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## The requirement of mobA for assimilatory and respiratory nitrate reduction in *Pseudomonas aeruginosa* PAO1

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THE REQUIREMENT OF *mobA* FOR ASSIMILATORY AND RESPIRATORY  
NITRATE REDUCTION IN *Pseudomonas aeruginosa* PAO1

Dissertation

Submitted to

The College of Arts and Sciences of the

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The Degree

Doctor of Philosophy in Biology

by

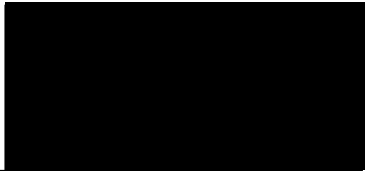


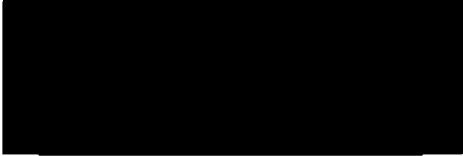
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## ABSTRACT

### THE REQUIREMENT OF *mobA* FOR ASSIMILATORY AND RESPIRATORY NITRATE REDUCTION IN *Pseudomonas aeruginosa* PAO1

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University of Dayton, 2006

Advisor: Dr. John J. Rowe

The requirement for *mobA* in key assimilatory and respiratory nitrogen metabolism of *Pseudomonas aeruginosa* (PAO1) was investigated. MobA is responsible for guanylation of the molybdopterin cofactor (MoCo), converting molybdopterin (MPT) to molybdopterin guanine dinucleotide (MGD). Until recently, it was thought that eukaryotes used the MPT form for all molybdoenzymes while prokaryotes used the MGD form. However, previous biochemical studies indicated that *P. aeruginosa* possesses both forms of the molybdenum cofactor. We show by mutational analysis of PA1779 (*nasA*; assimilatory nitrate reductase), PA3875 (*narG*; respiratory nitrate reductase) and PA3030 (*mobA*; MoCo guanylation enzyme), that a *mobA* mutant was deficient in both assimilatory and respiratory nitrate reductase activities, while xanthine dehydrogenase activity remained unaffected. Thus, *P. aeruginosa* requires both the MPT and MGD forms of the molybdenum cofactor for a complete spectrum of nitrogen metabolism and one form cannot substitute for the other. Regulation studies using a  $\Phi(mobA-lacZGm)$  reporter strain suggest that expression of *mobA* is not influenced by the type of nitrogen source or by

anaerobiosis. In relation to this, assimilatory nitrate reductase activity was present only in the presence of nitrate and diminished with the addition of ammonia and respiratory nitrate reductase was present only in the presence of nitrate anaerobically.

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## INTRODUCTION

Molybdoenzymes are ubiquitous in nature and found in all examined organisms from prokaryotes (eubacteria and archae) to eukaryotes (algae, fungi, higher plants, and humans). They are involved in maintaining the global balance of the carbon, nitrogen and sulfur cycles as well as in key reactions in fermentation and anaerobic respiration (102). With the exception of nitrogenases which contain a unique iron-molybdenum center (142), other molybdoenzymes have their molybdenum incorporated into a pterin complex of proteins known as the molybdenum cofactor (MoCo). In general, MoCo-containing enzymes catalyze the transfer of an oxygen atom to or from a substrate as an electron pair redox reaction cycling between Mo (VI) and Mo (IV) (78). Although other transition metals also have the capability to perform these reactions, Mo is the most abundant, found at about 110nM at sea (123). These MoCo containing enzymes are classified into three groups based on the types of reactions being catalyzed: the DMSOR Family (dimethyl sulfoxide reductase), the xanthine oxidase family, and the sulfite oxidase family. The DMSOR family consists of molybdoenzymes that serve as terminal reductases during respiration without oxygen. These enzymes include DMSOR, respiratory nitrate reductase, formate dehydrogenase, trimethylamine-N-oxide reductase (TMAOR), and biotin sulfoxide reductase. The xanthine oxidases

family is the largest and most diverse of all molybdoenzyme families, and is found in eukaryotic (including humans) and prokaryotic (archaea and eubacteria) organisms. These enzymes include xanthine dehydrogenases and aldehyde oxidases that are involved in purine and aldehyde metabolism, respectively. The third family represents sulfite oxidases and assimilatory nitrate reductases that are mainly found in eukaryotes such as algae and higher plants. However, assimilatory nitrate reductase is also found in prokaryotic organisms such *Klebsiella oxytoca*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (102, 119, 162). This enzyme is involved in the reduction of nitrate to nitrite, which is eventually converted to ammonium and incorporated into organic nitrogen-containing compounds such as amino acids. Sulfite oxidase is involved in the catabolism of sulfite-containing amino acids such as cysteine and methionine.

The synthesis of MoCo requires multiple steps beginning with the formation of precursor Z from a guanosine derivative by proteins encoded by the *moa* operon (134, 135, 143). Precursor Z is then converted into molybdopterin (MPT) by proteins encoded by both the *moa* and *moe* operons (57, 83, 143, 146, 147, 195). The *mod* locus encodes the proteins responsible for the transport of molybdenum and finally *mogA* is required for the attachment of molybdenum to MPT (71, 104, 115, 186). All eukaryotic enzymes utilize the MPT form of MoCo as the mature cofactor. In most prokaryotic molybdoenzymes such as nitrate reductase, an additional guanylation step, mediated by MobA, is required to form the molybdopterin guanine dinucleotide (MGD) form of MoCo (65). The synthesis of MoCo and the MGD form have been

thoroughly studied in *Escherichia coli*. Recently, however, two prokaryotic organisms, *Rhodobacter capsulatus* and *P. aeruginosa* have been shown to possess both the MPT and MGD forms of MoCo. The genetic studies in *R. capsulatus* established that xanthine dehydrogenase requires MPT (80). Biochemical studies in *P. aeruginosa* demonstrated the presence of both MPT and MGD in cell extracts (72) and specifically MPT from purified xanthine dehydrogenase (67), but no genetic studies have been forthcoming. It is particularly important to clarify the metabolic role for these cofactors in *P. aeruginosa* because the organism's ability to respire nitrate plays a major role in the last step of the nitrogen cycle (102, 132). In addition, *P. aeruginosa* infections of the lungs of cystic fibrosis patients are known to contain low concentrations of oxygen to induce anaerobic respiration as biofilms within the thick airway mucus (58, 190, 200). The mucoid form of *P. aeruginosa* becomes predominant in these biofilms which are resistant to antibiotics (48) and the high levels of nitrate found in the lungs of these patients provide a perfect environment for nitrate respiration (49). More importantly, the formation of the toxic intermediates such as nitrite during denitrification may also provide a novel approach for treatment of biofilms (199).

CHAPTER 1

LITERATURE REVIEW

PART I

**MOLYBDENUM COFACTOR SYNTHESIS**

## 1. Molybdenum Cofactor

Enzymes that are dependent on a molybdopterin (MPT) are ubiquitous, and have been identified in both prokaryotic and eukaryotic organisms where they serve diverse functions. For instance, in *Escherichia coli*, they are critical for the activity of many enzymes such as formate dehydrogenase, dimethyl sulfoxide reductase, trimethylamine N-oxide reductase, and nitrate reductase (128). All prokaryotes and eukaryotes (54) contain these enzymes. With the exception of nitrogenase, most known molybdoenzymes have a molybdenum (or tungsten) bound to a unique family of pterins to the *cis*-dithiolene group of one or two MPTs (Figure 1) (61, 129, 197). This dithiolene group is labile to oxygen and, thus, all structural characterization has been made with the inactive form (197).

Even though the tight association of molybdenum with MPTs had been long recognized, little was known about the nature of the bonding until pleiotropic effects of MoCo mutations were observed in *Aspergillus nidulans* (122). This led to the conclusion that a common cofactor served at the catalytic site of multiple enzymes. A series of studies performed in *Neurospora crassa* have also shown that the *nit-1* nitrate reductase mutant can be reconstituted by the addition of denatured molybdoenzymes from animal, fungal, or bacterial origin, thus indicating the existence of a dissociable element fitting a description of a cofactor (76, 112, 113). Unlike other cofactors, the molybdenum cofactor (MoCo) is not readily purified.



Rapid and irreversible loss of function during reconstitution due to its extreme sensitivity to air (76) has made work towards isolation of the active form difficult (Figure 1).

Variant forms of MoCo exist which include a molybdopterin cytosine dinucleotide (MCD) (129) and molybdopterin guanine dinucleotide (MGD). The distribution of MPT, MGD, and MCD amongst a number of different molybdoenzymes has been reported (129). It was initially proposed that the only form of MoCo present in prokaryotes was MGD. Recent studies however have demonstrated that *Rhodobacter capsulatus* and *Pseudomonas aeruginosa* contain both MPT and MGD (67, 72, 81, 117). For both organisms, xanthine dehydrogenase utilizes the MPT form and nitrate reductase utilizes the MGD form (67, 72, 81, 117). Thus, it appears that the same prokaryotic cell can possess more than one type of MoCo and that one form cannot substitute for the other.

In *E. coli*, the active form of MoCo for all molybdoenzymes is MGD (129). Several MoCo-deficient mutants of *E. coli* were isolated by screening for chlorate resistance, which led to the identification of multiple loci involved with different steps of MoCo biosynthesis. A summary of the genes isolated from the different loci is listed in Table 1 (160). The following section is a more detailed explanation of MoCo synthesis most of which are from data generated through studies in *E. coli*.

## 2. Molybdopterin Synthesis (*moa* operon)

The first step in the synthesis of MoCo is the conversion of a guanosine derivative to precursor Z (Figure 2). Labeling studies have indicated the first reaction to be the hydrolysis of the guanine ring, followed by linearization and rearrangement of the ribose group (195). The next series of reactions include a transfer of a formyl group followed by cleavage and cyclization to form precursor Z (195). The genes involved with these initial steps are *moaA*, *moaB*, and *moaC* (135), however, little is known regarding specific roles of each protein in the reaction mechanisms of precursor Z formation.

The MoaA amino acid sequence contains a cysteine motif at the N terminus that is known to form an Fe:S cluster and another cysteine motif at the C-terminus which is highly conserved and found in all known MoaA proteins (151). The crystal structure of the MoaB protein has recently been identified as a hexamer showing similarities to the MogA protein of *E. coli* (involved with molybdenum attachment to MPT) and domains of Cnx1 protein from *Arabidopsis thaliana* (also involved with molybdenum insertion) (7). The MoaC protein has also been successfully overexpressed and its structure also exists as a hexamer that belongs to a ferredoxin-like family (193).

The second step of MoCo synthesis is the formation of MPT from precursor Z by MPT synthase. The ability of crude extracts of a *moaA* mutant in *E. coli* to convert precursor Z to MPT was the first indication of the existence of MPT synthase (69). The reaction consists of the covalent attachment and reduction of two sulfur

atoms to create the two thiol groups in MPT. This step requires the genes *moaD*, *moaE*, and *moeB* (125). MoaD and MoaE encode for the small and large subunit of MPT synthase, respectively. The crystal structure of MPT synthase reveals a heterotetrameric enzyme which has the C terminus of a MoaD homodimer inserted into the MoaE homodimer subunit (143). The addition of sulfur to form the dithiolene group is carried as a thiocarboxylate at the MoaD C terminus (196). The *moeB* gene is part of the *moe* operon which includes another ORF called *moeA* (116). MoeB protein is used as a MPT synthase sulfurase, to regenerate the active sulfur of MPT synthase (125). The MoeA protein is thought to be involved in forming thiomolybdate during the formation of MoCo from a Mo-free MPT (57).

The third step involves the insertion of Mo into the cofactor. This process involves *mogA*, *moeA* (introduced above), and the *mod* locus. The *mod* locus is thought to be a transporter mechanism for Mo. The *modABC* encodes for an ABC-type (ATP-Binding Cassette) transport system in which the ModA protein binds molybdate in the periplasm (64). Fluorescent studies also indicate a conformational change with ModA during ligand binding, a characteristic seen with other periplasmic binding proteins (127). ModB is a 25 kDa hydrophobic protein that forms the membrane channel of the transporter. It has an inner membrane signature motif (5 predicted membrane spanning regions), as well as a permease signature sequence located in the cytoplasmic domain, characteristic of ABC transport proteins (105, 186). ModC is a 352-amino acid protein containing Walker A and B motifs along

with additional ABC conserved sequences found with ABC-ATPases (63, 186).

There is also an additional ORF labeled *modD*, with unknown function (186).

MogA and MoeA proteins have been shown to be responsible for the final step of MoCo formation by the attachment of Mo to MPT. High concentrations of molybdate were able to rescue *mod* and partially rescue *mog* mutants (71), suggesting that MogA is a molybdochelatease. In addition, human sulfite oxidase expressed in an *E. coli moeA* mutant could be reactivated *in vitro* in the presence of MoeA and low levels of Mo. However, MogA was incapable of activating sulfite oxidase expressed in an *E. coli mogA* mutant (115), suggesting that MoeA facilitates Mo attachment, thus, the specific role of MogA during *in vivo* Mo attachment remains unclear (115).

### **3. Conversion of MPT to MGD (*mob* genes)**

The final step of the synthesis of MGD involves a modification of MPT, which to date, has only been found in prokaryotes. Some prokaryotic organisms such as *R. capsulatus* and *P. aeruginosa* possess both the MPT and MGD forms of MoCo. In fact, *R. capsulatus* has been shown to synthesize several types of molybdoenzymes: dinitrogenase (FeMoco), DMSOR (MGD) (152), and xanthine dehydrogenase (MPT) (80). Likewise, two types of MoCo (MPT and MGD) were also identified in *P. aeruginosa* (72). In addition, AMP, CMP, or IMP can also be attached to the phosphate group of the MPT side chain to the phosphate group of the molybdopterin (MPT) side chain to add to the variability of MoCo. However, this

information is too broad for the scope of this review and, thus, will focus specifically on the formation of MGD.

In *E. coli*, the guanylation of MPT to MGD requires the *mob* locus, which consists of two genes, *mobA* and *mobB*. The *mobA* (originally called *chlB*) (129) gene was first identified by mutational analysis in *E. coli* and this specific mutant contained an inactive precursor form of the molybdoenzyme nitrate reductase (15). The mutant was reactivated with a soluble protein called FA which was in fact the product of *mobA* expression (15). Further studies using fluorescence and HPLC identified the wild type to contain both pterin forms and the mutant to solely express only the MPT form (68). MobA functions as a 21-kDa cytoplasmic protein that links a guanosine 5'-phosphate to MPT forming MGD. The crystal structure of the MobA-GTP complex reveals that the GTP-binding site is located in the N-terminal half of the molecule (79). The binding site for MPT is located in the C-terminus of the protein (79).

MobB has been characterized as a GTP-binding protein that is not essential for biosynthesis of an active molybdoenzyme (33). However, addition of a purified MobB protein enhances the level of nitrate reductase activation during incubation with cell extracts (33). More recently, the crystal structure of MobB has shown that it is capable of binding with the protein structure of MobA. Thus, MobB may act in concert with MobA to synthesize MGD more efficiently (96).

#### 4. MoCo attachment to the apoenzyme

Since there are numerous types of molybdoenzymes, the actual attachment of MoCo to each specific apoenzyme varies. This section of the review will focus mainly on the attachment of MoCo to nitrate reductase of *E. coli* and xanthine dehydrogenase of *R. capsulatus*, both of which are also present in *P. aeruginosa*.

MGD insertion has been investigated primarily in *E. coli*. This organism has the ability to respire anaerobically using nitrate with the initial enzymatic step consisting of the reduction of nitrate to nitrite by the enzyme nitrate reductase. The respiratory enzyme complex is encoded by *narGHJI* and termed the *nar* operon (164, 165). The NarG protein contains the MGD, which constitutes the catalytic site for the enzyme. The insertion of MGD into NarG has not yet been fully elucidated, but recent studies utilizing a bacterial two-hybrid approach to analyze *in vivo* protein interactions implicate a protein complex consisting of MobA, MobB, MoeA, and MogA interacting with an aponitrate reductase bound to the chaperone, NarJ (182).

MoCo insertion into the apoenzyme of *R. capsulatus* xanthine dehydrogenase has also been investigated. This enzyme is a molybdo/iron-sulfur/flavoprotein that catalyses oxidation of hypoxanthine to xanthine, which is subsequently oxidized to uric acid, followed by simultaneous reduction of NAD to NADH. The enzyme utilizes MPT rather than MGD (80). The xanthine dehydrogenase present in various bacterial strains allows these organisms to utilize purines as a sole source of nitrogen. In *R. capsulatus*, the enzyme is a heterotetrameric protein encoded by the genes *xdhA*

and *xdhB* (80). A third gene has been identified immediately downstream of *xdhAB*, appropriately designated *xdhC*, which may be involved in MPT attachment (82).

## 5. Regulation of MPT synthesis

One of the early regulation studies of MPT synthesis performed in *E. coli* utilizing *lacZ* fusions suggested regulation by Mo. The studies identified high expression of *modABCD* when *E. coli* cells were grown with less than 10nM Mo. Concentrations of Mo above that level decreased expression (100) possibly for preventing molybdate accumulation which could interfere with molybdoenzyme reactions. The presence of nitrate also increased *modABCD* expression and transcription was regulated via *narL* (100), which is part of the dual two-component signal transduction regulatory system of *E. coli* involved in the regulation of nitrate respiration. Additional studies showed no effect of nitrate or oxygen on the *mod* operon and a CATAA promoter sequence was identified as the molybdated dependent regulatory motif (131). The repression of *mod* by Mo was further explained by identifying another gene called *modR*, in which a mutation released *mod* repression regardless of Mo concentration (186). Thus, ModR may act as the repressor protein. An independent study also identified the repressor protein, alternatively labeled ModE, as a homodimer ModE-Mo complex which binds to the sequences TAYAT (Y=T or C) (50). Interestingly, this ModE-Mo complex has also been proposed to be a transcriptional activator of the *nar* operon (159). Functional dissection of the ModE

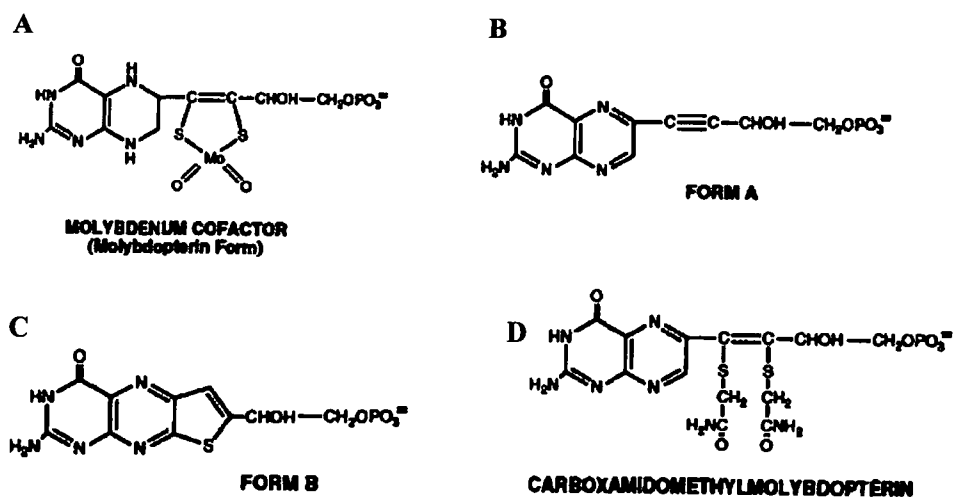
protein indicates that the DNA binding region is located at N-terminus and the C-terminus allows for efficient dimerization (97).

The *moa* operon involved with the synthesis of the Z precursor and sulfur attachment was shown to be under oxygen regulation. Expression was greatly enhanced by anaerobic conditions but repressed by the synthesis of MoCo (8). This regulation was originally suggested to be independent of *fnr* (formate, nitrate, reductase) which is a global transcriptional activator of anaerobic genes and repressor of aerobic genes (8). However, more recent studies have identified the distal region of the promoter as an Fnr binding site for anaerobic activation (2). In addition, the *moa* operon was also dependent on a ModE-Mo transcriptional activation through binding at the proximal region of its promoter (2).

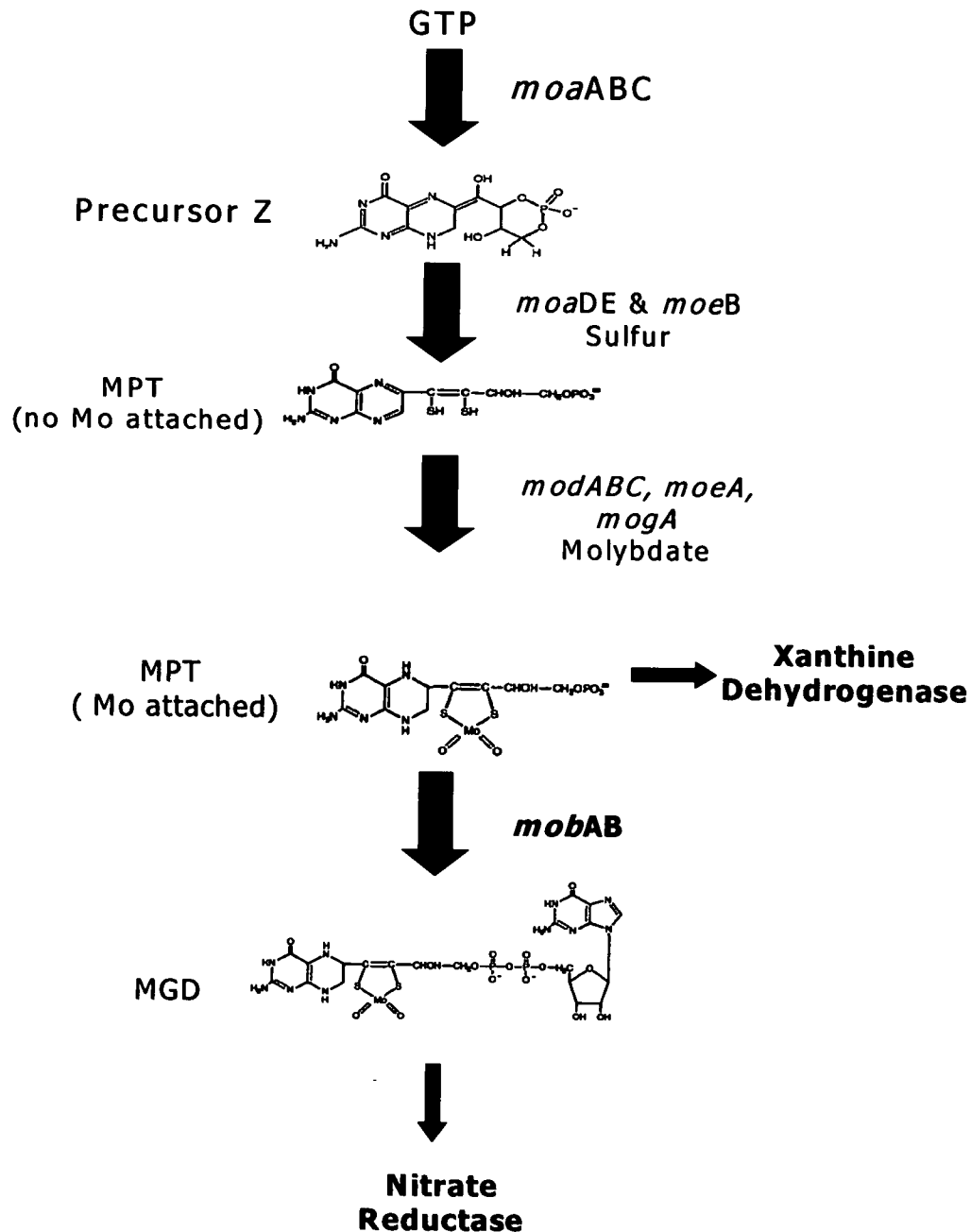
Like the *moa* operon, transcription of the *moe* operon was also activated by anaerobic conditions (56). In addition, the presence of nitrate and trimethylamine N-oxide enhances the transcriptional level of the locus (56). *moe* operon induction by nitrate required NarL and anaerobic activation required the Arc (anoxic redox control) regulatory proteins (56). However, nitrate-dependent regulation of *moe* was repressed by Fnr, thus, suggesting opposing modulation of MoCo by the two redox regulators (Arc and Fnr) (56). Therefore, transcriptional regulation of nitrate reductase and trimethylamine N-oxide reductase is related to the regulation of *moe*.

Currently, there little is known regarding regulation of *mog* or *mob* loci. Regulation of *mob* is further discussed in Chapter 2.





**Figure 1. Structures of MoCo (MPT form) and related compounds.** A) The proposed structure of MoCo depicted as an MPT. B) The earliest derivative isolated (Form A) was a result of vigorous oxidation at 100 °C in the presence of  $I_2/KI$ . C) Form B was identified through the same treatment carried out with air instead of  $I_2$ . D) Sulfite oxidase treated with SDS in the presence of iodoacetamide resulted with the isolation of carboxyamidoethyl MPT (129).



**Figure 2. MoCo synthesis pathway with intermediates and genes involved.** The first step of MoCo synthesis is the formation of precursor Z from guanosine followed by the attachment of sulfur groups and Mo respectively to form MPT. Depending on the enzyme, MPT can be further modified with additional guanylation (MGD).

**Table 1: The MoCo synthesis genes of *E. coli* and their specific functions.**

<b>Gene</b>	<b>Function</b>
<i>moaA</i>	Guanosine to Precursor Z
<i>moaB</i>	Guanosine to Precursor Z
<i>moaC</i>	Guanosine to Precursor Z
<i>moaD</i>	MPT synthase (small subunit), sulfur attachment
<i>moaE</i>	MPT synthase (large subunit), sulfur attachment
<i>moeA</i>	Molybdate attachment
<i>moeB</i>	MPT synthase sulfurase/sulfur attachment
<i>modA</i>	Mo ABC Transport/Periplasmic binding
<i>modB</i>	Mo ABC Transport/Membrane protein
<i>modC</i>	Mo ABC Transport/ATPase
<i>modD</i>	ORF unknown function
<i>modE</i>	Regulatory Protein
<i>mogA</i>	Molybdate attachment
<i>mobA</i>	GMP attachment to form MGD
<i>mobB</i>	GTP binding protein

## CHAPTER 1

### LITERATURE REVIEW

## PART II

# **TYPES OF MOLYBDOENZYMES**

Molybdenum-containing enzymes are utilized in three categories of redox enzymes: the dimethyl sulfoxide reductase (DMSOR) family, the xanthine oxidase family, and the sulfite oxidase family. All of the molybdoenzyme families with their respective enzymes are organized in Table 2.

### **1. Dimethyl Sulfoxide Reductase (DMSOR)**

The DMSOR family is exclusively found in eubacteria and includes enzymes such as dissimilatory nitrate reductase, formate dehydrogenase, trimethylamine-N-oxide reductase (TMAOR), and biotin sulfoxide reductase. All of the enzymes so far examined in this family have a modified MoCo called molybdopterin guanine dinucleotide (MGD). Most of them serve as terminal respiratory reductases utilized during oxygen limiting conditions. One of the representative enzymes in this family is (DMSOR). This enzyme is found in a variety of bacteria including *E. coli* (187), *Rhodobacter sphaeroides* (148), and *Rhodobacter capsulatus* (95). *E. coli* DMSOR, is membrane-bound enzyme which catalyzes the oxidation of menaquinol coupled with the reduction of dimethyl sulfoxide (DMSO) to dimethyl sulfide (DMS) and generates a proton gradient across the membrane. The enzyme consists of three subunits: the catalytic subunit containing the MGD cofactor (20), the electron transfer subunit containing four Fe-S clusters (20, 175), and a membrane anchor subunit (137). In the photosynthetic *R. capsulatus* and *R. sphaeroides*, DMSOR also reduces

DMSO to DMS as the terminal electron acceptor during respiration (103, 152). In *R. sphaeroides*, however, DMSOR is a cytosolic single subunit, which contains only MoCo as the only prosthetic group (148). The mechanism of DMSOR is divided into two half-cycles (Figure 3). The oxidation cycle consists of the reduced Mo (IV) form of the cofactor binding with the substrate and results in the donation of two electrons from Mo to DMSO to generate the reduced form, DMS. The second half consists of the regeneration of Mo (IV) by the transfer of two protons and two electrons yielding a water molecule (150, 152).

Another enzyme extensively characterized in the DMSOR family is formate dehydrogenase. When this enzyme was first isolated, it was found to be bound to the respiratory nitrate reductase and was proposed to be the primary electron donor to the respiratory nitrate reductase of *E. coli* (45). Formate dehydrogenase is involved in mixed acid fermentation in which formate is oxidized to carbon dioxide with the formation of NADH (174). The crystal structure analysis of formate dehydrogenase identified the presence of two MGD cofactors (16). This structure was also found to contain four domains and a protein ligand to Mo, a Se-cysteine, and an additional 4Fe-4S cofactor located near the N-terminal. The enzyme catalyzes the conversion of formate to CO<sub>2</sub> by a proton/electron transfer that is coupled to the reduction of the oxidized Mo (VI) center. The reaction mechanism is depicted in Figure 4.

In *P. aeruginosa*, the only enzymes of the DMSOR family that have been characterized are respiratory nitrate reductase and formate dehydrogenase. The genes that encode for the enzyme are organized as *fdnGHIE* (ORFs PA4812 to PA4809

respectively). In addition there are several ORFs with similarity (46 – 50%) to the regulatory protein DorX of *R. sphaeroides*, specifically PA1484, PA2591, and PA4806. The PA4892 ORF also has 54% similarity to C-terminus of DMSO/TMAO sensor kinase of *R. sphaeroides*. The role of formate dehydrogenase in this organism is the oxidation of formate to CO<sub>2</sub> for generation of NADH. The structure contains molybdenum, an Fe-S cluster and a cytochrome *b* (44, 45).

### **(1A) Respiratory Nitrate Reductase**

Although most of the respiratory nitrate reductase studies have been performed in *E. coli*, Nar enzymes have been purified from several bacterial strains and identified in others by gene/protein homology (203). In *E. coli*, there are two forms of respiratory nitrate reductase: NRA, which is expressed under anaerobic conditions in the presence of nitrate and NRZ which is constitutively expressed (11, 14). Nar enzyme complex of *E. coli* contains three subunits: the quinol-oxidizing 19 to 25 kDa membrane  $\gamma$  subunit (NarI), a 52 to 64 kDa soluble  $\beta$  subunit (NarH) containing one 3Fe-4S and three 4Fe-4S centers, and the 140 kDa catalytic  $\alpha$  subunit (NarG) containing the MGD cofactor. The Nar proteins utilize the quinol pool as the electron donor and generate PMF using a redox loop mechanism (10, 133). Specifically, the quinols are proposed to be on the periplasmic side of the membrane and are oxidized by the heme *b* of NarI which effectively releases two protons into the periplasmic space. The electrons are then passed to NarG by going through the Fe-S centers of NarH for the reduction of nitrate (Figure 5). A  $\delta$  polypeptide (NarJ)

is not part of the enzyme and appears to be involved in enzyme assembly possibly MoCo attachment (12, 32, 129). The respiratory nitrate reductase NRA is encoded by the *narGHJI* operon while the NRZ respiratory nitrate reductase is encoded by the *narZYWV* operon (14). An additional protein involved with respiratory nitrate reduction is NarK which is required for nitrate uptake possibly as a nitrate/nitrite antiporter (161) or as a nitrite extrusion protein (138).

- **Respiratory nitrate reduction in *P. aeruginosa***

The genes involved in *P. aeruginosa* respiratory nitrate reduction are located in the *nar* operon (PA3877 to PA3872) (Table 3). Much like *E. coli*, the structural genes encoding for respiratory nitrate reductase are named *narGHJI*. However, *P. aeruginosa* contains two *narK* genes (*narK1* and *narK2*) immediately upstream of the respiratory nitrate reductase genes. Disruption of both ORFs show that only the *narK2* is required for anaerobic growth in nitrate and that a *narK2* mutant loses the ability to take up nitrate indicating its role as a nitrate transporter (161). In addition, both *narK* ORFs are expressed as a polycistronic unit with *narGHJI* (161) as compared to the *E. coli narK* which is not part of the *nar* operon. Downstream of the *narGHJI* cluster is also an ORF potentially involved with molybdopterin synthesis (PA3870, *moaA1*). However, most of these ORFs have never been characterized and their putative functions are based purely on peptide sequence similarity. Besides the *narK1* and *narK2* genes (161), the only other confirmed ORF is *narG* which is presented in Chapter 2 (117).



- **A special case of non-MoCo nitrate reductase**

Together with molybdenum, tungsten is a group VI element of the periodic table with similar properties (70). Both have equal atomic and ionic radii, similar electronegativity, and can be modified by addition of polynucleotide molecules such as guanylation (MGD) (70). Tungsten also has the ability to replace molybdenum in enzymes forming inactive analogs (70). Thus, the possibility of non-MoCo nitrate reductases may exist specifically with strict anaerobic organisms such as hyperthermophilic archae. The lack of oxygen in primordial earth would have resulted in the formation of sulfides ( $\text{MoS}_2$  and  $\text{WS}_2$ ) rather than oxyanions ( $\text{MoO}_4$  and  $\text{WO}_4$ ) and  $\text{WS}_2$  is more soluble in water than  $\text{MoS}_2$  (106). One example is from the haloalkalophilic denitrifying bacterium *Halomonas* sp. strain AGJ 1-3 in which respiratory nitrate reductase was isolated and purified to homogeneity. The isolated enzyme belongs to a novel family of molybdenum-free nitrate reductases (4). In *Pseudomonas*, there is currently only one strain that has been shown to contain a molybdenum-free nitrate reductase. *P. isachenkovii* is a vanadate-reducing bacterium, which does not contain a MoCo in its periplasmic, and membrane bound respiratory nitrate reductase. Instead, the metal content consists of vanadium and chromium, respectively (3). Thus, the common MGD form of nitrate reductases found in *Pseudomonas* may be just one example of multiple types of cofactors that could exist in the environment out of which only two forms (vanadium and molybdenum) have been isolated.

- **Regulation of respiratory nitrate reduction**

In *E. coli*, the NarGHJI proteins are only synthesized during anaerobic conditions via FNR (fumarate and nitrate respiration) and with nitrate by a two-component regulatory system of NarXL. Although there is a NarPQ two-component regulatory system present for the *narZYWV* operon, this locus appears to be minimally expressed constitutively (11, 14).

The *E. coli* FNR protein is a major transcriptional regulator for the expression of genes involved with anaerobic metabolism (166). The Fe-S center of FNR is sensitive to oxygen. Under anaerobic conditions, FNR is a dimeric protein that binds to DNA and activates the *nar* operon and other essential genes. Under aerobic conditions, the 4Fe-4S centers are converted to either 3Fe-4S or 2Fe-2S centers, resulting in inactivation (77). Sequences similar to the FNR binding region upstream of *nar* have also been identified upstream of nitrate respiration genes in other bacterial strains as well as FNR-like regulators (87,100).

Mediation of *E. coli nar* by nitrate or nitrite is through the two-component regulatory mechanism of NarX and NarL proteins. NarX is a nitrate/nitrite sensor that responds through phosphorylation of NarL (166, 203). The activated NarL then binds to specific DNA regions to induce transcription (177). The isoform, NarP does not induce the *nar* operon; however, NarP can activate transcription of other loci such as *nap* (periplasmic nitrate reductase) and *nrf* (nitrite reductase) along with the NarX and NarL proteins (27). A distinguishing factor between the two proteins is that NarL recognizes all DNA heptamers, and NarP binds to only to heptamers with a 2-bp

spacing inverted repeat (28). Thus, both two-component regulatory systems have the ability to distinguish between nitrate and nitrite. NarL primarily serves as a nitrate regulator that is weakly phosphorylated in the presence of nitrite. NarX phosphorylates NarP but deactivates NarL and in the presence of both nitrate and nitrite, NarQ phosphorylates NarL and deactivates NarP (188). An additional element required for *nar* activation is an integration host factor which initiates DNA bending along with contact between NarL, FNR, and RNA polymerase (153, 201).

In *P. aeruginosa*, nitrate reduction is also under control of oxygen. Much like the FNR protein in *E. coli*, *P. aeruginosa* possesses ANR (arginine nitrate regulation) (40) and DNR (dissimilatory nitrate respiration regulator) (5) as part of the global oxygen regulation of denitrification genes (202). A vector carrying a *lacZ* under the control of a DNA sequence carrying an FNR binding motif first indicated the existence of an FNR-like element in *P. aeruginosa* (149). Eventually, a mutation in arginine degradation as well as anaerobic growth identified the *anr* locus (40). ANR is 51% identical with the *E. coli* FNR protein and carries the essential structures such as the N-terminal and central cysteine residues, a DNA-binding motif, a glycine-rich  $\beta$ -roll, and Fe-S clusters. The promoter region of the arginine degradation operon (*arcDABC*) carries a nucleotide sequence TTGAC....ATCAG, resembling the FNR box (40). In addition, an *anr* deletion also disrupts all the enzymes involved with the four steps of the denitrification pathway (40). DNR was initially identified in the vicinity of the structural genes for nitrite reductase (*nirS*) and nitric oxide reductase (*norCB*), and the gene for activation of the nitrite reductase (*nirQ*) in *P. aeruginosa*

(5). DNR was found to encode for a protein homologous with the CRP/FNR-family of transcriptional regulators (6). The promoter activities for *nirS*, *nirQ* and *norCB* were considerably reduced in the *dnr* mutant as well as in the mutant of *anr*. The regulation existed as a hierarchy such that *dnr* transcription was under the control of ANR. However, arginine degradation did not require DNR (6). Currently, the mechanism of interaction between oxygen and ANR/DNR is not well understood.

## **2. Xanthine Oxidase Family**

The xanthine oxidase family is the best studied and therefore the largest and most diverse family of all molybdoenzyme families. This division represents eukaryotic (including humans) and prokaryotic (archaea and eubacteria) organisms. Compared to the DMSOR family, the xanthine oxidase family in general is not directly involved in respiration but in catabolic and anabolic functions. Among the enzymes in this family are xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase (176). Xanthine oxidase and xanthine dehydrogenase are two different forms of enzymes that catalyze the same reaction, which is the conversion of xanthine to uric acid during purine metabolism. They only differ in the type of oxidizing agent that is utilized. Thus, in the xanthine oxidase form,  $O_2$  is utilized while in the xanthine dehydrogenase  $NAD^+$  is utilized. The reaction specifically involves the oxidation of xanthine to urate and the formation of NADH (Figure 6). Both enzymes are found in humans, and xanthine dehydrogenase is also utilized in organisms such as *R. capsulatus* (80) and *P. aeruginosa* (67). In fact, all molybdoenzymes in eukaryotes

have been proposed to utilize solely the MPT form and in prokaryotes, MoCo commonly undergoes an extra modification step such as addition of guanine or cytosine (MGD and MCD). Xanthine dehydrogenase is also of considerable medical interest, since this enzyme is implicated in gout and hyperuricaemia (80). This enzyme has broad substrate specificity, having the ability to hydroxylate various purines, pteridines, aromatic heterocycles, and aliphatic and aromatic aldehydes (61). The crystal structure of bovine xanthine dehydrogenase has identified the enzyme as a homodimer (34), in which each domain is arranged with two Fe-S clusters at the N-terminus with the neighboring FAD and MoCo cofactors.

Another enzyme classified under the xanthine oxidase family is aldehyde oxidase. This enzyme is involved in the conversion of an aldehyde, in the presence of oxygen and water, to an acid and hydrogen peroxide. It is found in both prokaryotes and eukaryotes. The prokaryotic form of the enzyme is modified by the attachment of cytosine to form (MCD) (136) instead of guanine. The reaction mechanism of this enzyme is a hydride transfer from the substrate to the sulfur group depicted in Figure 7. The crystal structure of *Desulfuvibrio desulfuricans* aldehyde oxidase shows a dimeric enzyme with three domains where each of the N-terminus is involved with coordination of a 2Fe:2S cluster.

### **3. Sulfite Oxidase Family**

Sulfite oxidase and assimilatory nitrate reductases mostly from eukaryotes such as algae and higher plants represent the majority of this family. These enzymes

catalyze the transfer of oxygen to or from nitrogen or a sulfur atom. Sulfite oxidase is located in the intermembrane space of mitochondria and catalyzes the oxidation of sulfite to sulfate. Specifically, it is involved in the terminal reaction in the degradation of the amino acids cysteine and methionine (43). This enzyme has been characterized in mammals (42, 43), bovine and chicken (9) and one prokaryote, *Thiobacillus novellus* (74), where the enzyme exists as a heterodimer as compared to the homodimer eukaryotic counterpart. In addition, the sulfite oxidase MoCo site of *T. novellus* was identified containing MPT not MGD as seen with most prokaryotic molybdoenzymes. In rat sulfite oxidase, the N-terminal domain contains cytochrome *b5* and the C-terminal domain harbors MoCo. The plant counterpart is similar except for the lack of the cytochrome *b5* domain, which makes plant sulfite oxidase different from the vertebrates. The crystal structure of chicken sulfite oxidase contains MoCo as a single MPT (78). The Mo is coordinated by the dithiolene sulfurs of MPT and by a cysteine side chain from the protein. In *P. aeruginosa*, there has been no characterization of a sulfite oxidase and no identification of any homologous sequences in the *Pseudomonas* genome database (171).

### **(3A) Assimilatory nitrate reductase**

Assimilatory nitrate reductase catalyzes the reduction of nitrate to nitrite, leading to the formation of ammonium that can be utilized in the formation of proteins and nucleic acids. Eukaryotic assimilatory nitrate reductases possess an MPT cofactor as compared to the MGD structure required in prokaryotes. Nitrate

assimilation has been studied in both biochemical and genetic levels in phototrophic and heterotrophic prokaryotes. Currently, there are two classes of assimilatory nitrate reductases found in bacteria: the ferredoxin- or flavodoxin-dependent Nas and the NADH-dependent enzyme (102). Both classes of enzymes contain the MGD cofactor and a sulfur group at the N-terminus, without the presence of heme groups, as compared to eukaryotic and other assimilatory nitrate reductases in prokaryotes (102). The flavodoxin-Nas of *A. vinelandii* is a polypeptide of 105 kDa (41), whereas the ferredoxin-Nas of cyanobacteria is a single subunit of 75 to 85 kDa (141). Purified Nas protein from *A. vinelandii* and *Plectonema boryanum* shows a combination of one Mo, four Fe, and four S atoms per molecule (41). The N-terminus also shows a Cys motif possibly for the binding of the Fe-S center. The NADH-Nas proteins of *Klebsiella pneumoniae* (84) and *Rhodobacter capsulatus* (13) are heterodimers consisting of a 45-kDa FAD-diophorase and a 95-kDa catalytic subunit with the MGD cofactor and a possible N-terminus with a Fe-S center.

The genes involved with the reduction of nitrate during assimilation are commonly clustered (Figure 8) which encodes for the structural and regulatory proteins as well as transport proteins. They have been characterized in several bacterial strains which resulted in confusing labels due to the inconsistencies in designation. In *K. pneumoniae*, the genes are classified as *nas* (84, 87). The *nasR* gene encodes for a transcription antiterminator involved with the expression of the *nasFEDCBA* operon (47, 84, 87, 88). The *nasB* encodes for the assimilatory nitrite reductase and both the *nasC* and *nasA* encodes for the NADH-dependent-nitrate

reductase. The *nasFED* genes are responsible for nitrate/nitrite transport. The *B. subtilis* designation has *nasBC* genes coding for nitrate reductase, *nasDE* coding for nitrite reductase, and *nasF* involved with siroheme cofactor synthesis (110). The nitrate transport protein is encoded by *nasA*. The structural gene encoding the flavodoxin-dependent Nas of *A. vinelandii* (*nasB*) is also cotranscribed with the nitrite reductase *nasA* gene (130). In cyanobacteria, the gene coding for the ferredoxin-dependent Nas is labeled *narB* (at present the *nar* designation is universally accepted as a reference to the respiratory enzyme) and has been sequenced in several strains, including the unicellular *Synechococcus*, filamentous nonheterocyst (*Oscillatoria*) and heterocyst-forming (*Anabaena*) cyanobacteria (18, 73, 140, 178). In most cases, the nitrite reductase *nirA* gene, the *nrtABCD* nitrate transport genes, and the nitrate reductase *narB* gene constitute an operon (18, 37, 73, 92, 120, 173).

- **Regulation of prokaryotic assimilatory nitrate reductase, *nas***

In *Klebsiella* the expression of the *nas* genes is subjected to a dual control: ammonia repression by the general nitrogen regulatory system (Ntr) and specific nitrate or nitrite induction (46, 89). The Ntr system is responsible for regulating the synthesis of most enzymes required for utilizing alternate nitrogen sources. During nitrogen-limiting growth conditions, the NtrB protein phosphorylates the NtrC protein which in turn binds upstream of specific promoter sequences namely, the promoters recognized by the alternate *rpoN*-encoded  $\sigma^N$  ( $\sigma^{54}$ ) factor and in the process activating transcription of the Ntr-regulated genes (99).



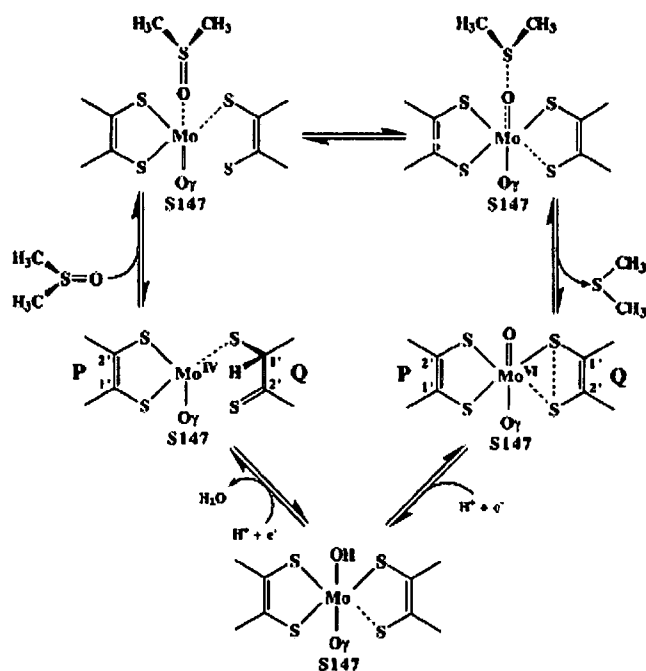
Nitrate and nitrite specific induction in *Klebsiella* is mediated by NasR, which specifically induces *nas* gene expression in the presence of nitrate or nitrite. NasR is a positive regulatory protein that acts by transcription attenuation. Thus, when nitrate or nitrite is present in the growth medium, the NasR protein promotes transcription antitermination in the *nasF* leader region and in the process increases the *nasF* operon expression. On the other hand, in the absence of nitrate or nitrite, a factor-independent transcription terminator present in the leader region prevents the transcription of *nas* genes. Therefore, nitrate regulation does not act by controlling transcription initiation but rather by controlling transcription termination (46, 86, 168).

Both the Ntr system and the positive regulation of nitrate assimilation by nitrate or nitrite have also been reported for photosynthetic bacteria. For instance, in *R. capsulatus*, the assimilatory nitrate reductase is induced by nitrate and repressed by low carbon-nitrogen ratios, probably through a balance of 2-oxoglutarate and glutamine, respectively. Ammonium also inhibits nitrate transport, avoiding nitrate reductase induction (21, 31). Further, in the cyanobacterium *Synechococcus* sp. strain PCC 7942, the nitrate assimilation gene cluster namely, *nirA-nrtABCD-narB*, is repressed by ammonium through its incorporation into glutamine. Positive regulation by nitrate requires nitrate reduction, and nitrite seems to be the actual activator of transcription of nitrate assimilation genes. In *cyanobacteria*, nitrogen control is mediated by the NtcA protein. The NtcA protein is a member of the Crp family of transcriptional activators (181). This organism possesses a second regulatory protein

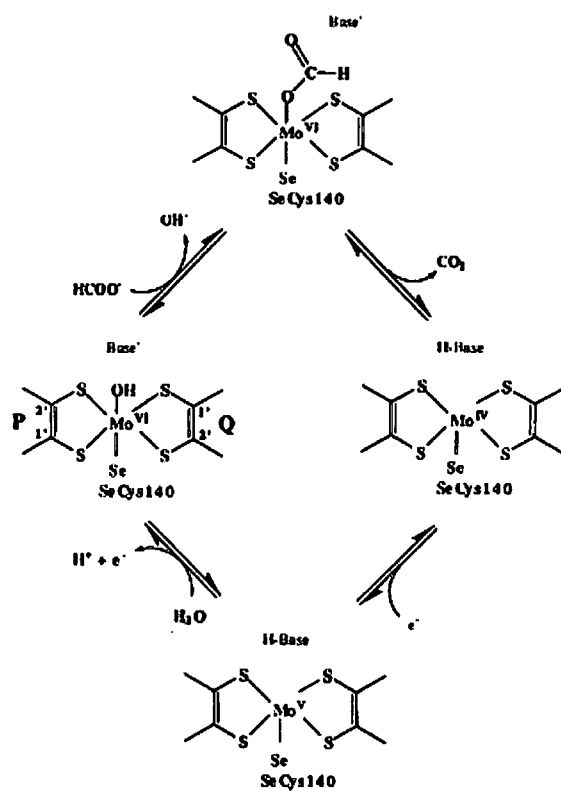
namely the NtcB protein which is required to activate the *nirA* operon in response to nitrite (1).

In *A. vinelandii*, the *ntr* genes are responsible for the induction or repression of the *nasAB* structural genes through nitrate/nitrite and ammonium, respectively (130). The organism also requires the *nasST* operon to control the expression of the *nasAB* operon. NasS is found to be similar to proteins involved in nitrate uptake but appears to negatively regulate the operon by blocking NasT (positive regulator) action in the absence of nitrate (52). NasT is found to be a homologue of dual two-component regulatory proteins and, thus, positively affects the *nasAB* operon expression. Additionally, the *nasAB* operon seems to be subjected to an autonomous regulation by the nitrate reductase protein, NasB, that negatively regulates the synthesis of nitrate and nitrite reductases (130). Finally, the metabolism of molybdenum may also be a regulatory factor in addition to a complex regulatory network involving the *nifO* gene (53).

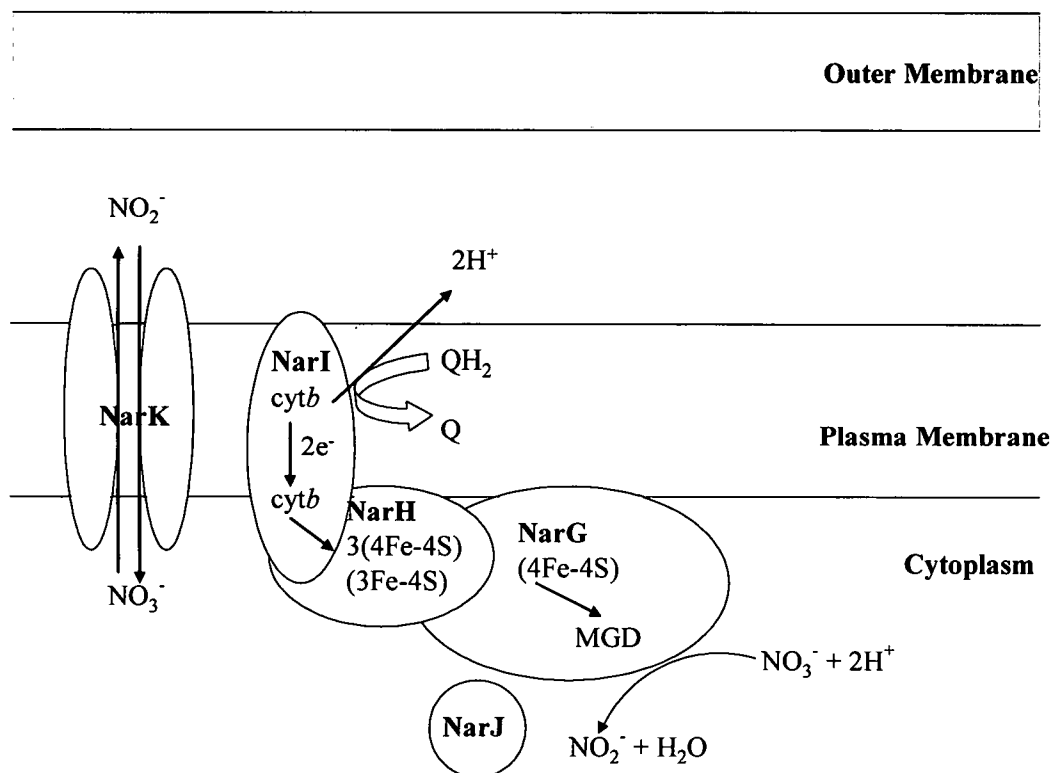
In contrast to other bacteria, the *Bacillus subtilis nas* operon is not subject to pathway-specific nitrate induction (109, 111). Moreover, this bacterium has no known system that is similar to the Ntr system, and nitrogen control is mediated by the TnrA protein, which binds to the DNA in order to activate the transcription of the *nas* operon and several nitrogen-controlled genes (109).



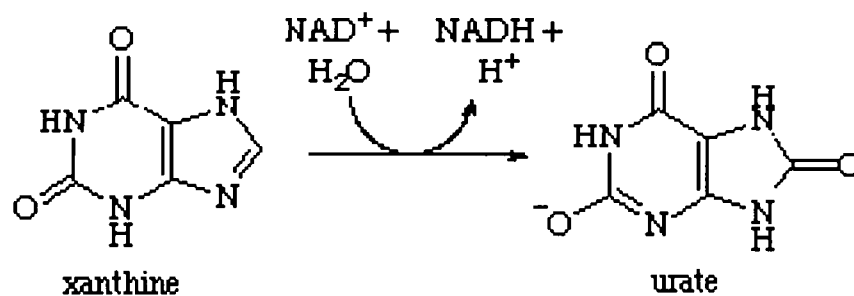
**Figure 3. Proposed mechanism of DMSO reduction to DMS by the catalytic site of DMSOR.** The binding of the substrate DMSO to Mo (IV) and the oxidation of Mo to form DMS constitutes step of the cycle. The second step consists of the regeneration of the reduced form of Mo (78).



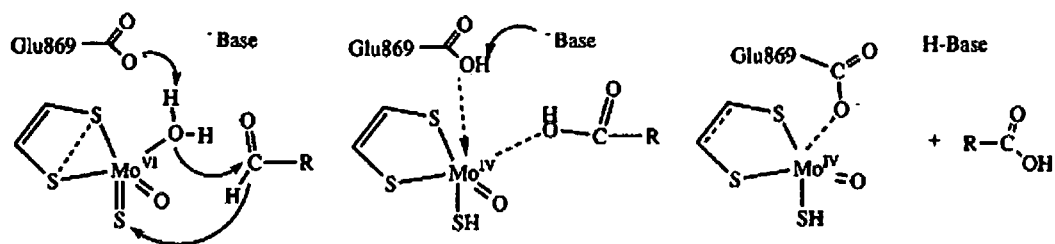
**Figure 4. Proposed mechanism of formate dehydrogenase.** The Mo (VI) form of the catalytic site allows for the oxidation of formate to CO<sub>2</sub>. The release of CO<sub>2</sub> forms the reduced form of Mo (IV) and regeneration of Mo (VI) occurs through a selenium-cysteine moiety utilizing a water molecule (151).



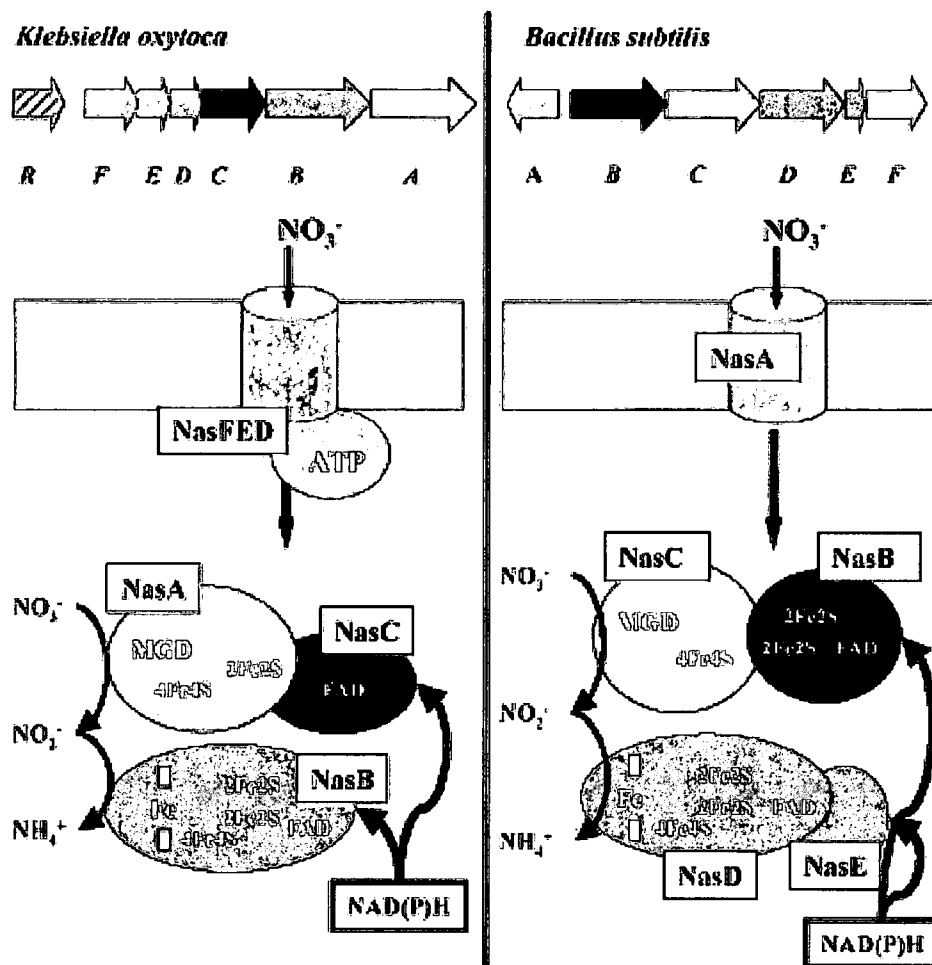
**Figure 5. Organization of respiratory nitrate reductase and potential locations and roles of the proteins.** The first step of respiratory nitrate reduction involves the transport of nitrate either as an anionporter or through two separate proteins, one involving nitrate uptake and the other extrusion of nitrite. The cytochrome *b* from NarI utilizes the electrons from the quinol loop, then donating the electrons to the Fe-S center of NarH. The catalytic component NarG reduces nitrate to nitrite from electrons donated by NarH. Drawing taken and modified from (132). The putative function of NarJ may be as a chaperone protein involved with the attachment of MoCo.



**Figure 6. The oxidation of xanthine to urate by xanthine dehydrogenase.**  $\text{NAD}^+$  acts as the electron acceptor forming NADH and a water molecule for the addition of the carbonyl group. Urate is further broken down to urea allowing the organism to assimilate the liberated nitrogen as  $\text{NH}_3$ .



**Figure 7. Degradation of an aldehyde via aldehyde oxidase.** The reaction occurs by the transfer of a hydride from the substrate which reduces the sulfido group and releases the oxidized substrate. The mechanism also utilizes a carboxylic acid group from the apoenzyme and a molecule of water for the electron transfer (151).



**Figure 8. Organization of assimilatory nitrate reductase genes and potential locations and roles of the proteins in *K. oxytoca* and *B. subtilis*.** In *K. oxytoca*, nitrate enters the cell by the ABC transport protein NasFED. The catalytic domain of nitrate reductase is NasA which contains a bis-MGD MoCo. The electron transfer is mediated by NasC via NAD(P)H. Nitrate is further reduced to nitrite by nitrite reductase NasB. In *B. subtilis*, nitrate enters the cell through the transport protein NasA and the catalytic nitrate reductase is designated as NasC. Electron transfer from NAD(P)H to NasC is mediated by NasB. Nitrate is reduced to nitrite by nitrite reductase, NasD and NasE (132).



**Table 2. Representatives of MoCo-Containing Enzyme Families (Modified from (78))**

Enzyme	Organism	Prokaryotic/Eukaryotic	MPT/MGD
<b>DMSO Family</b> DMSOR  TMAOR  Formate Dehydrogenase  Respiratory Nitrate Reductase	<i>Rhodobacter capsulatus</i>	Prokaryotic	MGD
	<i>Escherichia coli</i>	Prokaryotic	MGD
	<i>Shewanella massilia</i> <sup>26</sup>	Prokaryotic	MGD
	<i>Escherichia coli</i>	Prokaryotic	MGD
	<i>Alcaligenes eutrophus</i> <sup>38</sup>	Prokaryotic	MGD
	<i>Pseudomonas aeruginosa</i>	Prokaryotic	MGD
	<i>Escherichia coli</i>	Prokaryotic	MGD
	<i>Pseudomonas aeruginosa</i>	Prokaryotic	MGD
<b>Xanthine Oxidase Family</b> Xanthine oxidase/dehydrogenase    Aldehyde oxidase	<i>Rhodobacter capsulatus</i>	Prokaryotic	MPT
	<i>Pseudomonas aeruginosa</i>	Prokaryotic	MPT
	<i>Nitrobacter winogradskyi</i> <sup>39</sup>	Prokaryotic	?
	<i>Methanosarcina barkeri</i> <sup>75</sup>	Prokaryotic	?
	<i>Bos taurus</i>	Eukaryotic	MPT
	<i>Homo sapiens</i>	Eukaryotic	MPT
	<i>Desulfuvibrio gigas</i>	Prokaryotic	MCD (Cytosine)
	<i>Homo sapiens</i>	Eukaryotic	MPT
<b>Sulfite Oxidase Family</b> Sulfite Oxidase    Assimilatory Nitrate Reductase	<i>Thiobacillus novellis</i>	Prokaryotic	MPT
	<i>Escherichia coli</i> <sup>90</sup>	Prokaryotic	MPT
	<i>Paracoccus denitrificans</i> GB17 <sup>189</sup>	Prokaryotic	?
	<i>Gallus gallus</i>	Eukaryotic	MPT
	<i>Rattus norvegicus</i>	Eukaryotic	MPT
	<i>Homo sapiens</i>	Eukaryotic	MPT
	<i>Pseudomonas aeruginosa</i>	Prokaryotic	MGD
	<i>Neurospora crassa</i>	Eukaryotic	MPT

Additional classification of enzymes taken from (26, 38, 39, 75, 90, 189).

**Table 3. *Pseudomonas aeruginosa* respiratory nitrate reductase genes.**

PA# (Locus)	Gene Name	Homology
PA3877	<i>narK1</i>	49% similar to <i>Bacillus subtilis narK</i> gene product
PA3876	<i>narK2</i>	74% similar to <i>Escherichia coli narK</i> gene product
PA3875	<i>narG</i>	83% similar to <i>Escherichia coli narG</i> gene product.
PA3874	<i>narH</i>	86% similar to <i>Escherichia coli narH</i> gene product.
PA3873	<i>narJ</i>	63% similar to <i>Escherichia coli narJ</i> gene product.
PA3872	<i>narI</i>	68% similar to <i>Escherichia coli narI</i> gene product.
PA3878	<i>narX</i>	48% similar to <i>Escherichia coli narX</i> gene product
PA3879	<i>narL</i>	74% similar to <i>Escherichia coli narL</i> gene product
PA2376	Unnamed ORF	54% similar to <i>Pseudomonas aeruginosa</i>
PA1397	Unnamed ORF	56% similar to nitrate/nitrite response regulator
PA1544	<i>anr</i>	51% identity with the <i>Escherichia coli</i> FNR protein
PA0527	<i>dnr</i>	CRP/FNR-family regulatory proteins

## RESEARCH OBJECTIVES

### **Part I. MoCo synthesis in *P. aeruginosa*.**

In the published literature, there is little information about MoCo synthesis in *P. aeruginosa*. Most of the current data consists of protein sequence comparisons of this organism with *E. coli* (171). Although past studies have identified the presence of both MPT and MGD forms of MoCo (72), no studies have yet confirmed the function of MobA with guanylation of MPT in this organism. The focus of Chapter 2 is to establish the role of MobA in assimilatory/respiratory nitrate reductase and xanthine dehydrogenase in *P. aeruginosa*. In addition, a *lacZ* fusion of *mobA* was also investigated under different nitrogen supplements to assess the role of this gene in regulation between MPT and MGD. We hypothesized that MobA is required for nitrate reduction in *P. aeruginosa*, and the regulation between MPT and MGD is decided by the presence of MobA.

### **Part II. Regulation of nitrate assimilation in *P. aeruginosa*.**

Much of the studies on prokaryotic nitrate assimilation have been done in *A. vinelandii* (51, 91, 107), *K. pneumoniae* (19, 22, 23, 47, 84, 85, 87, 191, 192), and *B. subtilis* (108, 110, 119). The information on *P. aeruginosa* nitrate assimilation is lacking even with the availability of the *Pseudomonas* genome database (171). To

date, only ORF (PA1779) has been shown to be required for nitrate assimilation (Chapter 2, (117)). To this end, gene deletions of putative ORFs (PA1784-1786) involved with nitrate ssimilation was constructed, and the phenotypes of these strains are discussed in Part II of Chapter 3. We hypothesized that ORF PA1785 is involved with regulation of nitrate/nitrite assimilatory pathways.

### **Part III. The use of a modified pUCGM/*lox* for gene disruption in *P. aeruginosa*.**

In prokaryotes, manipulation of ORFs existing in polycistronic units presents a major challenge due to the possibility of creating polar effects. Thus, isogenic mutant construction in organisms such as *P. aeruginosa* can lead to polarity complications and can give the investigator a false phenotype. Currently there are no widely applicable gene tools that can alleviate this problem. In this regard, the pUCGM/*lox* vector (126) was modified by inserting a *Plac* element downstream of the *lox* sites flanking the gentamycin resistance marker. This modified vector allows the investigator to create gene disruptions by utilizing conventional techniques and at the same time gives them the freedom to induce/repress downstream ORFs in an operon, due to the presence of a regulatory element. The effectiveness of this tool is elaborated in Part III of Chapter 3.

## CHAPTER 2

THE *mobA* GENE IS REQUIRED FOR ASSIMILATORY AND  
RESPIRATORY NITRATE REDUCTION BUT NOT XANTHINE  
DEHYDROGENASE ACTIVITY IN *Pseudomonas aeruginosa*

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The *mobA* Gene is Required for Assimilatory and Respiratory Nitrate Reduction but not Xanthine Dehydrogenase Activity in *Pseudomonas aeruginosa*

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## ABSTRACT

The requirement for the *mobA* gene in key assimilatory and respiratory nitrogen metabolism of *Pseudomonas aeruginosa* PAO1 was investigated by mutational analysis of PA3030 (*mobA*; MoCo guanylate synthase), PA1779 (*nasA*; assimilatory nitrate reductase), and PA3875 (*narG*; respiratory nitrate reductase). The *mobA* mutant was deficient in both assimilatory and respiratory nitrate reductase activities, while xanthine dehydrogenase activity remained unaffected. Thus, *P. aeruginosa* requires both the MPT and MGD forms of the molybdenum cofactor for a complete spectrum of nitrogen metabolism and one form cannot substitute for the other. Regulation studies using a  $\Phi$ (PA3030-*lacZ*Gm) reporter strain suggest that expression of *mobA* is not influenced by the type of nitrogen source or by anaerobiosis, while assimilatory nitrate reductase activity was detected only in the presence of nitrate.

**Key words:** *mobA*, xanthine dehydrogenase, and nitrate reduction in *Pseudomonas aeruginosa*.

Molybdopterin (MPT) requiring enzymes are ubiquitous in nature, and are present in prokaryotes and eukaryotes. All of the molybdoenzymes, with the exception of nitrogenase, contain molybdenum bound to MPT or a slightly modified form of MPT. The synthesis of molybdenum cofactor (MoCo) requires several steps. First, a guanine nucleotide is rearranged to form precursor Z (135, 194, 195), followed by the attachment of two sulfur atoms to form a molybdenum-free MPT (69, 125, 143, 196). Then, the molybdenum is chelated to the pterin group to form MoCo (115). In the majority of prokaryotes, additional post-translational modifications are required which can add to the variability of MoCo such as the attachment of GMP to form molybdopterin guanine dinucleotide (MGD) catalyzed by the protein MobA (68, 79). This form of MoCo is found in numerous enzymes involved in carbon, sulfur, and nitrogen cycles such as dimethyl sulfoxide reductase (DMSOR), biotin sulfoxide reductase, trimethylamine *N*-oxide reductase and nitrate reductase (151).

In contrast to *E. coli*, *Rhodobacter capsulatus* has been shown to synthesize several types of molybdoenzymes: dinitrogenase (FeMoCo), DMSOR (MGD) (152) and xanthine dehydrogenase (MPT). Genetic evidence was presented that these forms are specific for different molybdoenzymes (80, 81). Two types of MoCo (MPT and MGD) were also identified in *Pseudomonas aeruginosa* (67, 72) and chemical evidence was presented to suggest the MPT form enables the bacteria to assimilate hypoxanthine as a sole nitrogen source through the enzyme xanthine dehydrogenase. However, aside from hypoxanthine, *P. aeruginosa* can also assimilate and denitrify nitrate. The first step of both pathways requires the reduction of nitrate to nitrite by



nitrate reductase. In nitrate assimilation, nitrite is further reduced to ammonia which is subsequently incorporated into amino acids (88). In denitrification, nitrate is utilized as an electron acceptor for anaerobic respiration (132) and is continually reduced to form nitrous oxide or dinitrogen gas. *P. aeruginosa* also possesses a third nitrate reductase located in the periplasm (*nap*) (184) which may be involved with redox balancing or nitrate scavenging when grown in reduced carbon sources or under nitrate limiting conditions respectively (132). The requirement for MobA in respiratory nitrate reductase activity was demonstrated in *E. coli* (121), assimilatory nitrate reductase in *Synechococcus* sp. (139), and periplasmic nitrate reductase in *Desulfovibrio desulfuricans* (30). Although these nitrate reductases are functionally different, there has been little experimental evidence, with the exception of *R. capsulatus*, establishing which forms of MoCo are required for these enzymes in bacteria containing multiple types of nitrate reductases and other molybdoenzymes requiring MoCo. Along with identification of the conserved amino acid motif for the Mo binding site, experimental evidence in distinguishing the specific MoCo required for different molybdoenzymes will be important in defining metabolic patterns. In addition, *P. aeruginosa* lung infections of cystic fibrosis patients are now known to undergo anaerobic metabolism in biofilms within the thick airway mucus (190). The high levels of nitrate and nitrite found in the lungs of these patients provide a perfect environment for anaerobic respiration (190). Thus, it is important to develop a better understanding of the specific proteins and factors such as MoCo that are involved in

the anaerobic metabolism of this organism. At the current time, only extrapolation by protein homology is available regarding *P. aeruginosa* MobA.

## **MATERIALS AND METHODS**

**Media and culture conditions.** All cultures were inoculated with overnight shaker-grown starter cultures on LB (Luria Bertani). Aerobic cultures were incubated at 37°C at 250 rpm using a culture volume of 50 ml grown in 500 ml Erlenmeyer flasks containing Vogel-Bonner (VB) minimal medium (183) with the appropriate nitrogen source (1% w/v KNO<sub>3</sub>, 0.025% w/v KNO<sub>2</sub>, 0.1% w/v NH<sub>4</sub>Cl, 0.1% w/v hypoxanthine). Anaerobic cultures were incubated at 37°C in a sealed 125 ml Erlenmeyer flask equipped with a gas release, filled to the top with LB supplemented with 1% w/v KNO<sub>3</sub> and flushed for 10 min with argon. In addition, complete anaerobiosis was ensured by the addition of 2% (w/v) Oxyrase enzyme (Oxyrase Inc., Mansfield, OH). Antibiotics from Bioworld (Dublin, OH) were used in *E. coli* cultures at the following concentrations: ampicillin, 100 µg/ml; gentamicin, 15 µg/ml. Gentamicin and carbenicillin were used at 300 µg/ml and 500 µg/ml, respectively, for *P. aeruginosa*.

**Manipulation of recombinant DNA and genetic techniques.** All plasmid and chromosomal nucleic acid manipulations were accomplished by standard techniques (144). Plasmid DNA was transformed into *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, MD), SM10, or *P. aeruginosa* PAO1. Restriction endonucleases,

Klenow fragment and T4 DNA ligase were used as specified by supplier (New England Biolabs, Beverly, MA). Plasmid DNA was isolated using a Qiagen (Valencia, Ca) plasmid preparation kit. DNA fragments were isolated from agarose gels using QBiogene (Carlsbad, Ca). PCR reactions were performed using Taq DNA polymerase, PCR buffer, and dNTP's (Sigma, St. Louis, Mo) in a Peltier Thermal Cycler.

**Construction of *mobA*, *nasA*, *narG* mutants in wild-type strains.** The PA3030 (*mobA*), PA1779 (*nasA*), and PA3875 (*narG*) mutants were constructed by using 5'-ACGACCTGCACGCTATCCTC-3' and 5'-ATTCTGACTCGACGTCCCTTC-3', 5'-TCGTTCTCCTCAGGCATTC-3' and 5'-ATAACAGGAGCCGAGCATG-3', 5'-CAAGGCGAAGAGCTACAAGAG-3' and 5'-TTGCCGAGGTCATCGTTC-3' primers respectively and disrupted with a *lacZ*Gm cassette from pZ1918G (156) using a gene replacement technique previously described (157, 163) (See Table 1).

**Measurement of enzyme activities.** Nitrate reductase levels were determined by methyl-viologen linked activity as previously described (93).  $\beta$ -Galactosidase levels were determined by the hydrolysis of *o*-nitrophenyl  $\beta$ -D galacto pyranoside (ONPG) as previously described (101).

## RESULTS AND DISCUSSION

**PA3030 (*mobA*) phenotypes.** The PA3030 (*mobA*) mutant was tested by its ability to grow aerobically in nitrate minimal and hypoxanthine minimal medium, and anaerobically in LB broth containing 1% nitrate (Fig. 1). PA1779 (*nasA*, assimilatory nitrate reductase by homology) and PA3875 (*narG*, respiratory nitrate reductase by homology) mutants were also measured as controls. The PA3030 (*mobA*) mutant showed minimal or no growth aerobically in nitrate minimal media and anaerobically in LB containing nitrate compared to wild type bacteria (Fig. 1A and B). In contrast, a *mobA* mutation did not affect the ability of *P. aeruginosa* to grow in hypoxanthine minimal media (Fig. 1C). Interestingly, the PA3875 mutant displayed a slight increase in growth aerobically in nitrate minimal media (Fig. 1A). One possible explanation is that even low expression of active respiratory nitrate reductase may limit the amounts of MGD required for nitrate assimilation, especially when cell densities begin reaching an OD<sub>660</sub> of 0.5 or higher when conditions may not truly remain aerobic. In fact, when grown beyond OD<sub>660</sub> of 0.6 in minimal conditions supplemented with nitrate and ammonia, there is detectable nitrate reductase activity much higher than those observed in Table 2 (data not shown).

**Assimilatory and respiratory nitrate reductase activities.** To ensure that the phenotypes observed were due to the lack of molybdoenzyme activity, nitrate reductase activity was measured in nitrate minimal medium aerobically (Table 2) and anaerobically in LB containing nitrate (Table 3). Both the PA3030 (*mobA*) and the

PA1779 (*nasA*) mutants expressed no detectable nitrate reductase activity when grown in minimal medium with both 0.025% KNO<sub>2</sub> and 1% KNO<sub>3</sub> aerobically compared to PAO1 and the PA3875 (*narG*) mutant (Table 2). Interestingly, the PAO1 strain used in this study only displayed nitrate reductase activity in the presence of nitrate without ammonia, contrary to past observations (162). We found variations of this effect between PAO1 (a gift from B. Iglewski) and our PAO1 strain (a gift from A. Darzins), but in all cases the activity observed in the absence of nitrate and presence of nitrite was lower (data not shown). In other organisms such as *Klebsiella pneumoniae* M5a1 and *Azotobacter vinelandii*, the *nas* operon is induced by either nitrite or nitrate (19, 51). Nitrate reductase activity was also measured in bacteria grown under anaerobic conditions with LB supplemented with 1% KNO<sub>3</sub> (Table 3). As expected, comparable nitrate reductase activities were detected with the PA1779 (*nasA*) mutant and with PAO1, while no activity was detected in the PA3030 (*mobA*) and PA3875 (*narG*) mutants (Table 3). These phenotypes confirm the proposed function which was based on homology and implies that the MGD is required for activity of both nitrate reductase enzymes.

Even though *P. aeruginosa* genome also contains periplasmic nitrate reductase, we did not attempt to explore this enzyme further since the levels of Nap were negligible and did not interfere with the determination and differentiation of respiratory and assimilatory nitrate reductase. No detectable enzyme activity was measured in the presence of ammonia (Table 2). In addition, no activity was detected in the PA3875 (*narG*) mutant in LB supplemented with nitrate under anaerobic

conditions, again indicating the minimal contribution of periplasmic nitrate reductase on the background of these experiments. However, due to the conserved nature of the nitrate reductase MGD binding site, it is very likely that periplasmic nitrate reductase would also be affected with a *mobA* mutation.

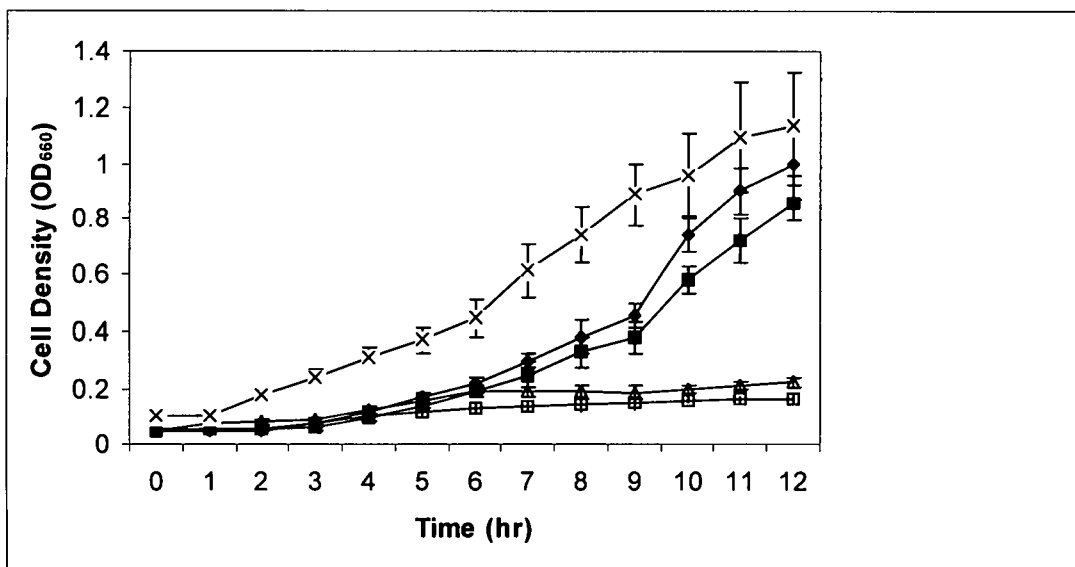
**Regulation of PA3030 (*mobA*).** Since *P. aeruginosa* contains both MPT and MGD cofactors, the expression of PA3030 (*mobA*) might be regulated, thus affecting the distribution of the two pools. To test this possibility,  $\beta$ -galactosidase activity was measured using a meridiploid  $\Phi$ (PA3030-*lacZ*Gm) transcriptional fusion with different nitrogen sources. However, no significant change in  $\beta$ -galactosidase activity was observed for any of the nitrogen sources tested (Table 4), indicating constitutive expression of PA3030 (*mobA*) in *P. aeruginosa* PAO1. It is possible that regulation may occur post-transcriptionally or other factors may be involved for the guanylation step, which are not expressed constitutively.

In conclusion, we have demonstrated that *mobA* is required for both assimilatory and respiratory nitrate reductases but not xanthine dehydrogenase in *P. aeruginosa*. In addition, we have confirmed by gene disruptions the requirement of *nasA* for assimilatory nitrate reductase and *narG* for respiratory nitrate reductase. Furthermore, the expression of MobA appears to be unaffected by nitrogen source.

## **ACKNOWLEDGEMENT**

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A



B

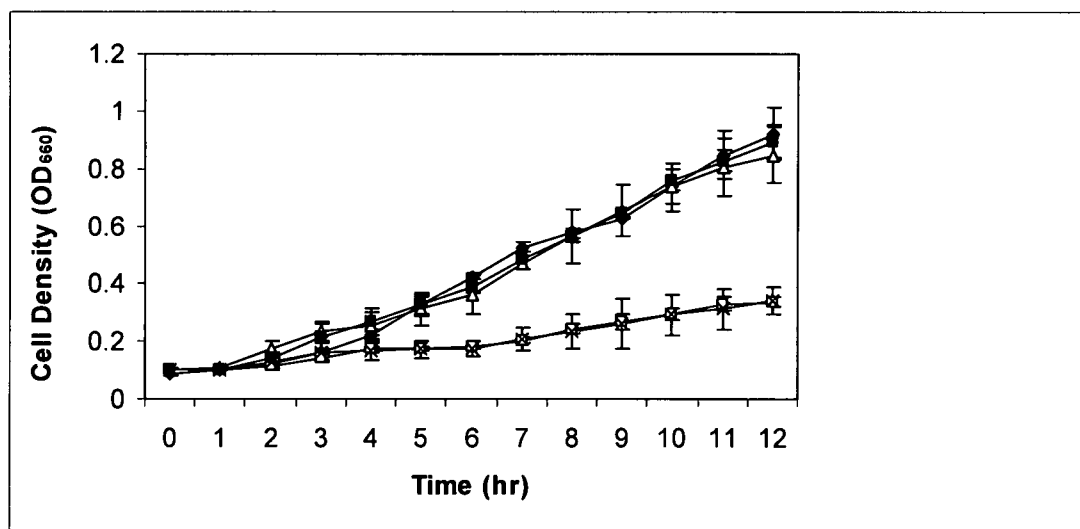
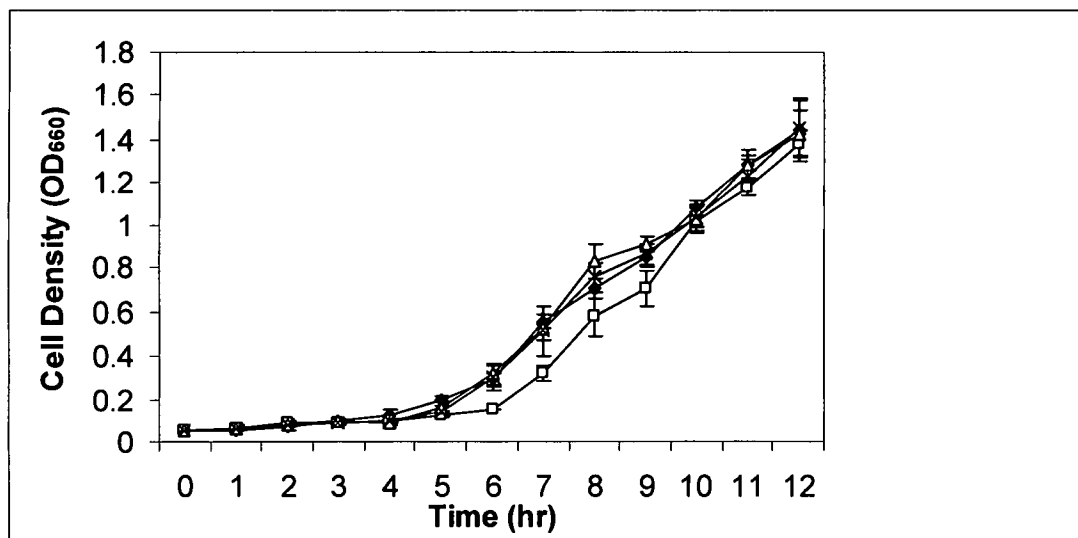


Figure 1. A and B



C



**Figure 1.** A) Aerobic growth of *P. aeruginosa*, (♦) PAO1 (wild type); (□) PA3030 (*mobA*), (■) PA3030 (*mobA*) complement, (Δ) PA1779 (*nasA*), and (X) PA3875 (*narG*) mutants in VB + 1% KNO<sub>3</sub> medium. B) Anaerobic growth of *P. aeruginosa*, (♦) PAO1 (wild type); (□) PA3030 (*mobA*), (■) PA3030 (*mobA*) complement, (Δ) PA1779 (*nasA*), and (X) PA3875 (*narG*) mutants in LB + 1% KNO<sub>3</sub> medium. C) Aerobic growth of *P. aeruginosa*, (♦) PAO1 (wild type); (□) PA3030 (*mobA*), (Δ) PA1779 (*nasA*), and (X) PA3875 (*narG*) mutants in VB + 1% Hypoxanthine medium. Growth rate was measured using optical density of cultures at 660 nm (OD<sub>660</sub>). Mean and standard deviations were calculated from three independent measurements.

**Table 1. Strains and plasmids**

Strains	Relevant genotype	Source or reference
<i>E. coli</i>		
DH5α	<i>recA1 endA1 gyrA96 thi-1 hsdR17supE44 relA1 ΔlacU169 (Φ80lacZΔM15)</i>	Gibco (Carlsbad, Ca)
SM10	Km <sup>r</sup> , Mobilizer strain	[163]
<i>P. aeruginosa</i>		
PAO1	Wild type	Al Darzins
PA3030	Gm <sup>r</sup> , Φ(PA3030- <i>lacZ</i> Gm)	This study
PA3030 Meridiploid	Gm <sup>r</sup> , Cb <sup>r</sup> , Φ(PA3030- <i>lacZ</i> Gm) meridiploid for transcriptional regulation study	This study
PA1779	Gm <sup>r</sup> , Φ(PA1779- <i>lacZ</i> Gm)	This study
PA1785	Gm <sup>r</sup> , Φ(PA3875- <i>lacZ</i> Gm)	This study
PA3030 Complement	Gm <sup>r</sup> Cb <sup>r</sup> , Φ(PA3030- <i>lacZ</i> Gm) with pUCP18:: <i>mobA</i>	This study
Plasmids		
pGem:: <i>mobA</i>	Ap <sup>r</sup> , 700bp fragment containing PA3030 in pGEM-T Easy vector (Promega)	This study
pCR:: <i>nasA</i>	Ap <sup>r</sup> , 2.7kb fragment containing PA1779 in pCR 2.1 vector (Invitrogen)	This study
pGem:: <i>narG</i>	Ap <sup>r</sup> , 1.5kb fragment of PA3875 in pGEM-T Easy vector (Promega)	This study
pEX100T	Ap <sup>r</sup> <i>oriT</i> <i>mob</i> <i>sacB</i> gene replacement vector	[157]
pEX100T:: <i>mobA</i>	Ap <sup>r</sup> , ligation of a blunt-ended 700 bp <i>EcorI</i> fragment containing PA3030 of pGem:: <i>mobA</i> into <i>SmaI</i> digest of pEX100T	This study
pEX100T:: <i>narG</i>	Ap <sup>r</sup> , ligation of a blunt-ended 1.5kb <i>EcorI</i> fragment of PA3875 of pGem:: <i>narG</i> into <i>SmaI</i> digest of pEX100T	This study
pEX100T:: <i>nasA</i>	Ap <sup>r</sup> , ligation of a blunt-ended 2.7kb <i>EcorI</i> fragment containing PA1779 of pCR:: <i>nasA</i> into <i>SmaI</i> digest of pEX100T	This study
pZ1918G	Ap <sup>r</sup> Gm <sup>r</sup> <i>lacZ-aacCI</i>	[156]
pEX100T:: <i>mobA</i> :: <i>lacZ</i> Gm	Ap <sup>r</sup> Gm <sup>r</sup> , ligation of a 4kb <i>PstI</i> fragment of PZ1918G containing <i>lacZ-aacCI</i> into <i>PstI</i> site of pEX100T:: <i>mobA</i>	This study
pEX100T:: <i>narG</i> :: <i>lacZ</i> Gm	Ap <sup>r</sup> Gm <sup>r</sup> , ligation of a 4kb <i>PstI</i> fragment of PZ1918G containing <i>lacZ-aacCI</i> into <i>PstI</i> site of pEX100T:: <i>narG</i>	This study
pEX100T:: <i>nasA</i> :: <i>lacZ</i> Gm	Ap <sup>r</sup> Gm <sup>r</sup> , ligation of a blunt ended 4kb <i>SmaI</i> fragment of PZ1918G containing <i>lacZ-aacCI</i> into <i>ApaI</i> of pEX18Ap:: <i>nasA</i>	This study
pUCP18	Ap <sup>r</sup> , broad-host-range cloning vector	Herbert P. Schweizer
pUCP18:: <i>mobA</i>	Ap <sup>r</sup> , ligation of a 700bp <i>EcorI</i> fragment of pGem:: <i>mobA</i> into <i>EcorI</i> site of pUCP18, complementation studies	This study

**Table 2. Nitrate reductase activities of aerobically grown strains in VB minimal media with different nitrogen sources.**

Strain	Nitrate reductase sp act <sup>a</sup>		
	VB NO <sub>2</sub> <sup>-</sup>	VB NO <sub>2</sub> <sup>-</sup> NO <sub>3</sub> <sup>-</sup>	VB NO <sub>2</sub> <sup>-</sup> NO <sub>3</sub> <sup>-</sup> NH <sub>3</sub>
PAO1	<0.1	15.7 ± 2.1	<0.1
PA3030 ( <i>mobA</i> )	<0.1	<0.1	<0.1
PA1779 ( <i>nasA</i> )	<0.1	<0.1	<0.1
PA3030 Complement	<0.1	16.9 ± 4.5	<0.1
PA3875 ( <i>narG</i> )	<0.1	16.0 ± 1.2	<0.1

<sup>a</sup> One Unit (U) of activity is defined as that amount of nitrate reductase required to produce 1 nmol nitrite/min. Specific activities were expressed as U/mg protein. Mean and standard deviation were calculated from three independent measurements.

**Table 3. Nitrate reductase activities of anaerobically grown strains on LB supplemented with 1% KNO<sub>3</sub>.**

Strain	Nitrate reductase sp act <sup>a</sup>
PAO1	273 ± 25
PA3030 ( <i>mobA</i> )	<1
PA1779 ( <i>nasA</i> )	209 ± 37
PA3875 ( <i>narG</i> )	<1
PA3030 Complement	295 ± 27

<sup>a</sup> One Unit (U) of activity is defined as that amount of nitrate reductase required to produce 1 nmol nitrite/min. Specific activities were expressed as U/mg protein. Mean and standard deviation were calculated from three independent measurements.

**Table 4. Expression of  $\Phi$ (PA3030-*lacZGm*) transcriptional fusion grown under the specified conditions.**

Nitrogen Source	$\beta$ -Galactosidase activity <sup>a</sup>
VB + 1%KNO <sub>3</sub>	4173 $\pm$ 773
VB + 0.1% Hypoxanthine	3610 $\pm$ 394
VB + 0.1%NH <sub>3</sub>	3090 $\pm$ 131
VB + 0.025% KNO <sub>2</sub>	3063 $\pm$ 466
LB + 1%KNO <sub>3</sub> Aerobic	3292 $\pm$ 244
LB + 1%KNO <sub>3</sub> Anaerobic	3640 $\pm$ 386

<sup>a</sup> One U of activity is defined as that amount of  $\beta$ -Galactosidase required to hydrolyze 1  $\mu$ mol ONPG/min. Specific activities were expressed as U/mg protein. All variables grown aerobically unless stated otherwise. Mean and standard deviations were calculated from three independent measurements.

## CHAPTER 3

### **SUMMARY, ONGOING RESEARCH AND FUTURE DIRECTIONS**

**Part I. The *mobA* gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*.**

The availability of MPT or MGD to apoenzymes specifically requiring one or the other is an important regulatory question. Without the cofactor, the molybdoenzymes of this organism are not functional thus; the amount of activity might be regulated at the last step (MobA) in the pathway. *P. aeruginosa* is very unusual as a prokaryote because we have shown that it possesses enzymes using both. Since the anaerobic metabolism depends on the availability of MGD, this might be a point at which antibiotics specific for anaerobic growth of the organism could be targeted. Preliminary results using *lacZ* fusions of *mobA* suggest that there is no obvious transcriptional regulation of the gene (117). Thus if regulation exists, it is likely to be post transcriptional in nature. Obviously the rate of anaerobic synthesis of the apoprotein of NarG will affect the pool of MGD. Regulation may also occur as a result of the differences in  $K_m$  for the last enzyme involved in the synthesis of MPT and the  $K_m$  for MobA. These have yet to be determined for any organism and this information may be helpful in sorting out physiological regulation at this level.

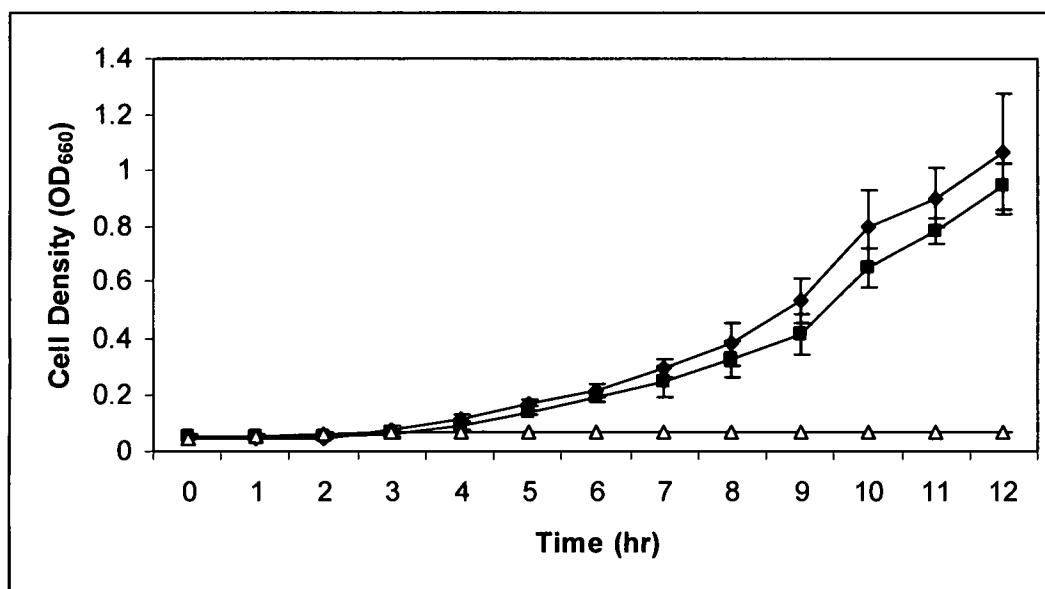
## **Part II. Regulation of nitrate assimilation in *P. aeruginosa*.**

Through the use of the *Pseudomonas* genome database (171), we have identified genes putatively involved with nitrate assimilation and these are listed in Table 1. These genes are designated as *nas* for assimilatory nitrate reductase and *nir* for assimilatory nitrite reductase. One *nas* ORF, PA1779, has been phenotypically verified to be required for growth in nitrate minimal medium and assimilatory nitrate reductase activity (117). However, disruption of another locus namely PA1783 which has 66% similarity to the nitrate transporter NasA in *B. subtilis* (171), did not result in the inability of the mutant to grow in nitrate minimal medium (Figure 1), thus pointing out the dangers of extrapolation by protein sequence similarity to function without phenotypic verification. In addition, a triple mutant deletion of PA1784, PA1785, and PA1786 resulted in the inability of the organism to grow in nitrate and nitrite minimal media. The deleted strain was complemented by a plasmid containing only PA1785 suggesting that ORFs PA1784 and PA1786 are not required for nitrate assimilation (Figure 1). Growth measurements on nitrite minimal media were monitored at the end of 12-18 hours (data not shown). Since PA1785 is 96% similar to the NasT regulatory protein of *A. vinelandi* (Table 1), it is likely that it plays a similar role in *P. aeruginosa*.



### **Future research**

To corroborate on the ability of PA1785 to act as a transcriptional activator of nitrate and nitrite assimilatory genes, assays detecting DNA-protein interactions can be carried out. To this end, a novel method, ChIP, can be used to identify the DNA binding site of a protein of interest (185). Briefly, the protein-DNA complex is crosslinked by formaldehyde following cell lysis. Antibodies directed towards the protein of interest are used for coimmunoprecipitation to identify the DNA fragment(s) bound to the regulatory protein. Utilization of this assay could provide the answers to the putative function of PA1785.



**Figure 1.** Aerobic growth of *P. aeruginosa*; (♦) PAO1 (wild type), (Δ) PA1785 mutant, and (■) PA1785 complementation in VB + 1% KNO<sub>3</sub> medium. Growth rate was measured using optical density of cultures at 660 nm (OD<sub>660</sub>). Mean and standard deviations were calculated from three independent measurements.

**Table 1. Putative *P. aeruginosa* Nitrate Assimilation Genes.**

PA# (Locus)	Gene Name	Homology
PA1779	<i>nasA ; nasC</i>	49% similar to nitrate reductase NasA of <i>Klebsiella pneumoniae</i> 49% similar to nitrate reductase NasC of <i>Bacillus subtilis</i>
PA1780	<i>nirD</i>	assimilatory nitrite reductase small subunit 62% similar to <i>Bacillus subtilis</i> nitrite reductase subunit protein NasE
PA1781	<i>nirB</i>	assimilatory nitrite reductase large subunit 69% similar to NirB (NasD), subunit of nitrite reductase <i>Bacillus subtilis</i> 53% similar to nitrite reductase, NasB <i>Klebsiella pneumoniae</i>
PA1783	<i>nasA</i>	66% similar to nitrate transporter NasA <i>Bacillus subtilis</i>
PA1785	<i>nasT</i>	96% similar to <i>Azotobacter vinelandii</i> NasT protein
PA1786	<i>nasS ; nasF</i>	76% similar to <i>Azotobacter vinelandii</i> NasS protein 69% similar to nitrate transporter NasF of <i>Klebsiella pneumoniae</i>
PA2329	Unnamed ORF	62% similar to putative nitrate transport protein NrtC in <i>Synechocystis sp.</i>
PA5124	<i>ntrB</i>	68% similar to <i>Vibrio alginolyticus</i> NtrB
PA5125	<i>ntrC</i>	79% similar to <i>Salmonella typhimurium</i> NtrC

### **Part III. The use of a modified pUCGm/*lox* for gene disruption in *P. aeruginosa*.**

#### **ABSTRACT**

Even with the knowledge provided by the *Pseudomonas aeruginosa* genome sequence, the availability of molecular tools for gene characterization relies on conventional technology which is not well developed for the *P. aeruginosa* system. Studies aimed at dissecting polycistronic loci may introduce polar effects, when creating gene disruptions. We describe here a modification of a pUCGm/*lox* vector to facilitate gene disruptions specifically, those involving polycistronic ORFs. This modified cassette minimizes polar effects due to an insertion of a *Plac* promoter. The introduction of the *Plac* promoter also allows the investigator to produce conditional mutations downstream of the target by utilizing a plasmid containing the *lacI<sup>q</sup>* gene (pREP). This is particularly useful when it necessary to deal with an operon or overcome transcriptional repression. We tested the functionality of this modified vector as well as the pREP vector by disrupting the *narK1K2* genes and inserting the *lac* promoter upstream of *narGHJI* respiratory genes in *P. aeruginosa*. We were able to conditionally express and overcome physiological control of the operon. One important outcome was the ability to express proteins required for anaerobic nitrate respiration under aerobic conditions.

## INTRODUCTION

The advent of genome sequencing has provided researchers with the advantage of viewing the complete nucleotide sequences of a variety of organisms including *Pseudomonas aeruginosa* (171). Even with the availability of such genome databases, the advances in technology for analyzing and characterizing the existence of numerous ORFs have been limited. In bacteria, studies of ORFs existing in polycistronic units present an additional obstacle due to polar effects. Thus, one approach for gene characterization is the construction of unmarked deletion mutants which have minimal or no effects on expression, downstream of the target gene. One existing technique utilizes an excisable cassette, the Flp-*FRT* system (24, 25), and was adapted from a procedure developed in *Saccharomyces cerevisiae* (62). This method allows for selection with an antibiotic marker and counter selection with sucros using *Bacillus subtilis sacB*. The Flp-*FRT* system allows for the removal of the antibiotic resistance marker, thus, reducing the possibility of polar effects. This method produces marker free gene disruptions, and provides the ability to create additional gene modifications in the same strain. Another similar approach is the Cre-*lox* system for the excision of the antibiotic resistance marker (94, 126). In addition, nonpolar gentamicin resistance cassettes have been previously described in *Pseudomonas aeruginosa* (155, 172).

Recently, vectors containing inducible/repressible promoters as part of genetic characterization have also been used to construct genetic disruptions. One example is pMUTIN in *B. subtilis* which utilizes a single crossover event that disrupts the target

gene and introduces a transcriptional termination site and a Pspac promoter which allows for the regulation of downstream genes by IPTG (179). However, the use of pMUTIN requires only a single crossover event and thus could create difficulties such as formation of merodiploids, when analyzing smaller ORFs requiring the presence of flanking sequences for homologous integration. A more widely applicable method involves the use of inducible/repressible promoters contained within the disrupting cassettes. An example of such a system in *Escherichia coli* utilizes a cassette containing the *lac* element to ensure transcription of genes downstream of the target sequence, as well as the FLP-*FRT* system for cassette removal. This method also uses a counter-selectable *sacB* marker within a suicide vector to ensure excision and gene replacement (98).

In *P. aeruginosa*, the presence of polycistronic ORFs also creates the possibility of polar effects during gene disruption procedures. Presently there are no cassettes available for specific targeting of gene disruption containing inducible/repressible elements. Although there is one technique which utilizes a promoter element as part of the transposon for mutagenesis (66), this system does not allow the investigator to utilize the promoter element as a cassette for targeting specific regions of the chromosome. In addition, the transposon utilizes a neomycin phosphotransferase promoter only to prevent polar effects but does not allow for conditional repression of genes downstream of the integration site. In this study, we modified a pUCGm/*lox* vector by inserting a *Plac* element outside of the *lox* excision site for gene disruption in *P. aeruginosa*. This modified cassette not only reduces the

possibility of polar effects when dealing with polycistronic ORFs but also provides the investigator with the ability to repress expression of downstream ORFs by regulating the *lac* promoter. The modified pUCGm/*lox* vector (pUCGmx*lac*) still contains the multiple flanking restrictions sites necessary to release the cassette. The *lox* sites flanking the gentamicin resistance marker also allows the investigator to integrate multiple *Plac* elements through Cre recombinase mediated excision. In addition, we also modified a pUCP18 *E. coli*-*P. aeruginosa* shuttle vector (158) by replacing the *Plac* promoter of pUCP18 with *lacI*<sup>q</sup> (pREP) for transcriptional repression downstream of the *Plac* insertion site. The *lacI*<sup>q</sup> is expressed in trans through a multi-copy plasmid, thus increasing the level of repression. The pREP vector still contains the original MCS site to insert the gene of interest during complementation studies. The combination of the pUCGmx*lac* vector and pREP allows gene characterization as well as operon identification via IPTG expression in *P. aeruginosa*.

The application of the Gmx*lac* cassette was demonstrated through the deletion of the *narK1K2* genes which are involved with nitrate/nitrite transport in *P. aeruginosa* (161). The *narK1K2GHJI* operon of *P. aeruginosa* contains the genes required for the first two steps of the denitrification pathway i.e., transport of nitrate/nitrite (161) and subsequent reduction of nitrate to nitrite (167). The denitrification pathway allows the organism to grow anaerobically using nitrogen oxides as terminal electron acceptors for respiration (167). During the first two steps, nitrate reductase reduces nitrate to nitrite which eventually, through a multi step

process, is ultimately reduced to dinitrogen gas. The conditional phenotype after introducing the pREP plasmid was measured through respiratory nitrate reductase activity using IPTG as the inducer. This use of the pUCGmx/*lac* and pREP vectors demonstrates the polycistronic expression of the *narK1K2* with *narGHJI*, further confirming these genes as an operon (161). In addition, the introduction of a *Plac* element upstream of *narGHJI* without the disruption of *narK2* allows investigators working with *P. aeruginosa* to override both oxygen and nitrate regulation of the expression of the gene products required for the reduction of nitrate to nitrite. This is demonstrated by the full activity of respiratory nitrate reductase under aerobic conditions in the absence of nitrate.

## **MATERIALS AND METHODS**

### **Bacterial growth conditions.**

All bacteria used in this study were grown in Luria Bertani (LB) broth (20g/L) which was supplemented with or without 1% w/v KNO<sub>3</sub>. Aerobic cultures were grown at 37°C with shaking at 250 rpm to exponential phase (*A*<sub>660</sub> of 0.4 to 0.6) without nitrate. Anaerobic growth cultures were inoculated with overnight shaker-grown starter cultures (LB only) and incubated at 37°C in a sealed 125 ml Ehrlenmeyer flask equipped with a gas release, filled to the top with LB + 1% w/v KNO<sub>3</sub> and flushed for 10 min with argon gas. In addition, complete anaerobiosis was ensured by the addition of 2% (w/v) Oxyrase enzyme (Oxyrase Inc., Mansfield, OH). For respiratory nitrate reductase activity, all strains were first grown aerobically at



37°C in LB containing 1% w/v KNO<sub>3</sub> to A<sub>660</sub> of 0.4 to 0.6, and transferred to anaerobic conditions in BBL Anaerobic GasPak System (Sparks, MD) for 3 h. Antibiotics from Bioworld (Dublin, OH) were used for *E. coli* at the following concentrations: ampicillin, 100 µg/ml; gentamicin, 15 µg/ml; tetracycline, 25 µg/ml. Gentamicin, carbenicillin, and tetracycline were used at 300 µg/ml, 500 µg/ml, and 25 µg/ml respectively for *P. aeruginosa*. When applicable, the appropriate concentrations of IPTG (Sigma, St. Louis, MO) were added immediately after inoculation.

#### **Manipulation of recombinant DNA and genetic techniques.**

All plasmid and chromosomal nucleic acid manipulations were by standard techniques (145). Plasmid DNA was transformed into *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, MD), SM10, or *P. aeruginosa* PAO1 (Manoil isolate), which was kindly donated by Elizabeth Sims (University of Washington, Seattle). Restriction endonucleases, Klenow fragment, and T4 DNA ligase were used as specified by New England Biolabs (Beverly, MA). Plasmid DNA was isolated using Qiagen (Valencia, Ca) plasmid preparation kit. DNA fragments were isolated from agarose gels using QBiogene (Carlsbad, Ca). PCR reactions were performed using Taq DNA polymerase, PCR buffer, dNTP's from Sigma (St. Louis, MO) in a Peltier Thermal Cycler.

### **Construction of pUCGMx*lac* and pREP plasmids (Figure 2).**

For the construction of pSOP, a 500 bp fragment containing the *lac* promoter was cloned out of pUCP18 using the following primers with the introduction of *Cla*I and *Sac*II restriction sites (underlined respectively) (5'-TGTATCGATTCGCCACCTCTGACTT-3' and 5'-CTCCGCGGCGTAATCATGGTCATAG-3') into pGEM T Easy (Promega, Madison, WI). The 500 bp *Cla*I & *Sac*II digest containing the *lac* promoter was directionally cloned into the *Cla*I & *Sac*II sites of pUCGm/*lox* (126), adjacent to the *loxP* site. For the construction of pREP, a 1 kb fragment containing the *lacI<sup>q</sup>* was cloned out of TOP10F' from Invitrogen (Carlsbad, Ca) using the following primers with the introduction of *Sap*I and *Eco*R I restriction sites (underlined respectively) (5'-GTGCAAAGCTCTTCGCGGTAT-3' and 5'-CGCGAATTCA CATTAAATTGCGTTG-3') into pGEM T Easy (Promega, Madison, WI). A 1 kb *Eco*R I digest containing the *lacI<sup>q</sup>* was cloned into the *Eco*R I site of pUCP18. Next, a *Sap*I digest, fill in, and blunt-end ligation removed the *lac* promoter to generate pREP.

### **Construction of $\Delta narK1K2$ -*lac* mutant in wild-type strain *P. aeruginosa* PAO1 (Figure 3).**

A 1.5 kb *Sma*I digest containing Gm*xlac* cassette was blunt ligated into a *Not*I-*Apa*I digest of pEX18Ap::*narK1K2* (161) releasing a 2.2 kb *narK1K2* fragment replaced by the insertion of the Gm*xlac* cassette. Single-copy chromosomal, gene disruptions were created using a gene replacement technique previously described

(157). *loxP* excision of the gentamicin resistance marker was conducted by transformation of pCM157 (126) containing a tetracycline resistance marker and the gene encoding for Cre recombinase into the  $\Delta narK1K2$ -Gm<sup>r</sup>lac *P. aeruginosa* strain. Several passages of growth in LB containing 25 µg/ml tetracyclin was followed by selection for the loss of growth in LB containing 300 µg/ml gentamicin. Constructs were confirmed by PCR using 5'-CCTGTCACCTCCAAAG-3' and 5'-TTGGCGCTGTAGATGTAC-3' primers (Sigma-Genosys, Woodlands, TX) (data not shown).

#### **Complementation studies.**

pUCP18 containing *narK1K2* and pREP-*K1K2* were constructed by ligating the 2.7 kb *EcoRI* fragment of pEX18Ap::*narK1K2* into the *EcoRI* site of both pUCP18 and pREP respectively.

#### **Measurement of nitrate reductase enzyme activities.**

Cell free extracts were prepared by sonication of the appropriate whole cell suspension and subsequently analyzed for methyl-viologen linked nitrate reductase activities as previously described (93). Specific activities were measured as nmol nitrite/min/mg protein. Mean and standard deviations were calculated from at least three independent measurements.

## **RNA isolation and reverse transcription**

Total RNA was isolated from 1 ml of aerobically grown cultures at  $A_{660}$  of 0.4 to 0.6 using TRI REAGENT (Molecular Research Center, Cincinnati, OH). Genomic DNA was eliminated by two consecutive DNase I treatments (Promega, Madison, WI). 0.5  $\mu$ g of total RNA was used for cDNA synthesis using random hexamers (iScript, BIO RAD, Hercules, CA). Concentrations of cDNA were normalized (Hitachi U-1100 Spectrophotometer).

## **Real-time PCR**

The following primers were used for *narG*, 5'-ACGACCTCAACACCTCCGAC-3' and 5'-GATCTCCCAGTCGCTCTTGG-3'. PCR was performed using iCycler iQ (BIO RAD, Hercules, CA) with iQ SYBR Green Supermix (BIO RAD, Hercules, CA). Master mixtures were prepared as recommended by manufacturer. PCR was accomplished by a 3 min denaturation step at 95.0 °C followed by 40 cycles of 30 s at 95.0 °C, 45 s at 60.0 °C and 45 s at 72.0 °C.

## **RESULTS AND DISCUSSION**

### **The use of the *Gmxlac* cassette for gene disruption in *P. aeruginosa*.**

The success of the *Gmxlac* cassette for gene disruption in *P. aeruginosa*, PAO1 was tested by targeting two ORFs in the *nar* locus, namely, *narK1* and *narK2* (Figure 3). These genes are contained in the *narK1K2GHJI* operon required for the

first two steps of the denitrification pathway in *P. aeruginosa* (161), providing the organism with the capability to respire anaerobically by using nitrate as the terminal electron acceptor. A *narK1K2* deletion with the *Gm<sub>x</sub>lac* cassette disrupts the function of *narK1K2* and introduces a *Plac* element upstream of *narGHJI*. When incubated under anaerobic conditions with nitrate, the  $\Delta narK1K2$ -*lac* was unable to grow compared to PAO1 (wild type) (Figure 4). Complementation of this mutant with pUCP18 containing *narK1K2* partially restored anaerobic growth. The incomplete restoration to wild type growth may be due to the over expression of membrane bound proteins (35) because pUCP18 is a multi-copy plasmid (118). This was confirmed by the limited growth of PAO1 containing pUCP18 with *narK1K2* genes (Figure 4). The introduction of pREP containing *narK1K2* also confirms that *narGHJI* is in an operon with *narK1K2*.

In addition, we were able to conditionally repress transcription of *narGHJI*, by modulating nitrate reductase activity via the *lac* repressor (Figure 5). Due to the dynamic nature of LacI and *lac* promoter interaction some minimal enzyme activity was still observed. Since *P. aeruginosa* also possesses a periplasmic nitrate reductase (184), a *narG* mutant (PA3875) (117) was used as a control to ensure that the enzyme activities being observed solely represent respiratory nitrate reductase, not that of periplasmic nitrate reductase or assimilatory nitrate reductase. Thus, the level of repression through the pREP plasmid caused decreased growth to a level observed in a *narK1K2* mutant (Figures 4 and 5). Interestingly, the level of nitrate reductase activity in  $\Delta narK1K2$ -*lac* was higher than that of PAO1 (Figure 5). One possible

explanation may be the presence of the *nar* promoter that is activated anaerobically (198). Therefore, the use of cassettes containing promoters such as *Plac* could result in altered transcriptional activity of downstream ORFs. The use of pREP with appropriate levels of IPTG to mimic wild type transcriptional levels may be necessary in some cases. In addition, the transcriptional noise observed with pREP may not always result as a conditional mutant phenotype.

With the *P. aeruginosa nar* locus, the phenotypes observed in Figures 4 and 5 demonstrate the polycistronic expression of *narK1K2* along with the *narGHJI* ORFs, thus, further confirming the genes to be in an operon. The additional use of pREP containing *narK1K2* genes also allows the investigator to identify involvement of the downstream genes, *narGHJI*, by transcriptional repression. Thus, the method described here allows the investigator to disrupt the gene(s) of interest, identify polycistronic ORFs, and introduce conditional mutations via a single insertion of the *Gm<sub>x</sub>lac* cassette.

### **Overcoming oxygen repression of respiratory nitrate reduction in *P. aeruginosa*.**

Under aerobic conditions, the *nar* cluster of *P. aeruginosa* is subject to two levels of regulation: at the transcriptional level and at the post-transcriptional level. At the transcriptional level, this control is exerted by two DNA-binding regulatory proteins namely, ANR and DNR, that are transcriptional activators for denitrification genes (6, 202). Both contain Fe-S sites which are thought to be inactivated by oxygen thus leading to the inactivation of Anr and Dnr. In addition, in organisms

such as *E. coli*, the presence of nitrate also induces the *nar* operon through the dual, two-component regulatory system *narXL* and *narQP* (154, 169, 170). However, in *P. aeruginosa*, functional characterization of *narX narL* and *narP narQ* has not been experimentally analyzed and it appears that *narQ* is not in the genome (171). The only study regarding *narX* and *narL* in a *Pseudomonas* strain has been shown in *Pseudomonas stutzeri* (55).

We hypothesized that nitrate reductase genes (*narGHJI*) would be transcribed aerobically in our genetically engineered strain due to the presence of *Plac* element. The results demonstrated that methyl viologens dependent respiratory nitrate reductase activity was present in crude extracts of the *narK1-lac* strain even under aerobic conditions without nitrate (Figure 6). In the presence of nitrate under aerobic conditions, the levels of nitrate reductase activity remained undetected in wild-type PAO1 contrary to recent findings (data not shown) (36). The derepression with IPTG demonstrated that the respiratory nitrate reductase activity was controlled by the *Plac* element (Figure 6). The methyl viologens activity of nitrate reductase in both the anaerobically grown wild-type and aerobically grown *narK1-lac* strain were the same. We conclude from this that the oxygen inhibition of transcription of the *nar* operon was completely overcome by the engineered strain.

Post-transcriptionally, oxygen also has the capacity to prevent nitrate reduction by two different modes: at the level of nitrate uptake (59, 60) and through diversion of electron flow (29). We have investigated whether or not the post-transcriptional effects of oxygen inhibition influenced physiological function in the

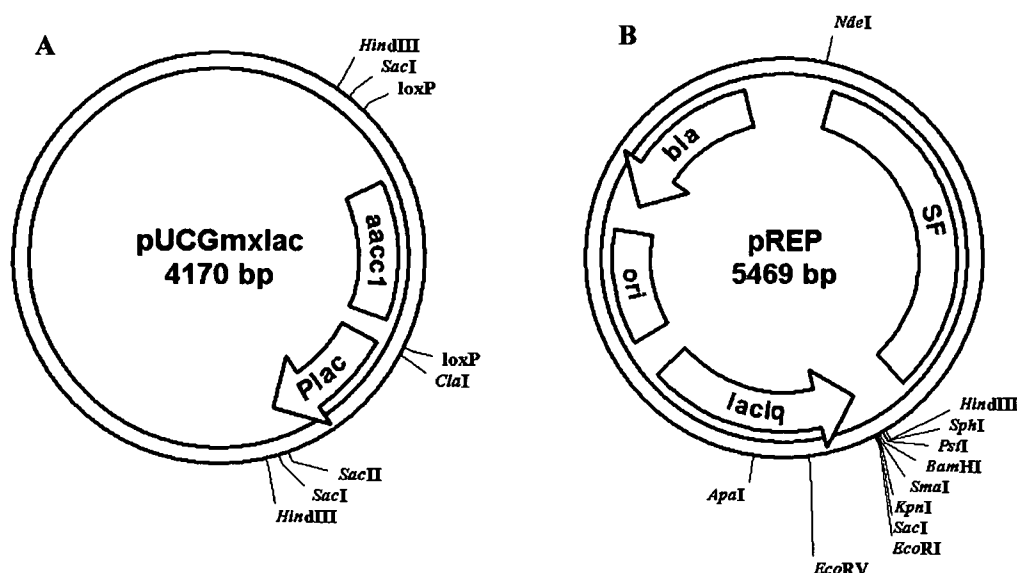
strain genetically engineered to transcribe the *nar* operon even in the presence of oxygen. We measured the ability of *narK1-lac* to reduce nitrate to nitrite during aerobic growth in a batch culture. The *narK1-lac* strain was grown aerobically in LB + 1% KNO<sub>3</sub> and harvested at A<sub>660</sub> of 0.5. Physiological nitrate reduction was measured indirectly by the levels of nitrite present in the external growth medium. The aerobically grown *narK1-lac* strain excreted nitrite at levels 10-fold higher than the aerobically grown wild-type strain (Figure 7). However, these levels are still significantly lower (60 fold, data not shown) than would be observed in the wild-type under anaerobic conditions. Thus, although the *Plac* insertion enabled the *narK1-lac* to transcriptionally overcome oxygen inhibition (Figure 6), post-transcriptional repression of nitrate respiration by oxygen was responsible for partially limiting nitrate to nitrite reduction during aerobic growth. Through the use of the *Gm<sub>x</sub>lac* cassette we were able to distinguish the levels of oxygen regulation transcriptionally and post-transcriptionally.

Finally, the levels of *narG* mRNA were measured by real-time PCR with the *narK1-lac* strain and compared to PAO1 under aerobic conditions in LB without nitrate (Figure 8). As expected, *narG* expression under the *Plac* promoter was approximately 30 fold higher as compared to wild-type. Thus, confirming constitutive transcriptional expression of the *narK2GHJI* genes under aerobic conditions.

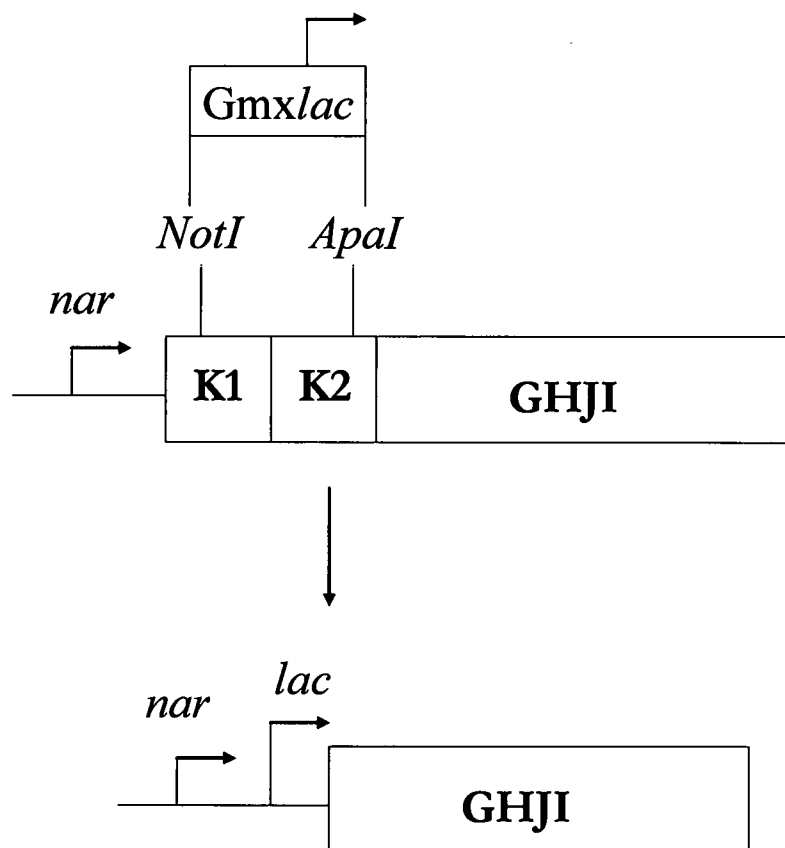


## CONCLUSIONS

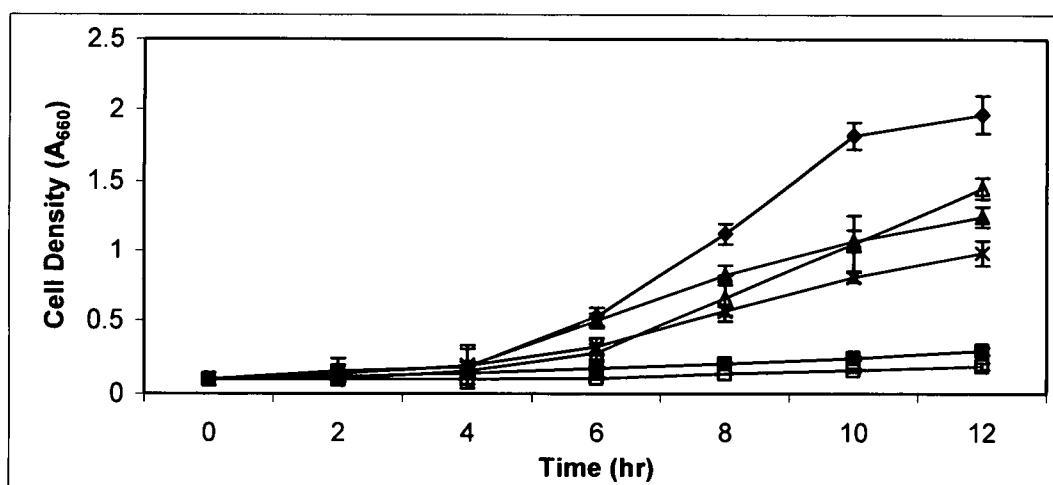
The *Gmxlac* cassette provides an efficient method to characterize and identify polycistronic ORFs in *P. aeruginosa*. Gene disruption by integration of the *lac* promoter also introduces a regulatory element for manipulating transcriptional activity of downstream ORFs, thus providing the investigator a tool to characterize essential genes. Another potential use for cassettes containing inducible/repressible elements is constitutive transcription of multiple genes within the chromosome, a technique already utilized in *E. coli* (180). By inserting multiple promoters throughout the chromosome, the researcher will be able to transcriptionally manipulate numerous genes, operons, and thus, possibly entire pathways. Along with greater understanding of different transcriptional regulation mechanisms, the use of inducible/repressible cassettes will aid in the construction of *P. aeruginosa* strains that can overcome environmental constraints.



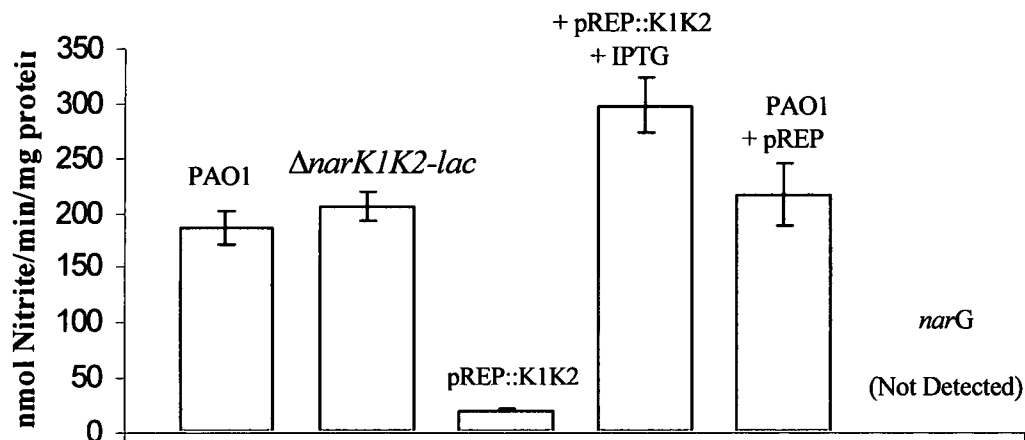
**Figure 2. Plasmids construction. 1.1.76 (<http://medlem.spray.se/acacalone/>).** A) Plasmid map of pUCGmxlac containing linked *Plac*. pUCGmxlac is derived from pUCGmlox (GenBank accession no. AY906831). The restriction sites to release the 1.5 kb Gmxlac cassette from pUCGmxlac vector are in the following order: *SacI-KpnI-XmaI-SmaI-SalI-HincII-PstI-SphI-HindIII*. B) Plasmid map of pREP containing the *lacI<sup>q</sup>* gene. pREP is derived from pUCP18 (GenBank accession no. U07164). ColE1 origin of replication (ORI) and a 1.8 kb stabilizing fragment (SF) are also shown. Due to the insertion site of the *lacI<sup>q</sup>* gene in pREP, blue-white screening is no longer applicable. The MCS in pREP is depicted above.



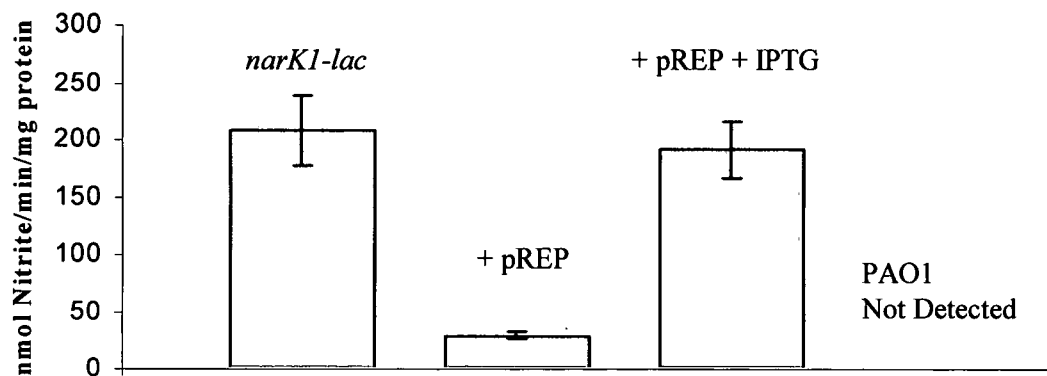
**Figure 3. A map of the *narK1K2GHJI* operon of *P. aeruginosa*.** The map shows the *narK1* and *narK2* genes to be upstream of the structural genes of nitrate reductase (*narGHJI*). Relevant restriction sites, used to create the deletion, are shown. The endogenous promoter for the *nar* cluster and the *lac* promoter are shown as *nar* and *lac* respectively. The direction of transcription of both the operon and the *lac* promoter are shown with the help of arrows. The  $\Delta narK1K2-lac$  mutant was created by blunt-ending the *Gmxlac* cassette into the *NotI-Apal* deletion site.



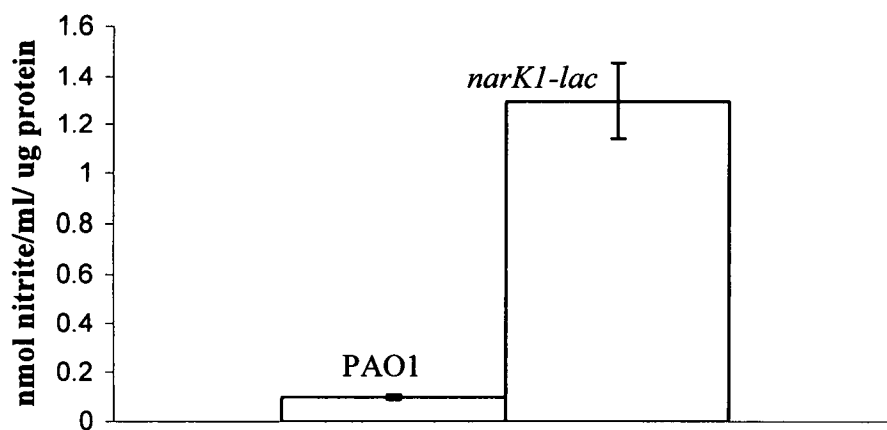
**Figure 4. Anaerobic growth of *P. aeruginosa* in LB supplemented with 1%  $KNO_3$ .** PAO1 (♦), PAO1 with pUCP18 containing *narK1K2* genes (Δ),  $\Delta narK1K2$ -lac (■),  $\Delta narK1K2$ -lac with pUCP18 containing *narK1K2* genes (▲), *narK1K2*-lac with pREP containing *narK1K2* genes (□), and *narK1K2*-lac and pREP containing *narK1K2* genes with 10mM IPTG (x). Cells were incubated aerobically overnight LB, transferred to LB supplemented with 1%  $KNO_3$  in anaerobic conditions (see Materials and Methods). Growth rate was measured using optical density of three independent cultures at 660nm ( $OD_{660}$ ).



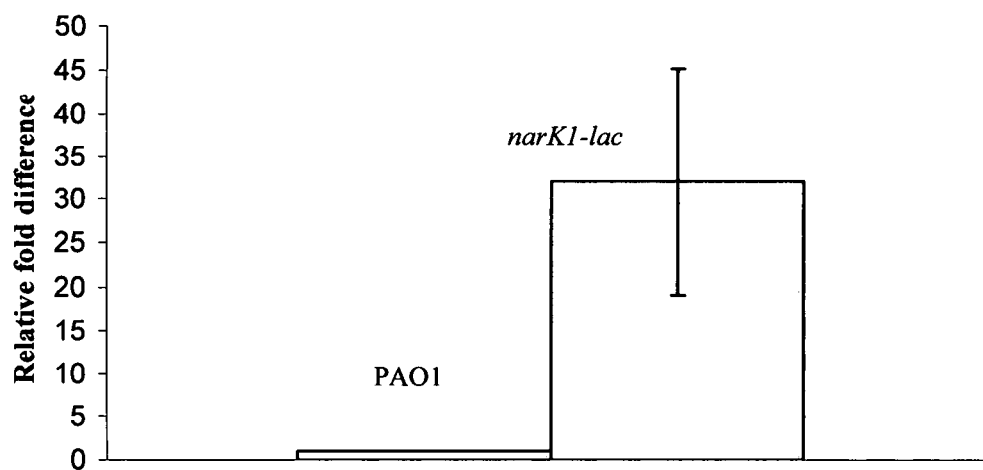
**Figure 5. Respiratory nitrate reductase activity in cell extracts under anaerobic conditions.** Strains used were: *P. aeruginosa* PAO1,  $\Delta narK1K1$ -lac,  $\Delta narK1K2$ -lac with pREP plasmid containing *narK1K2* genes (+ pREP::K1K2),  $\Delta narK1K2$ -lac with pREP plasmid containing *narK1K2* genes with 10mM IPTG (+ pREP::K1K2 + 10mM IPTG), *P. aeruginosa* with pREP (PAO1 + pREP), and *narG*-lacZGM (*narG*). Cells were incubated aerobically overnight in LB, transferred to LB supplemented with 1% KNO<sub>3</sub> in anaerobic conditions (see Materials and Methods).



**Figure 6. Respiratory nitrate reductase activity in cell extracts under aerobic conditions.** Strains used were: *P. aeruginosa* PAO1, *narK1-lac*, *narK1-lac* with pREP plasmid (+ pREP), and *narK1-lac* with pREP plasmid grown with IPTG indicated above (+ pREP + IPTG). All cultures were grown aerobically without the presence of nitrate.



**Figure 7. Levels of nitrite excreted during aerobic growth of PAO1 and *narK1-lac* strains.** Cells were grown aerobically in LB + 1% KNO<sub>3</sub> medium to an OD<sub>660</sub> of 0.5. Cells from 1 ml of media were harvested by centrifugation and the supernatant measured for nitrite by the method of Griess (114). Protein levels were measured by the method of Bradford (17). Mean and standard deviations were calculated from three independent measurements.



**Figure 8. Transcriptional activity of *narG* in the *narK1-lac* strain as compared to PAO1 under aerobic conditions without nitrate. *narG* expression of PAO1 was set at 1. Relative fold difference was calculated by the Pfaffl method (124).**



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