. *Pseudomonas aeruginosa* 3. *P. stutzeri* (J.F. Stolz) 4. *P. stutzeri* (Presque-Isle) 5. *P. fluorescens* (Biotype A) 6. *P. fluorescens* (Biotype C) 7. *Xanthomonas maltophilia* 8. *Azotobacter vinelandii* 9. *Rhizobium meliloti* 10. *Alcaligenes eutrophus* 11. *Paracoccus denitrificans* 12. *Proteus mirabilis* 13. *Staphylococcus aureus* 14. *Bacillus licheniformis* 15. *Rhodobacter capsulatus* 16. *Thiosphaera pantotropha* 17. *Hyphomicrobium vulgare* 18. *H. zavarzinii* 19. M91-3