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Nitrogen metabolism in iron-respiring bacteria

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ABSTRACT

Nitrogen metabolism was investigated in three species of iron respiring bacteria *Geobacter metallireducens*, *Geobacter sulfurreducens*, and *Sulfurospirillum barnesii*. *G. metallireducens* and *G. sulfurreducens* were shown to grow in medium containing N_2, NO_3^-, or NH_4 but nitrite inhibited growth. BLAST analysis of the draft genomic sequence of *G. metallireducens* revealed only Nar, Nrf, and Nif homologues but no assimilatory genes (i.e., Nas). Two *nar* operons were identified, *narCGHJIK*$_1$*K*$_2$, and *narCGHI*. A putative CAP/CRP recognition binding site was found upstream and two molybdenum biosynthetic genes were found downstream of the large operon. Both operons contain a novel cytochrome *c* (NarC) that has only been described in *Thermus thermophilus* strain HB8. An attempt was made to purify the periplasmic nitrate reductase (Nap) from *S. barnesii* without success. However, a method using lysozyme to solubilize Nap and a rapid activity assay for nitrate reductase using 96 well plates were developed.

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Introduction

Nitrogen cycle and the importance of nitrate and nitrite

Nitrogen is the most abundant gas in the atmosphere (approximately 80 %). Nitrogen can also exist in different oxidation states in the environment (Figure 1). Interconversion between these oxidation states is carried out by a variety of microorganisms and is known as the Nitrogen cycle (Figure 1). Nitrogen fixation converts gaseous dinitrogen to ammonia for assimilation. Microbial oxidation of ammonia provides energy for microbes and produces nitrite and nitrate. Denitrification (a type of dissimilatory nitrate reduction) is a bacterial respiratory process that reduces nitrate to nitrite, nitric oxide, then nitrous oxide, and finally dinitrogen gas under oxygen limiting conditions (75). Dissimilatory reduction of nitrate to nitrite then to ammonia occurs under anaerobic conditions, in which nitrate acts as the terminal electron acceptor. Assimilatory reduction of nitrate to ammonia on the other hand, can occur aerobically as well as anaerobically, and is done by plants, fungi, and algae as well as bacteria (75). Anaerobic ammonia oxidation (ANAMOX) is a relatively newly discovered process in which nitrite and ammonia combine to produce dinitrogen gas (10).

The balance of nitrogen in different oxidation states in the environment is a major concern for both humans and nature. For instance, excess agricultural nitrogen containing fertilizer may cause contamination of rivers and lakes by leaching from soil. Polluted freshwater systems and domestic wastewater are known to contain high concentrations of nitrite (80). Nitrate is a more mobile form of nitrogen compared to ammonia (or ammonium). It is because nitrate ions are not adsorbed by the negatively charged colloids

that dominate most soil (35). Thus the reduction of nitrate plays a central role in preventing leaching and polluting the groundwater (52).

Nitrate reduction also takes place in the human large intestine (49). Nitrate can have an inhibitory effect on methane production in the colon and may also have significant influence on human colonic fermentation (3). High dietary intake of nitrate may contribute to infantile methemoglobinemia and gastric/intestinal cancer through the production of carcinogenic N-nitrosoamine compounds (22). Food borne pathogens such as *Escherichia coli, Salmonella sp.* and *Proteus sp.* can utilize nitrate under anaerobic conditions. Thus even if meat is packaged anaerobically there still is a possibility that such pathogens can survive and enter the human body. Other pathogens such as *Neisseria gonococcus* (the cause of the sexualy transmitted disease gonorrhea) and *Neisseria meningitidis* (the cause of meningitis) are also able to survive anaerobic sites in human body by reducing nitrite to nitric oxide (54). Therefore studying how nitrate and nitrite are utilized by microbes is not only environmental significant but also has benefits for human health.

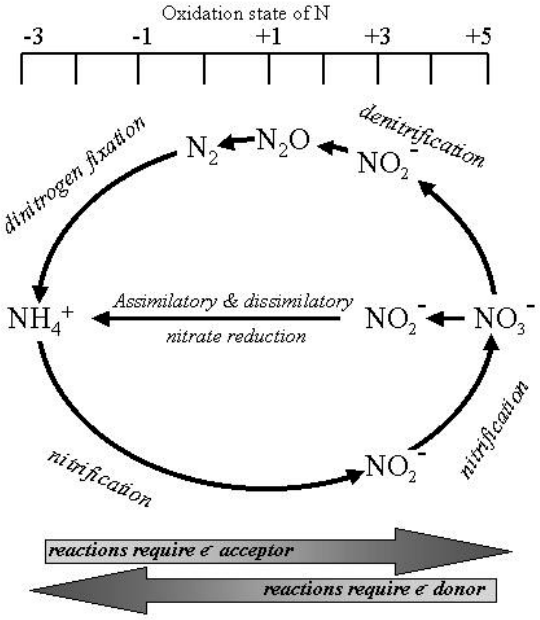


Figure 1: Nitrogen cycle (67)

Nitrate and nitrite reductase

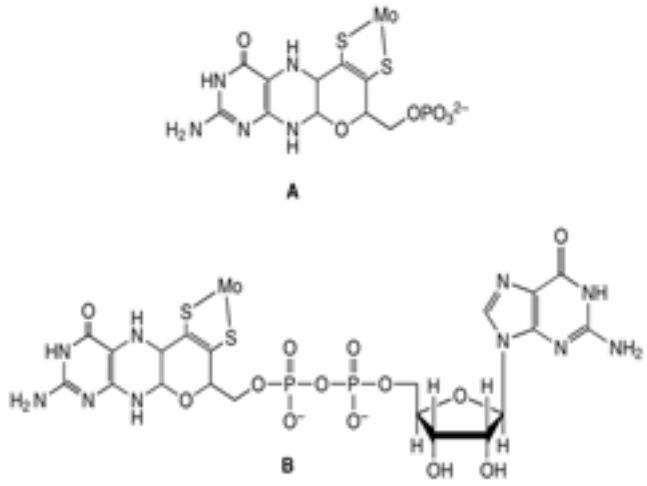
Denitrification, dissimilatory reduction of nitrate, and assimilatory reduction of nitrate are three processes of nitrate reduction that are known to exist in organisms (Fig 1). Four different classes of nitrate reductase have been found in plants, algae, fungi, archaea and bacteria (10, 62, 76). Cytoplasmic assimilatory nitrate reductases are known to exist in eukaryotes and prokaryotes. Membrane bound and periplasmic nitrate reductases are solely found in prokaryotes (10). All four types of nitrate reductase contain a molybdopterin cofactor that has a molybdenum atom bound to it (Figure 2). In bacterial nitrate reductase, the molybdopterin is further modified by the addition of a nucleotide (guanosine) and is termed molybdopterin guanine dinucleotide (MGD) (Figure 2).
A molybdenum free nitrate reductase has been isolated from *Pseudomonas isachenkovii*, a vanadate reducing bacterium (4). Molybdopterin containing nitrate reductases can be classified based on X-ray structural data, spectroscopic data, biochemical data, and sequence alignment (64). Eukaryotic nitrate reductases belong to the sulfite oxidase family whereas the other three prokaryotic dissimilatory reductases belong to Dimethyl Sulfoxide (DMSO) reductase family (64). Classification of these four nitrate reductases is summarized in Table 1.

Table 1: Summary of nitrate reductase

	Eukaryotic nitrate reductase	Prokaryotic nitrate reductase		
Organisms	Plants, algae, fungi	Bacteria, Archaea		
Molybdenum	Molybdopterin	Molybdopterin guanine dinucleotide		
Molybdoenzyme family	Sulfite oxidase	Dimethyl Sulfoxide reductase		
Location of the nitrate reductase	Cytoplasmic	Cytoplasmic	Membrane bound	Periplasmic
Assimilatory/Dissimilatory	Assimilatory	Assimilatory	Dissimilatory	Dissimilatory
Abbreviation	INR, NiR, Nit	Nas	Nar	Nap

Figure 2: Molybdenum cofactor

a) Molybdenum cofactor b) Molybdenum guanine dinucleotide (MGD) (67)



Molybdenum cofactor containing enzymes that are involved in nitrogen metabolism

Molybdenum cofactor containing enzymes are involved in nitrogen, sulfur and carbon cycles (34). These enzymes can be divided into four families, namely DMSO reductase family, Xanthine oxidase family, Sulfite oxidase family, and Aldehyde ferredoxin oxidoreductase family (Table 2) (31, 34).

Nitrate reductases and formate dehydrogenase are molybdoenzymes that are relevant to the nitrogen metabolism (xanthine oxidase is involved in uric acid production that is a part of purine and pyrimidine nucleotide degradation and will not be discussed here). Although nitrogenase is not classified as a molybdoenzyme, since it contains molybdenum and also is involved in nitrogen metabolism, it will be discussed here. Eukaryotic assimilatory nitrate reductase belongs to the sulfite oxidase family, whereas the other three prokaryotic dissmilatory nitrate reductases belong to DMSO reductase family.

Nitrogenase consists of two proteins. One is a homodimer Fe protein that contains an iron sulfur cluster (FeS) and is regarded as a transmitter of electrons from a donor protein, such as flavodoxin, to the second tetramer protein that contains molybdenum and iron (MoFe) (27). The structural genes of nitrogenase are *nifHDK*, which encode the homodimer Fe protein (*nifH*) and the tetramer MoFe (*nifDK*). Regulation of nitrogenase has been extensively studied in both phototrophic bacteria such as *Rhodobacter capsulatus* and enteric bacteria such as *Klebsiella sp.* (18). The regulatory cascade of nitrogenase is somewhat confusing due to the various nomenclatures used for the same regulatory system in different microorganisms. The regulatory cascade can be divided into three different levels, in the order of the Gln system (sensory transduction

components), Ntr (or NifR) system (general nitrogen regulation), and nifA-L (or nifAI, nifAII) system (18, 21). Nitrogenases with vanadium or iron replacing molybdenum are also known to exist (23, 27). Nitrogenase is known to be sensitive to oxygen and reduction of acetylene is a standard assay to test for nitrogenase activity. Thus, discovery of a novel nitrogenase that is oxygen tolerant and also utilizes even more reactive superoxide as its source of electron was remarkable (27, 61). Also interestingly, this nitrogenase does not reduce acetylene.

Formate dehydrogenase is known to be associated with nitrate reductase in *E. coli*. When *E. coli* is grown on media containing formate as electron donor and nitrate as electron acceptor, electrons are transported from formate to nitrate via formate dehydrogenase complex and nitrate reductase complex (33). Three different formate dehydrogenases are expressed in *E. coli*. Nitrate induced formate dehydrogenase (FdhN) is coupled to NarG that is a membrane bound nitrate reductase only expressed under anaerobic condition. FdhO is an isoenzyme of FdhN and is coupled to NarZ that is also a membrane bound nitrate reductase. Both FdhO and NarZ are expressed constitutively regardless of the presence of oxygen (1). FdhH is a membrane associated formate dehydrogenase involved in fermentation and is strongly repressed by nitrate (5, 18). Thus FdhH is not involved in nitrate reduction.

Table 2: Summary of molybdoenzymes (31, 34)

Enzyme family	DMSO reductase family	Xanthine oxidase family	Sulfite oxidase family	Aldehyde ferredoxin oxidoreducase family
Enzyme	DMSO reductase	Xanthine oxidase/dehydrogenase	Sulfite oxidase	Aldehyde ferredoxin oxidoreducase
	Trimethylamine-N-oxide reductase	Aldehyde oxidase	Nitrate reductase (Eukaryotic assimilatory)	Formaldehyde ferredoxin oxidoreductase
	Biotin sulfoxide reducase	Aldehyde oxidoreductase		Glyceraldehyde-3-phosphate ferredoxin oxidoreductase
	Nitrate reductase (Assimilatory) Nas	CO dehydrogenase		Carboxylic acid reductase
	Nitrate reductase (dissmililatory) Nar	Quinoline-2-oxidoreductase		Aldehyde hydrogenase
	Nitrate reductase (dissmililatory) Nap	Isoquinoline-1-oxidoreductase		Hydroxycarboxylate viologen oxidoreductase
	Formate dehydrogenase	Quinoline-4-carboxylate-2-oxidoreductase		
	Polysulfide reductase	Quinoline-4-oxidoreductase		
	Arsenite oxidase	Quinaldic acid 4-oxidoreductase		
	Formylmethanofuran dehydrogenase	Nicotinic acid hydrogenase/dehydrogenase		
		6-hydroxynictinate hydroxylase		
		nicotine dehydrogenase		
		Picolinate hydroxylase		
		2(R)-hydroxycarboxylate oxidoreductase		

Cytoplasmic assimilatory nitrate reductase

Eukaryotic nitrate reductase is either a dimer (plants) or tetramer (alga, *Chlorella*) (67). Each monomer contains the molybdopterin cofactor, cytochrome *b*, and FAD (67). It is thought that electrons are transferred in the order of NAD(P)H to FAD, cytochrome *b* then to molybdopterin cofactor (the active site) (67).

Prokaryotic assimilatory nitrate reductases (Nas) can be divided into two classes (46). One is ferredoxin or flavodoxin dependent such as the one that is found in cyanobacteria and the other is NADH dependent found in *Klebsiella* and *Rhodobacter (46).* Another group of scientists, however, divided prokaryotic assimilatory nitrate reductases into three classes with *Klebsiella pnuemoniae* (enterobacteria), *Bacillus*

subtilis (Gram positive bacteria) and *Synechococcus* (Cyanobacteria) as representative for each class (10, 62). Amino acid sequence comparison of the enzymes from these three representative bacteria indicates that the enzymes contain MGD (Figure 2) and an N-terminal iron sulfur cluster-binding site (10). Structurally, prokaryotic Nas can exist as monomeric or dimeric form (46, 67). Cyanobacteria and *Azotobacter* (nitrogen fixing bacteria) have a monomeric subunit whereas *Klebsiella pnuemoniae* and *Rhodobacter capsulatus* have heterodimers. The three representative Nas from *K. pnuemoniae, B. subtilis, Synechococcus* have been reviewed recently by Richardson et al. (62). The molybdenum binding subunit in each microorganism is termed NasA, NasC, and NarB respectively. Siroheme assimilatory nitrite reductase is also found in these organisms and the gene is located within the Nas cluster. They are termed *nasB* in *K. pnuemoniae*, *nasD* in *B. subtilis*, and *nirA* in *Synechococcus*. It is important to note that siroheme nitrite reductase can also be associated with dissimilatory nitrate reduction. In *E. coli*, a siroheme nitrite reductase termed NirB is associated with membrane bound dissimilatory nitrate reductase and is not involved in nitrogen assimilation (18).

Regulation of *nas* shares the same upstream regulatory systems that are observed in nitrogenase regulation. The general nitrogen regulatory Ntr system and a positive regulator (NasR) that acts by a transcription attenuation system are the two regulatory systems found in *Klebsiella* (36, 46). In *R. capsulatus*, *nas* is induced by nitrate and repressed at a low C/N ratio (i.e. 2-oxoglutarate/glutamine ratio), which is the same upstream regulatory system that regulates nitrogenase in this microorganism (48). Nitrogen regulation in cyanobacteria is regulated by the NtcA protein, a member of the Crp/Fnr family of transcription activators (36, 46). A second regulatory gene *ntcB* is

located immediate downstream of *ntcA* in *Synechococcus*, and its gene product is a LysR family transcription factor required to activate *nirA* operon in response to nitrite (2, 46). The Ntr system and a two component regulatory system NasS/T are responsible for Nas expression in *Azotobacter vinelandii* (46). In gram positive bacteria, *B. subtilis* has no analogue of Ntr system but rather has a positive regulator called TnrA, which is thought to bind DNA and activate transcription of nitrogen assimilation operons (36, 46). Recently, Nas has been purified from *Haloferax mediterranei*, a member of Archaea (45). This was the first Nas to be isolated from Archaea and is a heterodimer comprised of 105 and 50 KDa subunits.

As an additional note, all nitrate reductases are capable of using methyl viologen as an artificial electron donor. However, the ability to use bromophenol blue as an artificial electron donor is a characteristic of both eukaryotic and prokaryotic assimilatory enzymes (12, 30, 46).

Membrane bound cytoplasmic dissimilatory nitrate reductase

Membrane bound nitrate reductase (Nar) has a wide distribution and has been characterized from bacteria such as *E. coli* (52), *Bradyrhizobium japonicum* (28), and many others. Three subunits α (approximately 104-150 KDa), β (43-63 KDa), and γ (19-28 KDa) constitute the enzyme (Fig 3). These three subunits are also known as NarG, NarH, and NarI respectively. The α subunit contains molybdopterin and a high spin iron sulfur cluster. The β subunit contains four iron sulfur clusters and the γ subunit is a diheme cytochrome *b*. Membrane bound nitrate reductase is primarily involved in anaerobic respiration. This process is called dissimilatory reduction of nitrate. According

to the current model, the γ subunit accepts electrons from the quinol pool and passes them to the β subunit via its *b* hemes. Two protons are released to the periplasm, which generate transmembrane electrochemical potential. This proton gradient is utilized to generate ATP. After the electrons are passed from the γ subunit to the β subunit, they are then transferred from the β subunit's iron clusters to the molybdopterin in the α subunit. Based on the amino acid sequence of the α subunit, its N-terminal segment contains a His-X3-Cys-X3-Cys-Xn-Cys motif that bind a 4Fe-4S cluster (63). Some microorganisms have multiple membrane bound cytoplasmic nitrate reductase. Two isoenzymes are found in *E. coli*. One is only expressed under anaerobic growth conditions with nitrate (NarG) and the other is expressed constitutively (NarZ). In other words, NarZ is not under the influence of either oxygen or nitrate. Structurally, NarGHI and NarZYV are highly similar with 76 % identity for the catalytic subunit NarG and NarZ, 75 % identity for the β subunit (NarH and NarY), and 87 % identity for the NarI and NarY (11, 46). Genes encoding NarG comprises *narGHJI*, in which *narGHI* encode the enzyme and *narJ* may encode a chaperon that is involved in the assembly or stability of NarGH complex prior to its membrane attachment (11, 46). Upstream of the *narGHJI* is the *narK* gene encoding a putative nitrogen oxyanion transporter and further upstream are genes encoding the two component nitrate response sensor regulator system NarXL, in which NarX is the nitrate sensor and NarL is the DNA-binding regulator (46, 57, 62). NarZ on the other hand is encoded by the operon *narZYWV*, in which *narZYV* encode the enzyme and *narW* is the chaperon homologue to *narJ*. Upstream of *narZYWV* is *narU* gene that is the homologue to *narK*. No response sensory system genes are found further upstream of *narU*. However, a second set of the two component nitrate response sensor

regulator system NarQP does exist and is located at a different location on the *E. coli* chromosome (46).

Currently, the function of NarK and NarU as nitrite exporters to prevent accumulation of nitrite that is generated by nitrate reduction in the cell is widely accepted. They are also thought to transport nitrate into the cell. However, there has been no evidence that NarK and NarU are involved in nitrate transport into the cell until recently. A paper published by Clegg et al (16) showed that both NarK and NarU may be involved in nitrate uptake. Another oxyanion transporter, NirC, was also investigated in this paper. NirC is encoded by the *nir* operon (see nitrite section) and was determined to only transport nitrite.

Functions of the two component nitrate response sensor regulator system, NarXL and NarQP are summarized in table 3. It is important to note that the NarXL system is not found in all microorganisms that can reduce nitrate. For instance, denitrifiers such as *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, and *Paracoccus denitrificans* seem to lack this NarXL system (51). Another well studied regulator involved in expression of nitrate metabolism genes is Fnr. It is a transcriptional regulator involved in aerobic-anaerobic regulation by controlling the expression of anaerobic metabolism genes (7). Based on sequence analysis, Fnr is very similar to the catabolite activator protein (CAP) with one notable difference. Fnr has a ferredoxin-like four cysteine cluster, Cys-X3-Cys-X2-Cys-X5-Cys, at the N-terminal (7). Activation and deactivation of Fnr is due to the presence of an iron cluster (4Fe-4S). Under anaerobic conditions, Fnr is a dimer with one 4Fe-4S in each monomer. Upon exposure to oxygen, the 4Fe-4S center disassembles to form a 2Fe-2S cluster within minutes and prolonged

exposure to oxygen causes further disassembly of the 2Fe-2S center to form apoproteins that lack iron. Fnr like factors have been identified in various bacteria (46). For instance, an Fnr-like regulator recently identified in the denitrifying bacteria *Paracoccus pantotrophus* (previously classified as *Thiospaera panotropha*) is termed NarR and is involved in the maximal expression of membrane bound nitrate reductase (83). NNR, found in *P. denitrificans,* which controls nitrite and nitric oxide reduction, is another example of an Fnr-like regulator (78). The gram positive bacterium *B.subtilis* also has a gene encoding a transcription factor of the Fnr family (63). Additionally, two membrane bound cytoplasmic nitrate reductases have also been purified from *B. japonicum*. Based on SDS-PAGE analysis, one consists of three polypeptides of molecular masses 116, 68, and 56 KDa, and the other consists of four polypeptides of molecular masses 116, 68, 59, and 56 KDa (28). There was no cytochrome *b* detected in either enzyme, and resembles *Rhodobacter spaeroides, P. stutzeri,* and *Haloferax denitrificans* (28). Whether these isoenzymes function similarly to *E. coli* Nar is unknown.

Table 3: Overview of the two component nitrate response sensor regulator system and functioning details of the response sensor regulator system components (46)

	Two component nitrate response sensor regulator system	
	NarX and NarQ	NarL and NarP
Location	NarX: Upstream of *narGHJI* NarQ: 53 min of the *E. coli* genetic map	NarL: Up stream of *narGHJI* NarP: 46min of the *E. coli* genetic map
Function	Membrane sensor protein	Cytoplasmic response regulator
How do they work?	NO_3^- or NO_2^- binds to a periplasmic domain, altering the confirmation of the protein to allow autophosphorylation and subsequent phosphorylation of NarL and NarP	Activated proteins bind to specific DNA target sites, the so called NarL heptamers

	NarX	**NarQ**
Phosphatase activity	Dephosphorylates NarL	Dephosphorylates NarP
Kinase activity	Both NarX and NarQ can phophorylate NarL and NarP. Depends on nitrate/nitrite availability	
In the presence of nitrate	Phosphorylates both NarL and NarP, indistinguishable between NarX and NarQ	
In the presence of nitrite	Phosphorylates NarP but primarily acts as a NarL phosphatase	Same as in the presence of nitrate (I.e. Phosphorylates both NarL and NarP)

	NarL	**NarP**
Operons that it regulates	Periplasmic nitrite reductase *nrfABCDEFG*	Periplasmic nitrite reductase *nrfABCDEFG*
	Periplasmic nitrate reductase nap	Periplasmic nitrate reductase nap
	Nitrate reductase *narGHJI*	
	Fumarate reductase *frdABCD*	
	Nitrite exporter *narK*	

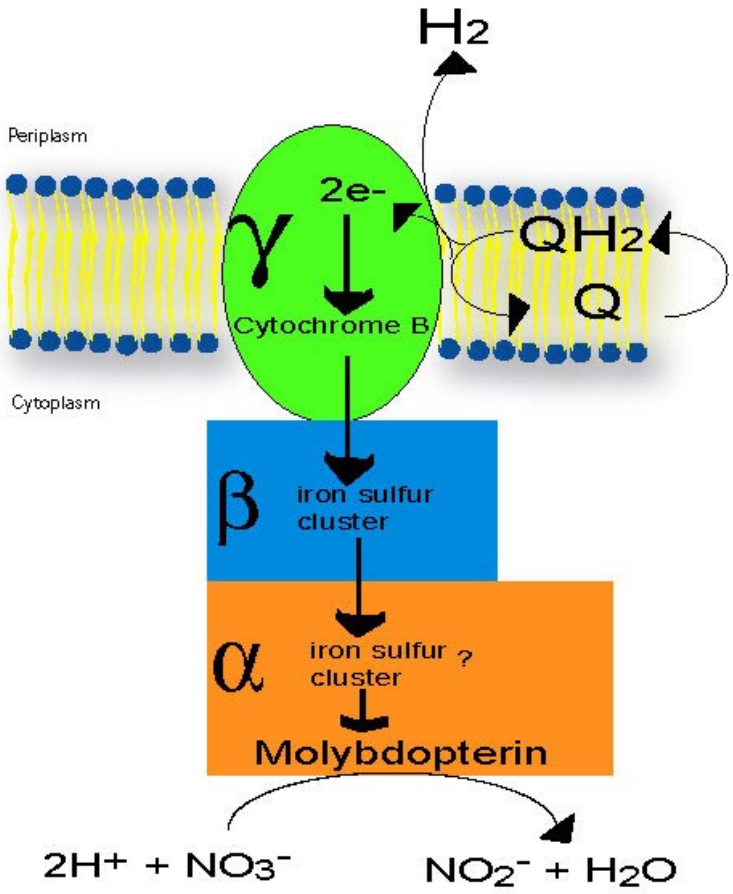


Figure 3: Schematic drawing of proposed *E. Coli* membrane bound cytoplasmic nitrate reductase model (52)

Periplasmic nitrate reductase

Periplasmic nitrate reductase (Nap) is a heterodimer consisting of a larger subunit termed NapA (63-93 KDa) that contains MGD and a smaller subunit termed NapB (13-19 KDa) which is a diheme cytochrome *c* (52). Nap is not primarily involved in nitrate assimilation or in anaerobic denitrification. However, a genetic study of *P. pantotrophus* lacking membrane bound nitrate reductase demonstrated denitrification under anaerobic

conditions (9, 10). This indicates that periplasmic nitrate reductase might be involved in anaerobic denitrification directly. *E. coli* with double mutations that inhibit generation of membrane bound nitrate reductases (*E. coli* has two membrane bound nitrate reductase enzymes and one periplasmic nitrate reductase) showed that nitrate reduction by Nap is half the total rate of parental wild type strain (55). *E. coli* mutants that express only one type of nitrate reductase grown in a nitrate scarce environment showed that Nap containing *E. coli* had a better growth rate than membrane bound nitrate reductase containing strain (54). Thus Nap is an effective enzyme when the microbe is living under low nitrate conditions. Studies on enzyme expression in *P. pantotrophus* and *P. denitrificans* has revealed that Nap is predominantly expressed under aerobic growth condition (62). Two promoters further regulate the Nap gene and are activated under growth on different carbon sources. These studies indicate that Nap maybe involved in redox balancing. The Nap gene cluster from various bacteria shows a variety of order. Based on a recent review by Philippot (51), there are at least 10 different variations in *nap* gene clusters known to exist (Figure 4). Four genes, *napABCD* are found in all the bacteria listed and are arranged in the order of *napDABC*, though in *E. coli*, *Magnetococcus MC-1*, and *Magnetospirillum magnetococcus MC-1*, *napGH* are located in between of *napA* and *B* (Figure 4) (51). Genes *napAB* encode for the heterodimer periplasmic nitrate reductase as described earlier. NapC is a membrane anchored tetraheme cytochrome *c* that is speculated to be involved in transferring electrons from the membranous quinols to NapAB. NapD is thought to be involved in the post-translational assembly of a functional molybdopolypeptide of NapA (51, 56). Five other genes, *napKEF* and *napGH*, can also be found in different combinations in the *nap*

clusters (51, 52). The *napG* gene encodes a 20 KDa soluble protein with four putative 4Fe-4S centers, and *napH* encodes a 32 KDa membrane protein that is predicted to bind two 4Fe-4S centers. It has been proposed that a putative NapGH complex could act as a redox sensor controlling the electron flow to NapA (10, 46). A more recent study by Brondijk et al showed that NapGH are essential for electron transfer from ubiquinol to NapAB (13). This study also addressed a possible role for NapF that is a soluble protein of 16 KDa with four putative 4Fe-4S clusters. The results of the study implicates NapF in an energy conserving role coupled to the oxidation of ubiquinol (13). There are three functional quinones in *E. coli*, namely ubiquinone (UQ), demethylmenaquinone (DMK), and menaquinone (MK) (13). Electrons from UQ and MK, but not DMK, can be used for nitrate reduction in *E. coli* (13, 77, 81, 82), and NapAB is capable of accepting electrons from either UQH_2 and MKH_2. NapK and NapE are small transmembrane proteins with unknown function (37, 51). Moreover, the Nap gene cluster is not always found in the genome. In bacteria such as *Ralstonia eutropha, Ralstonia. spaeroides,* and *Pseudomonas sp. G-147*, the *nap* genes are located on endogenous plasmids (51).

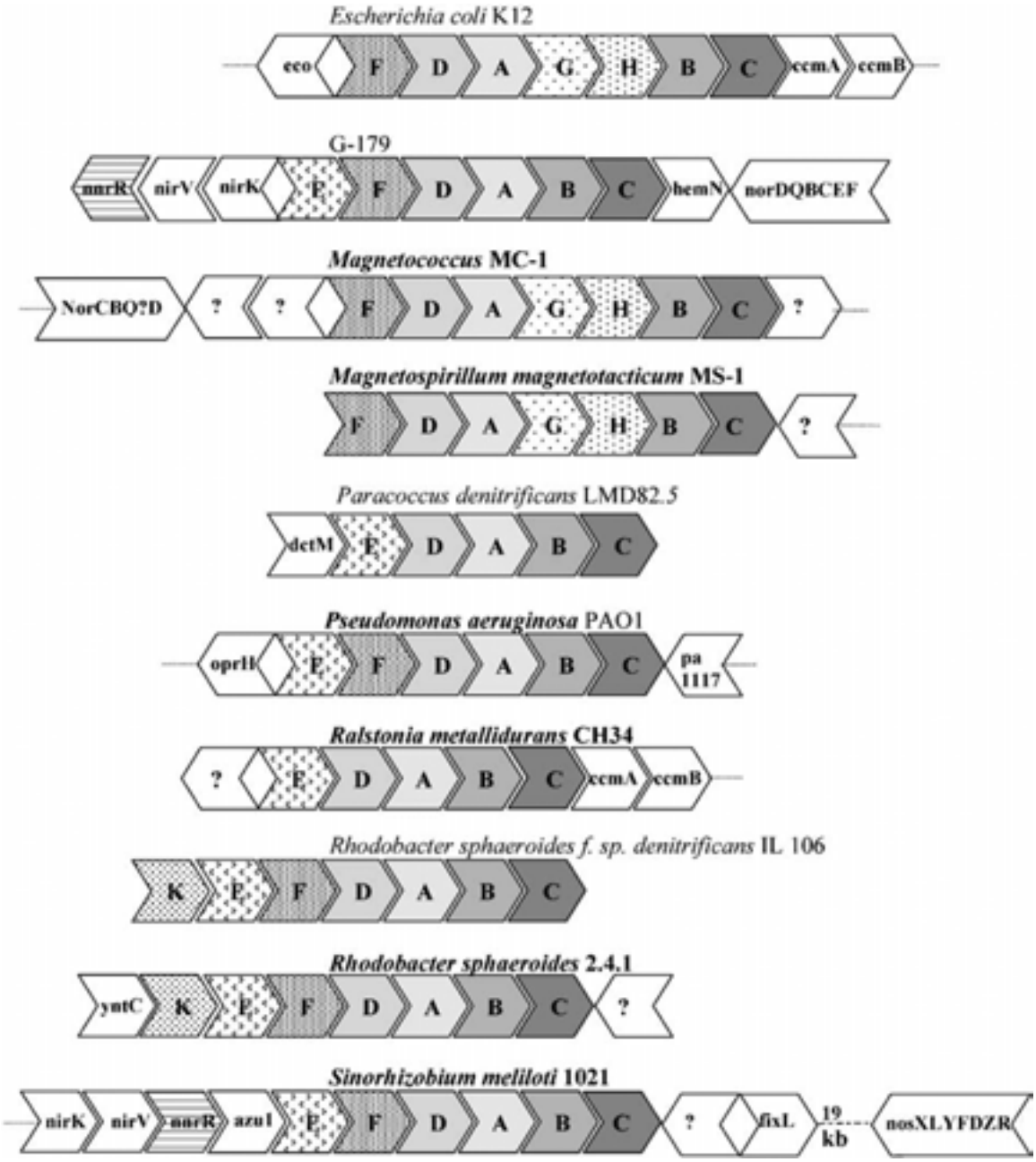


Figure 4: The nap operons (51)

Nitrite reductase

The product of nitrate reduction, nitrite is a toxic substance. In denitrifying bacteria, nitrite is rapidly converted to nitric oxide and eventually to dinitrogen gas. Whereas in assimilatory or dissimilatory nitrate reduction, nitrite is converted to ammonia.

Two nitrite reductases (NirK and NirS) are known to exist exclusively in denitrifying bacteria (67). The first one is a copper containing NirK, a trimeric enzyme with each subunit containing one type 1 Cu (blue copper) and one type 2 Cu (non-blue copper) (73). The second one (NirS) contains heme, which consists of a small cytochrome *c* containing subunit and a large cytochrome *d*1 containing subunit (29, 47, 73). Organization of *nirK* and *nirS* operons has been review recently by Philipott (51). Two other types of nitrite reductases siroheme and cytochrome *c* nitrite reductase reduce nitrite to ammonia. Siroheme containing nitrite reductases have been found to be involved in both assimilatory and dissimilatory nitrite reduction, whereas cytochrome *c* nitrite reductases has only been found to carry out dissimilatory nitrite reduction. According to Lin and Stewart (36) siroheme containing assimilatory nitrite reductase can be classified into two types; ferredoxin-dependent, found in cyanobacteria and plants; and NAD(P)H-dependent, found in other bacteria and fungi. NAD(P)H-dependent assimilatory nitrite reductase contains an iron sulfur cluster linked to siroheme and FAD. Electrons flow from NAD(P)H to FAD, the iron cluster, siroheme, and finally nitrite. On the other hand, the ferredoxin-dependent nitrite reductase contains an iron sulfur cluster linked to siroheme but lacks FAD. A siroheme containing nitrite reductase that carries out dissimilatory nitrite reduction, has been described in *E. coli*. It is a NADH-dependent

nitrite reductase termed NirB and is thought to be coupled to Nar. It is located in cytoplasm and reduces the nitrite generated by NarG to ammonia. Four genes *nirBDCcysG* are found in the nir operon (18). The structural genes *nirB* and *nirD* encode the subunits for the enzyme, *nirC* encodes a putative nitrite transport protein and the *cysG* gene product is required for the synthesis of the prosthetic group siroheme (18, 50). The most well studied cytochrome *c* containing nitrite reductase is found in *E. coli* and is formate dependent. It is located in the periplasmic space and is coupled to Nap. This cytochrome c nitrite reductase is termed NrfA, in which Nrf stands for "Nitrite reduction by formate". The X-ray crystal structure of NrfA from *Sulfurospirillum deleyianum* and *Wolinella succinogenes* revealed that the reductase is composed of two identical monomers each containing five hemes (25, 26). In these two nitrite reductases, they exhibit four conventional Cys-X1-X2-Cys-His and one unique Cys-X1-X2-Cys-Lys cytochrome *c* binding motifs. The X-ray crystal structure of NrfA from *E. coli* was made available recently and it also contains the novel Cys-X1-X2-Cys-Lys cytochrome c binding motifs (6). Seven genes, *nrfABCDEF* are found in the *nrf* operon in *E. coli* (18, 32). NrfB is a cytochrome c containing five heme groups, NrfC and NrfD are electron transfer proteins, and NrfEFG are involved in covalent attachment of heme c to a novel cysteine-lysine motif in NrfA (18, 24, 32). The *nrf* operon found in *W. succinogenes*, however, consists of *nrfHAIJ*, in which *nrfH* is a NapC type protein that transfer electrons from quinone pool to the catalytic subunit NrfA, and NrfIJ resemble proteins involved in cytochrome c biogenesis (66). According to Bamford et al, organization of *nrf* gene clusters can be divided into two groups among bacteria, which are *E. coli* like clusters

and *W. succinogenes* like clusters (6). Classification of the nitrite reductases is summarized in Table 4.

Table 4: Summary of nitrite reductase

Function	Denitrification		Assimilatory		Dissimilatory	
Organism	Denitrifying Bacteria		Bacteria/ Fungi	Cyano bacteria/ Plants	Bacteria	
Cofactor	Copper	Cytochrome *cd*1	Iron Sulfur Cluster	Iron Sulfur Cluster	Siroheme	Cytochrome *c*
Classification	Periplasmic denitrification	Periplasmic denitrification	NADP-dependent	Ferredoxin dependent	Cytoplasmic	Periplasmic
Abbreviation	NirK	NirS	Nas	Nir	NirB	NrfA

Phylogenetic diversity of iron reducing bacteria and nitrogen metabolism

The majority of microorganisms that have been identified as iron reducing bacteria are related to *Geobacter* and *Desulfuromonas* species (39). These microbes are strict anaerobes and based on 16S rRNA sequences they form a distinct clad (Figure 5). Other identified iron reducing bacteria such as *Shewanella putrefaciens*, *Shewanella alga*, and *Pseudomonas sp. stain Z-731* can utilize oxygen as an electron acceptor as well as iron (39). *Sulfurospirillum barnesii* (formally *Geospirillum barnesii*), which belongs to the Epsilon subgroup of the proteobacteria, is also capable of reducing iron coupled with oxidation of H_2 or lactate (68).

Nitrate reductases or putative nitrate reductases have been identified in some of these iron reducing bacteria. According to a recent review by Richardson et al (62), Delta

and Epsilon *Proteobacteria* only contain periplasmic nitrate reductase (Nap), whereas members of Gamma Proteobacteria can have different mixtures of assimilatory and dissimilatory nitrate reductases. This data was based on BLAST analysis of 77 complete or unfinished prokaryotic genome sequences at the NCBI Blast server at the time. However, only one bacterium each was listed as the representative for Delta and Epsilon, namely *Desulfovibrio desulfuricans* and *Campylobacter jejuni* respectively. Thus, it is possible that nitrate reductase other than Nap may be present in members of Delta and Epsilon *Proteobacteria*. *Geobacter metallireducens* strain GS-15 (referred as GS-15 beyond this point) is a member of the Delta *Proteobacteria* and is capable of reducing nitrate (41). Nitrate reductase activity has been found in its membrane fraction, which suggests that it has membrane bound nitrate reductase (58). Also a nitrate/nitrite complex has been characterized in GS-15 (44). This indicates that it may posses an alternative nitrate reducing pathway. Although, GS-15 is capable of reducing nitrate, a close relative of GS-15, *Geobacter sulfurreducens* is known not to reduce nitrate. Recently, five new bacteria were added to *Geobacter* genus, namely *Geobacter bermensis* (71), *Geobacter pelophilus* (71), *Geobacter hydrogenophilus* (17), *Geobacter chapelleim* (17), and *Geobacter grbiciae* (17). These new members of *Geobacter* are primarily characterized as iron reducing bacteria thus whether they reduce nitrate is yet to be studied. *S. barnesii* as mentioned earlier, belongs to the Epsilon *Proteobacteria*. A previous study has shown that nitrate reductase activity was found in the membrane fraction upon sonication treatment (69), however, only NapA has been found.

The presence of nitrite reductases in these iron reducing bacteria is relatively unclear compared to nitrate reductase. Cells of GS-15 harvested from media containing Fe (III) as

electron acceptor shows constitutive nitrite reductase activity but no nitrate reductase activity (58). This is also true with its close relative *G. sulfurreducens*. In the case for *S. barnesii*, a periplasmic nitrite reductase (NrfA) has been characterized from *S. deleyianum*, a close relative of *S. barnesii*. Thus it is very likely that *S. barnesii* also has NrfA (69).

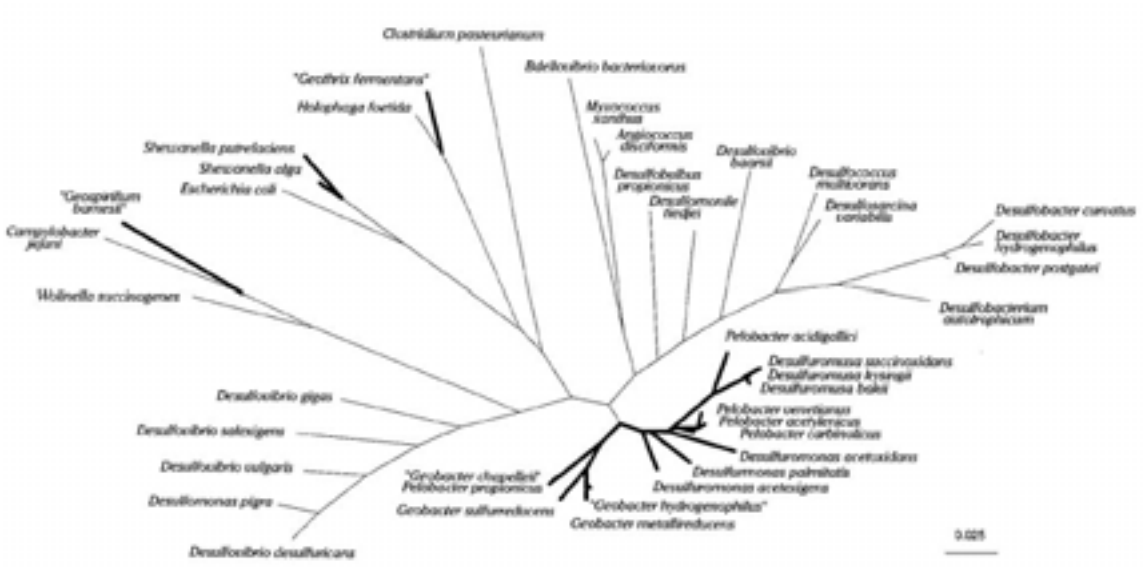


Figure 5: Phylogenetic tree inferred from 16s RNA sequences (39)

The bold lines indicate iron reducing bacteria. The phylogenetic tree was inferred from 1007 positions by using the maximum likelihood method. The sequence of *Clostridium pasteurianum* was included as an out group.

Geobacter metallireducens and *Geobacter sulfurreducens*

Ferric iron (Fe (III)) reduction may cause a ground water quality problem (40). Insoluble Fe^{3+} oxide is reduced to the more soluble ferrous iron (Fe (II)), which upon reaching the surface is then oxidized back to Fe (III) oxide. Fe (III) oxide can clog wells, causing staining problems and water discoloring if it enters the plumbing system. The

two most intensely studied Fe (III) reducers are *Geobacter* and *Shewanella,* which are in the Delta and Gamma subclass of the *Proteobacteria* respectively (40). GS-15 is capable of reducing nitrate to ammonia (42). A characterization study carried out by Lovley et al. (41) demonstrated GS-15 is capable of oxidizing short chain fatty acids, alcohols, and monoaromatic compounds with Fe (III) as the sole electron acceptor. The study also demonstrated reduction of manganese (Mn (IV)), uranium (U (VI)) and nitrate when acetate is utilized as electron donor. A dissimilatory nitrate reductase complex from GS-15 has been isolated and characterized previously (58). The presence of three indicative of *c*-type cytochrome absorption maxima at 552, 522, and 418nm were observed in the membrane fraction of nitrate grown cells. These three cytochrome *c* peaks were also observed in the membrane fraction of iron grown cells but at a low concentration suggesting that production of cytochrome *c* is enhanced when cells are grown in nitrate. A membrane bound cytochrome *c* containing enzyme complex exhibiting nitrate and nitrite reductase activity from GS-15 has also been characterized (44). The enzyme complex contains four different polypeptides with molecular weight of 62, 52, 36 and 16 KDa respectively. The 62 KDa polypeptide exhibited nitrite reductase activity. The known molybdoenzyme inhibitors do not inhibit nitrate reductase activity of the complex. This indicates an alternative nitrate reduction pathway may exist in GS-15. Additionally, the growth of GS-15 in the nitrate medium is significantly iron dependent (65). The nitrate/nitrite complex isolated from cells grown in iron enriched medium showed significant increase in cytochrome content (65).

Caccavo et al. (14) was the first to describe *G. sulfurreducens*. It was isolated from surface sediments of a hydrocarbon contaminated ditch in Norman, Okla. *G.*

sulfurreducens is capable of reducing iron, cobalt and sulfur coupled to the oxydation of acetate or hydrogen. However, *G. sulfurreducens* is unable to oxidize acetate when nitrate is used as the electron acceptor (20). A recent study demonstrated that *G. sulfurreducens* also reduces and precipitates technetium (Tc (VII)) (38). This discovery may lead to aid on controlling technetium-99, which is a fission product of uranium and is formed during nuclear reactions.

Sulfurospirillum barnesii

S. barnesii strain SES-3 (referred as SES-3 beyond this point) was isolated from a selenate contaminated slough in western Nevada (68). SES-3 is a motile, vibrioid shaped, gram negative bacterium, belonging the Epsilon subgroup of the *Proteobacteria*. SES-3 was first thought to reduce sulfate, because sulfate and selenate are closely related elements. However, the characterization of *S. barnesii* and other selenate reducing bacteria confirmed the reduction of selenate (Se(VI)) to selenium (Se(0)) is achieved by very different microbes using a different biochemical process (70). SES-3 can utilize selenate, arsenate, thiosulfate, elemental sulfur, trimethylamine oxide, Fe (III), nitrate, fumarate, aspartate and manganese as electron acceptor (68). Lactate, pyruvate or H_2 can be used as electron donors. Acetate is required a carbon source when grown with hydrogen (68, 70). SES-3 reduces nitrate to ammonium (68) indicating that both nitrate and nitrite reductases are expressed in SES-3. A NapA homologue has been cloned and sequenced from SES-3 (Stolz and Lisak unpublished). Although it is most likely that NapA is expressed in cells of SES-3 grown in media containing nitrate as electron acceptor, the existence of Nar and Nas is a possibility. A result of western blotting using

antibodies raised against *E. coli* NapA showed a positive band at around 50 KDa with SES-3 proteins prepared from whole cell (personal communication with Dr Sudesh). This may suggest the existence of Nas, because protein sequences of Nas and Nap are similar in various bacteria (67).

Objective

The recent release of the GS-15 draft genome was made available to the public at DOE Joint Genome Institute website. Although, the genomic sequence was not fully annotated and not fully completed, this enabled us to investigate the presence of nitrate and nitrite reductase homologues in the organism. Three hypotheses were proposed in this thesis. Based on previous studies carried out in our lab (44, 58), the first hypothesis is then GS-15 does not have Nar. This hypothesis was based on the finding that typical nitrate reductase inhibitor did not inhibit the nitrate reductase activity and the cytochrome contents of nitrate grown cells were higher compare to iron grown cells. Also, the growth of GS-15 in the nitrate-limited medium is significantly iron dependent (65). The second hypothesis predicted that GS-15 can assimilate nitrate and thus possibly possesses an assimilatory nitrate reductase. A close relative of GS-15, *G. sulfurreducens* is unable to grow in media containing nitrate as electron acceptor. Whether *G. sulfurreducens* possesses an assimilatory nitrate is unknown and was also investigated. The third hypothesis predicted that SES-3 has a periplasmic NapA homologue. This hypothesis is based on the fact that the NapA gene has been cloned and sequenced.

Experimental approach

To investigate the first hypothesis, sequences of all types of nitrate and nitrite reductase, and also nitrogenase (i.e. Nar, Nap, Nas, NirK, NirS, NrfA, NirB, NifD) were collected from National Center for Biotechnology Information (NCBI) website. These sequences were used to BLAST search homologues in GS-15 draft genome sequence. An attempt was made to partially purify membrane bound nitrate reductase from GS-15. To investigate the second hypothesis, growth experiments were carried out in media containing various nitrogen sources (N_2, NO_2^-, NO_3^-, and NH_4) and Fe (III) as electron acceptor. To investigate the third hypothesis, an attempt was made to purify NapA from SES-3.

Methods

A. Collection of enzyme sequences and BLAST search the draft genome of *G. metallireducens*

Sequences of enzymes involved in nitrogen metabolism (i.e. Nar, Nap, Nas, NirK, NirS, NrfA, NirB, NifD) were collected from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The list of the enzymes and organisms are summarized in Appendix I. These sequences were used to BLAST search possible genes encoding enzymes involved in nitrogen metabolism in the GS-15 database. The BLAST search was carried out on the DOE Joint Genome Institute website (http://www.jgi.doe.gov/JGI_microbial/html/geobacter/geobac_mainpage.html) that contained the genome of GS-15 (Feb 28th 2003 version). The identified open reading

frames of GS-15 were in turn BLAST searched at NCBI. Gene order was determined by obtaining the complete contigs.

B. Phylogenetic analysis of *nar* gene products

ClustalX was used for the alignment of the sequences (74). Phylogenetic trees were generated using Paup (72). Trees were generated based on neighbor joining method and maximum parsimony methods.

C. Cell culture

GS-15 was grown in fresh water acetate (FWA) with nitrate. The media were dispensed in 125 ml serum bottles and degassed (5 minutes medium, 2 minutes head space) with oxygen free CO_2 and N_2 gas (20:80 ratio). FWA-Fe (III) medium (Fe (III) 50mmol) was made separately for amending the FWA-nitrate media. After autoclaving, prior to inoculation of GS-15, FWA-Fe (III) medium was injected into FWA-Nitrate medium. The amount of FWA-Fe (III) added was calculated so that the final concentration of Fe (III) would be approximately 250 μmol in the FWA-nitrate medium.

For growth experiments, GS-15 was grown in fresh water acetate (FWA) with Fe (III) as the electron acceptors as described in Lovley and Phillips (42). FWA-Fe media were provided with ammonium salt only, ammonium salt with nitrite, nitrate only, nitrate with nitrite, or nitrite only, in different concentrations as nitrogen source (refer to Table 5 for concentration of each combination). To investigate if GS-15 contained nitrogenase, the microbe was also grown in FWA-Fe with N_2 nitrogen source. The media were dispensed in 125 ml serum bottles and degassed (5 minutes medium, 2 minutes head space) with oxygen free CO_2 and N_2 gas (20:80 ratio). The cells were transferred three

times subsequently to ensure the accuracy of the experiment. The inoculated medium bottles were incubated at 30 ^{o}C and color change started taking place on 4th or 5th day. If no growth was observed, the experiment was repeated to ensure the accuracy of the experiment. All experiments were done in triplicates.

Cells of SES-3 were grown in mineral salts medium (Appendix IV) containing lactate as the electron donor and nitrate as the electron acceptor. The medium was dispensed in 125 ml serum bottles and degassed (5 minutes medium, 2 minutes head space) with oxygen free CO_2 and N_2 gas (20:80 ratio). For large scale, a 14 Liter Microferm Fermenter (New Brunswich Scientific, Edison, NJ) was used. The medium was degassed longer (30 minutes medium, 15 minutes head space) and after autoclaving, prior to inoculation of cells, dithionite (10 ml of a 1 mg/ml solution) was injected to ensure the anoxic condition of the medium.

D. Solubilization of *G. metallireducens* NarG

Cells were harvested by centrifugation for 20 min at 14,000 x g and 4^{o}C then resuspended in Tris-HCl buffer (Appendix III). The suspended cells were lysed by sonication on ice for 3 minutes and lysates were centrifuged at 14,000 x g and 4^{o}C to remove unsonicated cells. The cell lysate was then centrifuged at 140,000 x g and 4^{o}C for 90 minutes. The cell pellets obtained were incubated in CHAPS for 24 hr and then centrifuged at 140,000 x g at 4^{o}C for 90 min to separate the CHAPS soluble fraction. The soluble fraction was treated with 10 % and 35 % ammonium sulfate to enrich for nitrate reductase.

E. Lysozyme treatment

The *S. barnesii* cell pellet was resuspended in buffer containing EDTA, 10 mM Tris PH8, protease inhibitor, 0.5 M sucrose, and treated with lysozyme (Table 5). Lysozyme treatment was done at room temperature for 1, 10, or 60 min. Spheroplasts and unbroken cells were separated from the supernatant by centrifugation at 12,500 x g at 4 ^{o}C for 15 minutes.

Table 5: Lysozyme treatment

Lysozyme concentration (mg/ml)	Incubation time (min)	EDTA (mM)	Temperature
0.5	30	1	Ambient
0.5	60	1	Ambient
1	10	1	Ambient
1	30	1	Ambient
1	60	10	Ambient
10	10	1	Ambient
25	10	1	Ambient

F. Solubilization of *S. barnesii* Nap

Cells were harvested by centrifugation for 20 min at 14,000 x g and 4 ^{o}C then resuspended in Tris-HCl buffer (appendix III). The suspended cells were lysed by sonication on ice for 15 cycles (30 sec on, 30 sec off) and lysates were centrifuged at 14,000 x g and 4 ^{o}C to remove unsonicated cells. The supernatant was then centrifuged at

140,000 x g and 4 ^{o}C for 90 min, to separate the soluble (i.e. cytoplasmic and periplasmic fraction) from the particulate fraction (i.e. membranes, ribosomes). Activity assays for nitrate resuctase activity was done on both soluble and particulate fraction. The soluble fraction contained about 50 % of the total activity and was used for the purification steps.

G. Protein concentration determination

The Lowry method (43) was used to determine protein concentration. Bovine serum albumin (BSA) was used to generate a standard curve (5, 25,50,75 and 100 mg/ml).

H. Electrophoresis

1. SDS-PAGE

Precast polyacrylamide Tris-HCl gels purchased from Bio-Rad (Hercules, CA) were used for SDS-gel electrophoresis. Approximately 20 μg of protein was suspended in sample buffer for 2 min at 90 ^{o}C and loaded to each lane. The gels were run at 150V until the dye front reached the bottom of the gel. The gels were developed for the methyl viologen zymogram assay, stained for heme, or stained with coomassie blue for protein detection.

2.Non denaturing (native) gel electrophoresis

Precast polyacrylamide Tris-HCl gels purchased from Bio-Rad (Hercules, CA) were used for native-gel electrophoresis. Native gel running buffer and sample buffer were used (Appendix III). 20 μg of protein was loaded to each lane. The gels were either

developed for the Methyl viologen zymogram assay, stained for heme, or stained with coomassie blue for protein detection.

I. Activity assay for nitrate and nitrite reductase activity

1. Zymogram

A 100 ml volume of 10 mM methyl viologen dissolved in 50 mM Tris-HCl pH8 was reduced with 100 mg sodium dithionite. After electrophoresis, the polyacrylamide gel was soaked in the methyl viologen solution for 2 minutes then replaced with 20 mM nitrate or 5 mM nitrite solution to detect nitrate or nitrite reductase activity respectively.

2. Spectroscopic photometric assay for nitrate and nitrite reductase activity

The reaction mixture contained 1.3 ml 50 mM Tris-HCl pH8, 150 μl of 50 mM electron acceptor (nitrate or nitrite), and 20 μl of protein. The reaction mixture was degassed for 2 minutes and the head space for 1 min and the bottle was sealed. The reaction was initiated by adding 30 μl of 50 mM methyl viologen and monitored over 10 minutes on a Perkin-Elmer (Pittsburgh, PA) Lamda2 dual beam spectrophotometer at 600 nm.

3. 96 wells methyl viologen assay

The methyl viologen spectrophotometric assay was modified for the 96 well format. 150 μl of 50 mM electron acceptor (nitrate or nitrite) and 20 μl of protein was added to each well. The plate was transferred to the anaerobic chamber and the reaction was initiated by adding 30 μl of 50 mM reduced methyl viologen. The color change was monitored. Both positive and negative controls were set up to ensure the accuracy of the

assay system. For the positive control, crude extract containing nitrate and nitrite reductase activity was used. The negative control contained no protein. To determine the sensitivity, different amounts of cell lysate with a known activity (in units) was used and the amount of time necessary for clearing was determined against control.

J. Heme stain

After proteins were run on the polyacrylamide gel, the gel was treated with 12.5 % trichloroacetic acid (TCA) for 30 min then rinsed with distilled water for 30 min to wash off excess TCA. While the gel was washing, 100 mg of DMB (o-Dianisidine) was mixed with 90 ml distilled water and stirred in the dark for 15 minutes. 10 ml of 0.5 M sodium citrate (pH 4.5) and 1 ml of hydrogen peroxide (H_2O_2) was added to the DMB and stirred for 2 minutes. The gel was treated with the mixed DMB solution for 1 hr in the dark. After the staining, the gels were rinsed in distilled water.

K. Dialysis

Dialysis cassettes purchased from Pierce (Rockford, IL) were used to dialyze the protein. The 35 % ammonium sulfate fraction was dialyzed in Tris-HCl buffer (Appendix III) for 24 hours to eliminate ammonium sulfate.

L.Column chromatography

1. *G. metallireducens*

The dialyzed 35 % ammonium sulfate fraction (8 mg in 2 ml) was loaded onto a DEAE-Toyopearl (Rohm and Haas, Montgomeryville, PA) ion exchange column (20 x 2 cm) with Buffer A (Appendix III) containing 1 % CHAPS (wt/vol) and eluted with a

linear NaCl gradient (0 - 0.5 M). The ion exchange column was connected to automated system Biologic LP (Bio-Rad, Hercules, CA) and 55 fractions of 1.5 ml were collected.

2. *S. barnesii*

3 ml of the soluble fraction (5 mg/ml concentration) was loaded onto a DEAE-650 M Toyopearl (Rohm and Haas, Montgomeryville, PA) ion exchange column (20 x 2 cm) with Buffer A containing 1 % Triton X-100 (wt/vol) and eluted with a linear NaCl gradient using Buffer B that contained the same ingredients as Buffer A and 0.5 M NaCl. The ion exchange column was connected to the automated system Biologic LP (Bio-Rad, Hercules, CA) and 55 fractions of 1.5 ml were collected. This step was repeated until the soluble fraction (60 ml) was all used. The pooled fractions were concentrated by centrifugal filter device (Millipore Billerica, MA) down to approximately 50 mg in 5 ml and loaded on to Sephacryl 300 HR (Sigma, St Louis, MO) size exclusion column. The column was eluted with Buffer A containing 0.25 M NaCl at a flow rate of 1 ml/min. 3 ml fractions were eluted. The fractions that contained protein (as determined by absorbance at 280 nm) were tested for nitrate and nitrite reductase activity using the 96 well plate assay and the zymogram assay.

Results

Physiological characterization of assimilatory nitrate/nitrite reductase in *G. metallireducens* and *G. sulfurreducens*

Growth of GS-15 and *G. sulfurreducens* was monitored by the color change of the media. Before inoculation, the color of the medium was dark brown (Fe (III)) and as the cells grew, the color of the medium turned to dark green then to clear. Growth results of both microbes in FWA-Fe medum amended with various nitrogen sources are summarized in Table 6. All media contained N_2 gas as it was used for degassing. Media containing NH_4 was used as the control. Nitrite concentration as low as 0.5 mM inhibited the growth of GS-15 and *G. sulfurreducens*. Both microbes were able to grow in media containing nitrate as nitrogen source and medum in which no solid nitrogen source (i.e. NH_4^+, NO_3^-, NO_2^-) was added. While these results could indicate the ability to assimilate NO_3^-, nitrogen fixation in both cases could not be eliminated. In fact, both microbes are capable of fixing nitrogen and nitrogenase activity and nitrogenase genes have been found in GS-15 and *G. sulfurreducens* respectively (8, 19). In the case for GS-15, the presence of nitrogenase was inferred by the reduction of acetylene and physiological characterization (8). The structural genes of nitrogenase were identified in *G. sulfurreducens* and were utilized as a reporter for constructing an expression vector (19). Growth rates for GS-15 and *G. sulfurreducens* have been reported (14, 42). According to the papers, the growth rate for GS-15 is 1.5×10^7 cells per day and for *G. sulfurreducens* is 1.7×10^7 cells per day at exponential growth phase.

Table 6: Physiological characterization of *G. metallireducens and G. sulfurreducens*

Nitrogen source	PCA-1 Growth	GS-15 Growth
NH_4(4mM)	Yes	Yes
NH_4(4mM) + NO_2^- (4mM)	N	N
NH_4(4mM) + NO_2^- (1mM)	N	N
NH_4(4mM) + NO_2^- (0.5mM)	N	N
NO_3^- (4mM)	Yes	Yes
NO_3^-(4mM) + NO_2^- (1mM)	N	N
NO_3^- (4mM) + NO_2^-(0.5mM)	N	N
NO_2^- (4mM)	N	N
NO_2^- (1mM)	N	N
NO_2^- (0.5mM)	N	N
None (N_2 gas)	Yes	Yes

Molybdoenzymes in *G. metallireducens*

A list of putative molybdoenzymes that were identified by using blastp from NCBI protein database is summarized in Table 7. The ten closest hits of the blastp result for each putative molybdoenzyme are listed in Appendix V. Two putative NarG homologues were identified in contig121 and 135. No other types of nitrate reductase (Nap or Nas) were identified. Four formate dehydrogenases homologues were identified. However, gene 2111 and 2112 are likely one piece rather than two because the size of gene 2111 is much too small to be a complete enzyme. The blastp results suggest gene 2111 and 2112 are fdh-N type formate dehydrogenase. Gene 498 and gene 3285 are also candidate formate dehydrogenases but which type is unclear based on the BLAST result. Gene 269 and gene 2012 are putative molybdopterin oxidoreductases.

Based on the phylogenetic tree analysis (Figure 6), gene 2111 and gene 2112 are closely related to Fdh-N and Fdh-O from *E. coli*, which is consistent with the blastp results. Gene 498 and gene 3285 are closely related to Fdh-H from *E. coli*. The two putative molybdopterin oxidoreductases, gene 269 and gene 2012 are placed closely to arsenate reductase from *Shewanella*. Gene 137 is likely a carbonmonoxide dehydrogenase, which is consistent with the BLAST result. The tree also indicates that gene 627 and gene 1636 are NarG homologues and are closely related to *Thermus thermophilus* strain HB8 (Here after strain HB8 is designated as *Thermus thermophilus* unless indicated).

Table 7: Putative molybdoenzymes in *G. metallireducens*

Putative enzyme	Contig number	Gene number
Carbonmonoxide dehydrogenase	108	137
Formate dehydrogenase (alpha)	140	2111
Formate dehydrogenase (alpha) nitrate inducible	140	2112
Formate dehydrogenase (alpha)	119	498
Formate dehydrogenase (alpha)	148	3285
Molybdopterin oxidoreductase	113	269
Molybdopterin oxidoreductase	139	2012
NarG	121	627
NarG	135	1636

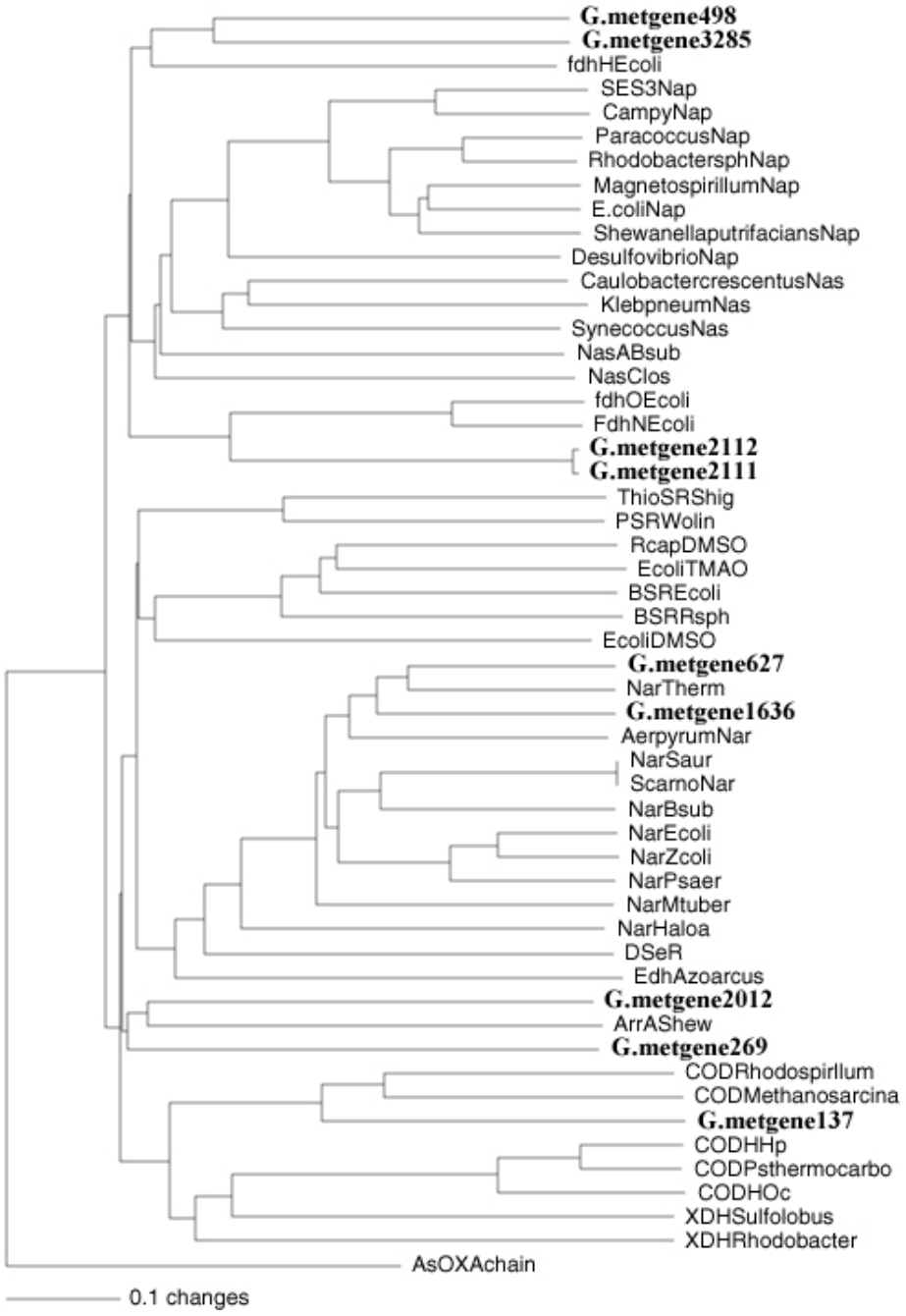


Figure 6: Phylogenetic tree of molybdoenzymes using the neighbor joining method.

Only the key abbreviations are explained. Representative from each group of the known molybdoenzymes including Nas (assimilatory), Nar (membrane bound) and Nap (periplasmic) nitrate reductases from various bacteria were collected from the NCBI database to generate the tree. Putative molybdoenzymes from GS-15 are listed as G.metgene followed by the gene number (e.g. G.metgene498). ArrA: Arsenate reductase,

COD: Carbon monoxide dehydrogenase, DMSO: DMSO reductase, Fdh: Formate dehydrogenase, XDH: Xanthine dehydrogenase,

Enzymes involved in nitrogen metabolism in *G. metallireducens*

A. Membrane bound nitrate reductase (Nar) homologues

Two putative *nar* operons were discovered in contig121 (*nar121*) and 135 (*nar135*). There are four ORFs in *nar121* and seven ORFs in *nar135* (Table 7). Both operons contain the putative membrane bound nitrate reductase structural genes *narGHI*. Secondary structure prediction suggests NarI in contig121 has 6 transmembrane domains whereas NarI in contig135 has 5 transmembrane domains (Appendix VI). A diheme cytochrome *c* was found immediate upstream of the putative *narG* gene. A novel cytochrome *c* (NarC) located upstream of *narG* has been described previously in *T. thermophilus* (84). *T. thermophilus* is the only other organism discovered with a cytochrome *c* located upstream of *narG* so far. A putative chaperon *narJ* was found only in *nar135*. Two putative *narK* homologues ($narK_1$ and $narK_2$) were found downstream of *narI* in *nar135*. This is similar to the *T. thermophilus nar* operon as there are two *narK* located downstream of NarI (59). Secondary structure predictions suggest the two putative NarKs have 12 putative transmembrane domains (Appendix VI), which supports the putative *narK* genes products are indeed NarKs. The ten highest hits of blastp result for each Nar subunit are listed in Appendix V.

B. Putative nitrite reductases

Over all two putative nitrite reductases were found in the GS-15 genome available at this stage (Table 7). A putative periplasmic dissimilatory nitrite reductase (NrfA) and a putative assimilatory/dissimilatory nitrite reductase were found in contig140 and contig146 respectively. The latter was uncertain because even though it has a conserved NirB domain (dissimilatory nitrite reductase domain), the BLAST result indicated that it could be either assimilatory or dissimilatory nitrite reductase. This BLAST result was due to its NADH binding site, because both assimilatory and dissimilatory nitrite reductase (NirB) have NADH binding site. However, it is unlikely a NirB because the expected molecular weight for the ORF is only 45.5 KDa, which is much too small compared to the size of NirB that have been characterized so far (e.g. *E. coli* NirB is approximately 93 KDa). It is also unlikely to be an assimilatory nitrite reductase because of its size (*B. subtilis* NasD is approximately 88 KDa) and also the fact that GS-15 was unable to grow in the presence of nitrite as nitrogen source. The presence of NrfA is intriguing, as it is known to couple with periplasmic nitrate reductase (NapA), but no NapA homologue was found in GS-15 genome. Molecular weight prediction of the putative NrfA is approximately 54 KDa, which is a reasonable size for a NrfA homologue. Upstream and down stream of the both putative nitrite reductases have been investigated. A putative promoter site was detected at upstream of gene 2131, which indicates that gene 2130 is unlikely to be transcribed together with gene 2131. Based on BLAST results, downstream genes 2132 and 2133 have a conserved domain containing NapH (iron-sulfur protein) and PAS respectively. PAS is a DNA binding domain and has been found in NifL, NtrB and NtrC and also various histidine kinase sensor molecules in bacteria (53). Ambiguity in

genome sequence prevented further analysis of the immediate downstream region. On the other hand, upstream and downstream of the putative NADH dependent nitrite reductase are genes encoding putative glutamate synthase (i.e. gene 2980, 2982 and 2983). BLAST results also indicate that all these genes have conserved glutamate synthase domains. This may suggest that the putative NirB (gene 2981) is actually a subunit of the putative glutamate synthase encoded by the nearby genes (i.e. NADH binding domain for the glutamate synthase).

C. Structural genes of nitrogenase

Structural gene homologues of nitrogenase were identified in Contig145 as summarized in Table 7. This corresponds with the physiological characterization, in which GS-15 was able to grow in FWA-Fe media without nitrogen source (i.e. only N_2 gas).

Table 8: Summary of all putative genes involved in nitrogen metabolism in *G. metallireducens*

Putative *nar* subunits	Contig number	Gene number	Contig number	Gene number
CytC (*narC*)	135	1635	121	626
narG	135	1636	121	627
narH	135	1637	121	628
narJ	135	1638		
narI	135	1639	121	629
narK$_1$	135	1640		
narK$_2$	135	1641		

Putative nitrite reductases	**Contig number**	**Gene number**
nrfA	140	2131
Assimilatory or *nirB*?	146	2981
Putative nitrogenase structural genes	**Contig number**	**Gene number**
nifH	145	2709
nifD	145	2710
nifK	145	2711
Missing	145	2712
Hypothetical	145	2713
Missing	145	2714
nifE	145	2715

Structure of *nar* operon in *G. metallireducens*

A schematic drawing of each *nar* operon is shown in Figure 7. The size and the order of the each putative *nar* genes in each operon is as follow; *nar* operon in contig121 contains *narC* (886bp), *narG* (3615bp), *narH* (2288bp), and *narI* (1179bp); *nar* operon in contig135 contains *narC* (789bp), *narG* (3576bp), *narH* (1455bp), *narJ* (620bp), *narI* (689bp), $narK_1$ (1320bp), and $narK_2$ (1428bp). No putative genes encoding the NarXL system was found in either upstream or downstream of the operons. The size of *narH* in *nar121* is significantly larger than the *narH* in *nar135*. The nucleotide sequence as well as deduced amino acid sequences for *nar121* and *nar135* are shown in Figure 8 and Figure 9. Promoter sites and transcriptional regulation sites were predicted using Neural Network program and IMD program respectively (15, 79). The Neural Network program predicted one putative promoter site and four putative promoter sites in contig121 and contig135 respectively. Only the promoter that is closest to the start codon ATG is indicated in Figure 8. No putative transcriptional regulation site was found upstream of

narC in *nar121*, whereas a putative CAP/CRP site was found in one of the putative promoter region of *nar135*. A putative Shine-Dalgarno sequence was found upstream of every *nar* gene in both *nar* operons. The start codon of *narJ* and *narI* overlaps with *narH* and *narJ* respectively in *nar135*. Thus *narHJI* are probably translated from the same mRNA. There is a 218 base pair gap between *narI* and $narK_1$ but no putative promoter region was found in this gap using the promoter prediction program. This is significantly different from *T. thermophilus*, in which the ATG start codon of $narK_1$ overlaps the last codon of *narI* and both are likely translated together from the same mRNA.

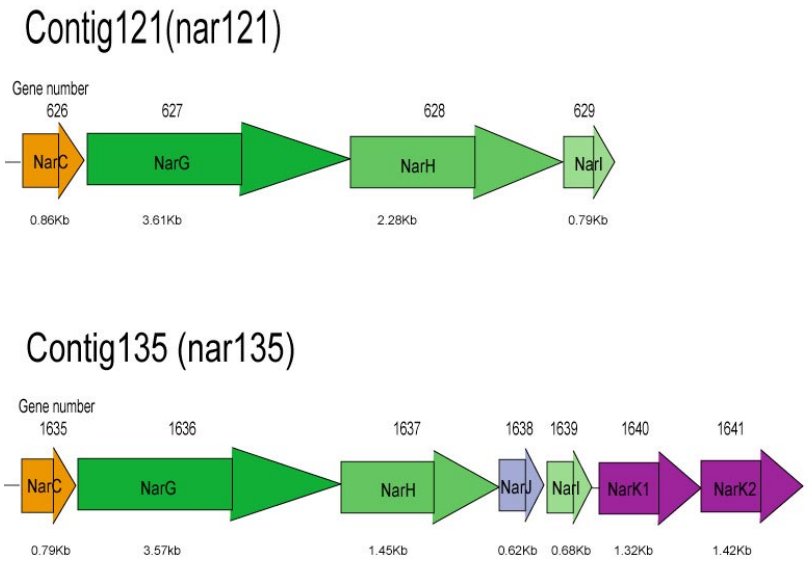
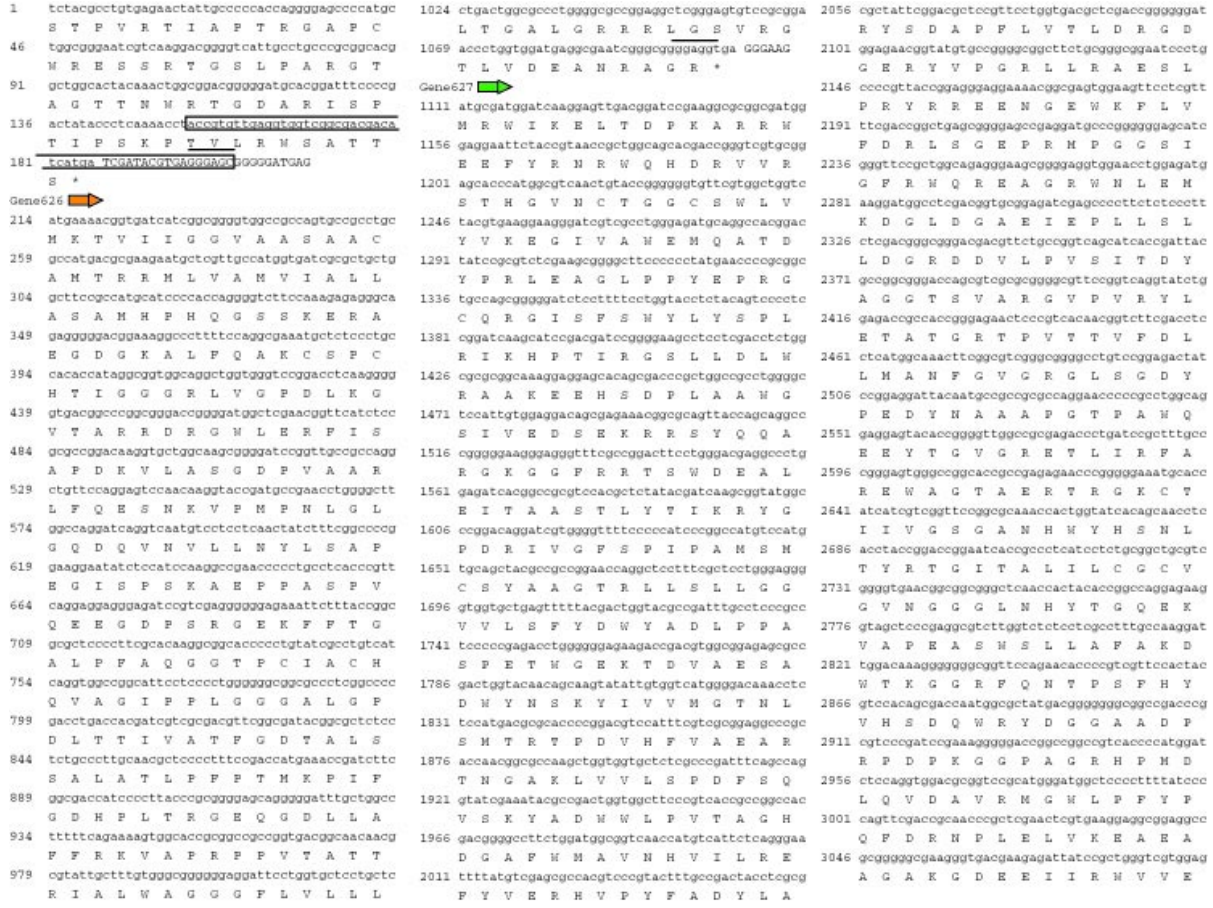
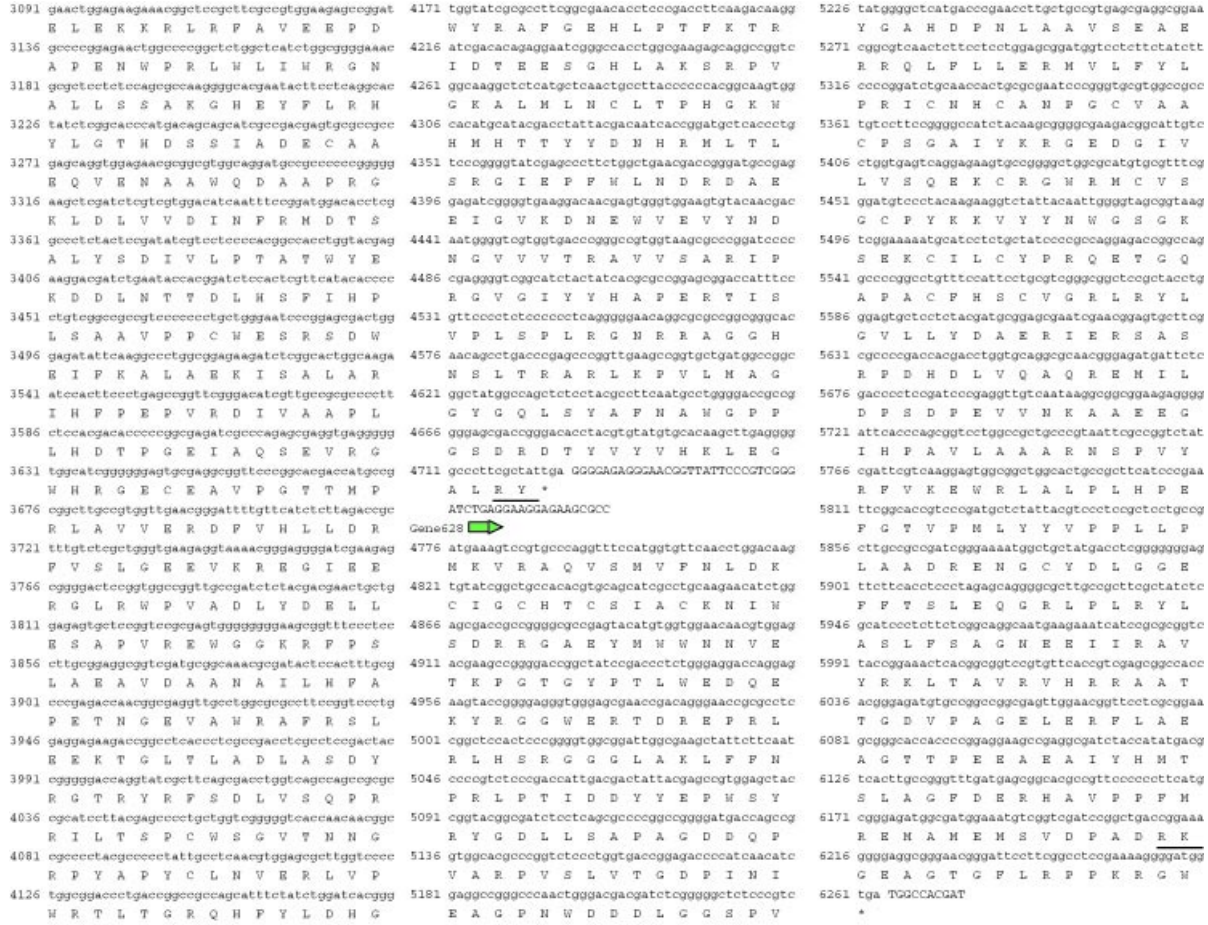


Figure 7: Gene order of *nar* operon in *G. metallireducens*





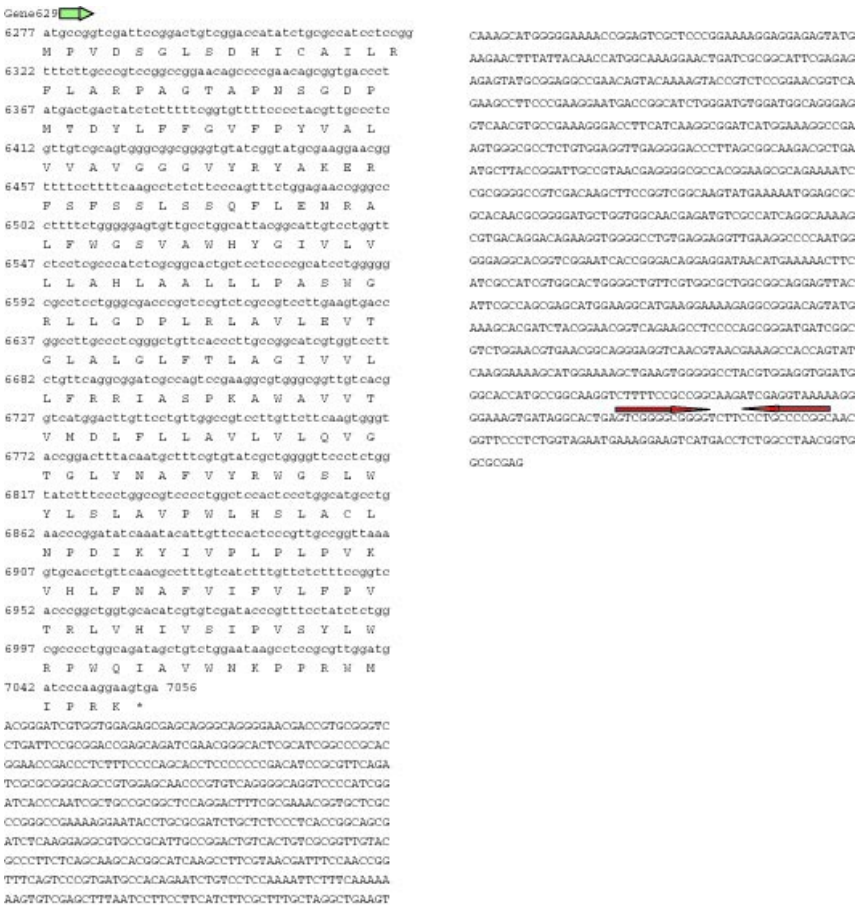
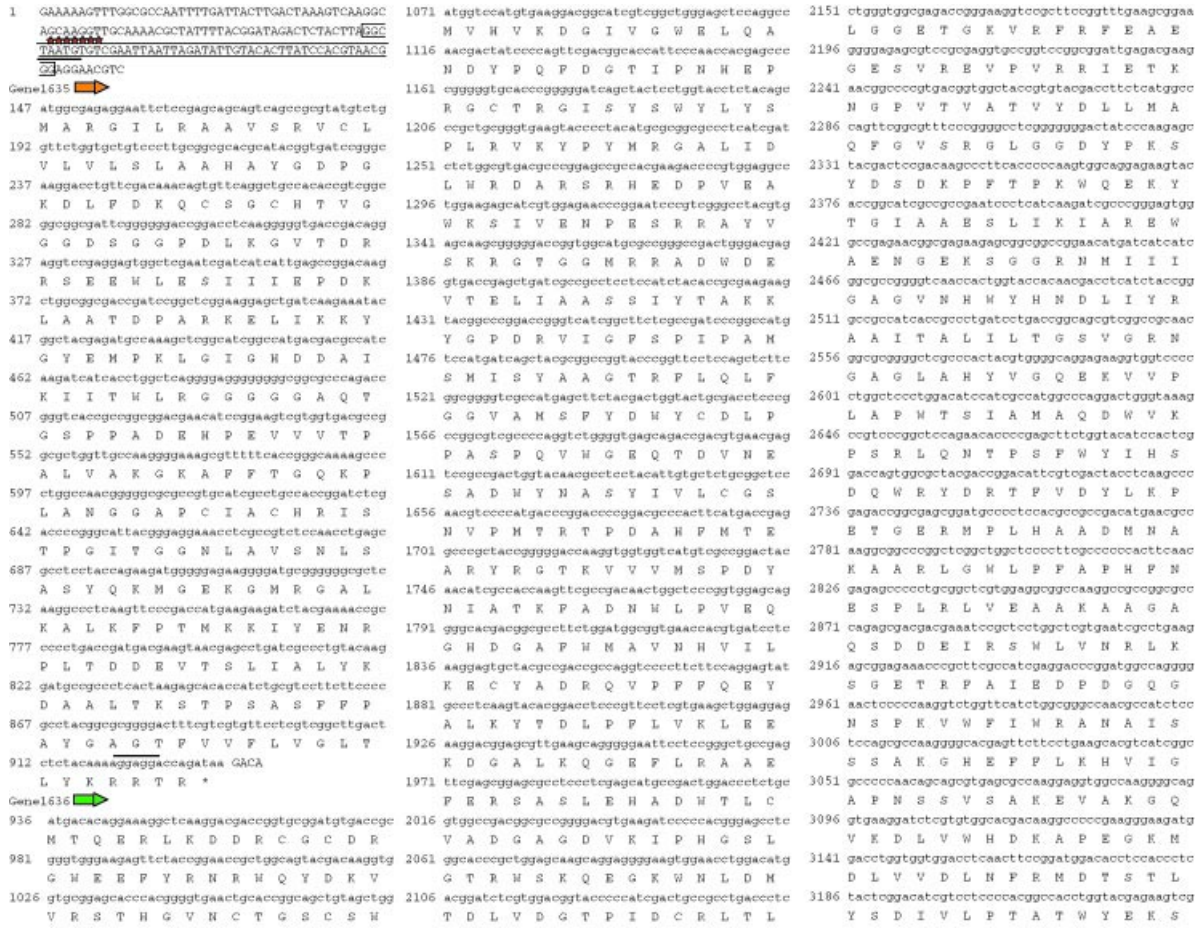
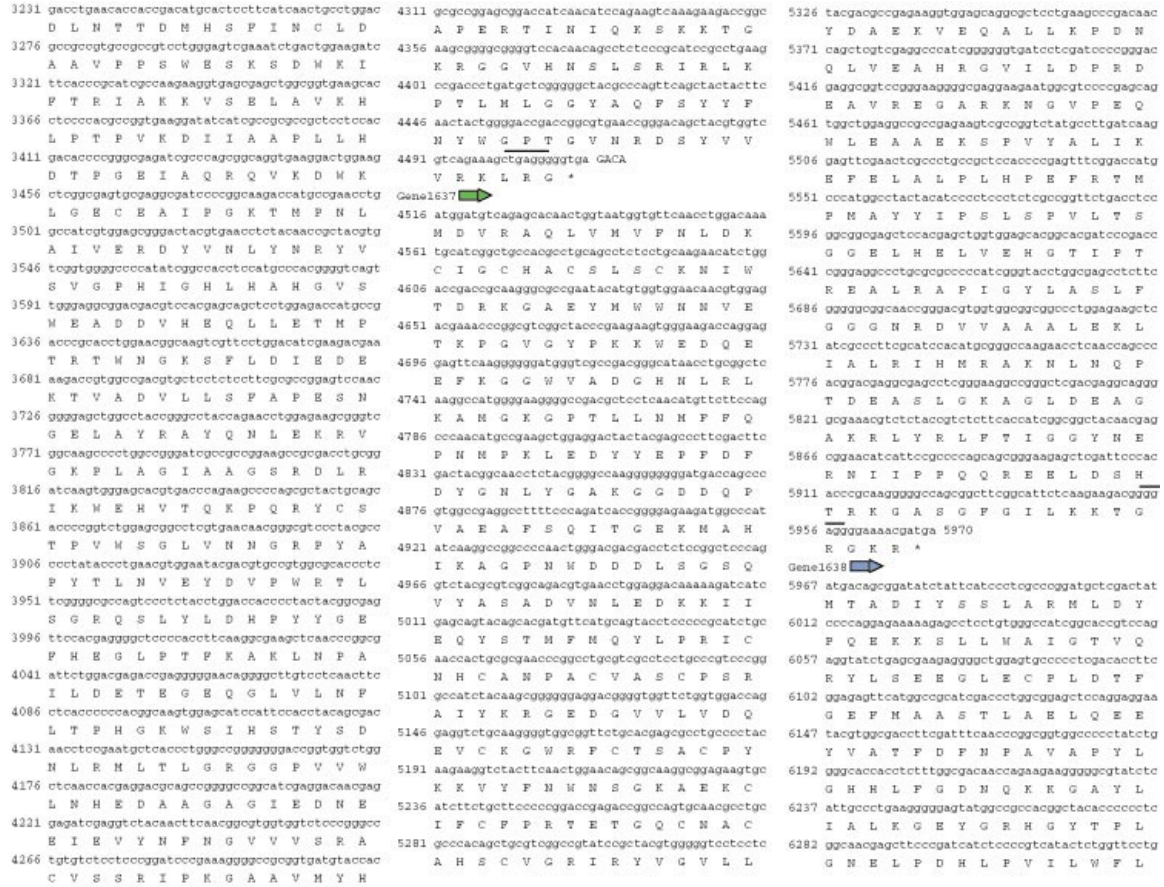


Figure 8: Nucleotide sequence of *nar* operon in contig121

The deduced amino acid sequences are indicated below the nucleotide sequence. The stop codon is designated by *. The beginning of each open reading frame is designated by the gene number and an colored arrow corresponding to Figure 7. The nucleotide sequence in the box is the putative promoter region predicted by Neural Network program. Bars above the sequence indicate putative Shine-Dalgarno sequences. Red arrows indicate the inverted repeat that can form a hairpin.





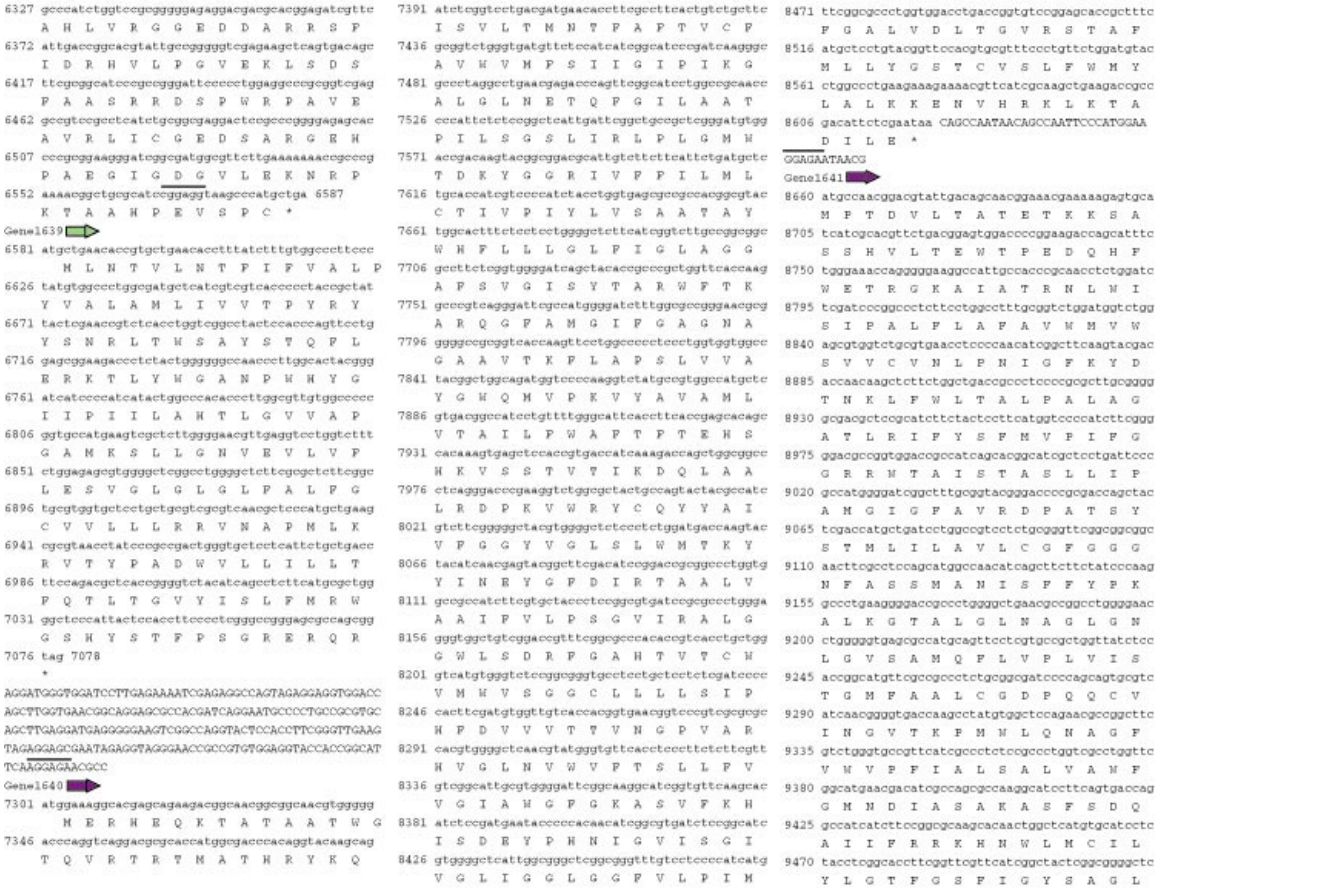


Figure 9: Nucleotide sequence of *nar* operon in contig135

The deduced amino acid sequences are indicated below the nucleotide sequence. The stop codon is designated by *. The beginning of each open reading frame is designated by the gene number and an colored arrow corresponding to Figure 7. The nucleotide sequence in the box is the putative promoter region predicted by Neural Network program. Red stars indicate a putative CAP/CRP recognition-binding site predicted by IMD program. Bars above the sequence indicate putative Shine-Dalgarno sequences. Red arrows indicate the inverted repeat that can form a hairpin.

Comparative analysis of the two putative Nar subunits and their similarities to *T. thermophilus* Nar subunits.

Sequences of NarCGHI from *nar121*, *nar135* and *T.thermophilus* were aligned using ClustalX and the percentage identity and similarity were calculated (Table 9). NarGHI from contig121 has a higher similarity to *T.thermophilus* NarGHI except NarC, in which contig 135 NarC has slightly higher similarity to *T.thermophilus*. The significantly low identity of contig135 NarI to *T.thermophilus* is very intriguing. Though this low identity did not affect its position in the phylogenetic tree, in which it is still significantly similar to *T.thermophilus* (Figure 10). The protein sequence alignment of NarC, NarG, NarH, and NarI is in Appendix VII.

Table 9: Similarities between the putative NarC, G, H and I in contig121 and 135 from GS-15 and the corresponding Nar subunits from *T.thermophilis.*

Numbers are in percentages. Abbreviations indicate the following; 121: *nar121*, 135: *nar135*, Therm: *T.thermophilis*

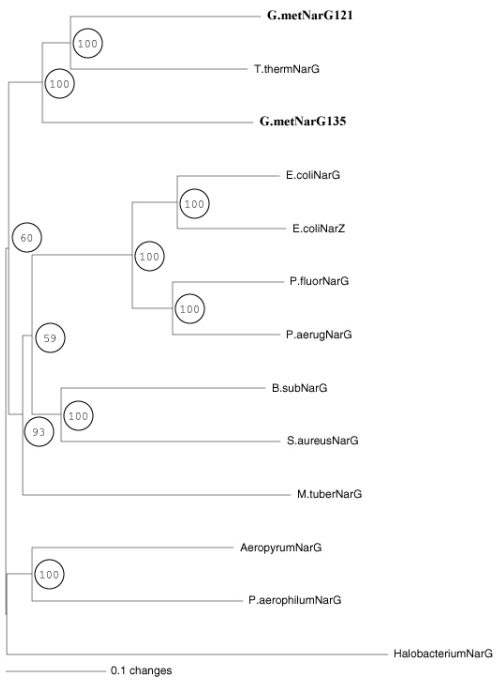
		121 vs 135	121 vs Therm	135 vs Therm	All 3
NarC	% identical	30.7	27.4	28.6	13.9
	% similar	60.5	56.4	61.8	37.8
NarG	% identical	56.2	62.7	58.3	46.8
	% similar	82.9	85.4	84.0	74.3
NarH	% identical	52.4	59.5	51.1	43.1
	% similar	79.1	83.0	77.7	69.3
NarI	% identical	15.8	40.0	12.3	5.4
	% similar	38.5	68.8	42.3	25.0

Phylogenetic trees of NarG, H, J, I, K_1, and K_2

All trees presented here were generated based on the neighbor joining method (Figure 10). The maximum parsimony method was also used on the same data set to confirm the accuracy of the neighbor joining trees (data not shown). The putative NarG, H, and I from both contigs form a distinctive clad with *T. thermophilus*. NarG, H and I from contig121 are closer related to *T. thermophilus* compared to the same subunits from contig135. NarJ from contig135 is placed in the same clad as *Haloarcula marismortui* (archaea) rather than *T. thermophilus*. The amino acid sequence comparison of NarJ between GS-15 and *T. thermophilus* is 13.9 % identical and 34 % similar. The putative $NarK_1$ and $NarK_2$ subunits were also placed closer to *Pseudomonas aeruginosa* rather

than *T. thermophilus*. A previous study has reported the high similarities of $NarK_1$ and $NarK_2$ between *P. aeruginosa* and *T. thermophilus*, in which $NarK_1$ protein from both organisms were 43 % identical and 54.5 % similar, and as for $NarK_2$ proteins the similarities were 41 % identical and 51.3 % similar (59). $NarK_1$ and $NarK_2$ proteins from GS-15 show much higher similarities to *P. aeruginosa* as compared to *T. thermophilus*. The comparison between GS-15 and *P. aeruginosa* $NarK_1$ proteins were 45.5 % identical and 67 % similar, and for the $NarK_2$ proteins, they were 63 % identical and 86 % similar. Comparison between GS-15 and *T. thermophilus* $NarK_1$ proteins were 30 % identical and 64 % similar, and for the $NarK_2$ proteins, they were 40 % identical and 72 % similar. The comparison was also made at the DNA level in which *narK₁* genes from GS-15 and *P. aeruginosa* were 52 % identical and *narK₂* genes were 70.5 % identical, whereas GS-15 versus *T. thermophilus narK₁* gene showed 45.5 % identity and 55.3 % identity for *narK2*. The phylogenetic tree using nucleotide sequences of *narK* from various bacteria also indicate the significant similarity between GS-15 and *P. aeruginosa*. This is an intriguing finding because in GS-15 and *T. thermophilus* the two in tandem *narK₁* and *narK₂* genes are located downstream of *narGHJI*, whereas in *P. aeruginosa* the *narK₁* and *narK₂* genes are located upstream of the corresponding *narGHJI* cluster. Yet both protein and DNA sequences of GS-15 *NarK₁* and *NarK₂* show higher similarities to *P. aeruginosa*.

A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T.thermNarG
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G.metNarG135
E.coliNarG
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E.coliNarZ
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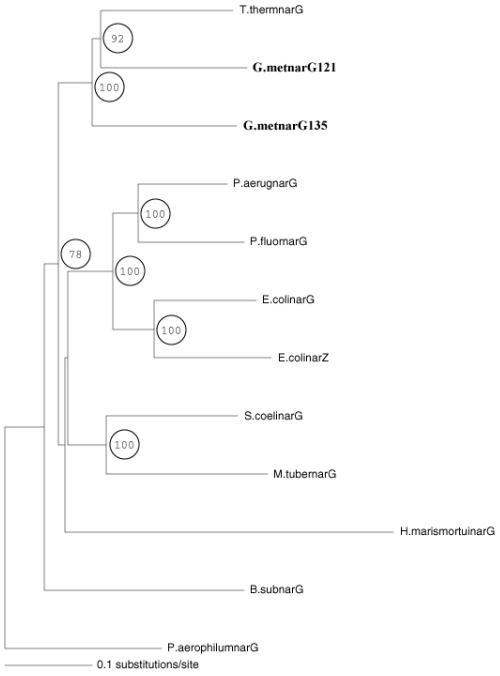
100
P.fluorNarG
100
P.aerugNarG
59
B.subNarG
100
93
S.aureusNarG
M.tuberNarG
AeropyrumNarG
100
P.aerophilumNarG
HalobacteriumNarG
0.1 changes

B


T.thermnarG
92
G.metnarG121
100
G.metnarG135
P.aerugnarG
100
P.fluornarG
78
100
E.colinarG
100
E.colinarZ
S.coelinarG
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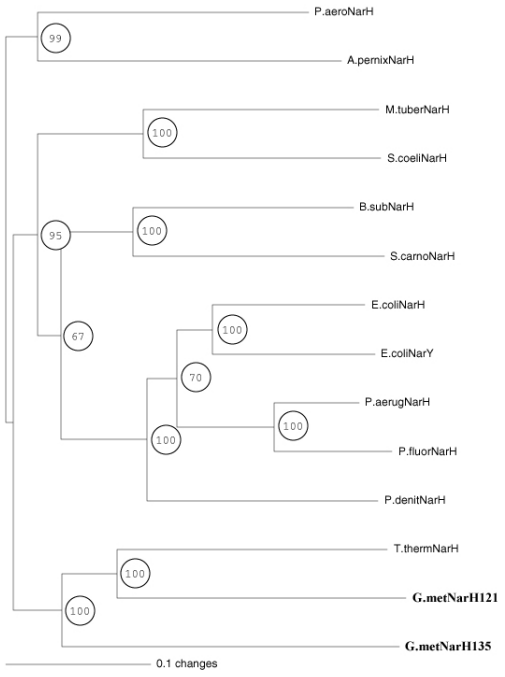
M.tubernarG
H.marismortuinarG
B.subnarG
P.aerophilumnarG
0.1 substitutions/site

C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A.pernixNarH
M.tuberNarH
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S.coeliNarH
B.subNarH
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S.carnoNarH
E.coliNarH
100
67
E.coliNarY
70
P.aerugNarH
100
100
P.fluorNarH
P.denitNarH
T.thermNarH
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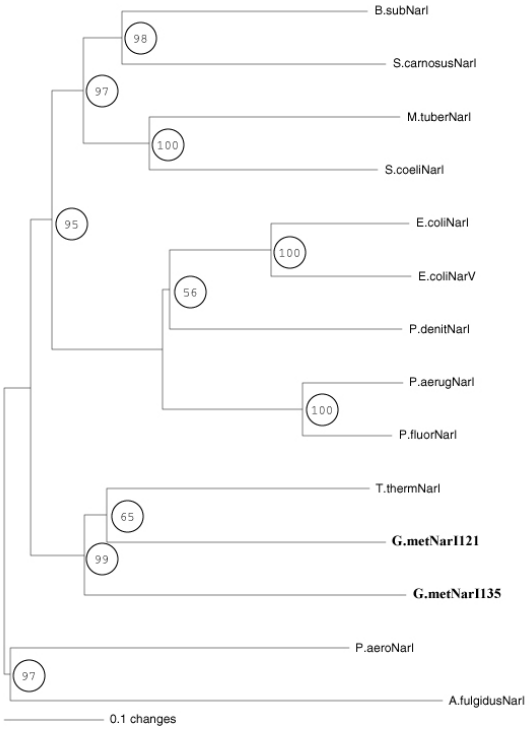
G.metNarH121
100
G.metNarH135
0.1 changes

D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S.coeliNarI
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E.coliNarI
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E.coliNarV
56
P.denitNarI
P.aerugNarI
100
P.fluorNarI
T.thermNarI
65
G.metNarI121
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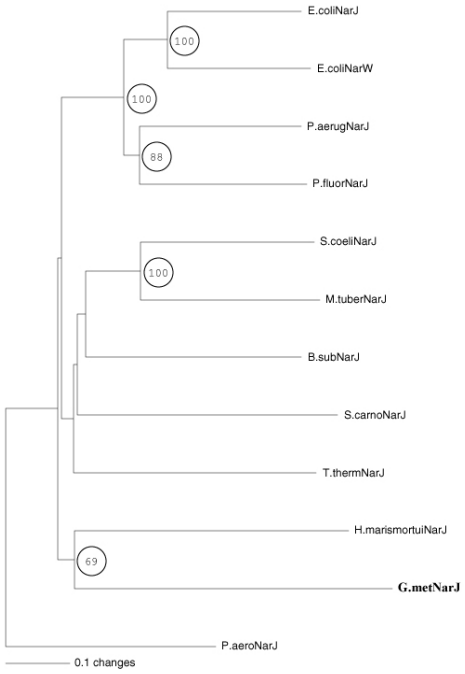
G.metNarI135
P.aeroNarI
97
A.fulgidusNarI
0.1 changes

E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E.coliNarW
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P.fluorNarJ
S.coeliNarJ
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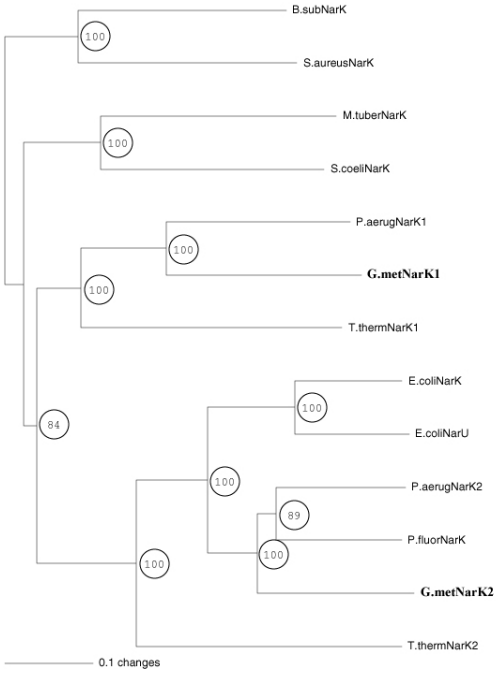
M.tuberNarJ
B.subNarJ
S.carnoNarJ
T.thermNarJ
H.marismortuiNarJ
69
G.metNarJ
P.aeroNarJ
0.1 changes

F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M.tuberNarK
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S.coeliNarK
P.aerugNarK1
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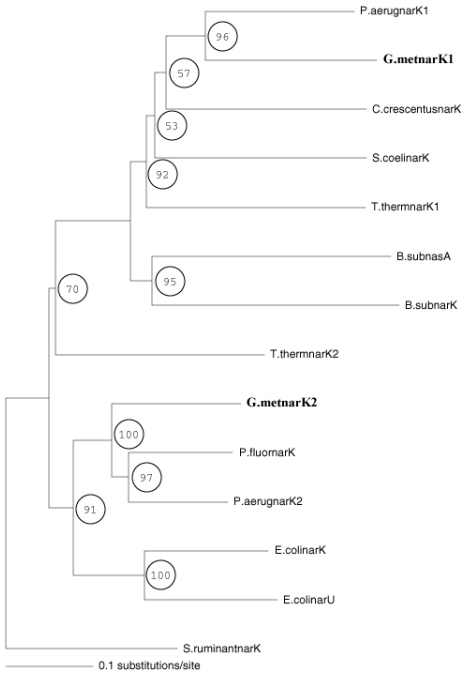
G.metNarK1
100
T.thermNarK1
E.coliNarK
100
84
E.coliNarU
100
P.aerugNarK2
89
P.fluorNarK
100
100
G.metNarK2
T.thermNarK2
0.1 changes

G


P.aerugnarK1
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G.metnarK1
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C.crescentusnarK
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S.coelinarK
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T.thermnarK1
B.subnasA
70
95
B.subnarK
T.thermnarK2
G.metnarK2
100
P.fluornarK
97
P.aerugnarK2
91
E.colinarK
100
E.colinarU
S.ruminantnarK
0.1 substitutions/site

Figure 10: Phylogenetic trees of NarG, H, I, J, K_1, and K_2.

A: NarG tree, B: *narG* tree, C: NarH tree, D: NarI tree, E: NarJ tree, F: $NarK_1$ and $NarK_2$, G: *narK*$_1$ and *narK*$_2$ tree

All trees presented here are generated based on the neighbor joining method. Bootstrap values are presented in circles (as percentages of 1000 replications). Organism names are abbreviated and the name of Nar subunit is listed after the abbreviation. “Nar” indicates protein sequence and “nar” indicates DNA sequence. The organisms’ name is listed below with their abbreviation.

Aeropyrum: *Aeropyrum pernix*
B.sub: *Bacillus subtilis*
C.crescentus: *Caulobacter crescentus*
C.perfringens: *Clostridium perfringens*
E. coli: *Escherichia coli*
G.met: GS-15
Halobacterium: *Halobacterium*
H.marismortui: *Haloarcula marismortui*
H.pylori: *Helicobacter pylori*
M.tuber: *Mycobacterium tuberculosis*
P.denit: *Paracoccus denitrificans*
P.aerug: *Pseudomonas aeruginosa*
P.fluor: *Pseudomonas fluorescens*
P.aerophilum: *Pyrobaculum aerophilum*
S.ruminant: *Selenomonas ruminantium*
S.aureus: *Staphylococcus aureus*
S.coeli: *Streptomyces coelicolor*
S.carnosus: *Staphylococcus carnosus*
T.therm: *Thermus thermophilus*

Additional analysis of *narG* gene products and putative nitrite reductases

Location of the putative enzymes

The location prediction program PSORT at Expasy website (http://us.expasy.org) was used for this analysis. NarG in contig121 is predicted to reside at the cytoplasmic inner membrane (certainty 0.153) whereas NarG in contig135 is predicted to reside in the cytoplasm (certainty 0.514).

Theoretical pI, and molecular weight

Theoretical pI and molecular weight of the two putative *narG* and the two nitrite reductases were determined using ProtParam at Expasy website.

Putative enzyme	Isoelectric point	Molecular weight (KDa)
NarG contig121	6.2	136
NarG contig135	6.59	134
Putative NrfA contig140	8.78	54
Putative NADH dependent nitrite reductase contig146	5.49	47

Partial purification of NarG from *G. metallireducens*

After the discovery of the two putative *nar* operons (*nar121* and *nar135*), partial purification was carried out to determine which *nar* is expressed in cells of GS-15 when grown in media containing nitrate as electron acceptor. Partial purification followed by N-terminus sequencing was planned as the strategy. However, the target NarG protein (approximately 130 KDa) was not found in the fractions that contained nitrate/nitrite reductase activity (Figure 11) followed by ion exchange column. Figure 12 shows a zymogram of nitrite reductase activity. A very faint nitrate reductase activity was also detected but was unable to be captured by the camera.

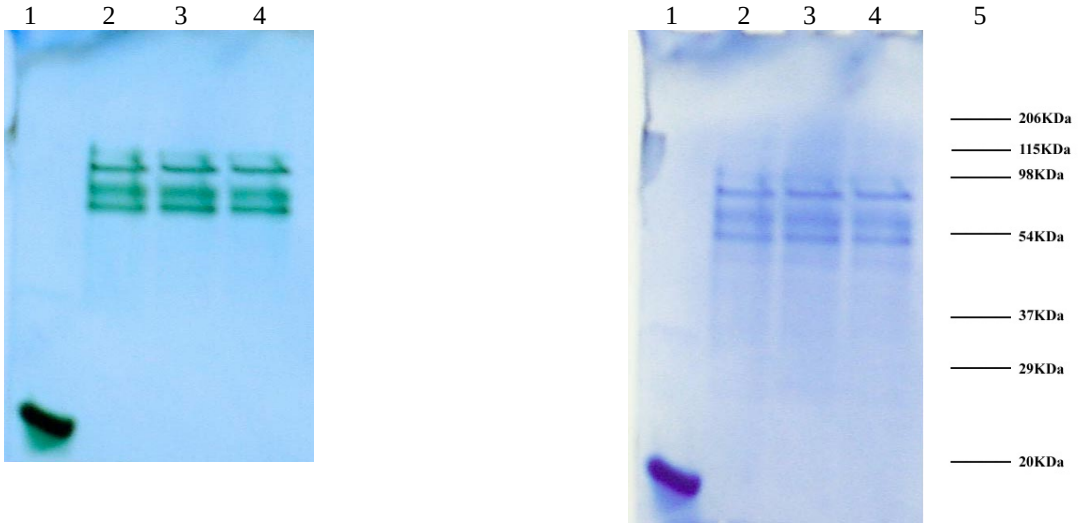


Figure 11: Heme stain and SDS-PAGE of *G. metallireducens* ion exchange fractions

The gel was heme stained followed by coomassie staining. Lane 1 is the heme stain standard (horse heart cytochrome *c*), lanes 2-4 are ion exchange fractions number 33-35, and lane 5 is the SDS-PAGE standards. Samples were denatured at 95 ^{o}C for 2 min. SDS-PAGE Standards: Myosin 206 KDa, β-galactosidase 115 KDa, Bovine serum albumin 98 KDa, Ovalbumin 54 KDa, Carbonic anhydrase 37 KDa, Soybean trypsin inhibitor 29 KDa, Lysozyme 20 KDa

Figure 12: Zymogram of nitrite reductase activity

The samples loaded were ion exchange fractions number 33-35 (lanes 1-3). Arrow indicates nitrite reductase activity.

Solubilization of NapA from *S. barnesii* using lysozyme treatment

Various lysozyme treatment conditions were tested as indicated in the method section. SES-3 NapA was solubilized using 10 mM EDTA and 1 mg/ml lysozyme concentration incubated at room temperature for 60 minutes. This was the most effective combination among the variety of combinations investigated. The results show that the activity of both periplasmic and spheroplast fractions were significantly greater than that determined for the whole cell sample. This indicates a permeability problem with the methyl viologen. The results also indicate that Nap complex is tightly anchored to the membrane.

Table 10: Summary table for lysozyme treated *S. barnesii* fractions' nitrate reductase activity

	Volume (ml)	Protein Volume (µl)	Activity (µmol/min)	Total activity (µmol/min)	Protein (µg/ul)	Specific activity (U/mg)
Whole cell	0.09	20	0.011	0.55	6.6	0.083
Periplasm	0.81	20	0.023	1.15	1.4	0.82
Spheroplast	1.3	20	0.063	3.15	2.4	1.31

Purification of NapA from *S. barnesii*

An attempt was made to purify NapA. Sonication was utilized as an initial step to solubilize NapA instead of lysozyme treatment. This was because of the high EDTA concentration used in lysozyme treatment. The possibility of EDTA disrupting the molybdenum cofactor in NapA was the main concern. Nitrate and nitrite reductase activity was monitored by zymogram. 60 ml of solubilized fraction (10 mg/ml) was loaded to an ion exchange column followed by size exclusion column were utilized for the purification. However, only partial purification was completed. Figure 13 shows the nitrite reductase activity detected by zymogram. Nitrate reductase activity was too faint to be captured by the camera. Figure 14 shows the corresponding fraction on a SDS-PAGE.

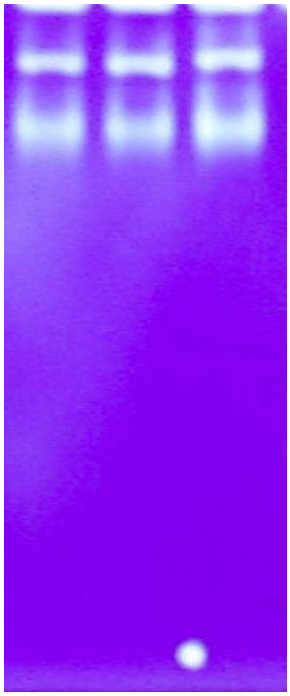


Figure 13: Zymogram of nitrite reductase activity

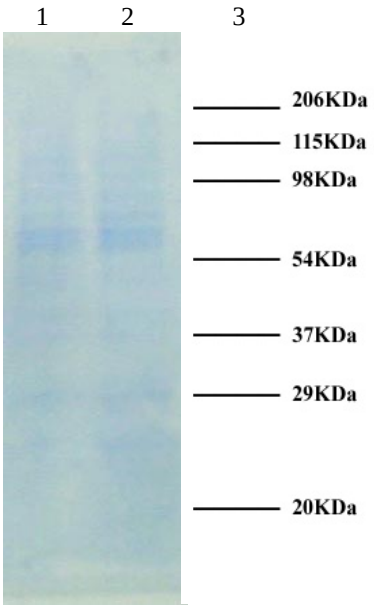


Figure 14: SDS-PAGE of *S. barnesii* size exclusion column fractions

Figure 13: *S. barnesii* size exclusion fraction that contained nitrite reductase activity was detected by zymogram. Figure 14: Size exclusion column fractions that contained nitrite reductase activity were denatured by heating in a sand bath for 2 min and run on a SDS-PAGE. Lane 1 and 2 are the fractions. Lane 3 is the SDS-PAGE molecular weight standard. SDS-PAGE Standards: Myosin 206 KDa, β-galactosidase 115 KDa, Bovine serum albumin 98 KDa, Ovalbumin 54 KDa, Carbonic anhydrase 37 KDa, Soybean trypsin inhibitor 29 KDa, Lysozyme 20 KDa

Development of 96 well assay

A 96 well assay was developed by modifying the spectrophotometric methyl viologen assay protocol (Figure 15). Several false positives were detected in some fractions collected from ion exchange column. No protein bands could be detected using SDS-PAGE from these fractions. Native gel assay also indicated there was no enzyme activity from these fractions. However, the probability of getting false positive results

was relatively low (approximately 5 %). The positive control (containing crude extract) and negative control (containing no enzyme) used in the 96 well assay did not cause any problems. Depending on the condition of the anaerobic glove box (i.e. oxygen contamination), the color of methyl viologen in the negative control did not change for at least 10 – 20 min. This indicates that development of the assay system was successful.

A titration study was carried out to investigate the sensitivity of the 96 well assay. Serial dilution was carried out using the crude extract. During this experiment it was discovered that letting the plate to sit in the anaerobic glove box for several minutes before adding methyl viologen would result in a more accurate results. Color change of the media was difficult to quantify by sight, although wells containing more proteins did have a more rapid color change. The negative control for nitrate lasted for 22 min and for nitrite it lasted for 14 min before clearing. The overall sensitivity result is summarized in Table 11.

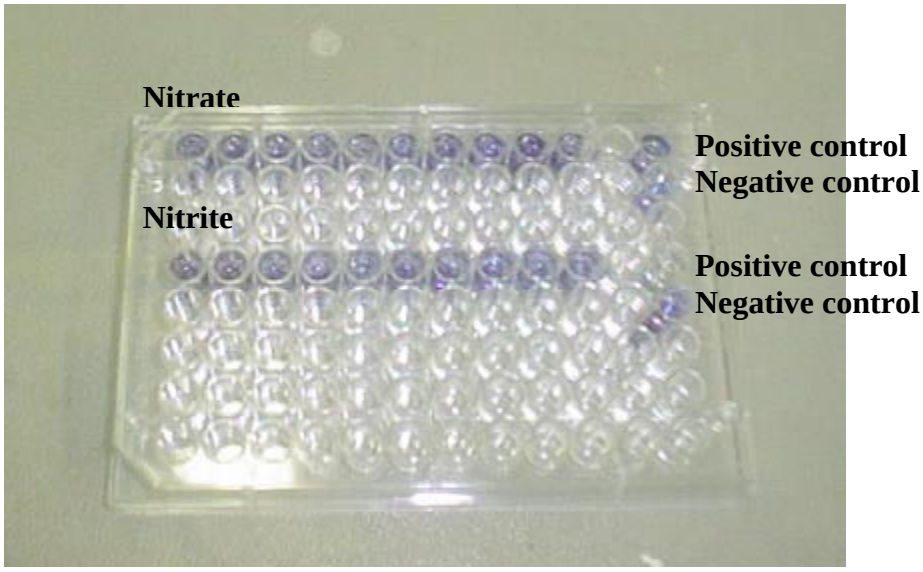


Figure 15: 96 well assay

Table 11: Summary of 96 well assay sensitivity

Nitrate reductase Units of activity (μmol/min)	**Time taken for complete methyl viologen oxidation (min)**
0.0076	14
0.015	10
Nitrite reductase Units of activity (μmol/min)	**Time taken for complete methyl viologen oxidation (min)**
0.2	2
0.4	1
0.6	1
0.8	0.5

Discussion

Physiological characterization of assimilatory nitrate and nitrite reduction in *G. metallireducens* and *G. sulfurreducens*

Both GS-15 and *G. sulfurreducens* were able to grow in media containing ammonia, nitrate, and N_2 (Table 6). Due to the fact that both organisms have nitrogenase genes and the activity has been confirmed previously (8, 19), it is likely that cells grown in nitrate were utilizing nitrogen gas rather than nitrate as the nitrogen source. Whether these two organisms have the ability to assimilate is still uncertain. Based on the BLAST search carried out in this study, however, no putative Nas homologue was identified. However, in order to confirm the absence of Nas in GS-15, a mutant strain lacking nitrogenase activity is required, or degassing the medium with a gas other than N_2.

Molybdoenzymes in *G. metallireducens*

Nine putative molybdoenzymes were identified in the current version of GS-15 genome draft. The presence of putative nitrate induced formate dehydrogenase and fdh-H type formate dehydrogenase genes is intriguing, because GS-15 was unable to grow in media containing formate as electron donor with nitrate or Fe (III) as electron acceptor (data not shown). Three explanations can be proposed. First, the ORFs may not encode formate dehydrogenase. It is difficult to argue this unless complete protein and nucleotide analysis using prediction programs are carried out. Second, the ORFs are indeed formate dehydrogenase genes but have and cannot be transcribed or even they can be transcribed, they are not functional. Third, the growth experiments carried out did not induce a certain

factor that initiates transcription of the genes. Positions of the two putative molybdopterin oxidoreductases (gene 269 and gene 2012) in the phylogenetic tree suggest GS-15 may posses arsenate reductases (Figure 5). Currently, there is no data that suggests GS-15 can grow on arsenate thus it will be interesting to determine this.

Putative *nar* operons and their evolutionary process

Discovering the existence of the two putative nar operon in GS-15 is intriguing. Multiple membrane bound nitrate reductase isoenzymes have been discovered previously in various bacteria, though these bacteria are not strictly anaerobes like GS-15. In *E. coli*, one of the membrane bound nitrate reductase is expressed constitutively to ensure a smooth transition from aerobic respiration to nitrate anaerobic respiration. It is possible that GS-15 also express one of the Nar isoenzyme constitutively, but the fact that no nitrate reductase activity was found present in cells grown in Fe (III) media (58) proves this to be unlikely. Putative promoter sites were found in both operons indicating that transcription can potentially be carried out on both *nar* operons. Four putative promoter sites were found upstream of *nar135* as oppose to only one putative promoter region upstream *nar121*. A putative CAP/CRP site was found and overlaps the fourth promoter that is closest to the ATG start codon of NarC in *nar135* (Figure 8). Thus it is likely that *nar135* can potentially be expressed by different stimuli (such as transcription induced by heat response sigma factor) and also is regulated CAP/CRP. One of the *nar* operons (*nar121*) in GS-15 lacks the putative *narJ* genes suggesting that the enzyme that it encodes may not be functional. The gene product of *narJ* is a chaperone like component required for assembling functional NarGHI complex, thus lacking the gene may result in

nonfunctional enzyme. All the functional *nar* operons that have been characterized so far contain the *narJ* gene. Although a *nar* operon lacking *narJ* has been found in *Pseudomonas stutzeri ZoBell* (51), it is possible that the gene is located nearby and is yet to be discovered. Discovery of genes encoding a putative cytochrome *c* that has only been found in *T. thermophilus* is novel. Based on the BLAST search results, GS-15 and *T. thermophilus* are the only two organisms with *narC* located upstream of *narG*. *T. thermophilus* with mutated *narC* was unable to generate functional Nar enzyme and respire nitrate (84).

It is tempting to conclude that GS-15 may have acquired a *nar* operon from *T. thermophilus*. This hypothesis can also be supported by the high similarities between *narGHI* from the two organisms based on the results of amino acid identity/similarity comparison and the phylogenetic trees. Also, characterization of the *nar* operon from *T. thermophilus* has revealed that the operon is a part of a conjugative plasmid integrated into its chromosome (60). Also, growth experiment with other strains of *Thermus* indicated no anaerobic growth in nitrate (60). Moreover, the study also indicated that the *nar* operon could be horizontally transferred to other strains of *Thermus*. To add more complexity to the discussion, the amino acid sequence of *narJ* from GS-15 and *T. thermophilus* showed only a 13.9 % identity and 34 % similarity. Also, $narK_1$ and $narK_2$ genes and their translated amino acid sequence analysis from GS-15 showed that GS-15 *narK1* and *narK2* are closer to *P. aeruginosa* rather than *T. thermophilus*. Another difference found between *narK* genes of GS-15 and *T. thermophilus* was that the start codon ATG of $narK_1$ in *T. thermophilus* overlaps with the last codon of *narI* gene (59), whereas a 218 base gap is located in between *narI* stop codon and $narK_1$ start codon in

GS-15 (Figure 8). Taking all these findings into account, plus the phylogenetic trees results, in which all of *nar121* genes are closer to *T. thermophilus,* one can argue that *nar* operon in GS-15 was originally from *T. thermophilus*. In other words, *nar121* might have been the copy that was acquired from *T. thermophilus,* and during the evolutionary process, *nar135* was duplicated from *nar121,* and both operons were eventually modified to their current state in GS-15. Finally, it would be interesting to purify NarG from GS-15 cells grown in FWA-nitrate media and sequence the N-terminus of the purified NarG. This might help determine which *nar* operon is transcribed.

Upstream and downstream of *nar121* and *nar135*

BLAST search results of three ORFs upstream of *nar135* indicate that these three genes may encode a membrane bound three subunit enzyme (results not shown). According to the BLAST results, gene 1634 is a putative molybdopterin oxidoreductase membrane binding subunits, gene 1633 is a putative molybdopterin oxidoreductase, iron-sulfur binding subunit, and gene 1632 is a molybdopterin with very low E values. A putative Integration host factor (IHF) binding site was found at the upstream of gene 1632. Since there is a gap in between the stop codon of the three ORF and start codon of *narC*, this putative enzyme complex is not likely to be transcribed together with *nar* operon. Down stream of *nar135* on the other hand, are gene 1642 and gene 1643 encoding molybdenum cofactor biosynthesis enzyme.

Upstream of *nar121* are two hypothetical genes (gene 625 and gene 624) then a gene encoding aconitase (gene 623). Genes located down stream are three genes encoding hypothetical proteins (gene 630-632) then NtrC and NtrB (gene633-4). Overall,

no Fnr or NarXL like genes were identified upstream or downstream of *nar121* and *nar135*. Though BLAST search using amino acid sequence of NarX and NarL from *E. coli* did generate a dozen of positive hits. It will be interesting to find out whether any of these putative two component response sensor regulators is involved in nitrate reduction in GS-15.

Solubilization of NapA utilizing lysozyme treatment and 96 wells assay development

Requirement of high EDTA concentration to solubilize NapA may infer an involvement of a metallic binding between NapA to the periplasmic membrane. Although lysozyme treatment seems to be successful, breakage of spheroplasts needs to be investigated. Freezing and thawing was also utilized to solubilize NapA with no success. Longer sonication time may be an alternative to lysozyme treatment. It was discovered that 15 sonication cycles (30 seconds on 30 seconds off) was an effective way to solubilize NapA. The reason is likely due to the heat that causes the dissociation of the Nap complex. Additional experiments are required to confirm the effectiveness of longer sonication time.

The success of 96 well assay development enabled the screening of many samples in a short time. However, from the fact that it sometimes gives false positive results, the assay should be used in conjunction with other assay methods.

Conclusion

Based on the growth experiment of GS-15 and *G. Sulfurreducens* in media containing different nitrogen source, assimilation of nitrate was unable to be confirmed due to the nitrogenase activity present in both organisms.

Nine putative molybdoenzymes were identified in the GS-15 draft genome using BLAST search. The BLAST analysis also identified putative Nar, Nrf, Nir, and Nif genes. Two putative *nar* operons were identified with *nar121* consists of four genes and *nar135* consists of seven genes. A novel cytochrome *c* was identified immediately upstream of *narG* in both *nar* operons. This novel cytochrome *c* has only been identified in *T. thermophilus* previously. Protein sequence comparison between *nar* operons from GS-15 and *T. thermophilus* exhibited an interesting profile. Overall, *nar121* exhibited a higher identity and similarity to *T. thermophilus* rather than *nar135*. This was also consistent with the phylogenetic tree analysis. Protein and DNA sequence of putative $narK_1$ and $narK_2$ from *nar135* showed a higher similarity to *P. aeruginosa* rather than *T. thermophilus* based on phylogenetic tree analysis. A hypothesis that *nar* operons in GS-15 was originally from *T. thermophilus* was proposed.

Partial purification of NarG from GS-15 was attempted to investigate which *nar* operon is transcribed. However, only partial purification was completed. Thus cloning *narG* and over expressing the gene in *E. coli* may be an alternative to the direct purification of NarG from GS-15.

Purification of NapA from SES-3 was attempted but only partial purification was completed. However, cell yield was increased by successfully growing SES-3 in a fermenter. Also, it was determined that lysozyme treatment can solubilize NapA. Thus

NapA is likely a periplasmic protein as predicted. During the course of purification, a new assay system using 96 well assay plate was developed to assist a rapid screening of fractions collected from ion exchange and size exclusion column.

Appendix I

Enzyme	Enzyme subunit	Organism
Periplasmic nitrate reductase	NapA	*Desulfuro Vibrio*
Periplasmic nitrate reductase	NapA	*Klebsiella Pneumoniae*
Periplasmic nitrate reductase	NapB	*E. coli*
Periplasmic nitrate reductase	NapC	*E. coli*
Periplasmic nitrate reductase	NapF	*E. coli*
Membrane bound nitrate reductase	NarG	*E. coli*
Membrane bound nitrate reductase	NarH	*E. coli*
Membrane bound nitrate reductase	NarI	*E. coli*
Membrane bound nitrate reductase	NarJ	*E. coli*
Membrane bound nitrate reductase	NarK	*E. coli*
Assimilatory nitrate/nitrite reductase	NasA	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	Nas	*klebsiella pneumoniae*
Assimilatory nitrate/nitrite reductase	NasA	*Vibrio diazotrophicus*
Assimilatory nitrate/nitrite reductase	NasA	*Azospirillum brasilense*
Assimilatory nitrate/nitrite reductase	NasA	*Psudoalteromonas*

Assimilatory nitrate/nitrite reductase	NasB	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	NasC	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	NasD	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	NasE	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	NasF	*Klebsiella oxytoca*
Assimilatory nitrate/nitrite reductase	NasA	*Bacillus subtilis*
Assimilatory nitrate/nitrite reductase	NasB	*Bacillus subtilis*
Assimilatory nitrate/nitrite reductase	NasC	*Bacillus subtilis*
Assimilatory nitrate/nitrite reductase	NasD	*Bacillus subtilis*
Assimilatory nitrate/nitrite reductase	NasE	*Bacillus subtilis*
Assimilatory nitrate/nitrite reductase	NarB	*Synechococcus sp*
Assimilatory nitrate/nitrite reductase	NirA	*Synechococcus sp*
Periplasmic nitrite reductase	NrfA	*E. coli*
Periplasmic nitrite reductase	NrfB	*E. coli*
Periplasmic nitrite reductase	NrfC	*E. coli*
Periplasmic nitrite reductase	NrfD	*E. coli*
Periplasmic nitrite reductase	NrfE	*E. coli*

Periplasmic nitrite reductase	NrfF	*E. coli*
Periplasmic nitrite reductase	NrfG	*E. coli*
Periplasmic nitrite reductase	NrfA	*Sulfurospirillum deleyianum*
Periplasmic nitrite reductase	NrfH	*Sulfurospirillum deleyianum*
Cytoplasmic dissimilatory nitrite	NirB	*E. coli*
Cytoplasmic dissimilatory nitrite	NirC	*E. coli*
Cytoplasmic dissimilatory nitrite	NirD	*E. coli*
Cytoplasmic dissimilatory nitrite	CysG	*E. coli*
Cytochrome *cd*1 nitrite reductase	NirS	*Paracoccus denitrificans*
Cytochrome *cd*1 nitrite reductase	NirI	*Paracoccus denitrificans*
Cytochrome *cd*1 nitrite reductase	NirE	*Paracoccus denitrificans*
Cytochrome *cd*1 nitrite reductase	NirT	*Pseudomonas*
Copper nitrite reductase	NirK	*Bladyrhizobium japonicum*
Nitrogenase	NifA	*Klebsiella Pneumoniae*
Nitrogenase	NifD	*Klebsiella Pneumoniae*
Nitrogenase	NifH	*Klebsiella Pneumoniae*
Nitrogenase	NifK	*Klebsiella Pneumoniae*

Appendix II

Distilled Water	980 ml
Ammonium Chloride	0.225 g
Sodium Phosphate (Monobasi	0.6 g
Potassium Chloride	0.1 g
Sodium Acetate	6.8 g
Sodium Bicarbonate	2.5 g
Vitamin Mix	10 ml
Trace Elements	10 ml
Electron acceptor	
Ferric Citrate	13.7 g(50mmol)
Sodium Nitrate	1.7 g (20mmol)

Vitamin Mix

Biotin	2 mg/L
Folic Acid	2 mg/L
Pyridoxine HCl	10 mg/L
Riboflavin	5 mg/L
Thiamine	5 mg/L
Nicotinic Acid	5 mg/L
Pantothenic Acid	5 mg/L
p-Aminobenzoic Acid	5 mg/L
Thioctoc Acid	5 mg/L
B12	0.1 mg/L

Mineral Mix

Nitrilotriacetic Acid	1.5 g/L
Magnesium Sulfate	3.0 g/L
Manganous Chloride	0.444 g/L
Sodium Chloride	1.0 g/L
Ferric Chloride	0.067 g/L
Calcium Chloride; Dihydrate	0.1 g/L
Cobalt Chloride	0.1 g/L
Zinc Sulfate 7-Hydrate	0.274 g/L
Cupric Sulfate 5-Hydrate	0.01 g/L
Aluminum Potassium Sulfate	0.01 g/L
Boric Acid; Granular	0.01 g/L
Sodium Molybdate	0.025 g/L
Nickelous Chloride 6-Hydrate	0.024 g/L
Sodium Tungstate; Hydrate	0.025 g/L

Appendix III

Washing buffer (1 liter)	
Tris-HCl	10 mM
EDTA	1 mM
Protease inhibitor	1:50 volume ratio
Distilled Water	985 ml

Native gel running buffer	
Glycin	45 g
Tris-HCl	9 g
H2O	2000 ml

Native gel sample buffer	
Tris-HCl PH6.8 0.5M	6 ml
Glycerol	4 ml
Bromophenol Blue	Few crystal

Buffer A	
Tris-HCl	10 mM
EDTA	1 mM
PMSF	10 μM
Distilled Water	985 ml

Appendix IV

Mineral salt media

980 ml	Distilled water
0.225 g	K_2HPO_4
0.225 g	KH_2PO_4
0.46 g	NaCl
0.225 g	$(NH_4)_2SO_4$
0.117 g	$MgSO_4$
1 g	Yeast extract
2.24 g	Sodium lactate
4.2 g	$NaHCO_3$
0.5 g	cysteine
10 ml	trace elements
10 ml	vitamine mix

Appendix V

Molybdoenzymes BLAST results

Contig108gene137

Sequences producing significant alignments:	Score (bits)	E Value
gi|23055047|gb|ZP_00081176.1| hypothetical protein [Geobact...	1123	0.0
gi|23050770|gb|ZP_00077622.1| hypothetical protein [Methano...	357	4e-97
gi|21226223|ref|NP_632145.1| Carbon monoxide dehydrogenase ...	349	8e-95
gi|23114032|ref|ZP_00099357.1| hypothetical protein [Desulf...	345	1e-93
gi|20092097|ref|NP_618172.1| carbon-monoxide dehydrogenase,...	341	2e-92
gi|23473779|ref|ZP_00129074.1| hypothetical protein [Desulf...	325	1e-87
gi|23103291|ref|ZP_00089776.1| hypothetical protein [Azotob...	310	4e-83
gi|23111777|ref|ZP_00097361.1| hypothetical protein [Desulf...	304	3e-81
gi|15826756|pdb|1JJY|A Chain A, Crystal Structure Of A Ni-C...	304	3e-81
gi|11095246|gb|AAG29809.1|AF249899_2 carbon monoxide dehydr...	299	1e-79

Contig140gene2111

Sequences producing significant alignments:	Score (bits)	E Value
gi|23054053|gb|ZP_00080232.1| hypothetical protein [Geobact...	363	e-100
gi|16605575|emb|CAC86667.1| formate dehydrogenase alpha sub...	182	2e-45
gi|28948357|pdb|1H0H|A Chain A, Tungsten Containing Formate...	177	9e-44
gi|23474797|ref|ZP_00130089.1| hypothetical protein [Desulf...	165	4e-40
gi|23473410|ref|ZP_00128706.1| hypothetical protein [Desulf...	164	6e-40
gi|23474726|ref|ZP_00130018.1| hypothetical protein [Desulf...	162	2e-39
gi|23112916|ref|ZP_00098340.1| hypothetical protein [Desulf...	160	1e-38
gi|24113106|ref|NP_707616.1| formate dehydrogenase-N, nitra...	133	2e-30
gi|26247761|ref|NP_753801.1| Formate dehydrogenase, nitrate...	132	4e-30
gi|16129433|ref|NP_415991.1| formate dehydrogenase-N, nitra...	132	4e-30
gi|15801663|ref|NP_287681.1| formate dehydrogenase-N, nitra...	131	4e-30

Contig140gene2112

Sequences producing significant alignments:	Score (bits)	E Value
gi|23054054|gb|ZP_00080233.1| hypothetical protein [Geobact...	1640	0.0
gi|23474796|ref|ZP_00130088.1| hypothetical protein [Desulf...	834	0.0
gi|16605575|emb|CAC86667.1| formate dehydrogenase alpha sub...	810	0.0
gi|28948357|pdb|1H0H|A Chain A, Tungsten Containing Formate...	806	0.0
gi|23474726|ref|ZP_00130018.1| hypothetical protein [Desulf...	803	0.0
gi|23473409|ref|ZP_00128705.1| hypothetical protein [Desulf...	798	0.0
gi|16129433|ref|NP_415991.1| formate dehydrogenase-N, nitra...	733	0.0
gi|2506864|sp|P24183|FDNG_ECOLI Formate dehydrogenase, nitr...	733	0.0
gi|15801663|ref|NP_287681.1| formate dehydrogenase-N, nitra...	733	0.0
gi|20150976|pdb|1KQF|A Chain A, Formate Dehydrogenase N Fro...	732	0.0

Contig119gene498

Sequences producing significant alignments:	Score (bits)	E Value
gi|23054608|gb|ZP_00080755.1| hypothetical protein [Geobact...	1546	0.0
gi|14250944|emb|CAC39239.1| FdhA-II protein [Eubacterium ac...	454	e-126
gi|14717798|gb|AAB18330.2| formate dehydrogenase alpha subu...	449	e-124
gi|16263281|ref|NP_436074.1| NuoG2 NADH I CHAIN G 2 [Sinor...	416	e-115
gi|14250938|emb|CAC39234.1| FdhA-I protein [Eubacterium aci...	415	e-114
gi|5650750|emb|CAB51635.1| putative NADH-ubiquinone oxidore...	414	e-114
gi|29375957|ref|NP_815111.1| NAD-dependent formate dehydrog...	409	e-112
gi|15679548|ref|NP_276665.1| formate dehydrogenase, alpha s...	401	e-110
gi|1361902|pir||S57430 probable formate dehydrogenase (EC 1...	386	e-105
gi|26988909|ref|NP_744334.1| formate dehydrogenase, alpha s...	379	e-103

Contig148gene3285

Sequences producing significant alignments:	Score (bits)	E Value
gi|14250944|emb|CAC39239.1| FdhA-II protein [Eubacterium ac...	556	e-157
gi|14250938|emb|CAC39234.1| FdhA-I protein [Eubacterium aci...	527	e-148
gi|14717798|gb|AAB18330.2| formate dehydrogenase alpha subu...	520	e-146
gi|29375957|ref|NP_815111.1| NAD-dependent formate dehydrog...	502	e-141
gi|23054608|gb|ZP_00080755.1| hypothetical protein [Geobact...	500	e-140
gi|15679548|ref|NP_276665.1| formate dehydrogenase, alpha s...	478	e-133
gi|1361902|pir||S57430 probable formate dehydrogenase (EC 1...	451	e-125
gi|16263281|ref|NP_436074.1| NuoG2 NADH I CHAIN G 2 [Sinor...	442	e-122
gi|26988909|ref|NP_744334.1| formate dehydrogenase, alpha s...	417	e-115
gi|22987538|ref|ZP_00032616.1| hypothetical protein [Burkho...	409	e-112

Contig113gene269

Sequences producing significant alignments:	Score (bits)	E Value
gi|23055700|gb|ZP_00081789.1| hypothetical protein [Geobact...	1837	0.0
gi|23115051|ref|ZP_00100326.1| hypothetical protein [Desulf...	479	e-134
gi|23115047|ref|ZP_00100322.1| hypothetical protein [Desulf...	413	e-114
gi|11498802|ref|NP_070031.1| molybdopterin oxidoreductase, ...	339	1e-91
gi|23113320|ref|ZP_00098706.1| hypothetical protein [Desulf...	337	6e-91
gi|23115035|ref|ZP_00100310.1| hypothetical protein [Desulf...	327	5e-88
gi|23114874|ref|ZP_00100154.1| hypothetical protein [Desulf...	326	1e-87
gi|23113724|ref|ZP_00099076.1| hypothetical protein [Desulf...	309	1e-82
gi|23115033|ref|ZP_00100308.1| hypothetical protein [Desulf...	309	2e-82
gi|23113735|ref|ZP_00099086.1| hypothetical protein [Desulf...	296	2e-78

Contig139gene2012

Sequences producing significant alignments:	Score (bits)	E Value
gi|23053111|gb|ZP_00079320.1| hypothetical protein [Geobact...	1419	0.0
gi|23014021|gb|ZP_00053862.1| hypothetical protein [Magneto...	216	9e-55
gi|23135169|ref|ZP_00116925.1| hypothetical protein [Cytoph...	202	1e-50
gi|23473865|ref|ZP_00129160.1| hypothetical protein [Desulf...	192	1e-47
gi|24215966|ref|NP_713447.1| molybdopterin oxidoreductase, ...	182	2e-44
gi|11498802|ref|NP_070031.1| molybdopterin oxidoreductase, ...	179	1e-43
gi|23474822|ref|ZP_00130114.1| hypothetical protein [Desulf...	176	1e-42
gi|22970536|gb|ZP_00017603.1| hypothetical protein [Chlorof...	166	1e-39
gi|24375549|ref|NP_719592.1| polysulfide reductase, subunit...	161	4e-38
gi|23112121|ref|ZP_00097650.1| hypothetical protein [Desulf...	160	9e-38

Contig121gene627

Sequences producing significant alignments:	Score (bits)	E Value
gi|23054016|ref|ZP_00080195.1| hypothetical protein [Geobac...	2337	0.0
gi|2105093|emb|CAA71210.1| nitrate reductase alpha subunit ...	1481	0.0
gi|23056188|ref|ZP_00082242.1| hypothetical protein [Geobac...	1337	0.0
gi|14601314|ref|NP_147849.1| nitrate reductase alpha chain ...	1177	0.0
gi|30020253|ref|NP_831884.1| Respiratory nitrate reductase ...	1129	0.0
gi|21399996|ref|NP_655981.1| molybdopterin, Molybdopterin o...	1128	0.0
gi|1009366|emb|CAA62926.1| Respiratory nitrate reductase [B...	1111	0.0
gi|16080781|ref|NP_391609.1| nitrate reductase (alpha subun...	1108	0.0
gi|21698999|dbj|BAC02732.1| nitrate reductase alpha subunit...	1103	0.0
gi|18314193|ref|NP_560860.1| nitrate reductase alpha subuni...	1102	0.0

Contig135gene1636

Sequences producing significant alignments:	Score (bits)	E Value
gi|23056188|ref|ZP_00082242.1| hypothetical protein [Geobac...	2476	0.0
gi|2105093|emb|CAA71210.1| nitrate reductase alpha subunit ...	1418	0.0
gi|23054016|ref|ZP_00080195.1| hypothetical protein [Geobac...	1365	0.0
gi|14601314|ref|NP_147849.1| nitrate reductase alpha chain ...	1261	0.0

gi|21399996|ref|NP_655981.1|	molybdopterin, Molybdopterin o...	1237	0.0
gi|30020253|ref|NP_831884.1|	Respiratory nitrate reductase ...	1237	0.0
gi|1009366|emb|CAA62926.1|	Respiratory nitrate reductase [B...	1212	0.0
gi|16080781|ref|NP_391609.1|	nitrate reductase (alpha subun...	1210	0.0
gi|21698999|dbj|BAC02732.1|	nitrate reductase alpha subunit...	1206	0.0
gi|18314193|ref|NP_560860.1|	nitrate reductase alpha subuni...	1185	0.0

nar121, 135 operon Blast results

Contig121gene626

Sequences producing significant alignments:		Score (bits)	E Value
gi|23054015|gb|ZP_00080194.1|	(NZ_AAAS01000003) hypothetica...	466	e-130
gi|23056187|gb|ZP_00082241.1|	(NZ_AAAS01000025) hypothetica...	130	2e-29
gi|23058888|gb|ZP_00083905.1|	(NZ_AABA01000091) hypothetica...	94	2e-18
gi|23135168|gb|ZP_00116924.1|	(NZ_AABE01000014) hypothetica...	59	9e-08
gi|15594131|emb|CAC69865.1|	(Y10124) periplasmic cytochrome...	57	2e-07
gi|23136139|gb|ZP_00117866.1|	(NZ_AABE01000043) hypothetica...	54	3e-06
gi|23136218|gb|ZP_00117943.1|	(NZ_AABE01000045) hypothetica...	49	7e-05
gi|575386|emb|CAA57910.1|	(X82563) c-type cytochrome [Synec...	44	0.003
gi|17549783|ref|NP_523123.1|	(NC_003296) PROBABLE CYTOCHROM...	41	0.015
gi|23055766|gb|ZP_00081850.1|	(NZ_AAAS01000016) hypothetica...	41	0.022

Contig121gene627

Sequences producing significant alignments:		Score (bits)	E Value
gi|23054016|ref|ZP_00080195.1|	hypothetical protein [Geobac...	2337	0.0
gi|2105093|emb|CAA71210.1|	nitrate reductase alpha subunit ...	1481	0.0
gi|23056188|ref|ZP_00082242.1|	hypothetical protein [Geobac...	1337	0.0
gi|14601314|ref|NP_147849.1|	nitrate reductase alpha chain ...	1177	0.0
gi|30020253|ref|NP_831884.1|	Respiratory nitrate reductase ...	1129	0.0
gi|21399996|ref|NP_655981.1|	molybdopterin, Molybdopterin o...	1128	0.0
gi|1009366|emb|CAA62926.1|	Respiratory nitrate reductase [B...	1111	0.0
gi|16080781|ref|NP_391609.1|	nitrate reductase (alpha subun...	1108	0.0
gi|21698999|dbj|BAC02732.1|	nitrate reductase alpha subunit...	1103	0.0
gi|18314193|ref|NP_560860.1|	nitrate reductase alpha subuni...	1102	0.0

Contig121gene628

Sequences producing significant alignments:		Score (bits)	E Value
gi|23054017|gb|ZP_00080196.1|	(NZ_AAAS01000003) hypothetica...	962	0.0
gi|2105094|emb|CAA71211.1|	(Y10124) nitrate reductase beta ...	608	e-173
gi|97384|pir||S21535	nitrate reductase (EC 1.7.99.4) beta c...	607	e-172
gi|23056189|gb|ZP_00082243.1|	(NZ_AAAS01000025) hypothetica...	508	e-143
gi|15801456|ref|NP_287473.1|	(NC_002655) nitrate reductase ...	474	e-133
gi|16129188|ref|NP_415743.1|	(NC_000913) nitrate reductase ...	473	e-132
gi|16080780|ref|NP_391608.1|	(NC_000964) nitrate reductase ...	472	e-132
gi|21699000|dbj|BAC02733.1|	(AB087407) nitrate reductase be...	470	e-131
gi|1001849|emb|CAA59372.1|	(X85014) nitrate reductase beta ...	470	e-131
gi|21399997|ref|NP_655982.1|	(NC_003995) hypothetical prote...	465	e-130

Contig121gene629

Sequences producing significant alignments:		Score (bits)	E Value
gi|23054018|gb|ZP_00080197.1|	(NZ_AAAS01000003) hypothetica...	359	2e-98
gi|23056191|gb|ZP_00082245.1|	(NZ_AAAS01000025) hypothetica...	101	1e-20
gi|2105096|emb|CAA71213.1|	(Y10124) nitrate reductase gamma...	100	1e-20
gi|21699002|dbj|BAC02735.1|	(AB087407) nitrate reductase ga...	59	7e-08
gi|21399999|ref|NP_655984.1|	(NC_003995) Nitrate_red_gam, N...	57	2e-07
gi|3929525|gb|AAC82545.1|	(AF029224) NarI [Staphylococcus c...	57	2e-07
gi|15925384|ref|NP_372918.1|	(NC_002758) nitrate reductase ...	56	4e-07
gi|15599067|ref|NP_252561.1|	(NC_002516) respiratory nitrat...	55	1e-06

gi|16760125|ref|NP_455742.1|	(NC_003198) respiratory nitrat...	54	2e-06
gi|16765102|ref|NP_460717.1|	(NC_003197) nitrate reductase ...	54	2e-06

Contig135gene1635

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056187|gb|ZP_00082241.1|	(NZ_AAAS01000025) hypothetica...	444	e-124
gi|23054015|gb|ZP_00080194.1|	(NZ_AAAS01000003) hypothetica...	113	3e-24
gi|15594131|emb|CAC69865.1|	(Y10124) periplasmic cytochrome...	62	8e-09
gi|23058888|gb|ZP_00083905.1|	(NZ_AABA01000091) hypothetica...	41	0.014
gi|23136139|gb|ZP_00117866.1|	(NZ_AABE01000043) hypothetica...	33	2.4

Contig135gene1636

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056188|ref|ZP_00082242.1|	hypothetical protein [Geobac...	2476	0.0
gi|2105093|emb|CAA71210.1|	nitrate reductase alpha subunit ...	1418	0.0
gi|23054016|ref|ZP_00080195.1|	hypothetical protein [Geobac...	1365	0.0
gi|14601314|ref|NP_147849.1|	nitrate reductase alpha chain ...	1261	0.0
gi|21399996|ref|NP_655981.1|	molybdopterin, Molybdopterin o...	1237	0.0
gi|30020253|ref|NP_831884.1|	Respiratory nitrate reductase ...	1237	0.0
gi|1009366|emb|CAA62926.1|	Respiratory nitrate reductase [B...	1212	0.0
gi|16080781|ref|NP_391609.1|	nitrate reductase (alpha subun...	1210	0.0
gi|21698999|dbj|BAC02732.1|	nitrate reductase alpha subunit...	1206	0.0
gi|18314193|ref|NP_560860.1|	nitrate reductase alpha subuni...	1185	0.0

Contig135gene1637

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056189|gb|ZP_00082243.1|	(NZ_AAAS01000025) hypothetica...	1029	0.0
gi|97384|pir||S21535	nitrate reductase (EC 1.7.99.4) beta c...	542	e-153
gi|23054017|gb|ZP_00080196.1|	(NZ_AAAS01000003) hypothetica...	540	e-152
gi|2105094|emb|CAA71211.1|	(Y10124) nitrate reductase beta ...	535	e-151
gi|17989296|ref|NP_541929.1|	(NC_003318) NITRATE REDUCTASE ...	513	e-144
gi|15599069|ref|NP_252563.1|	(NC_002516) respiratory nitrat...	511	e-144
gi|16129188|ref|NP_415743.1|	(NC_000913) nitrate reductase ...	511	e-143
gi|15801456|ref|NP_287473.1|	(NC_002655) nitrate reductase ...	509	e-143
gi|22978247|gb|ZP_00024009.1|	(NZ_AAAI01000312) hypothetica...	508	e-143
gi|21284047|ref|NP_647135.1|	(NC_003923) nitrate reductase ...	507	e-142

Contig135gene1638

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056190|gb|ZP_00082244.1|	(NZ_AAAS01000025) hypothetica...	419	e-117
gi|17549197|ref|NP_522537.1|	(NC_003296) PUTATIVE RESPIRATO...	69	3e-11
gi|22978246|gb|ZP_00024008.1|	(NZ_AAAI01000312) hypothetica...	67	1e-10
gi|21699001|dbj|BAC02734.1|	(AB087407) nitrate reductase pr...	64	9e-10
gi|16080779|ref|NP_391607.1|	(NC_000964) nitrate reductase ...	59	4e-08
gi|1075873|pir||S52917	nitrate reductase delta chain - Baci...	59	5e-08
gi|16765103|ref|NP_460718.1|	(NC_003197) nitrate reductase ...	57	1e-07
gi|2105095|emb|CAA71212.1|	(Y10124) chaperone protein [Ther...	57	1e-07
gi|2147971|pir||S61308	nitrate reductase assembly protein n...	57	2e-07
gi|16760124|ref|NP_455741.1|	(NC_003198) respiratory nitrat...	57	2e-07

Contig135gene1639

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056191|gb|ZP_00082245.1|	(NZ_AAAS01000025) hypothetica...	353	9e-97
gi|23054018|gb|ZP_00080197.1|	(NZ_AAAS01000003) hypothetica...	111	9e-24
gi|2105096|emb|CAA71213.1|	(Y10124) nitrate reductase gamma...	96	4e-19
gi|21224834|ref|NP_630613.1|	(NC_003888) putative nitrate r...	95	7e-19
gi|21399999|ref|NP_655984.1|	(NC_003995) Nitrate_red_gam, N...	91	9e-18
gi|3929525|gb|AAC82545.1|	(AF029224) NarI [Staphylococcus c...	88	1e-16

gi|19552410|ref|NP_600412.1|	(NC_003450) COG2181:Nitrate re...	85	7e-16
gi|15925384|ref|NP_372918.1|	(NC_002758) nitrate reductase ...	84	2e-15
gi|21218773|ref|NP_624552.1|	(NC_003888) putative nitrate r...	78	7e-14
gi|16080778|ref|NP_391606.1|	(NC_000964) nitrate reductase ...	77	1e-13

Contig135gene1640

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056192|gb|ZP_00082246.1|	(NZ_AAAS01000025) hypothetica...	914	0.0
gi|11344599|gb|AAG34371.1|AF197465_3	(AF197465) putative ni...	436	e-121
gi|22978250|gb|ZP_00024012.1|	(NZ_AAAI01000312) hypothetica...	434	e-120
gi|17549193|ref|NP_522533.1|	(NC_003296) PUTATIVE NITRITE/N...	412	e-114
gi|15599072|ref|NP_252566.1|	(NC_002516) nitrite extrusion ...	391	e-107
gi|17989293|ref|NP_541926.1|	(NC_003318) NITRITE EXTRUSION ...	308	2e-82
gi|18413616|dbj|BAB84307.1|	(AB076402) putative nitrate tra...	279	7e-74
gi|14348603|gb|AAK61313.1|AF295359_4	(AF295359) NarK [Parac...	269	6e-71
gi|16264170|ref|NP_436962.1|	(NC_003078) putative nitrate t...	256	8e-67
gi|6689412|emb|CAB65479.1|	(Y10124) NarK1 protein [Thermus ...	246	4e-64

Contig135gene1641

Sequences producing significant alignments:		Score (bits)	E Value
gi|23056193|gb|ZP_00082247.1|	(NZ_AAAS01000025) hypothetica...	804	0.0
gi|11344600|gb|AAG34372.1|AF197465_4	(AF197465) putative ni...	527	e-148
gi|22978249|gb|ZP_00024011.1|	(NZ_AAAI01000312) hypothetica...	518	e-146
gi|17549194|ref|NP_522534.1|	(NC_003296) PROBABLE NITRITE/N...	499	e-140
gi|15599071|ref|NP_252565.1|	(NC_002516) nitrite extrusion ...	491	e-138
gi|2765453|emb|CAA75539.1|	(Y15252) nitrate extrusion prote...	491	e-138
gi|17989293|ref|NP_541926.1|	(NC_003318) NITRITE EXTRUSION ...	469	e-131
gi|14348603|gb|AAK61313.1|AF295359_4	(AF295359) NarK [Parac...	432	e-120
gi|16765106|ref|NP_460721.1|	(NC_003197) MFS superfamily, n...	400	e-110
gi|16760121|ref|NP_455738.1|	(NC_003198) nitrite extrusion ...	399	e-110

Appendix VI

Transmembrane prediction for NarI in *nar121*

TMpred output for NarI contig121
[ISREC-Server] Date: Sat Jul 26 0:02:37 MET 2003

Sequence: MPV...PRK, length: 260
Prediction parameters: TM-helix length between 17 and 33

1.) Possible transmembrane helices
The sequence positions in brackets denominate the core region.
Only scores above 500 are considered significant.

```
Inside to outside helices :   6 found
      from        to    score center
  36 (  36)  55 (  53)   1990     45
  88 (  88) 105 ( 105)   2021     96
 120 ( 120) 138 ( 138)   1987    128
 146 ( 150) 170 ( 167)   2178    159
 177 ( 177) 197 ( 195)    981    185
 212 ( 214) 232 ( 230)   1615    222

Outside to inside helices :   6 found
      from        to    score center
  36 (  36)  58 (  54)   2260     45
  86 (  86) 103 ( 103)   2138     95
 120 ( 120) 138 ( 138)   2475    128
 146 ( 148) 173 ( 165)   1886    156
 172 ( 175) 193 ( 193)   1174    183
 212 ( 214) 230 ( 230)   1487    222
```

2.) Table of correspondences
Here is shown, which of the inside->outside helices correspond to which of the outside->inside helices.

Helices shown in brackets are considered insignificant.
A "+"-symbol indicates a preference of this orientation.
A "++"-symbol indicates a strong preference of this orientation.

```
         inside->outside | outside->inside
   36-  55 (20) 1990     |    36-  58 (23) 2260 ++
   88- 105 (18) 2021     |    86- 103 (18) 2138  +
  120- 138 (19) 1987     |   120- 138 (19) 2475 ++
  146- 170 (25) 2178 ++  |   146- 173 (28) 1886
  177- 197 (21)  981     |   172- 193 (22) 1174  +
  212- 232 (21) 1615  +  |   212- 230 (19) 1487
```

```
--------------------------------------------------------------------------
-
```

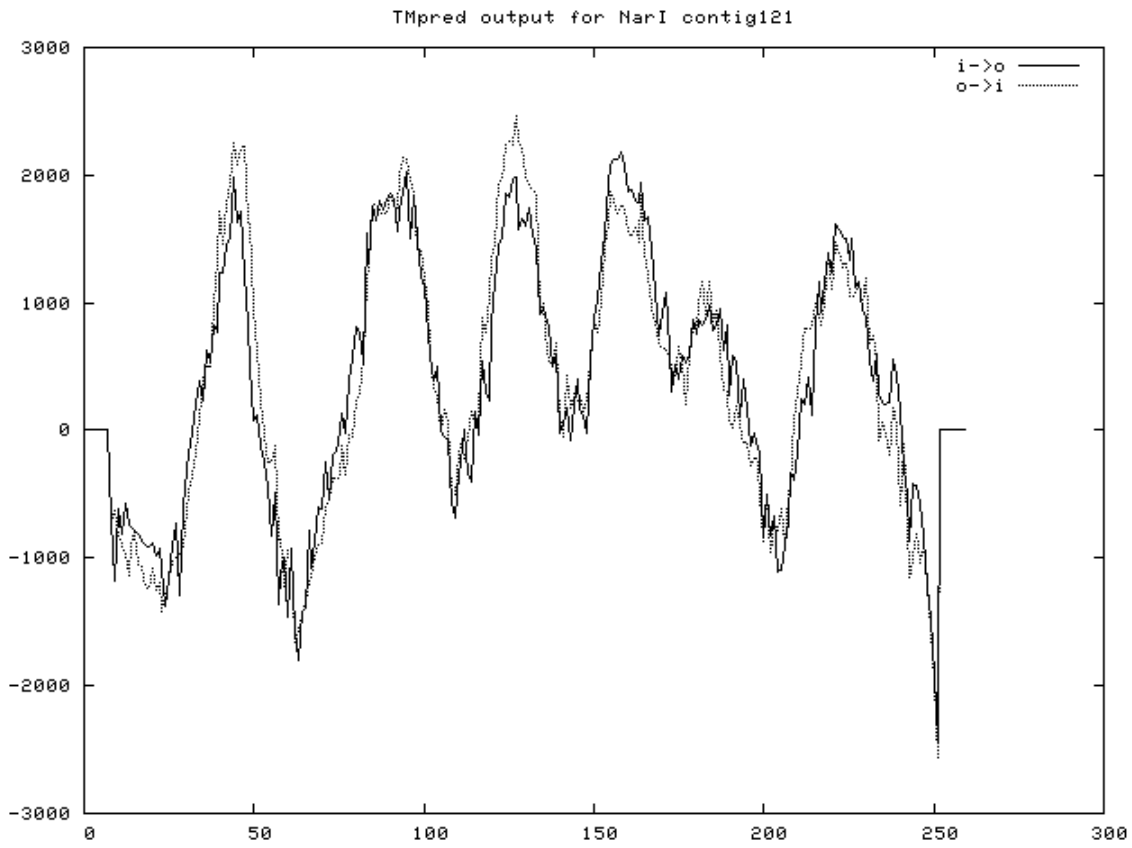
3.) Suggested models for transmembrane topology

These suggestions are purely speculative and should be used with **extreme caution** since they are based on the assumption that all transmembrane helices have been found.
In most cases, the Correspondence Table shown above or the prediction plot that is also created should be used for the topology assignment of unknown proteins.

```
2 possible models considered, only significant TM-segments used

-----> STRONGLY prefered model: N-terminus outside
 6 strong transmembrane helices, total score : 11723
 # from   to length score orientation
 1   36   58 (23)    2260 o-i
 2   88  105 (18)    2021 i-o
 3  120  138 (19)    2475 o-i
 4  146  170 (25)    2178 i-o
 5  172  193 (22)    1174 o-i
 6  212  232 (21)    1615 i-o

------> alternative model
 6 strong transmembrane helices, total score : 10469
 # from   to length score orientation
 1   36   55 (20)    1990 i-o
 2   86  103 (18)    2138 o-i
 3  120  138 (19)    1987 i-o
 4  146  173 (28)    1886 o-i
 5  177  197 (21)     981 i-o
 6  212  230 (19)    1487 o-i
```



Transmembrane prediction for NarI in *nar135*

TMpred output for NarI contig135
[ISREC-Server] Date: Sat Jul 26 0:05:27 MET 2003

Sequence: MLN...KVE, length: 231
Prediction parameters: TM-helix length between 17 and 33

1.) Possible transmembrane helices
The sequence positions in brackets denominate the core region.
Only scores above 500 are considered significant.

```
Inside to outside helices :   5 found
      from        to    score center
   5 (   5)  24 (  24)   2070     14
  59 (  59)  78 (  76)   1158     67
  93 (  93) 111 ( 111)   1990    101
 128 ( 128) 148 ( 148)   2021    138
 185 ( 187) 203 ( 203)   1593    195

Outside to inside helices :   6 found
```

```
      from        to    score center
   9 (  13)  32 (  32)   2224     21
  59 (  59)  76 (  76)   1307     68
  93 (  93) 111 ( 109)   2478    101
 128 ( 128) 146 ( 146)   1803    138
 152 ( 152) 169 ( 169)    438    160
 185 ( 187) 203 ( 203)   1715    195
```

2.) Table of correspondences
Here is shown, which of the inside->outside helices correspond to which of the outside->inside helices.

Helices shown in brackets are considered insignificant.
A "+"-symbol indicates a preference of this orientation.
A "++"-symbol indicates a strong preference of this orientation.

```
          inside->outside | outside->inside
    5-  24 (20) 2070      |     9-  32 (24) 2224  +
   59-  78 (20) 1158      |    59-  76 (18) 1307  +
   93- 111 (19) 1990      |    93- 111 (19) 2478 ++
  128- 148 (21) 2021 ++   |   128- 146 (19) 1803
                         |(  152- 169 (18)  438 ++ )
  185- 203 (19) 1593      |   185- 203 (19) 1715  +
```

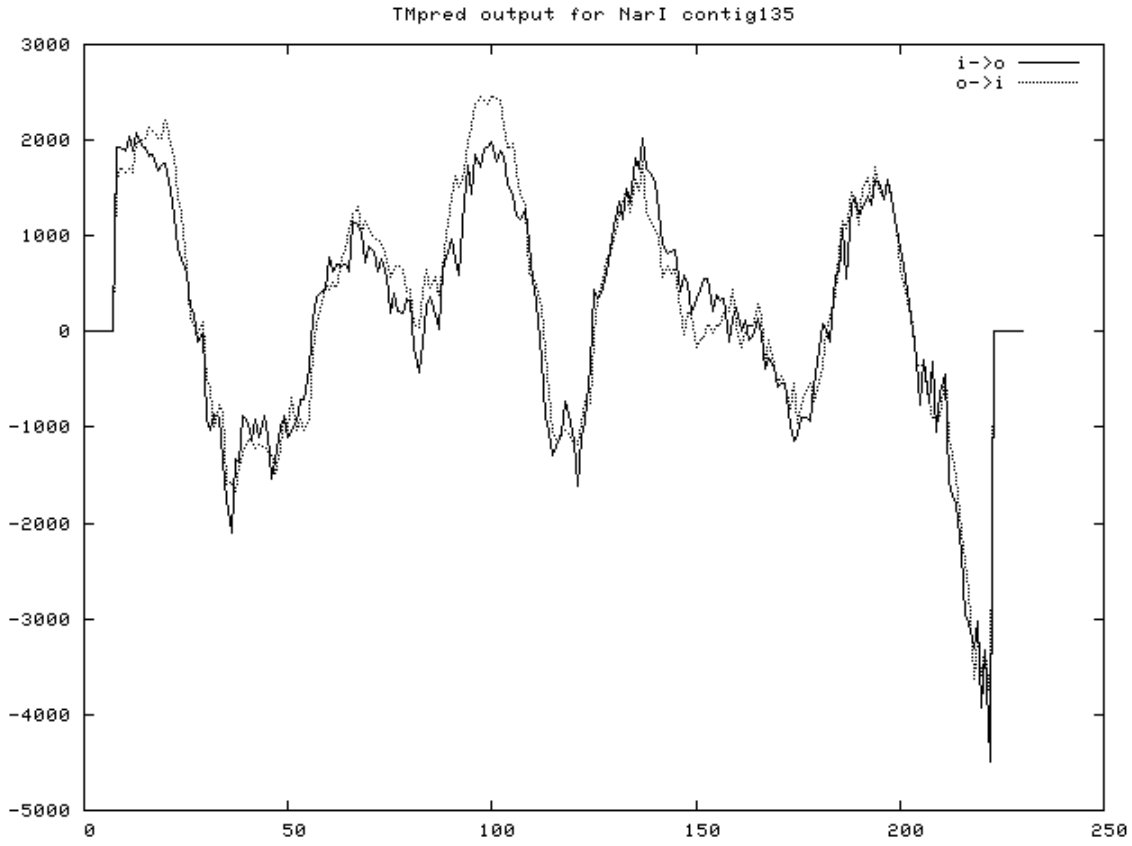
3.) Suggested models for transmembrane topology
These suggestions are purely speculative and should be used with **extreme caution** since they are based on the assumption that all transmembrane helices have been found.
In most cases, the Correspondence Table shown above or the prediction plot that is also created should be used for the topology assignment of unknown proteins.

```
2 possible models considered, only significant TM-segments used

-----> STRONGLY prefered model: N-terminus outside
 5 strong transmembrane helices, total score : 9596
 # from   to length score orientation
 1    9   32 (24)    2224 o-i
 2   59   78 (20)    1158 i-o
 3   93  111 (19)    2478 o-i
 4  128  148 (21)    2021 i-o
 5  185  203 (19)    1715 o-i

------> alternative model
 5 strong transmembrane helices, total score : 8763
 # from   to length score orientation
 1    5   24 (20)    2070 i-o
 2   59   76 (18)    1307 o-i
```

```
3   93  111 (19)    1990 i-o
4  128  146 (19)    1803 o-i
5  185  203 (19)    1593 i-o
```



Transmembrane prediction for $NarK_1$ in *nar135*

TMpred output for NarK1 in contig135
[ISREC-Server] Date: Sat Jul 26 0:07:47 MET 2003

Sequence: VVP...ILE, length: 525
Prediction parameters: TM-helix length between 17 and 33

1.) Possible transmembrane helices
The sequence positions in brackets denominate the core region.
Only scores above 500 are considered significant.

```
Inside to outside helices :  12 found
      from        to    score center
 125 ( 125) 144 ( 142)   2333    134
 154 ( 158) 176 ( 176)    962    166
 180 ( 180) 200 ( 200)   2475    190
```

```
 209 ( 209) 227 ( 227)   2395    217
 241 ( 243) 269 ( 260)   1159    252
 275 ( 275) 293 ( 293)   1860    284
 320 ( 323) 343 ( 341)   1375    333
 352 ( 352) 370 ( 368)   1400    360
 382 ( 382) 400 ( 398)   1991    390
 417 ( 420) 438 ( 436)   2590    428
 458 ( 458) 477 ( 477)   2234    467
 490 ( 490) 507 ( 507)   1714    499

Outside to inside helices :  12 found
      from        to    score center
 125 ( 125) 144 ( 142)   2069    133
 153 ( 153) 171 ( 171)    944    163
 184 ( 184) 200 ( 200)   2290    192
 209 ( 209) 227 ( 227)   2496    219
 241 ( 243) 267 ( 259)   1054    251
 275 ( 275) 292 ( 292)   1945    283
 323 ( 323) 341 ( 341)   1643    333
 352 ( 352) 369 ( 369)   1710    360
 383 ( 383) 400 ( 400)   1884    391
 418 ( 420) 438 ( 438)   2123    429
 455 ( 457) 473 ( 473)   2354    465
 489 ( 489) 509 ( 509)   1977    499
```

2.) Table of correspondences

Here is shown, which of the inside->outside helices correspond to which of the outside->inside helices.

Helices shown in brackets are considered insignificant.
A "+"-symbol indicates a preference of this orientation.
A "++"-symbol indicates a strong preference of this orientation.

```
          inside->outside | outside->inside
   125- 144 (20) 2333 ++   |   125- 144 (20) 2069
   154- 176 (23)  962      |   153- 171 (19)  944
   180- 200 (21) 2475  +   |   184- 200 (17) 2290
   209- 227 (19) 2395      |   209- 227 (19) 2496  +
   241- 269 (29) 1159  +   |   241- 267 (27) 1054
   275- 293 (19) 1860      |   275- 292 (18) 1945  +
   320- 343 (24) 1375      |   323- 341 (19) 1643 ++
   352- 370 (19) 1400      |   352- 369 (18) 1710 ++
   382- 400 (19) 1991  +   |   383- 400 (18) 1884
   417- 438 (22) 2590 ++   |   418- 438 (21) 2123
   458- 477 (20) 2234      |   455- 473 (19) 2354  +
   490- 507 (18) 1714      |   489- 509 (21) 1977 ++
```

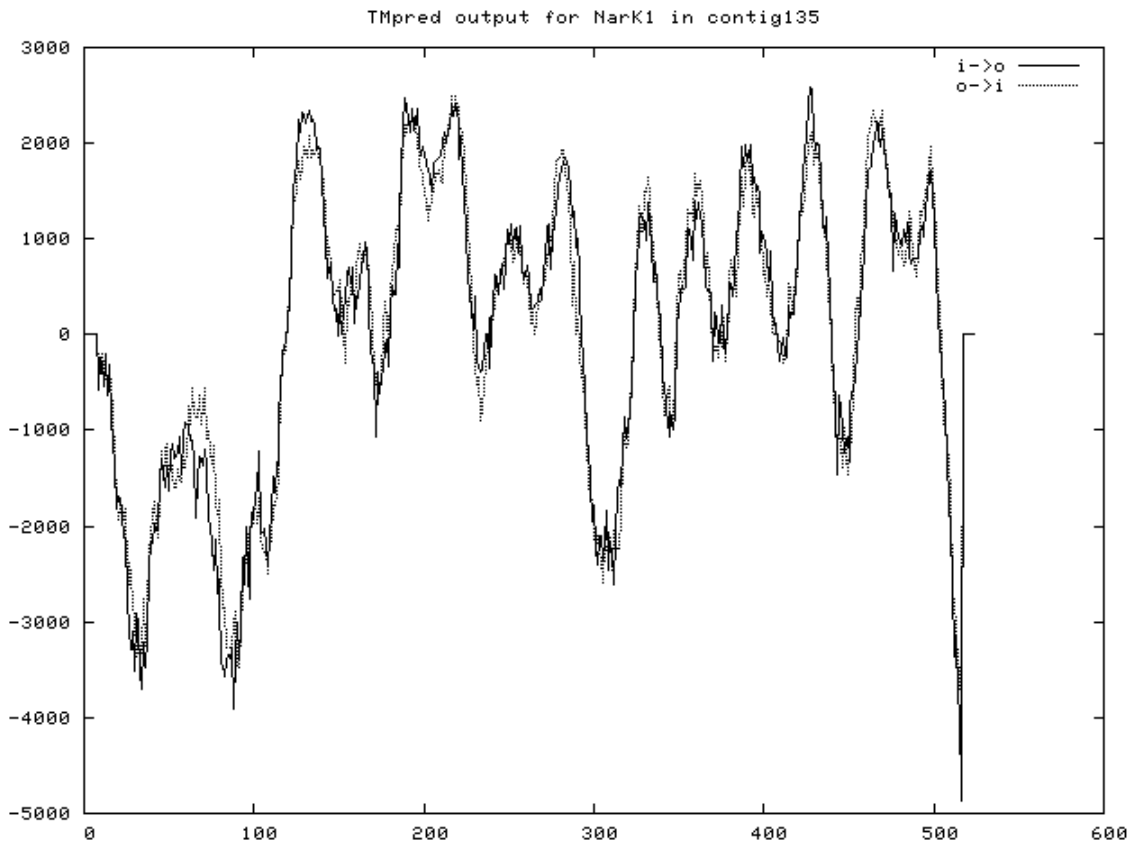
3.) Suggested models for transmembrane topology

These suggestions are purely speculative and should be used with **extreme caution** since they are based on the assumption that all transmembrane helices have been found.
In most cases, the Correspondence Table shown above or the prediction plot that is also created should be used for the topology assignment of unknown proteins.

```
2 possible models considered, only significant TM-segments used

-----> STRONGLY prefered model: N-terminus inside
12 strong transmembrane helices, total score : 22762
 # from   to length score orientation
 1  125  144 (20)    2333 i-o
 2  153  171 (19)     944 o-i
 3  180  200 (21)    2475 i-o
 4  209  227 (19)    2496 o-i
 5  241  269 (29)    1159 i-o
 6  275  292 (18)    1945 o-i
 7  320  343 (24)    1375 i-o
 8  352  369 (18)    1710 o-i
 9  382  400 (19)    1991 i-o
10  418  438 (21)    2123 o-i
11  458  477 (20)    2234 i-o
12  489  509 (21)    1977 o-i

------> alternative model
12 strong transmembrane helices, total score : 22215
 # from   to length score orientation
 1  125  144 (20)    2069 o-i
 2  154  176 (23)     962 i-o
 3  184  200 (17)    2290 o-i
 4  209  227 (19)    2395 i-o
 5  241  267 (27)    1054 o-i
 6  275  293 (19)    1860 i-o
 7  323  341 (19)    1643 o-i
 8  352  370 (19)    1400 i-o
 9  383  400 (18)    1884 o-i
10  417  438 (22)    2590 i-o
11  455  473 (19)    2354 o-i
12  490  507 (18)    1714 i-o
```



Transmembrane prediction for NarK$_2$ in *nar135*

TMpred output for NarK2 contig135
[ISREC-Server] Date: Sat Jul 26 0:10:00 MET 2003

Sequence: MPT...VPC, length: 475
Prediction parameters: TM-helix length between 17 and 33

1.) Possible transmembrane helices
The sequence positions in brackets denominate the core region.
Only scores above 500 are considered significant.

```
Inside to outside helices :  12 found
      from        to    score center
  44 (  44)  64 (  61)   2517     53
  78 (  81) 106 ( 100)   1267     90
 111 ( 111) 128 ( 128)   1321    120
 137 ( 137) 153 ( 153)   1882    145
 185 ( 185) 203 ( 203)   1503    194
 223 ( 225) 242 ( 242)   2309    233
 265 ( 265) 283 ( 283)   2214    275
```

```
 301 ( 301) 319 ( 319)    696    311
 328 ( 330) 348 ( 346)   2535    338
 350 ( 352) 372 ( 369)   2340    361
 414 ( 414) 434 ( 432)   1556    424
 446 ( 446) 464 ( 464)   2326    456

Outside to inside helices :  12 found
      from        to    score center
  42 (  45)  65 (  62)   2498     54
  79 (  79) 104 (  97)   1465     89
 109 ( 111) 127 ( 127)   1534    119
 138 ( 142) 164 ( 164)   1914    152
 181 ( 183) 203 ( 200)   1300    192
 223 ( 225) 242 ( 242)   2006    233
 265 ( 265) 288 ( 283)   1701    275
 301 ( 301) 318 ( 318)    766    309
 333 ( 333) 352 ( 352)   2132    343
 352 ( 352) 372 ( 372)   2013    362
 415 ( 415) 431 ( 431)   1366    423
 446 ( 446) 466 ( 464)   2558    456
```

2.) Table of correspondences

Here is shown, which of the inside->outside helices correspond to which of the outside->inside helices.

Helices shown in brackets are considered insignificant.
A "+"-symbol indicates a preference of this orientation.
A "++"-symbol indicates a strong preference of this orientation.

```
          inside->outside | outside->inside
   44-  64 (21) 2517      |    42-  65 (24) 2498
   78- 106 (29) 1267      |    79- 104 (26) 1465  +
  111- 128 (18) 1321      |   109- 127 (19) 1534 ++
  137- 153 (17) 1882      |   138- 164 (27) 1914
  185- 203 (19) 1503 ++   |   181- 203 (23) 1300
  223- 242 (20) 2309 ++   |   223- 242 (20) 2006
  265- 283 (19) 2214 ++   |   265- 288 (24) 1701
  301- 319 (19)  696      |   301- 318 (18)  766
  328- 348 (21) 2535 ++   |   333- 352 (20) 2132
  350- 372 (23) 2340 ++   |   352- 372 (21) 2013
  414- 434 (21) 1556  +   |   415- 431 (17) 1366
  446- 464 (19) 2326      |   446- 466 (21) 2558 ++
```

-

3.) Suggested models for transmembrane topology

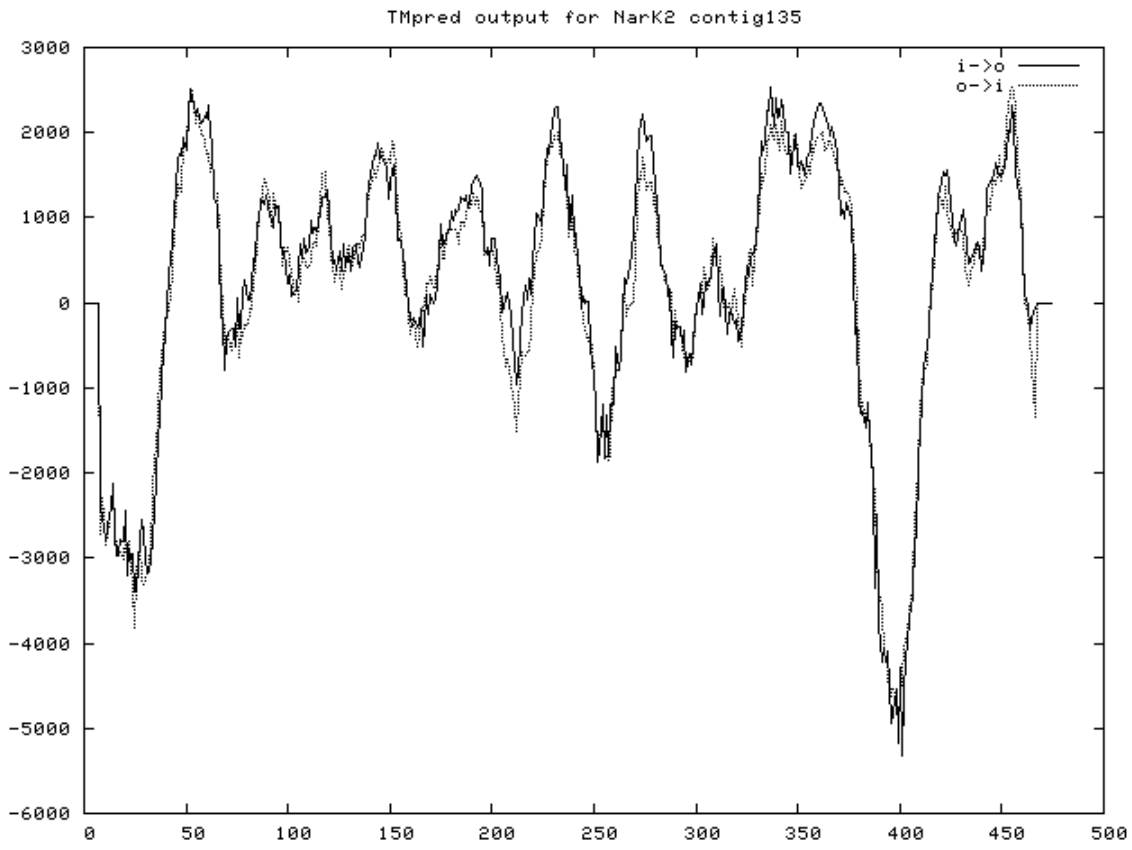
These suggestions are purely speculative and should be used with **extreme caution** since they are based on the assumption that all transmembrane helices have been found.
In most cases, the Correspondence Table shown above or the prediction plot that is also created should be used for the topology assignment of unknown proteins.

```
2 possible models considered, only significant TM-segments used

*** the models differ in the number of TM-helices ! ***

-----> STRONGLY prefered model: N-terminus inside
12 strong transmembrane helices, total score : 22368
 # from   to length score orientation
 1   44   64 (21)    2517 i-o
 2   79  104 (26)    1465 o-i
 3  111  128 (18)    1321 i-o
 4  138  164 (27)    1914 o-i
 5  185  203 (19)    1503 i-o
 6  223  242 (20)    2006 o-i
 7  265  283 (19)    2214 i-o
 8  301  318 (18)     766 o-i
 9  328  348 (21)    2535 i-o
10  352  372 (21)    2013 o-i
11  414  434 (21)    1556 i-o
12  446  466 (21)    2558 o-i

------> alternative model
11 strong transmembrane helices, total score : 19433
 # from   to length score orientation
 1   42   65 (24)    2498 o-i
 2   78  106 (29)    1267 i-o
 3  109  127 (19)    1534 o-i
 4  137  153 (17)    1882 i-o
 5  181  203 (23)    1300 o-i
 6  223  242 (20)    2309 i-o
 7  265  288 (24)    1701 o-i
 8  301  319 (19)     696 i-o
 9  333  352 (20)    2132 o-i
10  414  434 (21)    1556 i-o
11  446  466 (21)    2558 o-i
```


TMpred output for NarK2 contig135
i->o
o->i
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
0
50
100
150
200
250
300
350
400
450
500

Appendix VII

Protein sequence alingment of NarC from *G. metallireducens* and *T. thermophilus*

```
G.metacytC121      MKTVIIGGVAASAACAMTRRMLVAMVIALLASAMHPHQGSSKERAEGDGKALFQAKCSPC 60
G.metacytC135      --------MARGILRAAVSRVCLVLVLSLAAHAYG-----------DPGKDLFDKQCSGC 41
T.thermcytC        ----------------MARRLLPLALAFLLGLSLAQ-----------DGKALYGTHCASC 33
                                  . *:    :  * . :              ** *:  :*: *

G.metacytC121      HTIGGGRLVG--PDLKGVTARRDRGWLERFISAPDKVLASGDPVAARLFQESNKVPMPNL 118
G.metacytC135      HTVGGGDSGG--PDLKGVTDRRSEEWLESIIIEPDKLAAT-DPARKELIKKYG-YEMPKL 97
T.thermcytC        HGAEGQGIPGAIPPLAGNPRVQDEAYVVRAVREG-----LSGPLEVGGVTYDG-VMPPLP 87
                   *   *    *  * * * .  :.. ::   :          .*     .   .    *

G.metacytC121      GLGQDQVNVLLNYLSAPEG---ISPSKAEPPASPVQEEGDPSRGEKFFTGALPFAQGGTP 175
G.metacytC135      GIGHDDAIKIITWLRGGGGGAQTGSPPADEHPEVVVTPALVAKGKAFFTGQKPLANGGAP 157
T.thermcytC        QVSEAEARAIAQYLKGLGG----APAQAAPAPQVQ---GDPALGRALYLGQKALKNGGAP 140
                    :.. :.  :  :* .  *    ... *   ..     .  : *. :: *  .: :**:*

G.metacytC121      CIACHQVAGIPPLGGGALGPDLTTIVATFG-DTALSSALATLPFPTMKPIFGDHPLTRGE 234
G.metacytC135      CIACHRISTPGITGGNLAVSNLSASYQKMG-EKGMRGALKALKFPTMKKIYENRPLTDDE 216
T.thermcytC        CQACHTVAGVGFFGGGALGKDLTDAAKRLGGEAGLAALVENPAFPVMREAYKGKPLTKEE 200
                   * *** ::     **.    :*:     :* : .: . :    **.*:  : .:***  *

G.metacytC121      QGDLLAFFRKVAPRPPVTATTRIALWAGGGFLVLLLLTGALGRRRLGSVRGTLVDEANRA 294
G.metacytC135      VTSLIALYKDAALTKSTPSASFFPAYGAGTFVVFLVGLTLYKRRTR-------------- 262
T.thermcytC        ATALAAFLVQVSQEAPRPSSLYVGRFLVAGLVVLALLLLYQAVLWQLRPRSLAERIRAQL 260
                      * *:  ..:   . .::  .  :  . ::*: :

G.metacytC121      GR 296
G.metacytC135      --
T.thermcytC        RR 262
```

Protein sequence alingment of NarG from *G. metallireducens* and *T. thermophilus*

```
T.thermNarG         -MRDWIKEVENPAERKWEEFYRNRFQHDKRVRTTHGVNCTGSCSWEVFVKDGIVTWELQA 59
G.metaconti121      -MR-WIKELTDPKARRWEEFYRNRWQHDRVVRSTHGVNCTGGCSWLVYVKEGIVAWEMQA 58
G.metaconti135      MTQERLKDDRCGCDRGWEEFYRNRWQYDKVVRSTHGVNCTGSCSWMVHVKDGIVGWELQA 60
                      :  :*:      * ********:*:*: **:********.*** *.**:*** **:**

T.thermNarG         TDYPSLEAGLPPYEPRGCQRGISFSWYLYSPIRVKYPYAKGALLDLWREAKKAHGDPVAA 119
G.metaconti121      TDYPRLEAGLPPYEPRGCQRGISFSWYLYSPLRIKHPTIRGSLLDLWRAAKEEHSDPLAA 118
G.metaconti135      NDYPQFDGTIPNHEPRGCTRGISYSWYLYSPLRVKYPYMRGALIDLWRDARSRHEDPVEA 120
                    .*** ::. :* :***** ****:*******:*:*:*  :*:*:**** *:. * **: *

T.thermNarG         WEAIQNDPHKRKRYQKARGKGGFRRASWDEVLELIAAAVVSTVKRYGPDRVIGFSPIPAM 179
G.metaconti121      WGSIVEDSEKRRSYQQARGKGGFRRTSWDEALEITAASTLYTIKRYGPDRIVGFSPIPAM 178
G.metaconti135      WKSIVENPESRRAYVSKRGTGGMRRADWDEVTELIAASSIYTAKKYGPDRVIGFSPIPAM 180
                    * :* ::...*: * . **.**:**:.***. *: **: : * *:*****::********

T.thermNarG         SQISYAAGSRFLSLLGGVPMTFYDWYCDLPNASPEIWGEQTDVHESADWYNARFIAVMGS 239
G.metaconti121      SMCSYAAGTRLLSLLGGVVLSFYDWYADLPPASPETWGEKTDVAESADWYNSKYIVVMGT 238
G.metaconti135      SMISYAAGTRFLQLFGGVAMSFYDWYCDLPPASPQVWGEQTDVNESADWYNASYIVLCGS 240
                    *  *****:*:*.*:*** ::*****.*** ***: ***:*** *******: :*.: *:

T.thermNarG         NLNMTRTPDTHFIAEVRHAGAKLTVFSPDFSQVSKYADWWIPIHPGQDGAFWMAVNHVLL 299
G.metaconti121      NLSMTRTPDVHFVAEARTNGAKLVVLSPDFSQVSKYADWWLPVTAGHDGAFWMAVNHVIL 298
G.metaconti135      NVPMTRTPDAHFMTEARYRGTKVVVMSPDYNIATKFADNWLPVEQGHDGAFWMAVNHVIL 300
                    *: ******.**::*.*  *:*:.*:***:. .:*:** *:*:  *:***********:*

T.thermNarG         KEFYADREVPYFQDYLKRYTDAPFLVEIKDGR-----PGRYLRANRLAEYAEEENGDFKL 354
G.metaconti121      REFYVERHVPYFADYLARYSDAPFLVTLDRGDGERYVPGRLLRAESLPRYRREENGEWKF 358
G.metaconti135      KECYADRQVPFFQEYALKYTDLPFLVKLEEKDG-ALKQGEFLRAAEFERSASLEHADWTL 359
                    :* *.:*.**:* :*  :*:* **** :.         *. ***  : .    *:.::.:

T.thermNarG         LVFDEAKG-PRMPMGTLGFRWQKEKGKWNLKLEDPRTGEPLNPRLSLLGVEDEVVLVEFD 413
```

```
G.metaconti121      LVFDRLSGEPRMPGGSIGFRWQREAGRWNLEMKDGLDGAEIEPLLSLLDGRDDVLPVSIT 418
G.metaconti135      CVADGAGD-VKIPHGSLGTRWSKQEGKWNLDMTDLVDGTPIDCRLTLLGG--ETGKVRFR 416
                     * *    .  ::* *::* **.:: *:***.: *   *  ::  *:**.   :.  * :

T.thermNarG         DFAADRRLKRGVPVKYVVTKDGERVAVATVFDLLMAQFGVGRGLPGDYPASYEDDLPYTP 473
G.metaconti121      DYAGGTSVARGVPVRYLETATG-RTPVTTVFDLLMANFGVGRGLSGDYPEDYNAAAPGTP 477
G.metaconti135      -FEAEGESVREVPVRRIETKNG-PVTVATVYDLLMAQFGVSRGLGGDYPKSYDSDKPFTP 474
                     : .     * ***: : *  *  ..*:**:*****:***.*** **** .*:   * **

T.thermNarG         AWQEKWTGIHRDTLLKYARAWGENGLKTKGRNLIIIGAGINHWYHNNLMYRAGIVALMLT 533
G.metaconti121      AWQEEYTGVGRETLIRFAREWAGTAERTRGKCTIIVGSGANHWYHSNLTYRTGITALILC 537
G.metaconti135      KWQEKYTGIAAESLIKIAREWAENGEKSGGRNMIIIGAGVNHWYHNDLIYRAAITALILT 534
                     ***:**:  ::*:: ** *. .. :: *:  **:*:* *****.:* **:.*.**:*

T.thermNarG         GSVGVNGGGLAHYVGQEKLANQASWAPIAFATDWGYPPRQQNAPSFHYVHSDQWRYERGF 593
G.metaconti121      GCVGVNGGGLNHYTGQEKVAPEASWSLLAFAKDWTKGGRFQNTPSFHYVHSDQWRYDGGA 597
G.metaconti135      GSVGRNGAGLAHYVGQEKVVPLAPWTSIAMAQDWVKPSRLQNTPSFWYIHSDQWRYDRTF 594
                    *.** **.** **.****:.  *.*: :*:* **    * **:*** *:*******:

T.thermNarG         SAYDKTAKG--LSDHTIDHQVRAVRKGWLPFFPQFNKSPLEVVAEAEAKGAKTEAEIVQY 651
G.metaconti121      ADPRPDPKGGPAGRHPMDLQVDAVRMGWLPFYPQFDRNPLELVKEAEAAGAKGDEEIIRW 657
G.metaconti135      VDYLKPETGERMPLHAADMNAKAARLGWLPFAPHFNESPLRLVEAAKAAGAQSDDEIRSW 654
                           .*     *. * :. *.* ***** *:*:..**.:*  *:* **: : **  :

T.thermNarG         VVEALKRGDS-FAVEDPDAPENWPRVWFIWRGNAIGTSAKGHEYFLKHYLGTHTNAI-AE 709
G.metaconti121      VVEELEKKRLRFAVEEPDAPENWPRLWLIWRGNALLSSAKGHEYFLRHYLGTHDSSI-AD 716
G.metaconti135      LVNRLKSGETRFAIEDPDGQGNSPKVWFIWRANAISSSAKGHEFFLKHVIGAPNSSVSAK 714
                    :*: *:     **:*:**.  * *::*:***.**: :******:**:* :*:  .:: *.

T.thermNarG         EVAEGHVTEVVYRKPAPEGKLDLVVDLNFRMDTSALYSDIVLPAATWYEKDDLNTTDLHT 769
G.metaconti121      ECAAEQVENAAWQDAAPRGKLDLVVDINFRMDTSALYSDIVLPTATWYEKDDLNTTDLHS 776
G.metaconti135      EVAKGQVKDLVWHDKAPEGKMDLVVDLNFRMDTSTLYSDIVLPTATWYEKSDLNTTDMHS 774
                    * *  :* : .::. **.**:*****:*******:********:******.******:*:

T.thermNarG         FINPLQAAVPPAWESKPDWEIFKAIAKKVSELARVHLPRPVKDLVMIPLQHDTPDELAQL 829
G.metaconti121      FIHPLSAAVPPCWESRSDWEIFKALAEKISALARIHFPEPVRDIVAAPLLHDTPGEIAQS 836
G.metaconti135      FINCLDAAVPPSWESKSDWKIFTRIAKKVSELAVKHLPTPVKDIIAAPLLHDTPGEIAQR 834
                    **: *.*****.***:.**:**. :*:*:* **  *:* **:*::  ** ****.*:**

T.thermNarG         EDRDWKRGEVEPIPGKTMPKFRVVERDYTKLYEKFVTLGPTVEKVGVGMHGLTIPVEDFY 889
G.metaconti121      EVRGWHRGECEAVPGTTMPRLAVVERDFVHLLDRFVSLGEEVKREGIEERGLRWPVADLY 896
G.metaconti135      QVKDWKLGECEAIPGKTMPNLAIVERDYVNLYNRYVSVGPHIG--HLHAHGVSWEADDVH 892
                    : :.*: ** *.:**.***.: :****:.:* :::*::*  :    :  :*:   . *.:

T.thermNarG         KELAERQP-RLYEGEKRPSLEEARQVAEAILLLDPVSNGELAYRAFLDEEKKTGVKLTDL 948
G.metaconti121      DELLESAPVREWGGKRFPSLAEAVDAANAILHFAPETNGEVAWRAFRSLEEKTGLTLADL 956
G.metaconti135      EQLLETMPTRTWNGKSFLDIEDEKTVADVLLSFAPESNGELAYRAYQNLEKRVGKPLAGI 952
                    .:* *  * * : *:   .: :   .*:.:* : * :***:*:**: . *::.*  *:.:

T.thermNarG         AEGNRHVRISFKDIVAQPRRQLTTPTWSAIINHGRAYSPYTLNVERLVPWRTLTGRQHFY 1008
G.metaconti121      ASDYRGTRYRFSDLVSQPRRILTSPCWSGVTNNGRPYAPYCLNVERLVPWRTLTGRQHFY 1016
G.metaconti135      AAGSRDLRIKWEHVTQKPQRYCSTPVWSGLVNNGRPYAPYTLNVEYDVPWRTLSGRQSLY 1012
                    * . *  *  :..:. :*:*  ::* **.: *:**.*:** ****  ******:*** :*

T.thermNarG         LDHPNYLAWGEHLPTYKPRPEVHMLQETEKAAKEAQGKLMNYITPHGKWSIHSTYSENHR 1068
G.metaconti121      LDHGWYRAFGEHLPTFKTRIDTEESGHLAKSRPVGKALMLNCLTPHGKWHMHTTYYDNHR 1076
G.metaconti135      LDHPYYGEFHEGLPTFKAKLNPAILDETEGE----QGLVLNFLTPHGKWSIHSTYSDNLR 1068
                    ***  *  : * ***:*.: :     .        :. ::* :****** :*:** :* *

T.thermNarG         MMTLSRGGYPVWLNDKDAAELGIRDNDWVELFNDNGVFVQRAIVSARIPRGTVFVYHATE 1128
G.metaconti121      MLTLSRGIEPFWLNDRDAEEIGVKDNEWVEVYNDNGVVVTRAVVSARIPRGVGIYYHAPE 1136
G.metaconti135      MLTLGRGGPVVWLNHEDAAGAGIEDNEEIEVYNFNGVVVSRACVSSRIPKGAAVMYHAPE 1128
                    *:**.**   .***..**   *:.**: :*::* ***.* ** **:***:*. . ***.*

T.thermNarG         RTVGIPKSPLRG-KRAGMNNSITRARLKPVLMSGGYAQFTYAFNYWGPVGVNRDTWVYVR 1187
G.metaconti121      RTISVPLSPLRGNRRAGGHNSLTRARLKPVLMAGGYGQLSYAFNAWGPPGSDRDTYVYVH 1196
G.metaconti135      RTINIQKSKKTG-KRGGVHNSLSRIRLKPTLMLGGYAQFSYYFNYWGPTGVNRDSYVVVR 1187
                    **:.:  *   * :*.* :**::* ****.** ***.*::* ** *** * :**::* *:

T.thermNarG         KLETPPEW 1195
G.metaconti121      KLEGALRY 1204
G.metaconti135      KLRG---- 1191
                         **.
```

Protein sequence alingment of NarH from *G. metallireducens* and *T. thermophilus*

```
T.thermNarH        MKVRAHMSMLFHLDKCIGCHTCSIACKNLWTDRKGAEYMWWNNVETRPGAGYPTGWEDQE 60
G.mata121NarH      MKVRAQVSMVFNLDKCIGCHTCSIACKNIWSDRRGAEYMWWNNVETKPGTGYPTLWEDQE 60
G.meta135NarH      MDVRAQLVMVFNLDKCIGCHACSLSCKNIWTDRKGAEYMWWNNVETKPGVGYPKKWEDQE 60
                   *.***:: *:*:********:**::***:*:**:************:**.***. *****

T.thermNarH        RFRGGWEYKDGHLDLRLHSRTQGLFRLFFNPALPSLDDYYEPFTFRYADLFTAPEGEDQP 120
G.mata121NarH      KYRGGWERTDREPRLRLHSRGGGLAKLFFNPRLPTIDDYYEPWSYRYGDLLSAPAGDDQP 120
G.meta135NarH      EFKGGWVADGHNLRLKAMGKGPTLLNMFFQPNMPKLEDYYEPFDFDYGNLYGAKGGDDQP 120
                   .::***   . .  *:  .:   * .:**:* :*.::*****: : *.:*  *  *:***

T.thermNarH        TAVPVSMITGEPMT-PEAGPNWDDDLSGSPLYAKNDPNLKALAPEVRAQLEEIEGVVFQY 179
G.mata121NarH      VARPVSLVTGDPIN-IEAGPNWDDDLGGSPVYGAHDPNLAAVSEAERRQLFLLERMVLFY 179
G.meta135NarH      VAEAFSQITGEKMAHIKAGPNWDDDLSGSQVYASADVNLE-----DKKIIEQYSTMFMQY 175
                   .* ..* :**: :   :*********.** :*.  * **       :  :   . :.: *

T.thermNarH        LPRICNHCLNPACVAACPSGAIYKRAEDGVVLVNENKCKAWRMCVAACPYKKVYYNWATG 239
G.mata121NarH      LPRICNHCANPGCVAACPSGAIYKRGEDGIVLVSQEKCRGWRMCVSGCPYKKVYYNWGSG 239
G.meta135NarH      LPRICNHCANPACVASCPSRAIYKRGEDGVVLVDQEVCKGWRFCTSACPYKKVYFNWNSG 235
                   ******** **.***:*** *****.***:***.:: *:.**:*.:.*******:** :*

T.thermNarH        KSEKCILCFPRLETGQAPACAHSCVGRIRYMGVLLYDADRIPEAAMVPDEELVESQLSLI 299
G.mata121NarH      KSEKCILCYPRQETGQAPACFHSCVGRLRYLGVLLYDAERIERSASRPDHDLVQAQREMI 299
G.meta135NarH      KAEKCIFCFPRTETGQCNACAHSCVGRIRYVGVLLYDAEKVEQALLKPDNQLVEAHRGVI 295
                   *:****:*:** ****. ** ******:**:*******::: .:   **.:**:::  :*

T.thermNarH        LDPFDPEVIAAAKAEGIDEGWIQAAQKSPIYKFVKTWGLALPLHPEYRTLAMMFYVPPLS 359
G.mata121NarH      LDPSDPEVVNKAAEEGIHPAVLAAARNSPVYRFVKEWRLALPLHPEFGTVPMLYYVPPLL 359
G.meta135NarH      LDPRDEAVREGARKNGVPEQWLEAAEKSPVYALIKEFELALPLHPEFRTMPMAYYIPSLS 355
                   *** *  *   *  :*:    : **.:**:* ::* : ********: *:.* :*:*.*

T.thermNarH        PVVATVEKALRAGRGGEGLVRLDIPETDLDFEVYAALEKARMPVKYLANLFAAGNEALIV 419
G.mata121NarH      PLAADRENGC----------------YDLGGEFFTSLEQGRLPLRYLASLFSAGNEEIIR 403
G.meta135NarH      PVLTSGGELH----------------ELVEHGTIPTREALRAPIGYLASLFGGGNRDVVA 399
                   *: :   :                   :     .: *  * *: ***.**..**. ::

T.thermNarH        PILKKMLAVRILKRQESLEGRPTEKALKVLADAGLTLEEAEAIYRLTTLPTLEERFVLPP 479
G.mata121NarH      AVYRKLTAVRVHRRAATTGDVPAGELERFLAEAGTTPEEAEAIYHMTSLAGFDERHAVPP 463
G.meta135NarH      AALEKLIALRIHMRAKNLN-QPTDEAS--LGKAGLDEAGAKRLYRLFTIGGYNERNIIPP 456
                   .  .*: *:*:  *  .    *: :    *..**     *: :*:: ::   :**  :**

T.thermNarH        YHREMAAEVWKDPLAHKGEAGLGYIQPPLRGE- 511
G.mata121NarH      FMREMAMEMSVDPADRKGEAGTGFLRPPKRG-- 494
G.meta135NarH      QQREE-----LDSHTRKGASGFGILKKTGRGKR 484
                     **       *.  :** :* * :: . **
```

Protein sequence alingment of NarI from *G. metallireducens* and *T. thermophilus*

```
T.thermNarI        MKWNAAL-----------------------------FGVFPYIALTLAVAVTAYRMVYR 30
G.mata121NarI      MPVDSGLSDHICAILRFLARPAGTAPNSGDPMTDYLFFGVFPYVALVVAVGGGVYRYAKE 60
G.meta135NarI      ----------------------------------MTADIYSSLARMLDYPQEKKSLLWA 25
                                                     .::. :*  :

T.thermNarI        PFSVS-AQSSQLLEQKRLFFGSTAMHWGLVIVLLGHLLALLVPKGLLLWNAVPLRLYLLE 89
G.mata121NarI      RFSFS-SLSSQFLENRALFWGSVAWHYGIVLVLLAHLAALLLPASWGRLLGDPLRLAVLE 119
G.meta135NarI      IGTVQRYLSEEGLECPLDTFGEFMAASTLAELQEEYVATFDFNPAVAPYLGHHLFGDNQK 85
                     :..   *.: **     :*.      :. :   :: :: .  .     .  *     :

T.thermNarI        ITGLGLGLWALFGTYVLLARRIAVARVRAASTPMDYLVLVVVFVSALTGVLTAVLYRYGS 149
G.mata121NarI      VTGLALGLFTLAGIVVLLFRRIASPKAWAVVTVMDLFLLAVLVLQVGTGLYNAFVYRWGS 179
G.meta135NarI      KGAYLIALKGEYGRHGYTP----LGNELPDHLPVILWFLAHLVRGGEDDARRSFIDRH-- 139
                     .  :.*    *            .  .    :   .*. :.     .   :.: *

T.thermNarI        FWFPAVMTPYLWSVLTLNPRPELLQDLPFWTKLHVFNFWLFLALFPFSRLVHIITVPLGY 209
G.mata121NarI      LWYLSLAVPWLHSLACLNPDIKYIVPLPLPVKVHLFNAFVIFVLFPVTRLVHIVSIPVSY 239
G.meta135NarI      ---VLPGVEKLSDSFAASRR-----DSPWRPAVEAVRLICGEDSARGEHPAEGIGDGVLE 191
                         .  * .    .         *    :. ..          : .. :   :
```

```
T.thermNarI         VVRPWQIVVWVRNWRGEL--- 227
G.mata121NarI       LWRPWQIAVWNKPPRWMIPRK 260
G.meta135NarI       KNRPKTAAHPEVSPC------ 206
                      **    .
```

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