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DISSIMILATORY REDUCTION OF NITRITE AND NITRIC OXIDE BY DENITRIFYING BACTERIA

BY

Weizhang Ye

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

ABSTRACT

DISSIMILATORY REDUCTION OF NITRITE AND NITRIC OXIDE BY DENITRIFYING BACTERIA

BY

Weizhang Ye

Denitrifying bacteria usually prefer oxygen as the electron acceptor and use other nitrogen oxides only when oxygen is limited. Understanding of denitrification is of importance for agriculture and environment. The key step of denitrification is the reduction of nitrite since it converts a mineral form of nitrogen to gaseous forms. This thesis is focused on understanding the pathway, mechanism, and regulation of nitrite and nitric oxide reduction. Cytochrome *cd1* nitrite reductase purified from *Pseudomonas stutzeri* JM300 produced NO as the major product and N_2O as a minor product. With the addition of Fe(II), both production of NO and N_2O was enhanced. Similar enhanced effects of Fe(II) was also observed for the copper-containing nitrite reductase from *Achromobacter cycloclastes*. The mechanism of NO reduction was studied with ^{18}O labeled water in organisms containing copper or cytochrome *cd1* nitrite reductases. It was found that ^{18}O from water was incorporated into both the product and the substrate, producing $N_2{}^{18}O$ and $N^{18}O$. This exchange reaction reached equilibrium rapidly and could be inhibited by electron donors added to the crude cell extract. These results suggest that an enzyme nitrosyl complex exists during reduction of NO,

similar to that found in the reduction of nitrite. The findings that N_2O is the major product of nitrite reductase and that ^{18}O exchange during NO reduction, raise questions on the hypothesis that N_2O is formed via the direct attack of the second nitrite. To study the relationship between the NO_2^- and NO reduction, mutants deficient in nitrite reduction (Nir^-) were obtained with Tn*5* mutagenesis in *Pseudomonas fluorescens* which contains a cytochrome *cd1* nitrite reductase, and in *Pseudomonas* sp. G-179 which contains a copper nitrite reductase. Mutants in the nitrite reductase structural gene from both strains showed not only a loss of nitrite reductase activities, but also a reduction in NO reductase activities. In *P. fluorescens*, the ^{18}O exchange reaction was abolished in all Nir^- mutants. These results suggest that although reduction of NO is distinct from the nitrite reduction, mutation in the nitrite reduction step had physiological and /or genetic effects on NO reduction.

By isolating Tn*5* Nir^- mutant, a copper nitrite reductase gene was isolated from *Pseudomonas* sp. G-179 and showed strong homology with other denitrifiers that contains the copper-type nitrite reductase. Analysis of the upstream region of this gene indicates that its expression may be under the control of FNR and σ^{54}. The role of FNR is further supported by studies with an FNR^- mutant from *P. aeruginosa* , which showed no growth on nitrate, nitrite and nitrous oxide.

in the hope that all these years' effort will help understand somethings in the nature

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Chapter One

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a review

Introduction

Denitrification is the dissimilatory reduction of nitrate to nitrogen gases. It is carried out by a variety of bacteria that occupy a wide range of natural habitats, including soil, water, foods and digestive tract (78). These organisms are facultative and prefer oxygen as their electron acceptor, but in the absence of oxygen they can obtain energy from electron transport phosphorylation coupled to the denitrification. Besides the traditional concern over the loss of fertilizer as a result of denitrification, another great emphasis has been on the negative impact of NO and N_2O evolution, which has been found to contribute to ozone destruction and global warming(11,34,51). At the same time, denitrification has been very useful in the treatment of waste discharges. Utilization of nitrate as the terminal electron acceptor may often be a better way to degrade contaminants by bacteria under oxygen limiting conditions, such as in the aquifers (33). A growing number of denitrifiers with these properties are being discovered(14,52).

Four enzymatic steps are required to convert nitrate to dinitrogen gas (Figure 1). In gram-negative bacteria, nitrate reductase is bound to the cytoplasmic membrane with its active site facing the cytoplasm (61). Nitrate is reduced at the cytoplasmic site of the membrane and therefore has to be transported inside the cell. However, nitrite reductase is located in the periplasmic space. It is believed that import of nitrate and export of nitrite is carried out via an antiport system. There are two major types of dissimilatory nitrite reductases: those containing

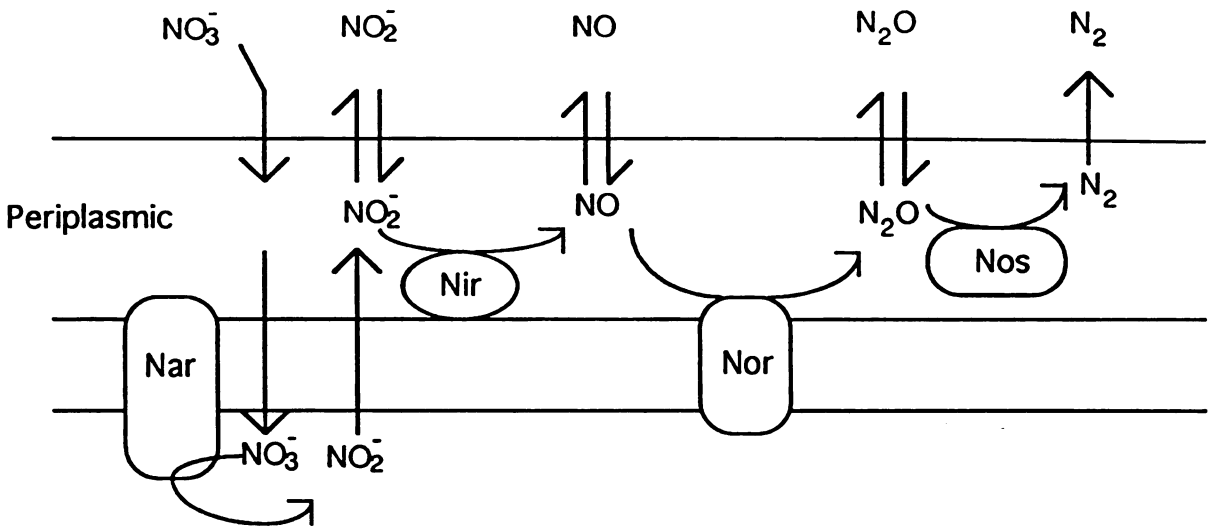

Figure 1. Denitrification pathway in gram-negative bacteria. Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase.

cytochrome cd_1 (cd_1-dNirs) and those containing copper (Cu-dNirs) in the active site (10,30,54). The major product of nitrite reduction by nitrite reductase is nitric oxide, which is subsequently reduced to nitrous oxide by the nitric oxide reductase. Nitric oxide reductases purified so far are membrane-bound(8,12,29). In organisms like *P. aureofaciens* and *P.chlororaphis*, N_2O formation is the last step of denitrification (16). Loss of N_2O reduction ability can also be found in some soil isolates that have been cultured for many generations in laboratories. Reduction of N_2O is accomplished by nitrous oxide reductase which is located in the periplasmic space. Nitrous oxide reductase is usually isolated under anaerobic conditions and exposure to oxygen inactivates most of the known enzymes (30,61).

The focus of research on denitrification has been on the process of nitrite reduction to nitrous oxide for the following reasons. 1) Dissimilatory reduction of nitrite is the key point of divergence from assimilatory nitrogen metabolism because the products of dissimilatory nitrite reduction leads to formation of gases (Fig.1). 2) Evolution of NO and N_2O has significant environmental impact (34). 3) The mechanism of formation of the N=N bond in nitrous oxide was unknown (1), and whether NO is a free and obligate intermediate of denitrification has been controversial. During the past few years, a great deal of information has been accumulated to support the idea that nitric oxide is indeed an obligate intermediate in denitrification. Nitric oxide reductases have been successfully isolated (8,12,29), the crystal structure of the copper dNir from *Achromobacter cycloclastes* has been elucidated (23), and great progress has been made in the studies of genes

involved in nitrite reduction from denitrifiers containing cd_1 or Cu-dNirs (39,57,59,65). The genes involved in nitric oxide reduction in denitrifiers containing cd_1 nitrite reductases has also been identified (5,6). These findings provide a much better understanding of denitrification and point to new directions for future research.

Establishment of NO as an obligate intermediate of denitrification.

Nitric oxide has been proposed as an intermediate in denitrification for several decades (16,50). However, there has been uncertainty as to the true role of NO in the reductive pathway. It was reported that there was a lack of scrambling between the N species from nitrite and added nitric oxide at high cell density in several denitrifiers (20). Unlike other intermediates in the pathway, NO is present only at very low steady state levels (4,26). No denitrifiers are able to grow on NO alone except *Thiobacillus denitrificans* (35), probably due to the toxic nature of NO. The nitric oxide reduction system was poorly studied and the existence of enzymatic NO reduction was questionable. One group suggested that nitrous oxide could be formed from nitrite directly by a single enzyme, the nitrite reductase (1,67).

NO is the major product of dissimilatory reduction of nitrite.

Purified nitrite reductases studied so far produce NO as the major product and N_2O as the minor one (7,43,69). The N_2O produced can be abolished by chelators such as EDTA, suggesting chemical conversion of NO to N_2O by a trace amount of Fe contamination(77). Shapleigh *et al* used Triton X-100 to inhibit NO reduction in crude extracts of several denitrifiers (55). In this

system, 80-95% of the nitrite was recovered as NO. Addition of CHAPS-soluble extract with nitrite reductase but devoid of NO reductase restored the capacity to convert NO_2^- to N_2O. Production of NO also depends on the pH of the growth media (66). Between pH 6.5 and 7.0, reduction of nitrite or nitrate leads to accumulation of NO in in resting cells of *P. aeruginosa*. At pH above 7.5, only N_2O is detected.

To quantify the amount of NO diffusible out of the cell during reduction of nitrite or nitrate, extracellular hemoglobin(Hb) was used to trap NO in several denitrifiers (24). About 2 N atoms in 3 were trapped irreversibly as NO. The rate of HbNO formation was approximately zero order at the diffusion controlled limits. Since the formation of HbNO was irreversible, the denitrification pathway was short-circuited. To better study the production of NO and its subsequent reduction to N_2O, Zafirou, *et al*, used gas sparging to measure the extracellular NO (74). This process permits kinetic measurements under partially reversible conditions. The yield of NO gas depends on the rate of gas flow in this system and the maximum scavengeable NO was estimated to be 78% with a simplified Michealis-Menten equation (4,74), in agreement with the Hb trapping experiment. All the above experiments demonstrated that NO can diffuse out of the cell and that a majority of the N atoms from nitrite or nitrate are found in NO *in vivo* before it is reduced to N_2O.

If NO is the intermediate during the reduction of nitrite to nitrous oxide, exogenous NO would have two effects: (i) increased accumulation of endogenous NO as the exogenous NO increased due to competition for NO reductase or even inhibition; and (ii) a nearly completely random

combination (scrambling) between the exogenous N and endogenous N to form N_2O during simultaneous reduction of nitrite and NO. These two phenomena were observed by Firestone *et al* (16) in ^{13}N labelling experiments with *P. aureofaciens* and *P. chlororaphis*. Complete scrambling was also found in *Paracoccus denitrificans* (25). Early observations that there was a lack of extensive scrambling between ^{14}N and ^{15}N with a high density of cells might have been due to lack of equilibration between the gas and liguid phases (25). The result of random scrambling is not consistent the hypothesis that the bulk of N_2O is formed directly by the nucleophilic attack of a second nitrite molecule on the nitrosyl complex in the nitrite reductase(1,67).

However, the above experiments do not rule out the possibility that some of the N_2O produced comes from the direct reduction of nitrite without the formation of NO. The ultimate proof that NO is the only product of nitrite reductase comes from the isolation of Nor^- mutants which lack of the ability to convert NO to N_2O. These mutants were created by gene replacement by Zumft and his colleaques (5). No N_2O was produced by the cd_1 nitrite reductase from reduction of nitrite in these mutants and thus suggests that NO is the obligate intermediate *in vivo*. The Nor^- mutant is conditionally lethal under anaerobic conditions in the presence of nitrate due to the accumulation of toxic NO. A double mutations in both nitrite and NO reduction rendered the bacteria again viable.

To address the question of a low steady-state levels of NO, Gorestski, *at el*, (25,26) showed that a low level of NO allows NO to be an intermediate without reaching toxic steady-state levels. This is accomplished by a very

low apparent Km of less than 10 uM and a higher Vmax of the nitric oxide reductase than the nitrite reductase. It is estimated that the Vmax for of NO uptake in five denitrifiers at low cell density condition exceeded the Vmax for nitrite uptake. Steady state concentration of NO under denitrifying conditions ranged from 1 to 65 nM. Once established, the amount of NO does not change with time and is independent of the initial concentration of nitrate or nitrite. This level was re-established after addition of exogenous NO or loss of NO by sparging. Formation of nitric oxide complex of cytochrome *c'* in cells of denitrifying bacteria may play a role in reducing the level of NO in conjunction with the NO reductase (73).

Purification of NO reductase. Most of the NO reductase activity is found in the membrane fraction (28,77). Choices of suitable detergents are essential for purification of the enzyme. Grant, *et al*, used CHAPSO to extract NO reductase from the membrane fraction of *Paracoccus halodenitrificans,* resulting in the separation of nitrite and nitric oxide reductase activities (28). Cytochromes were found in the nitric oxide reductase fraction. NO reductase from *P. stutzeri* was purified with Triton X-100 (29), while NO reductase from *Paracoccus denitrificans* was purified with dodecyl maltoside (8). A single purification scheme with octyl glucoside is also reported to purify the NO reductase from *Paracoccus denitrificans*(12) Characteristics of these two NO reductases are summarized in Table 1. They are both cytochrome complexes

Table 1. Characteristics of NO Reductase

	P. stutzeri (28)	*Pa. denitrifican* (8,12)
MW(kDa)	38,17	37,18,45,29
Heme types	b,c	b,c
Specific Activity	12.7	11
Km		< 1 uM
Iron/*Mr*	6	5.2-6.1
pH optimum	4.8	
Absorption maxima	420.5,522.5,552.5	420.5,552.2,558.8

containing heme b (associated with 37 or 38 kDa subunit) and heme c (associated with the 17 or 18 kDa subunit).

Each NO reductase molecule contains two heme groups in both organisms, but there are 6 Fe molecules in the enzyme isolated from *P. stutzeri* . The number of nonheme Fe molecules in the enzyme from *Pa. denitrificans* is around 2.2 to 3.1. The role of these heme or nonheme iron atoms is unknown.

The activity of NO reductase from *P. stutzeri* was markedly enhanced by the addition of lipids, such as soybean lipids or detergents (e.g. n-octyl-b-d-glucopyranoside) (29). The enzyme activity is inhibited by cyanide with a K_i of 2.6 mM. NO reductase can be inhibited by addition of high concentration of NO. Inhibition of NO reduction activity by nitrate and nitrite was also been reported (50).

Reduction of NO is energy-conserving. As the intermediate of denitrification, one important function is to serve as the electron acceptor and conserve energy. Proton translocation unique to NO reduction under denitrifying conditions has been shown in a number of denitrifiers (53). Values obtained were consistent with the expected ratios of 0.5 mol of H^+/mol of NO for reduction of NO to N_2O in *Paracoccus denitrificans* (7). Antimycin A strongly inhibited the NO-dependent proton translocation, further suggesting that a proton electrochemical gradient is generated. NO reduction with inverted membrane vesicle preparations from *P. denitrificans* give a ratio of 0.75 Pi per pair of electrons, comparable to that

found in the reduction of nitrate (7). However, an NO-dependent H^+-gradient was not observed in either *P. aeruginosa* or *A. brasilense*(66). This result challenges the significance of NO reduction for the purpose of energy generation in these two organisms. More experiments are needed to confirm these observations, because a small amount of O_2 was present in the assay system as indicated by the authors (66).

Mechanism of NO reduction. Our studies on the mechanism of NO reduction show(71): 1) exchange with $H_2{}^{18}O$, resulting in production of $N_2{}^{18}O$; 2) competition of ^{18}O exchange by availability of electrons ; 3) attacked of a N intermediate by nucleophilic compounds; 4) formation of $N^{18}O$ due to the reversible reactions before NO is reduced. These findings suggest that the formation of an enzyme nitrosyl, E-NO^+, during dissimilatory reduction of NO, similar to the intermediate proposed for reduction of nitrite by nitrite reductase (21). The site of ^{18}O exchange during NO reduction is presumed to be the NO reductase. The ^{18}O exchange reaction during reduction of NO differs among different denitrifiers studied. Those with the heme *cd1* -dNiRs exhibited relatively less exchange, while organisms with Cu-dNiRs gave generally higher extents of exchange but over a broader range of values. In two organism containing Cu-dNirs,*P. aureofaciens* and *A. cycloclastes*, very little ^{18}O exchange was found during reduction of NO_2^- to N_2O, but a significant amount of exchange was observed during reduction of NO to N_2O. This suggests that there are some mechanistic differences in the reduction of extracellular NO as compared to the reduction of the intracellular pool of NO.

The realization that NO, as a denitrification intermediate, can undergo ^{18}O exchange was completely unexpected and raises questions about previous studies on the mechanism of N=N bond formation from nitrite (21,67). In these studies, ^{18}O exchange was determined by measuring the ^{18}O enrichment in N_2O product using nitrite as the substrate. As a result, it is impossible to determine whether the observed ^{18}O exchange occurred during reduction of NO_2^- to NO or reduction of NO to N_2O. In nitrosyl trapping experiments with azide or hydroxylamine and whole cell or crude extracts, it is also impossible to determine at which step the nucleophilic attack took place. It was shown that catalysis of nitrosyl transfer seemed to depend on NO when nitrite and azide were used, since removal of NO by CrSO4 eliminated the nitrosation reaction(27). It is possible that nucleophilic attack by azide or hydroxylamine occurred after formation of NO from nitrite in these previous experiments. Therefore, we conclude that the evidence does not support formation of the N=N bond by attack of a second nitrite molecule (67).

Nitrite and nitric oxide reductions are two distinct processes. Purified *cd*$_1$-dNirs do not have the ability to convert NO to N_2O (43). In crude extracts, when Cu-type nitrite reductases were inhibited by the chelator, DDC, the ability to convert NO to N_2O was not affected (54). In *P. stutzeri,* which contains a *cd*$_1$-dNir, a normal rate of NO conversion was observed in Nir$^-$ mutants(75). These results indicate that nitrite and NO reductions are two distinct processes. However, this does not imply that these two steps do not influence each other. Tn*5* Nir$^-$ mutants obtained from *P. fluorescens* AK-15 showed a significant decrease in the rate of NO reduction (72). Furthermore, the oxygen atom exchange with $H_2{}^{18}O$ during

reduction of NO was abolished. This suggests that there is a functional or genetic interdependence of these two steps in this organism. In the case of *P. stutzeri*, two Nir$^-$ mutants have been found to have effects on the production of cytochrome c_{552} and soluble alpha-type cytochromes (75).

Cytochrome *cd1* Nitrite Reductase

cd1-dNirs have been isolated from a large number of bacteria including *Pseudomonas aeruginosa*, *Thiobacillus denitrificans*, *Paracoccus denitrificans*, *Pseudomons stutzeri* , *Paracoccus halodenitrificans*, and *Alcaligenes faecalis* (30). These enzymes are composed of two identical subunits with a molecular mass of 60 kDa, and each contains one heme c prosthetic group covalently linked to the polypeptide chain and one heme *d1* moiety noncovalently associated with the protein. Heme c binding ligands (39,56,69) are located near the N-terminus of the protein. Antibodies raised against the dNir from *P. aeruginosa* cross-react strongly with those from *P. fluorescens* and *Alcaligenes* strains, which are commonly found in natural environments (10). Comparison of the amino acid sequences of nitrite reductases from *P. aeruginosa* and *P. stutzeri* strain Zobell reveals 56.4% identity (39). The heme *c* binding regions near the N-termini show strong homology (39,69). All dNir gene sequences reveal the existence of a signal peptide, in agreement with location of these enzymes in the periplasm (39,56,30). Incorperation of both heme c and heme *d1* into the apoprotein is proposed to occur inside the periplasm (49).

The precence of heme d_1 is unique in denitrifiers with cytochrome cd_1 nitrite reductases. Chang and Wu proposed a porphyrindione (dioxoisobacteriochlorin) structure for this green colored chromophore (9). The apoprotein lacking the heme d_1 could be reconstituted with the synthetic heme d_1 and about 80% of enzyme activity could be restored, indicating the correct structural model of heme d_1 and the key role of this heme in the conversion of nitrite to NO (69). The detailed kinetics of nitrite reduction by cytochrome cd_1 was studied by stopped-flow and rapid freezing EPR (57). The first step involved electron transfer from reduced d_1 to nitrite and dehydration, resulting in the binding of oxidized heme d_1 to NO species. This step is very fast, being lost in the dead time of rapid mixing. In the second step, the electron is passed from the heme *c* to heme d_1 with a rate constant of 1 s^{-1}. When heme d_1 is NO-bound, the rate at which heme *c* can accept electrons from ascorbate is remarkably increased as compared to the oxidized enzyme and this electron transfer results in the formation of $c^{+2}d_1^{+2}$-NO, which can be detected by EPR. The binding of NO to the reduced heme d_1 is very tight. Since this study was done at pH 8.0, the kinetic data under physiological conditions (pH 7.0) may be different.

Cu-type nitrite reductases

Denitrifiers with copper dNirs occur at the frequency of 32% among numerically dominant isolates from soil (10). These denitrifiers include species from *Pseudomonas*, *Alcaligenes*, *Corynebacterium*, *Bacillus*, *Rhizobium*, *Agrobacterium*, and *Rhodobacter*. Most of the Cu-dNirs cross-react with the polyclonal antibodies raised against copper dNir from *A. cycloclastes* (10) or from *R. sphaeroides* fp. *denitrificans* (47). These results

suggest that most Cu dNirs share substantial similarity. Cross-reaction is not found in some *Bacillus* and *Rhizobium* species(10).

The nitrite reductase from *A. cycloclastes* is best studied (15,23,36). The enzyme is a trimer with the molecular weight of 36 kDa per monomer and it has a total of six copper atoms per molecule (15,23). The amino acid sequence (15) and 2.3 Å X-ray stucture (23) reveal that the two copper atoms in the monomer comprised of one type 1 copper site and one putative type 2 copper site, which are about 12.5 Å apart. The type 2 copper is bound with nearly perfect tetrahedral geometry by residues not within a single monomer, but from each of two monomers of the the trimer. Amino acid residues 8-175 folds into domain I with a Greek-key ß-barrel structure. Domain I contains the type 1 copper held by ligands of Cys136, His145, Met150, and His95. Residues from 175-340 fold into the domain II which is also a ß-barrel similar to the type 1 copper binding domain. The type 2 copper binding ligands are comprised of His100 and His135 from the domain I of one subunit and His306 from domain II of the second.

It has been reported that the active site of Cu dNirs is the type 2 copper, since only type 1 copper was detected in the copper dNirs isolated from *Pseudomonas aureofaciens* (76) and *Alcaligenes xylosoxidans* (46) and the nitrosyl complex formed upon the addition of NO was found at the type 1 site (62). However, the following evidence strongly suggests that type 2 copper site is required for enzymatic activity and it may even be the active site of the enzyme. (i). Nitrite binds to the type 2 copper site, not to the type 1 Cu. This is observed with competition experiments between nitrite and azide (Hulse, C. and B. Averill, submitted for publication) and with studies of 2.3 Å X-ray

stucture of the enzyme (23). (ii). Type 2 Cu can be removed from the enzyme and reconstituted, and poprotein with very small amount of type 2 Cu has very low activity (Libby, E and B. Averill, submitted for publication). The reconstituted enzymes showed increased activity as the ratio of type 2 copper atoms per enzyme molecule increased. (iii) Ascorbate oxidase has a type 2 Cu in its active site and its location is very similar to the dNir from *Achrombacter cycloclastes*(23). (iv). The amino acid sequence of the copper dNir from *Pseudomonas aureofaciens* reveals type 2 copper binding site (22). It is possible that the type 2 copper is weakly held by its ligands and thus can be removed from the enzyme molecule during purification. It appears that the role of type I Cu is to accept electrons from its physiological donor and pass them to the type II copper active site, similar to the relationship between the heme *c* and heme d_1 in the cytochrome cd_1 enzyme.

The reduced form of azurin or pseudoazurin, the blue copper protein, is found to serve as an effective physiological electron donor for copper dNirs from *A. faecalis* strain S-6 (36), *Achromobacter cycloclastes* (45) and *P. aureofaciens* (76). This small blue copper protein has molecular weight of 12 to 15 kDa and has one Cu atom per molecule. Three absorption maxima are found in the oxidized form: 453, 595, and 750 nm and only one peak at 278 nm is found when reduced. The EPR spectrum is typical of those containing type I Cu. The blue protein can be reduced by various reducing agents including dithionite, ascorbate, cysteine, 2-mercaptoethanol, dithiothreitol, glutathione, and hydroquinone. The reduced protein is autooxidized very slowly. The electron transfer from blue protein to dNir was studied (42). When the concentration of reduced pseudoazurin was

higher than that of oxidized dNir, the reduction of the latter occurred in two steps: a burst phase followed by steady-state kinetics. However, when the concentration of pseudoazurine to dNir was 1:1, only the burst kinetics was observed.

Inhibitors of Cu-dNirs include metal chelators such as diethyldithiocarbamate (DDC), thioglycollate, o-phenanthroline, 8-hydroxyquinoline, ethylene diamine tetraacetic acid (13) and sulfhydryl group inhibitors such as N-ethylmaleimide and p-chloromercuribenzene sulfonate or p-chloromercuribenzoate (36). Electron transport chain inhibitors, KCN and CO, also inhibit the Cu-dNir activity.

Genetics of nitrite reduction

Characterization of genes involved. An operon containing the cd_1-dNir structural gene (*nirS*) is found in *P. stutzeri* (39,59), *P. aeruginosa* (56) and *P. fluorescens* (72)(Fig. 2). This operon starts with

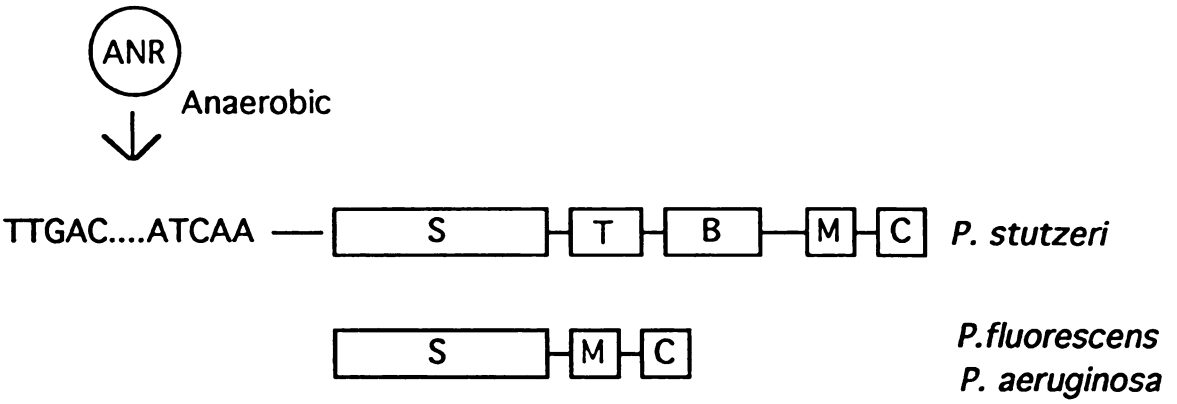

Fig. 2. Gene organization of the operon containing cd1 nitrite reductase. S: nitrite reductase structural gene; T: tetraheme; B: cytochrome C_{552} M: cytochrome C_{551}; C: another monoheme.

nirS. Immediately downstream are *nirM* , encoding the cytochrome c_{551}, and *nirC* (ORF5) encoding an unknown heme protein in *P. aeruginosa* and *P. fluorescens*. In *P. stutzeri nirT,* encoding an unknown tetra-heme protein, and *nirB* , encoding cytochrome c_{552}, are interchelated between *nirS* and *nirM* (39). This difference in gene organization raises questions with regards to the role of cytochrome c_{551} and cytochrome c_{552} in nitrite reduction among different denitrifiers. In *P. aeruginosa* and *P. fluorescens*, the location of the cytochrome c_{551} gene is consistent with the *in vitro* results showing that reduced cytochrome c_{551} can serve as electron donor to nitrite reductase. However, in *P. stutzeri*, cytochrome c_{551} is not regulated by anaerobic condition. There is a 320 base pair intergenic region with multiple inverted repeats between the *nirB* and *nirM*. Thus cytochrome c_{551} may belong to a separate operon and its exclusive role in nitrite reduction is questionable in *P. stutzeri* (39). Instead, cytochrome c_{552} is produced under anaerobic conditions. The location of its structural gene (*nirB*) in the *nir* operon implies its role as an electron carrier for nitrite reduction. Since mutation in *nirT* leads to a defect in electron donation but has no effect on the production of active nitrite reductase, the authors propose that the hydrophobic portion at the mature N-terminus of the *nirT* protein might anchor the cytochrome in the membrane and allow electron flow between the respiratory chain and a periplasmic acceptor. Electrons could pass directly from NirT to NirS, or via cytochrome c_{551}. The function of NirC is unknown, although it is also has a heme *c* binding motif. Tn*5* insertion in this region resulted in production of a defective nitrite reductase in *P. fluorescens*, suggesting

Table 2. characteristics of genes involved in nitrite reduction

genes	Protein	Function	heme c motifs	Mr
nirS	reductase	nitrite reduction	1	59,532
nirM	C_{551}	e^- transport	1	8,563
nirC(ORF5)	-	-	1	9,202
nirT	-	e^- transport	4	19,738
nirB	C_{552}	e^- transport	2	28,180
nirD	?	heme d_1 synthesis	-	-

that this region, or gene(s) downstream, are important for the production of active nitrite reductase (72). Possible function of cytochromes encoded by *nir* operons is summarized in Table 2.

Apart from those in the *nir* operon, gene(s) responsible for heme d_1 synthesis (*nirD*) has been identified. In *P. stutzeri*, two mutations were found the region containing *nirD*, leading to the production of an inactive nitrite reductase due to a lack of heme d_1 prosthetic group. Cosmid-mapping and Southern hybridization revealed a close linkage of the genes *nirS* ,*nosZ* and *nor*(6).

Structural gene for Cu-dNir (*nirA*) has been isolated from *Pseudomonas*. sp. G-179 (Ye,et al, submitted for publication) and *P. aureofaciens* (65). The deduced amino acid sequences appears homologous to that of *Achromobacter cycloclastes*.

Regulation of gene expression.

Regulation by oxygen. Although *E. coli* and other enteric bacteria can not denitrify, they can respire nitrate and nitrite under anaerobic conditions. The *fnr* gene is essential for the expression of genes involved in fumarate and nitrate reduction (38,60) under anaerobic conditions. A conserved symmetrical sequence, $TTGATN_4ATCAA$, the FNR box, is located upstream of the FNR-dependent genes and operons and plays an important role in regulation of gene expression. Under low oxygen tension, the FNR protein acts as a transcriptional activator by binding to the FNR box. A similar regulatory mechanism was found for the anaerobic

metabolism of arginine, nitrate and nitrite in *P. aeruginosa*(17). With chemical mutagenesis, van Hartingsveldt *et al*, isolated a mutant which results in the inability of *P. aeruginosa* to dissimilate nitrate and nitrite (64). It was found later that this strain also lacks the ability to metabolize arginine under anaerobic condition (17). The gene is thus renamed as *anr* for anaerobic regulation of arginine deiminase and nitrate reduction. Haas and his colleaques showed that *anr* encodes an FNR-like protein that acts on the consensus FNR box to regulate the gene expression under anaerobic condition in *P. aeruginosa* (17,19). The *arcDABC* operon responsible for enzymes in the arginine deiminase pathway of *P. aeruginosa* was rendered virtually noninducible by a deletion or an insertion in the -40 FNR binding site and by a mutation in the *anr* gene. Anaerobic induction requires the FNR box in *cis* and *anr* protein in *trans*. This *arc* operon can be expressed at a low level in *E.coli* under anaerobic conditions. Introduction of an anr^+ plasmid had a larger positive effect. The FNR-dependent promoter containing the consensus -40 sequence from *E. coli* was expressed well in *P. aeruginosa* and was regulated by oxygen limitation. *anr* and *fnr* proteins have 51% sequence identity, and several amino acid residues known to be essential for FNR function are strictly conserved in *anr*. Thus, ANR and FNR appear to have similar functions.

Table 3. Putative regulatory sequences from denitrifying bacteria.[a]

Strains	Genes	*fnr* box	*ntrA* box
P. sp. strain G-179	Cu-*nir*	TTGAT....ATCAA	TTGGAGCAAAC ATGCT GTGGAGCCGAGGTTGCT
P. aeruginosa (56)[b]	*cd1 -nir*	UN[c]	CGGGAGTTCCCGACGCA AAGGGAGCGCC TCGCA
P. stutzeri JM300 (59)	*cd1 -nir*	TTGAT....GTCAA TTGAC....ATCAA	UN
P. stutzeri Zobell (59)	*cd1 -nir*	TTGAT....ATCAA TTGAT....GTCAA	UN
P. stutzeri Zobell (65)	*nos*	UN	GTGGAACCCTGAGCGCG
P. aeruginosa (31)	*azu*	TTGAC....ATCAG	GCGGCACATCT GTGCT
Alcaligenes denitrificans (31)	*azu*	TTGAT....GTCAA	CAGGCATGTGCCTGGCG
Alcaligenes faecalis S-6(70)	Peudo-*azu*	TTGAT....ATCAA	GTGGCGTGTTGAGGCC

[a] References are given in the parenthesis followoing the strain names. Abbreviations are *nir*: nitrite reductase gene; *nos*: nitrous oxide reductase gene; *azu*: azurin gene.

[b] The presence of possible regulatory regions were identified in this work based on published results.

[c] UN=Unknown.

Putative FNR boxes have been found in the upstream region of the promoter of dNir structural gene from *P. stutzeri* (59) and *Pseudomonas* sp. G-179 (Ye, et al, submitted for publication). This box is also found in the upstream region of the gene encoding the pseudo-azurin, which is believed to be the physiological donor of the Cu-dNir in *Alcaligenes faecalis* S-6 (70). The presence of FNR boxes and the pleotrophic effects of the*anr*⁻ mutation on nitrate and nitrite dissimilation strongly suggest that the *anr* protein regulates the genes involved in denitrification under oxygen limited conditions. However, the interaction between the *anr* protein and the operator region for nitrite reduction or the denitrification pathway has not been characterized.

Another regulatory protein *ntrA*, which encodes σ^{54} has been found to be involved in diverse physiological processes, including nitrogen fixation and nitrate assimilation (37). The binding site for σ^{54} usually has the GG and GC doublet separated by 10 bp. Analysis of the promoter regions of some genes involved in denitrification or anaerobic metabolism reveals putative *ntrA* boxes (Table 3). It has been found in genes for hemecd_1 or Cu-dNirs, nitrous oxide reductase, pseudo-azurin, and azurin. These lines of evidence suggest that σ^{54} may facilitate the recognition of RNA polymerase to promoters involved in denitrification.

Regulation by substrates. Substrates for the denitrification pathway, such as nitrate, nitrite and N_2O are required for the full expression of enzymatic activities. This is further supported at the gene level. Hirouki, *et al*, introduced the *xylE* gene which encodes catechol 2,3-dioxygenase

(C23O), into the *nir* operon containing the nitrite reductase gene and promoter region, and studied gene expression under different conditions by measuring C23O enzyme activity (3). When cells were grown on arginine anaerobically in the absence of nitrate, the promoter activity of the operon was approximately one-fifth of that under anaerobic denitrifying conditions with nitrate as the electron acceptor. This experiment suggests that substrates activate the transcription of the genes involved in denitrification.

Conclusions

Evidence from several experimental approaches have now shown that nitric oxide is an intermediate in the denitrification pathway. Key evidence is that Nor$^-$ mutants accumulate NO as the only product of nitrite reduction. NO reductases are cytochromes and contains heme *c* and *b* moieties. The mechanism of NO reduction is proposed to involve a nitrosyl complex based on the ^{18}O exchange studies. Cu nitrite reductases may require both type 1 and type 2 Cu for their activities. Type 1 Cu is bound within a monomer whereas type 2 Cu is bound between two monomers. Regulation of nitrite reduction by oxygen may be under the control of an FNR protein and σ^{54}. This is supported by FNR$^-$ mutants which are inactive in denitrification and FNR boxes present upstream of the promoter of the nitrite reductase genes studied.

Many areas of nitrite and nitric oxide reduction remain as possible focuses for study. Although NO reductases have been isolated and characterized from two organisms containing cd_1 nitrite reductases, they have not been isolated from organisms with Cu-type nitrite reductases. It

is still unclear whether all the NO reductases are similar. The mechanism of NO reduction is poorly understood. The major role of NO reduction (detoxification or/and energy generation) in denitrification pathway remained to be clarified. In recent years, gene regulation has been one of major foci of research of prokaryotic genetics. However, very little information is available on regulation of denitrification.It is of particular interest since it regulated by oxygen. The roles of *fnr* and σ^{54} -like proteins need further investigation. Another aspect of gene regulation is the possible factors involved in signal transduction. Finally, studies on the components involved in electron transport including electron donors for specific steps and those further up in the cascade needs more emphasis.

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Chapter Two

Enhanced Effect of Fe(II) on Nitric Oxide and Nitrous Oxide Production by Cytochrome cd_1 Nitrite Reductase from *P. stutzeri* JM300.

Abstract

We examined the effects of Fe(II) on the production of NO and N_2O from nitrite under routine assay conditions for nitrite reductase from *Pseudomonas stutzeri* JM300. The major product of the enzymatic reduction of nitrite by the purified nitrite reductase was NO and a minor product was N_2O. The enzyme did not appear to have the ability to convert NO to N_2O. However, with the addition of 10 uM Fe (II), as much as 30% of the product was N_2O. Fe(II) at this concentration also enhanced NO production. This dual catalytic capability of Fe (II) led to an increase in the rate of nitrite reduction. The enhanced NO and N_2O production was specific for Fe(II) and could not be obtained with other metal ions. Fe chelators diminished the production of N_2O by nitrite reductase in the presence of Fe (II). EDTA and NTA had little effect on NO production, but when Fe(II) was added NO production increased markedly. The Cu-containing dissimilatory nitrite reductase from *Achromobacter cycloclastes* also showed a similar increase in both NO and N_2O production with the addition of Fe(II). Under the assay conditions of this experiment, the increased amount of N_2O production from nitrite by 10 uM Fe(II) could not be accounted for by the chemical reduction of NO from the gas phase. Instead, the role of Fe(II) may be to intercept NO directly from the enzyme and efficiently reduce it to N_2O .

Introduction

The denitrification pathway has been established as : NO_3^- --> NO_2^- --> NO and N_2O --> N_2. The key ecological step in denitrification is the dissimilatory reduction of nitrite since the subseqent products are unavailable to the biota. Two types of dissimilatory nitrite reductases in denitrifying bacteria: one type contains cytochrome*c* and d_1 and the other contains copper(4,9). Organisms containg the cytochrome cd_1 nitrite reductase are more frequently isolated from nature, whereas Cu-type nitrite reductases are found in orgaisms that exhibit more phylogenic diversity and occupy a wider range of ecological niches (4). The product of nitrite reduction, NO, exists at very low steady-levels during denitrification (1). Nitric oxide reductases have been isolated from *P. stutzeri* strain Zobell (8) and *Paracoccus denitrificans* (3,5). Mutations in the structural genes for nitric oxide reductase in *P.stutzeri* Zobell generate only NO from nitrite and Nor$^-$ mutants can not grown on nitrite due to the toxicity of NO (2). ^{18}O exchange studies of N=N formation during reduction of NO indicates the existance of an enzyme-nitrosyl complex, similar to that found in the reduction of nitrite (13).

Assay systems for nitrite or nitric oxide reductases *in vitro* include ascorbate - PMS, NADH-FMN, or NADH - PMS. Zumft and Frunzke (14) found that NO can be converted to N_2O nonenzymatically by Fe(II) in the assay systems with ascorbate. Chelators for Fe(II) eliminate this nonenzymatic activity and allow the distinction between the enzymatic and chemical conversion of NO in the assay systems with membrane fractions

containing nitric oxide reductase. These findings lead these authors to suggest that the presence of adventitious iron and the ubiquitous use of ascorbate might have contributed to the nonenzymatic conversion of NO to N_2O in previous work. However, their experiments were done with higher concentrations of Fe(II) (1.0 mM) and of NO (41.1 umol in 12 ml flasks) than normally present in enzymatic assays for nitrite reductase. While their study was focused on characterizing the nonenzymatic reaction of Fe (II)-ascorbate with NO and the assay for nitric oxide reductase in membrane fractions, the effect of Fe(II) on N_2O production in assays for nitrite reductase was not characterized in detail.

In this experiment we further investigated the effects of Fe(II) on the production of both NO and N_2O in assay systems for nitrite reductases without ascorbate. Under these conditions gas phase NO was not reduced to N_2O, but low concentration of Fe (II) increased both NO and N_2O production from nitrite.

Materials and Methods

Enzymatic assay. The heme-type nitrite reductase (>95% pure) was purified as previously described from *Pseudomonas stutzeri* JM 300 (12). The assay for nitrite reductase activity was routinely carried out in an 8-ml serum bottle with a 1 ml solution containing 50 mM HEPES (pH 7.3), 4 mM NADH , 0.12 mM PMS and 1.0 mM $NaNO_2$ and 30 ug of enzyme. All water used was from reverse osmosis and Millicue treatment. Anaerobic conditions were established by repeatedly evacuating and flushing the gas phase with argon. Assay mixtures were incubated at 25°C with constant shaking (100 rpm) with the serum bottles laid on their sides to ensure equilibration of dissolved gases between the liquid and gas phase. NO and N_2O was analyzed by gas chromatography (10). Standards for N_20 were purchased from Matheson (New Jersey, USA). NO standards were freshly prepared from pure NO. Fresh solutions of $FeSO_4$ or FeCl were prepared in degased water. Fe(II) was added after PMS and NADH were present in the reaction mixture. For assays of the chemical reduction of NO, the same assay conditions were used except that 1.0 umol of NO was used as the substrate. Results were presented as average of duplicates.

Isotope experiment. $Na^{15}NO_2$ (99% ^{15}N) was purchased from Cambridge Isotope Laboratory (Massachusetts, USA) . Gas samples were analyzed with an HP 5985 gas chromatography/mass spectrometry system equipped with a Porapak Q column (13). The mass spectrometer was operated using electron impact and selective ion monitoring.

Results

Stimulatory effects of Fe (II) on NO and N_2O production. Without the addition of Fe (II), purified nitrite reductase produced mainly NO from nitrite (Figure 1A). N_2O was a minor product and made up no more than 10 % of the total nitrogen converted . With the addition of 10 uM of Fe(II), N_2O production rate increased markedly and reached up to 30% of the total nitrogen converted. The rate of NO production was also increased, especially in the early stages of the reaction. As a result, the presence of Fe (II) approximately doubled the rate of nitrite reduction (Figure 1 B).

The enhanced effects of Fe(II) on both NO and N_2O productin could be observed at Fe(II) concentrations from 1 to 100 uM, with 10 uM being the optimum concentration for total N-gas production (Figure 2). At 1 mM, the enhanced effect of Fe(II) was reduced for both gases.

Other metal ions tested individually were $MnCl_2$, $CuSO_4$, $ZnSO_4$, $CaCl_2$, $MgSO_4$, and $CoCl_2$ at 10 or 20 uM. They showed no effect on NO or N_2O production under the standard assay condition.

Chemical conversion of NO to N_2O. The nonenzymatic conversion of NO to N_2O depends on the concentration of both NO and Fe(II) (14). Since 1.0 umol of nitrite was used in the enzymatic reaction, 1.0 umol NO was used to evaluate the chemical conversion of NO added to the gas phase. The combination of PMS, NADH, and nitrite produced a very trace amount of N_2O after 30 min without the addition of Fe(II) (Table 1), indicating that NO

was fairly stable under these assay conditions. In contrast to the reduction of nitrite by nitrite reductase, the addition of 10 uM of Fe(II) did not markedly enhance N_2O production from NO.

The nonenzymatic NO reduction catalyzed by Fe (II) was inhibited at low pH (Figure 3 A). The optimal conversion was observed at pH 8 . At pH values higher than 9, the chemical conversion was slow. The pH optimum for nitrite reductase activity was around pH 7 (Figure 3 B). At pH 8 or higher, the enzymatic activity dropped sharply. The Fe(II) enhanced NO and N_2O production from nitrite paralleled the pH profile of the enzyme and was different from that of the nonenzymatic N_2O production.

Effect of chelators. All iron chelators inhibited N_2O production by nitrite reductase in the presence of 10 uM Fe(II) (Table 3). DDC, a specific chelator for Cu, also diminished the N_2O production. Specific Fe(II) chelators 2,2'-bipyridyl, o-phenathroline, 8-hydroxyquinoline and EGTA inhibited NO production as well. EDTA and NTA did not have any effects on NO production. However, combinations of EDTA and Fe(II) or NTA and Fe(II) has a stimulatory effects on NO production.

The Fe(II) enhanced NO and N_2O production from nitrite was not unique to heme-type nitrite reductase. This phenomenon was also observed with a Cu-type nitrite reductase purified from *A. cycloclastes* (Table 3). If Cu was removed by the Cu-specific chelator DC, the presence of Fe(II) catalyzed no NO production.

Isotope experiment. The chemical reduction of NO by 10 uM Fe (II) did not appear to account for the marked increase of N_2O production from 1 umol of nitrite in the presence of 10 uM of Fe (II) and purified nitrite reductase (Table 1). To further test whether the chemical reduction of N_2O under standard assay conditions was due to the chemical reduction of NO that was released to the gas phase and then equilibrated back to the solution, ^{14}NO was added in the gas phase and used as a tracer. In this experiment, 357 nmol of ^{14}NO was added to serum bottle 1 min before the start of the reaction with 1.0 umol of $Na^{15}NO_2$. All the reaction vials were under constant shaking. Chemical reduction of nitric oxide from the gas phase will be a random process, producing $^{14}N^{15}NO$ proportional to the amount of ^{14}N and ^{15}N. In the experiment with 10 uM of Fe(II), 33% of the headspace was ^{14}N and 77% was ^{15}NO of the total nitric oxide in the gas phase after 30 min of incubation. As a result, chemical reduction of nitric oxide in the gas phase should yield at least 33 % of ^{14}N out of the total amount of nitrogen in nitrous oxide produced. The experiment yielded only about 5 %, inconstant with the above calculation. A similar result was obtained with 200 uM Fe(II) except that more ^{14}NO from the gas phase was converted to nitrous oxide. This result further indicates that most of N_2O produced from nitrite by nitrite reductase under standard assay conditions plus Fe(II) was not due to the reduction of NO from the gas phase.

Discussion

Zumft and Frunzke showed the catalytic ability of Fe(II) to reduce NO to N_2O with PMS/ascorbate system in the absence of any enzyme (14). Our study extends information on the special catalytic role of Fe(II) by showing that both NO and N_2O production from nitrite are enhanced when Fe(II) was added to both heme c,d_1 and Cu nitrite reductases. Under our assay conditions (PMS/NADH, 1 umol of NO), little NO was reduced to N_2O in the absence of enzyme. The amount of Fe(II) need for stimulatory effect on NO and N_2O production from nitrite by nitrite reductases was 10 uM, which is equivalent to approximately 40 mol of Fe(II) per mol of enzyme. In the chemical reduction system studied by Zumft and Frunzke, reduction of NO to N_2O increases as Fe(II) concentration increases, ranging from 50 uM to 1 mM. However, lower concentration of Fe(II) did not have a high capacity to convert added NO to N_2O in the standard assay systems used in this experiment (Table 1). Thus it could not account for the marked increase of N_2O produced by the enzyme when Fe(II) was present at least at concentrations below 200 uM. This is further supported by isotope experiment in which a random scrambling between the ^{14}N and ^{15}N from the gas phase was not observed (Table 4). These lines of evidence suggests that the majority of the N_2O produced in the presence of Fe(II) in the assay systems with nitrite and nitrite reductase was not due to the chemical reduction of NO released to the gas phase. One simple interpretation is that there is a lack of equilibration between the gas phase and the solution in the assay condition. Such a possibility has been proposed during the reduction of NO with high cell density (7). However, it is also possible that Fe(II)

molecules has a very high affinity for NO and thus is able to intercept its release into the gas phase. Direct channeling of NO from the nitrite reductase to Fe(II) molecules in the solution may increase the efficiency of NO reduction to N_2O.

The possible catalytic mechanism responsible for the dual role of Fe(II) is unclear. The Fe(II) enhanced effect on NO production may be different from that on N_2O production since EDTA and NTA chelators inhibited the later but not the former. Fe(II) may increase the efficiency of delivering electrons to nitrite reductase to enhance the NO production. Fe(II) may also able to relieve any inhibitory effect of NO by remove the NO from the enzyme. The complexes of EDTA-Fe(II) and NTA-Fe(II) may even make Fe(II) more available for these purposes, while inhibiting its catalytic ability to convert NO to N_2O.

Table 1. Conversion of nitric oxide or nitrite to nitrous oxide in different assay conditions.[a]

Assay mixture	N_2O (nmole-N)
PMS + NADH + nitrite + NO	4
PMS + NADH + enzyme + NO	16
PMS + NADH + enzyme + Fe + NO	24
PMS + NADH + enzyme + EDTA + NO	13
PMS + NADH + enzyme + nitrite	37
PMS + NADH + enzyme + Fe + nitrite	243

[a] Results presented were obtained after 30 min incubation. The amount of nitric oxide and nitrite used in the assay system were 1.0 umole. EDTA and Fe (II) used were at 1.0 mM and 10 uM, respectively. Results were average of duplicates.

Table 2. Production of nitric oxide and nitrous oxide from nitrite by nitrite reductase in the presence of different chelators.[a]

Assay mixture	NO	N_2O
	(nmol-N)	
Control	17	0.2
+ Fe	50	11.4
EDTA	13	0
EDTA + Fe	110	0.3
EGTA	3	0
EGTA + Fe	5	0
NTA	28	0
NTA + Fe	123	1.4
DDC	23	0
DDC + Fe	31	0
Bipyridyl	3	0
Bipyridyl+ Fe	2	0
o-phenanthroline	3	0
o-phenanthroline + Fe	1	0
8-hydroxyquinoline	2	0
8-hydroxyquinoline + Fe	2	0

[a] Concentration for chelators was 1.0 mM. The Fe (II) concentration was 10 uM. NO or N_2O was measured after 2 min of reaction. Reactions were carried out according to standard assay conditions.

Table 3. Effect of Fe (II) on the production of nitric oxide and nitrous oxide from nitrite by the Cu-nitrite reductase of *A. cycloclastes*[a]

	(nmole)					
	2 min			30 min		
Assay mixture	NO	N_2O	N_T[b]	NO	N_2O	N_T
Control	106	0.4	106	873	32	909
+ Fe	442	72	514	629	356	985
+DDC	0.5	0	0.5	4	0	4
+DDC + Fe	1.6	0	1.6	7	0	7

[a]Assay mixtures contained 1.0 mM of DDC or 10 uM of Fe(II) if used.

[b] N_T = Total amount of nitrogen.

Table 4. Isotope study on Fe(II)-enhanced production of N_2O by nitrite reductase.[a]

Fe (II) (uM)	^{14}NO (nmol)	^{15}NO (nmol)	% ^{14}NO	$^{14}N^{14}NO$ (nmol)	$^{14}N^{15}NO$ (nmol)	$^{15}N^{15}NO$ (nmol)	%^{14}N
20	355	713	33%	0.8	3.6	88	5%
200	350	620	37%	1.5	12.6	124	9%

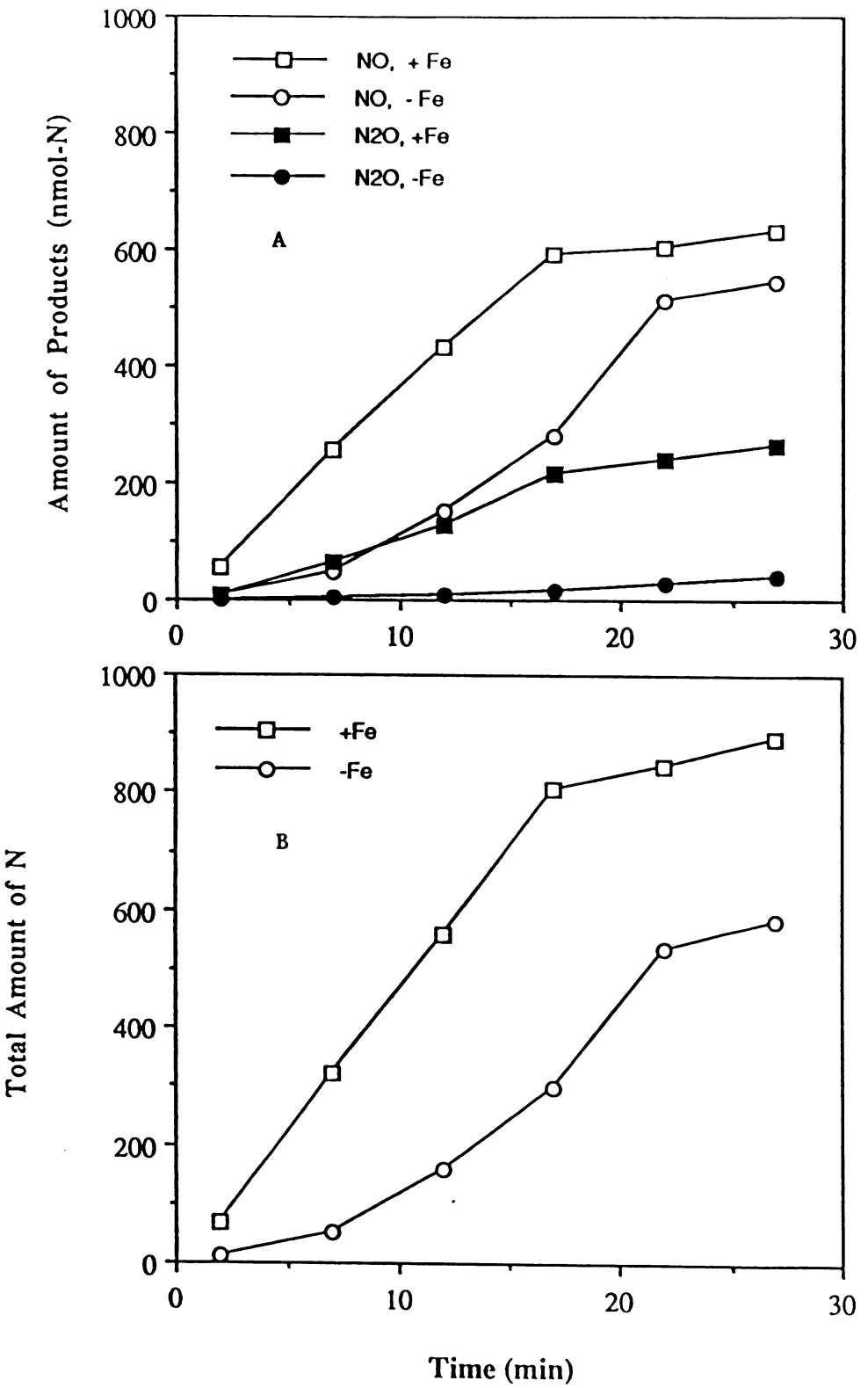
[a] Different NO and N_2O species are presented as nmol produced in 30 min. 357 nmole of ^{14}NO was added to the reaction mixture 1 min before the addition of 1 mM $Na^{15}NO_2$ (99% ^{15}N) to start the reaction. Percent ^{14}NO or $^{14}N^{15}NO$ was calculated based on the total amount of nitric oxide or nitrous oxide.

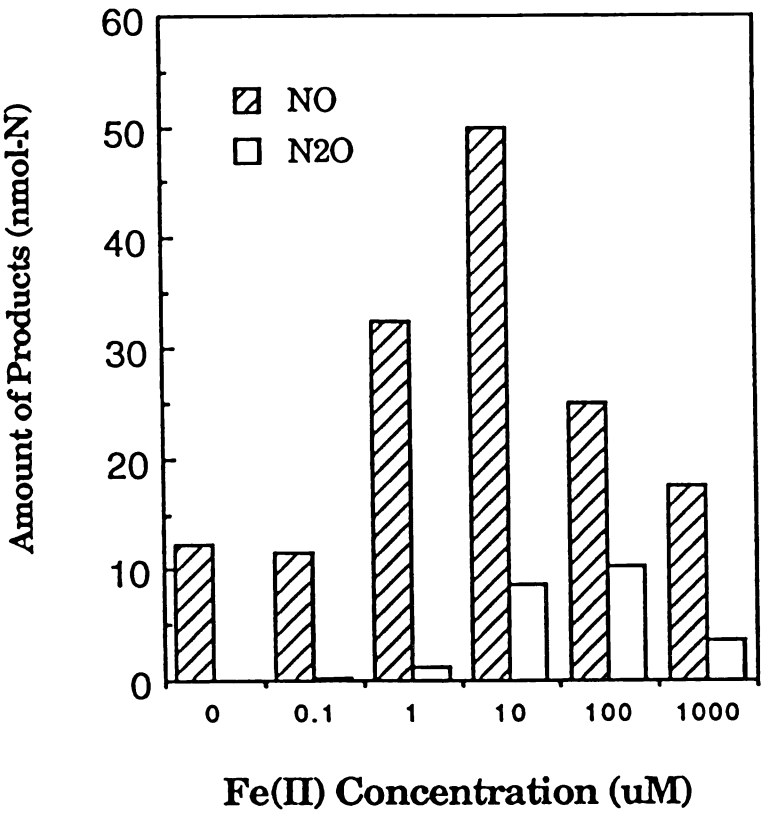
Figure legends

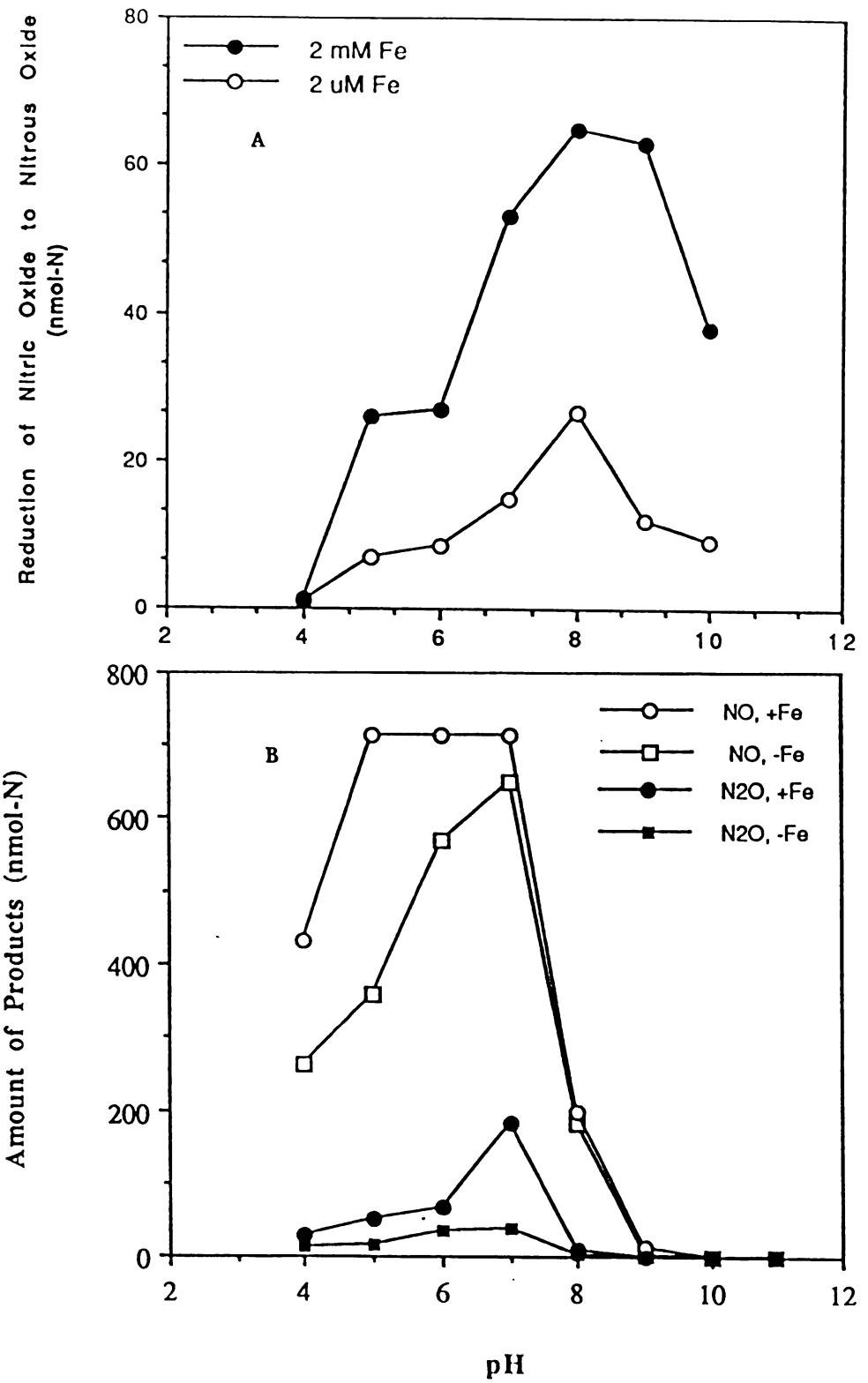
Figure 1. Fe (II) enhanced NO and N_2O production by purified nitrite reductase from *P. stutzeri* JM 300. Panel A: evolution of NO and N_2O during reduction of NO_2^-. Panel B: total amount of nitrogen production calculated from panel A. The reaction was carried out under the standard assay conditions with 1 umol of nitrite. The Fe (II) concentration was 10 uM if used.

Figure 2. Production of NO and N_2O from NO_2^- by nitrite reductase in the presence of different concentrations of Fe(II). The reaction was carried out under the standard assay condition with 1 umol of nitrite. Results were obtained after 2 min of reaction.

Figure 3. Effect of pH on the conversion NO to N_2O by Fe(II) without nitrite reductase (panel A) or NO_2^- to NO and N_2O by nitrite reductase (panel B). Reactions were carried out under the standard assay conditions in HEPES buffer adjusted to the indicated pH values. Results were obtained after 30 min of reaction.


1000
800
600
400
200
0
Amount of Products (nmol-N)
NO, + Fe
NO, - Fe
N2O, +Fe
N2O, -Fe
A
0
10
20
30
1000
800
600
400
200
0
Total Amount of N
+Fe
-Fe
B
0
10
20
30
Time (min)


60
50
40
30
20
10
0
NO
N2O
Amount of Products (nmol-N)
0
0.1
1
10
100
1000
Fe(II) Concentration (uM)


Reduction of Nitric Oxide to Nitrous Oxide (nmol-N)
2 mM Fe
2 uM Fe
A
80
60
40
20
0
2
4
6
8
10
12
Amount of Products (nmol-N)
B
NO, +Fe
NO, -Fe
N2O, +Fe
N2O, -Fe
800
600
400
200
0
pH

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Chapter Three

$H_2{}^{18}O$ isotope exchange studies on the mechanism of reduction of nitric oxide and nitrite to nitrous oxide by denitrifying bacteria

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$H_2{}^{18}O$ Isotope Exchange Studies on the Mechanism of Reduction of Nitric Oxide and Nitrite to Nitrous Oxide by Denitrifying Bacteria

EVIDENCE FOR AN ELECTROPHILIC NITROSYL DURING REDUCTION OF NITRIC OXIDE*

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Reduction of NO and NO_2^- by whole cells of eight strains of denitrifying bacteria known to contain either heme cd_1 or copper-containing nitrite reductases (NiRs) has been examined in the presence of $H_2{}^{18}O$. All organisms containing heme cd_1 NiRs exhibited relatively large extents of exchange between NO_2^- and $H_2{}^{18}O$ (39–100%), as monitored by the ^{18}O content of product N_2O. Organisms containing copper NiRs gave highly variable results, with *Achromobacter cycloclastes* and *Pseudomonas aureofaciens* exhibiting no ^{18}O incorporation and *Rhodopseudomonas sphaeroides* and *Alcaligenes entrophus* exhibiting complete exchange between NO_2^- and $H_2{}^{18}O$. Organisms containing heme cd_1 NiRs exhibited significant but lower levels of exchange between NO and $H_2{}^{18}O$ than between NO_2^- and $H_2{}^{18}O$, while organisms containing copper NiRs gave significantly higher amounts of ^{18}O incorporation than observed for the heme cd_1 organisms. These results demonstrate the existence of an NO-derived species capable of undergoing O-atom exchange with $H_2{}^{18}O$ during the reduction of NO. Trapping experiments with ^{18}NO, $^{14}N_3^-$, and crude extracts of *R. sphaeroides* support the electrophilic nature of this intermediate and suggest its formulation as an enzyme nitrosyl, E-NO$^+$, analogous to that observed during reduction of NO_2^-. The observation of lower levels of ^{18}O incorporation with NO_2^- than with NO as substrate for *A. cycloclastes* and *P. aureofaciens* indicates that, for these organisms at least, a sequential pathway involving free NO as an intermediate is significantly less important than a direct pathway in which N_2O is formed via reaction of two NO_2^- ions on a single enzyme.

The mechanism of microbial denitrification remains a controversial subject, despite a wealth of detailed studies on both intact bacteria and isolated enzymes (1–4). It is now generally accepted that denitrifying bacteria possess a nitric oxide reductase activity that is distinct from the nitrite reductase (NiR)[1] activity. The latter are typically soluble enzymes that are rather easily purified and have been shown to be of two distinct types: a cytochrome cd_1-containing dimer of ~60-kDa subunits and a copper-containing enzyme that is more variable in both subunit size and degree of oligomerization (1, 5). The membrane-bound nature of the nitric oxide reductase activities has hindered their purification and characterization, but recently nitric oxide reductases have been purified to apparent homogeneity from two organisms (6–8) and shown to contain both heme *b* and *c* prosthetic groups (7, 8).

Virtually all workers in the field now agree that at least a significant portion of the total nitrogen flux occurs via a stepwise pathway with NO as an intermediate (Equation 1, where NoR is nitric oxide reductase),

$$NO_3^- \rightarrow NO_2^- \xrightarrow{NiR} NO \xrightarrow{NoR} N_2O \rightarrow N_2 \qquad (1)$$

rather than via the direct pathway previously proposed (Equation 2), in which two NO_2^- ions are reduced to N_2O on a single enzyme (NiR) (9).

$$NO_3^- \rightarrow NO_2^- \xrightarrow{NiR} \tfrac{1}{2}N_2O \rightarrow \tfrac{1}{2}N_2 \qquad (2)$$

Indeed, quantitative studies of NO levels during denitrification by several denitrifiers have been interpreted as indicating that only the former pathway (Equation 1) is operative and that NO is a free obligatory intermediate in denitrification (10–13). This conclusion is consistent with the observed lack of reduction of NO by isolated NiRs, with the fact that most isolated NiRs produce predominantly NO upon reduction of nitrite, and with the fact that mutants lacking either the heme cd_1 (14) or copper NiRs[2] are still capable of reducing NO. It fails, however, to account for the following observations. (i) Purified NiRs do, in at least some cases, produce significant amounts of N_2O that cannot be attributed to chemical reduction (15–17); (ii) NO_2^- and reagents such as N_3^- and $H_2{}^{18}O$ that are known to react with a nitrosyl intermediate derived from NO_2^- exhibit apparent competitive behavior (18, 19); (iii) the ^{18}O content of N_2O produced from nitrite is about half that of nitrosation products derived from the nitrosyl intermediate, suggesting that unlabeled oxygen from a second nitrite enters the reaction (19); and (iv) the magnitude of the ^{15}N isotope effect increases with increasing nitrite concentration, suggesting that two nitrite ions react with the enzyme prior to the first irreversible step (20, 21). Consequently, the relative importance of the routes shown in Equations 1 and 2 remains unclear.

In this communication, we present evidence that the NO reductase exhibits a remarkable and previously unsuspected similarity to the NiRs in that an *electrophilic* species derived from NO can be trapped during the reduction of NO. Further,

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[1] The abbreviations used are: NiR, nitrite reductase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] R. Ye and J. M. Tiedje, unpublished results.

TABLE I

Percent exchange with $H_2{}^{16}O$ during dissimilatory reduction of NO and nitrite by whole cells of denitrifying bacteria containing either heme cd_1 (1–4) or copper (5–8) nitrite reductases

Acetylene (0.2 atmosphere) was added to the gas phase to block nitrous oxide reductase activity. Cell density corresponded to a protein concentration of 11 mg/ml. 1.0 μmol of NO or nitrite was used as substrate. $H_2{}^{16}O$ was present at the range of 8–13%. After completion of the reaction (50 min), 0.1 ml of 10 N NaOH was added to the reaction vial. Data are means of three replicates from two independent experiments.

Strain	Substrate	
	Nitrite	NO
1. *P. denitrificans* ATCC 19367	59 ± 5	11.3 ± 1.2
2. *P. aeruginosa* PAO 1	76 ± 7	19.0 ± 0.2
3. *P. stutzeri* JM300	58 ± 14	4 ± 1
4. *P. fluorescens* AK-15	39 ± 1	15.0 ± 1.2
5. *A. eutrophus* ATCC 17699	94 ± 5	84 ± 6
6. *A. cycloclastes* ATCC 21921	4 ± 2	30 ± 7
7. *P. aureofaciens* ATCC 13985	6.0 ± 0.2	37 ± 14
8. *R. sphaeroides* forma sp. *denitrificans*	90 ± 15	61 ± 19

we demonstrate via comparison of the amount of ^{18}O incorporated into N_2O from either NO_2^- or NO that, in certain bacteria at least, reduction of NO_2^- may not proceed entirely according to the stepwise pathway shown in Equation 1.

MATERIALS AND METHODS

Bacterial Strains—*Pseudomonas aeruginosa* PAO1 was from B. W. Holloway, Monash University, Clayton, Australia; *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* was from T. Satoh; and *Pseudomonas stutzeri* JM 300 was from J. Ingraham. *Pseudomonas fluorescens* AK-15 was a soil isolate obtained in this laboratory.[3] The rest of the strains used were from ATCC. Characterization of copper-type or cytochrome cd_1 nitrite reductases in some of these strains has been described (22); classification of *Alcaligenes eutrophus* ATCC 17699 as a copper NiR-containing organism was performed using *N,N*-dimethyl dithiocarbamate as described (22).

Sample Preparation—Cultures were grown anaerobically in 3% tryptic soy broth (Sigma) containing 0.15% potassium nitrate in 110-ml serum bottles. *P. aeruginosa* PAO1 was grown overnight at 37 °C, and the rest of the cultures were grown at 30 °C. Cells were harvested, washed, and resuspended in tryptic soy broth in an 8-ml serum bottle. These bottles were made anaerobic by flushing with argon, and nitrite or NO was added to start the reaction; suspensions were maintained at room temperature. 100 μl of 10 N NaOH was added to stop the reaction and absorb CO_2. Crude extracts were prepared by sonication followed by centrifugation at 10,000 × *g* for 30 min to remove cell debris.

Isotopes and Their Analysis—^{15}NO was prepared by mixing 1 ml of 100 mM H_2SO_4, 1 ml of 100 mM KI, and 1 ml of 299 mM $Na^{15}NO_2$ (99.9 atom ^{15}N%) in a 25-ml serum bottle (23). Gas chromatography/mass spectrometry measurements and the calculation of the extent of ^{18}O exchange were performed as described (19, 23).

RESULTS AND DISCUSSION

Exchange with $H_2{}^{18}O$ during Nitrite Reduction—The results obtained for four denitrifiers known to contain heme cd_1 NiRs and four denitrifiers known to contain copper NiRs are presented in Table I, column 1. As expected based upon previous work demonstrating the existence of an electrophilic nitrosyl intermediate during reduction of nitrite by the heme cd_1 NiRs (18, 19, 23–25), organisms containing such enzymes exhibited relatively large amounts of ^{18}O incorporation from $H_2{}^{18}O$ (extents of exchange ranging from 39 to 80%) during reduction of NO_2^- to N_2O. In fact, strain 2 exhibited significantly more than 50% exchange. This is significant because our original hypothesis regarding the direct pathway (9) (Equation 2) postulated that the N–N bond was formed by attack of a second NO_2^- upon an electrophilic nitrosyl intermediate, *E*-NO^+, derived from the first nitrite, which is known to undergo facile ^{18}O exchange by a reversible hydration/dehydration process (23–25). Shearer and Kohl (21) have shown that the NiR from *P. stutzeri* JM300 is a "sticky" enzyme and that NO_2^- is committed to reduction once bound. Hence, ^{18}O-labeled NO_2^- does not accumulate and ^{18}O incorporations of >50% are not consistent with the direct pathway (Equation 2), unless the NiR in organism 2 differs substantially from that in *P. stutzeri* JM 300 (organism 3).

The organisms containing copper NiRs exhibited significantly different behavior in two cases (6 and 7), where essentially no ^{18}O incorporation into N_2O product was observed. This result is consistent with previous work on the *Achromobacter cycloclastes* system, which showed undetectable amounts of ^{18}O exchange (26). In contrast, *R. sphaeroides* exhibited virtually complete exchange between $H_2{}^{18}O$ and product N_2O, suggesting the presence of an electrophilic nitrosyl intermediate in these organisms that differs dramatically in its reactivity with $H_2{}^{18}O$ from the other organisms containing a copper NiR. Such behavior is perhaps not surprising, given the extreme variability in subunit size and immunoreactivity observed with copper NiR-containing organisms (22).

Exchange with $H_2{}^{18}O$ during Reduction of NO—As shown in column 2 of Table I, substantial amounts of ^{18}O were also observed in N_2O produced by reduction of NO. The extent of ^{18}O incorporation observed with NO tended to be lower than that observed with NO_2^- for the same organism (except for 6 and 7, see below) but was well above background levels for all but one case. The four organisms with copper NiRs gave

TABLE II

Exchange with $H_2{}^{18}O$ during reduction of NO by crude extracts of R. sphaeroides forma sp. denitrificans in the presence of added electron donor or mediator

0.5 μmol of NO and 4.24% of $H_2{}^{18}O$ were used in the reaction mixtures, which contained cell extracts equivalent to a protein concentration of 2.4 mg/ml in 30 mM HEPES buffer, pH 7.0, and 10 mM EDTA. Nitrous oxide species were measured after the reaction was completed (180 min). Conditions otherwise as in Table I. PMS, phenazine methosulfate.

Sample	$N_2{}^{16}O$	$N_2{}^{18}O$	Exchange
	nmol	*nmol*	%
Crude extract	23.0 ± 0.3	477 ± 0.3	100
Crude extract + PMS (40 μM)	23.2 ± 0.3	477 ± 0.3	100
Crude extract + NADH (4 mM)	13 ± 1.2	487 ± 1	56
Crude extract + NADH (4 mM) + PMS (40 μM)	3.2 ± 0.3	497 ± 0.3	9

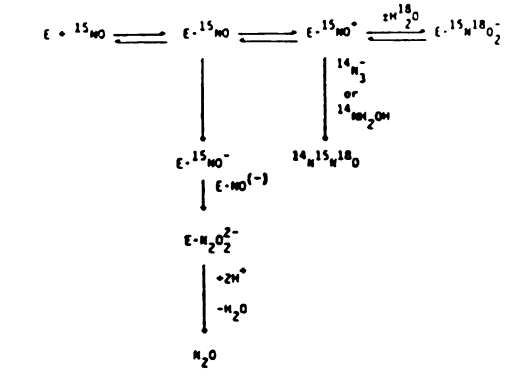
TABLE III

Nitrosyl transfer from ^{15}NO to $^{14}N_3^-$ in reaction containing $H_2{}^{18}O$ and R. sphaeroides crude extracts

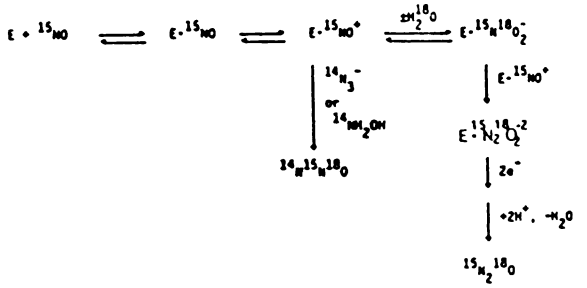
Reaction mixtures contained 2 μmol of ^{15}NO and 1.0 mM NaN_3; all other conditions were as in Table II. Reactions were stopped after 30 min. Percent nitrosation was calculated based on the ratio of $^{14,15}N_2O$ and total amount of nitrous oxide produced. Data are for duplicate experiments at 1 mM azide, but similar results were obtained with duplicates run at both 2.5 and 5 mM azide as well.

Isotope	Amount	Exchange	Nitrosation
	nmol	%	%
$^{14,15}N_2{}^{16}O$	0.74, 0.88		
$^{14,15}N_2{}^{18}O$	20, 24	80, 79	13, 14
$^{15,15}N_2{}^{16}O$	5.1, 5.8		
$^{15,15}N_2{}^{18}O$	134, 136	82, 87	

[3] A. Arunakumari and J. M. Tiedje, unpublished results.



SCHEME 1



SCHEME 2

significantly higher extents of ^{18}O exchange (30–84%) than did those known to contain a heme cd_1 NiR (4–19%), but the origin of this difference is unclear since an NO reductase has yet to be purified from any of the former.

The data in Table I, column 2, demonstrate the presence of an NO-derived species capable of undergoing O-atom exchange with $H_2{}^{18}O$ during the reduction of NO, which would not be expected *a priori* to proceed via an electrophilic species. The overall reaction can be written as

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \quad (3)$$

indicating that water is produced during the reaction (presumably by protonation and dehydration of a hyponitrite level species containing two N atoms, such as $N_2O_2^{2-}$), suggesting a possible route for ^{18}O incorporation if the final dehydration step were reversible. As a control experiment, cells of *Pseudomonas aureofaciens* were grown on NO_3^-, suspended in medium containing 10% $H_2{}^{18}O$, and incubated anaerobically for 5 h at room temperature with 0.1 ml of N_2O (8.8 μmol) in an 8-ml bottle. The ^{18}O content of the N_2O was measured and did not differ from the natural abundance. Thus, the observed ^{18}O incorporation during reduction of NO must occur *prior* to reduction to the N_2O level.

This conclusion is also supported by the data in Table II, which demonstrate that for *R. sphaeroides* at least the extent of exchange with $H_2{}^{18}O$ decreased as the concentration of electron donor/mediator was increased. This suggests strongly that ^{18}O exchange occurs via a relatively oxidized nitrogen intermediate.

Trapping with Azide during NO Reduction—If an electrophilic NO-derived species is indeed present during reduction of NO, it might be expected to react with nucleophiles other than $H_2{}^{18}O$. For example, N_3^- and NH_2OH have been reported to trap the electrophilic nitrosyl produced by the heme cd_1 NiRs (19, 20, 23–25). Thus, crude extracts of *R. sphaeroides* were treated with ^{15}NO in the presence of $^{14}N_3^-$ and $H_2{}^{18}O$; the amounts of the various isotopically labeled forms of N_2O formed are given in Table III. It is clear that substantial (~14%) amounts of a nitrosation product $^{14,15}N_2O$ were observed even at the relatively low azide concentration used (1 mM) and that both the nitrosation product and NO reduction product exhibited comparable ^{18}O incorporation.

Implications for the Mechanism of NO Reduction—The $H_2{}^{18}O$ exchange and N_3^- trapping results presented above strongly imply the presence of an electrophilic mononitrogen intermediate during reduction of NO. The simplest such species is an enzyme nitrosyl, E-NO^+, analogous to that observed for the heme cd_1 NiRs. This is a most unexpected result, since it is not obvious why an *oxidized* NO species should be an intermediate in its reduction to N_2O. We note that similar results observed earlier for *P. stutzeri* JM 300 in the presence of NO and $H_2{}^{18}O$ or ^{15}NO and $^{14}NH_2OH$ (19) are also completely consistent with the results reported here (although they were initially attributed to an NO complex of the heme cd_1 NiR in this organism).

Two possible tentative explanations for the data are shown in Schemes 1 and 2. In Scheme 1, the ^{18}O exchange and nucleophilic trapping occur via a species that is not on the catalytic pathway for NO reduction but is rather on an oxidized "shunt." Given the redox potentials for synthetic heme nitrosyls (27–29), it might not be surprising if electron transfer to another center on the enzyme or elsewhere were to generate a transient oxidized species that, as shown, has nothing to do with catalysis. The other extreme is represented in Scheme 2, in which the E-NO^+ species is an obligatory intermediate, possibly reacting via a hypothetical enzyme-bound NO_2^- with a second E-NO^+ in a fashion analogous to that postulated by us earlier for the reduction of NO_2^- by NiR (9). Available data do not permit us to distinguish between these alternatives or the many possible variants thereof.

Implications for the Pathway of Denitrification—Examination of the data in columns 1 and 2 of Table I reveals that, in most cases, our results are fully compatible with NO as an obligatory intermediate in denitrification, *i.e.* the sequential pathway shown in Equation 1. That is, for organisms 1–5 and 8, the extent of ^{18}O incorporation observed for NO_2^- as substrate is greater than that observed for NO, reflecting the fact that for these organisms ^{18}O exchange can occur at either the $NO_2^- \rightarrow NO$ or $NO \rightarrow N_2O$ steps. These data do not, however, constitute proof that the bulk of the nitrogen flux proceeds via Equation 1 rather than Equation 2, although they are not inconsistent with this view. In contrast, the data for organisms 6 and 7 (*A. cycloclastes* and *P. aureofaciens*) are *not* compatible with the view that NO is an obligatory free intermediate, because the amount of ^{18}O exchange observed with NO_2^- as substrate is far less than with NO. (If the situation shown in Equation 1 were to obtain, the amount of ^{18}O incorporated into N_2O derived from NO_2^- would always have to be at least equal to that in N_2O derived from NO.) Thus, for these organisms at least, the sequential pathway of Equation 1 appears to be significantly less important than a direct pathway as indicated in Equation 2.

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Addendum to Chapter Two -- A study on the reversibility of ^{18}O exchange during reduction of NO.

One of the important features of a nitrosyl complex is its exchange of oxygen with H_2O. This exchange activity has been well demonstrated during the reduction of NO_2^-. As indicated in Chapter Two, the finding of ^{18}O exchange during the reduction of NO to N_2O was completely unexpected. We suggested that an enzyme nitrosyl complex may also form during the reduction of NO, similar to that which is involved in the reduction of NO_2^-. One of the characteristics of ^{18}O exchange during the reduction of NO_2^- is the reversibility of the reaction, i.e. the formation of $N^{18}O_2^-$ in the reaction with $H_2{}^{18}O$. To test whether NO will also be released from the enzyme after exchange with H_2O, we used $H_2{}^{18}O$ as a tracer to measure the accumulation of $^{15}N^{18}O$ in the gas phase during reduction ^{15}NO by *Alcaligenes eutrophus*. Experimental conditions were the same as described in Chapter Two except that time points were taken in this experiment. Results are presented as percent ^{18}O in the total amount of nitric oxide or nitrous oxide present in the system at the time of measurement (Figure 1).

Indeed, accumulation of $^{15}N^{18}O$ was observed in the gas phase (Figure 1). The amount of $^{15}N^{18}O$ increased in a linear fashion as the reaction progressed. At late stage of reaction, the nitric oxide contained 5.2% ^{18}O, which is equal to the amount of $H_2{}^{18}O$ used (5.2%). This suggests that all oxygen atoms in nitric oxide that remained in the gas phase had been replaced by the oxygen atom

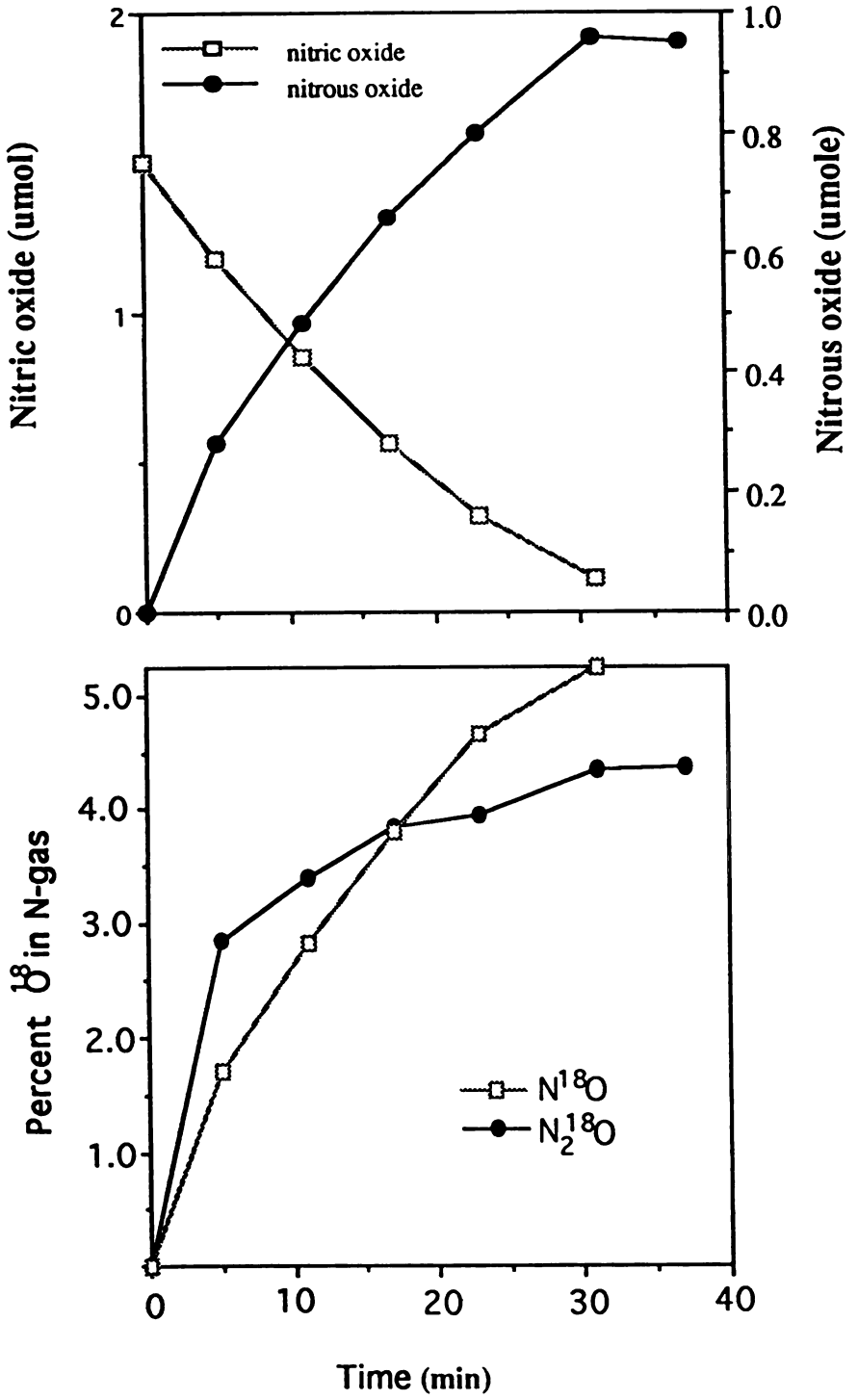
from water. Release of $^{15}N^{18}O$ to the gas phase implies that ^{18}O exchange occurred before the committed step of N=N bond formation and further supports the hypothesis that an enzyme-nitrosyl complex ($E\text{-}NO^{+}$) is formed during dissimilatory reduction of NO.

The N_2O contained about 2.6% ^{18}O in an early stage of reaction, indicating that the extent of exchange was above 50% and that the exchange reaction is very fast. The result found for *P. fluorescens*AK-15 during the reduction of nitric oxide also supports the rapid equilibration of ^{18}O into nitrous oxide (see Chapter Three). However, we could not detect a pool of $N^{18}O$ with this organism. Thus *P. fluorescens* AK-15 could be considered to have a 'sticky' NO reductase, while *Alcaligenes eutrophus* had a 'loose' one. Another difference between these two organism is that the extent of exchange in *P. fluorescens* was about 25% (see Chapter Three), while in *Alcaligenes eutrophus*, it reaches above 80%. *P. fluorescens* AK-15 contains a heme c,d_1 nitrite reductase, while *A. eutrophus* has a Cu nitrite reductase.

In summary, denitrification of NO has the following features. i) ^{18}O from $H_2{}^{18}O$ can be incorporated into the product, nitrous oxide; ii) a pool of $N^{18}O$ can be detected in the gas phase at least in some organisms; iii) the incorporation of ^{18}O into nitrous oxide is very fast and it reaches equilibrium rapidly; iv) abundance of electron donor abolishes the ^{18}O reaction *in vitro*; A possible explanation of mechanism of NO reduction that is consistent with these features is shown in scheme I and II. The nitrosyl complex is possibly formed

by the NO reductase: E-NO^+, which can undergo an oxygen exchange reaction with H_2O (supported by features i and ii). Formation of nitrosyl complex and exchange with H_2O are reversible, leading to the release of the substrate from the enzyme. The exchange reaction likely occurs before the electron accepting step(s) and the formation of N=N bond, based on features ii and iv. Available data do not permit us to draw the conclusion as to whether E-NO^+ is obligatory for N=N bond formation and thus other routes may also possible.

Figure 1. Accumulation of ^{18}O labelled products during dissimilatory reduction of ^{15}NO in *Alcaligenes eutrophus*. The reaction mixture contained 5.2% $H_2{}^{18}O$ and 1.5 umol of ^{15}NO in 1ml final volume of TSB.


nitric oxide
nitrous oxide
Nitric oxide (umol)
Nitrous oxide (umole)
2
1
0
1.0
0.8
0.6
0.4
0.2
0.0
Percent 18 in N-gas
5.0
4.0
3.0
2.0
1.0
N18O
N218O
0
10
20
30
40
Time (min)

Chapter Four

Mutants of *Pseudomonas fluorescens* deficient in dissimilatory nitrite reduction are also altered in nitric oxide reduction

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Mutants of *Pseudomonas fluorescens* Deficient in Dissimilatory Nitrite Reduction Are Also Altered in Nitric Oxide Reduction

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Five Tn*5* mutants of *Pseudomonas fluorescens* AK-15 deficient in dissimilatory reduction of nitrite were isolated and characterized. Two insertions occurred inside the nitrite reductase structural gene (*nirS*) and resulted in no detectable nitrite reductase protein on a Western immunoblot. One mutant had Tn*5* inserted inside *nirC*, the third gene in the same operon, and produced a defective nitrite reductase protein. Two other mutants had insertions outside of this *nir* operon and also produced defective proteins. All of the Nir$^-$ mutants characterized showed not only loss of nitrite reductase activity but also a significant decrease in nitric oxide reductase activity. When cells were incubated with ^{15}NO in $H_2{}^{18}O$, about 25% of the oxygen found in nitrous oxide exchanged with H_2O. The extent of exchange remained constant throughout the reaction, indicating the incorporation of ^{18}O from $H_2{}^{18}O$ reached equilibrium rapidly. In all nitrite reduction-deficient mutants, less than 4% of the ^{18}O exchange was found, suggesting that the hydration and dehydration step was altered. These results indicate that the factors involved in dissimilatory reduction of nitrite influenced the subsequent NO reduction in this organism.

Dissimilatory reduction of nitrite is the key step in the denitrification pathway; it is the point of divergence from assimilatory nitrogen metabolism (24). There are two types of nitrite reductases: one contains the cytochromes cd_1, and the other contains copper (6, 14, 20). Organisms containing the cytochrome cd_1 nitrite reductases are more frequently isolated from nature, whereas Cu-type nitrite reductases are found in organisms that exhibit more phylogenic diversity and occupy a wider range of ecological niches (6, 9).

Nitric oxide is the major product of nitrite reduction by purified nitrite reductases, and nitrous oxide is a minor product (4, 14, 17, 26). NO is generally accepted as one of the free and obligatory intermediates in the denitrification pathway (2, 5, 8, 10, 14, 20, 28), and NO reductases have been purified from *Pseudomonas stutzeri* Zobell (13) and *Paracoccus denitrificans* (4, 7). We recently showed that many denitrifiers containing Cu or cd_1 nitrite reductases are capable of undergoing O-atom exchange with $H_2{}^{18}O$ during the reduction of NO to N_2O (27) and that a labeled intermediate can be trapped with azide. These results suggest that a nitrosyl complex is formed during the reaction. It was also shown that the extent of the O-atom exchange reaction depended on the availability of electrons. The dependence of nitrosyl transfer upon the presence of NO during reduction of NO_2^- has been demonstrated (11).

Tn*5* was used by Zumft et al. (29) to generate mutants deficient in dissimilatory nitrite reduction (Nir$^-$) in *P. stutzeri* Zobell. All of the mutants isolated possessed normal NO reduction activity, indicating that NO reduction and nitrite reduction are distinct. The nitrite reductase gene (*nirS*) from *Pseudomonas aeruginosa* (21) and from two strains of *P. stutzeri* (Zobell [15] and JM 300 [23]) has been cloned. *nirM* encoding cytochrome c_{551} and *nirC* encoding a heme protein (also named ORF5 in *P. stutzeri*) are located immediately downstream of the nitrite reductase gene in *P. aeruginosa* (1, 15, 19). This is not the case in *P. stutzeri* (15), where *nirT* encoding a tetraheme protein and *nirB* encoding cytochrome c_{552} are located between the *nirS* and *nirM* genes.

We used Tn*5* to generate Nir$^-$ mutants in *Pseudomonas fluorescens* and found that, in contrast to *P. stutzeri* Zobell, all five Nir$^-$ mutants characterized not only lacked the ability to reduce nitrite but also showed a decreased ability to reduce NO. Furthermore, the extent of ^{18}O exchange with $H_2{}^{18}O$ during reduction of ^{15}NO to $^{15,15}N_2O$ was reduced to background levels in the mutants. These results suggest that the dissimilatory reductions of NO_2^- and NO are linked in this organism.

MATERIALS AND METHODS

Bacterial strains. *P. fluorescens* AK-15 is a gram-negative rod isolated from Capac loam soil from the Kellogg Biological Station, Kalamazoo County, Mich. This strain was identified based on its denitrifying ability, production of fluorescent pigments on King medium, growth at 4°C, lack of growth at 37°C, and fatty acid profiles. This strain was chosen for these studies because *P. fluorescens* strains are the most prevalent denitrifiers in nature and this strain has a high frequency of Tn*5* mutagenesis. Bacteria were grown at 25°C in tryptic soy broth (TSB; Difco Laboratories) supplemented with 0.15% KNO_3. A rifampin-resistant clone, YT101, was isolated from a spontaneous mutation.

Conjugation and isolation of mutants. The rifampin-resistant strain of wild-type *P. fluorescens*, YT101, was used as the recipient, and *Escherichia coli* S-17 carrying the pSUb2021 plasmid containing Tn*5* was used as the donor (22). Mating was carried out at 25°C overnight. Exconjugants were selected on TSB plates supplemented with rifampin (50 μg/ml) and kanamycin (50 μg/ml). Colonies were replica plated onto TSB plates containing 0.15% KNO_3 and grown

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overnight in an anaerobic glovebox. Plates were then taken out of the anaerobic glovebox, and a piece of Whatman no. 42 filter paper was laid on top of each plate and covered with a thin layer of cooled agar. N_2 gas bubbles appeared over denitrifying colonies in 30 to 60 min. If a thick top agar layer was used without the Whatman paper, it took more than 12 h before N_2 gas bubbles appeared and they were more difficult to distinguish. Colonies that did not produce gas bubbles were picked and further tested for their ability to produce bubbles by growth in culture tubes containing inverted Durham tubes.

Growth conditions and activity assays. Strain YT101 was grown in the presence of rifampin (50 μg/ml). Mutant strains were grown in the presence of both kanamycin (50 μg/ml) and rifampin (50 μg/ml). Anaerobic cells were grown overnight at 25°C in serum bottles containing 100 ml of TSB and 0.15% KNO_3. They were harvested by centrifuging at 8,000 × *g* for 15 min and were washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.3). Crude extracts were prepared by sonication and subsequent centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant was assayed for nitrite and NO reductase activities by measuring the rate of NO and N_2O appearance. The reaction mixture for NO reduction by the crude extract included 10 mM EDTA, 40 μM phenazine methosulfate (PMS), 4 mM NADH, and 50 mM HEPES (pH 7.3). To assay the NO reduction activities in whole cells, anaerobically grown cells were suspended in 1 ml of TSB in an 8-ml serum bottle with an anaerobic atmosphere created by repeated evaculation and filling with argon. NO was injected to start the reaction. All reaction vials were shaken on their sides at 100 rpm to facilitate the rate of gas-liguid transfer. NO and N_2O were detected by gas chromatography with ^{63}Ni electron capture detectors (16). The protein concentration of the crude extract was determined by the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co.). The amount of protein in whole cells was estimated with the Folin and Ciocalteu phenol reagent (Sigma) after alkaline lysis with 1 N NaOH (12).

DNA sequencing. To determine the Tn*5* insertion site in the Nir^- mutants, subclones in pUC18 containing the left inverted repeat region of Tn*5* were sequenced with a primer made to a region near the end of the inverted repeat. DNA was sequenced by the dideoxy method with a sequencing kit (U.S. Biochemical Corp.).

Western and Southern blots. Western immunoblots were developed with antibodies against nitrite reductase from *P. aeruginosa* as described previously (6). Genomic DNA isolation, restriction enzyme digestion, electrophoresis, and Southern blotting were done as described by Maniatis et al. (18). Plasmid pSUP2021 containing Tn*5* was isolated by alkaline lysis (18) and labeled by nick translation (Boehringer Mannheim). The nitrite reductase gene from *P. aeruginosa* in a 3.5-kb *Eco*RI fragment in pEMBL18 was provided by M. C. Silverstrini (21).

Isotope analysis. ^{15}NO preparation, gas chromatography-mass spectrometry measurements, and calculation of ^{18}O exchange were performed as described previously (25, 27).

RESULTS

Isolation of Nir^- and Nos^- mutants. Deficiency in any step of denitrification leads to a lack of N_2 formation. The top agar method used previously to isolate denitrifying deficient mutants in *P. stutzeri* (29) did not work well with *P. fluorescens* AK-15, perhaps because of the slower rate of N_2 production in strain AK-15. Whatman filter paper applied on top of colonies before a thin layer of top agar was added proved to be more efficient in trapping the N_2 gas. Mutants that could not produce bubbles were categorized as Nir^- (deficient in NO_2^- reduction) or Nos^- (deficient in N_2O reduction) based on the characteristics summarized in Table 1. The frequencies of Nir^- and Nos^- phenotypes were about 0.032 and 0.024%, respectively. No nitric oxide reduction-deficient (Nor^-) mutants were found. All Nir^- and Nos^- mutants could grow with NO_3^- as the electron acceptor, but they could not produce N_2 bubbles because of a block in either nitrite reduction or N_2O reduction. Mutants deficient in nitrite reduction produced bubbles in the presence of both NO_3^- and N_2O, suggesting that these mutants could convert N_2O to N_2. Nitrate was included in this test because *P. fluorescens* AK-15 could not grow well on N_2O alone. After overnight growth on nitrate, Nir^- mutants accumulated NO_2^-, whereas Nos^- mutants accumulated N_2O. Two Nos^- mutants (YT15 and YT25) were used for control studies.

TABLE 1. Characteristics of Nir^- and Nos^- mutants from *P. fluorescens* AK-15

Strain	Accumulation of NO_2^- from NO_3^-[a]	Bubble formation from:		Accumulation of N_2O from NO_3^-[a]
		NO_3^-	N_2O plus NO_3^-	
YT101	−	++	++	−
YT2511	+	−	++	−
YT2471	+	−	++	−
YT3221	+	−	++	−
YT31	+	−	++	−
YT4221	+	−	++	−
YT25[b]	−	−	−	++
YT15	−	−	−	++

[a] Accumulation of NO_2^- and N_2O was tested after overnight growth.
[b] Four additional mutants with the same phenotype were also isolated.

Physical characterization of Nir^- mutants. To detect Tn*5* in these mutants, Tn*5* was labeled and used to probe the genomic DNA from the wild-type and mutant strains. When genomic DNA was cut with *Eco*RI and *Bam*HI, two bands, which varied in sizes in different mutants, hybridized to the Tn*5* probe, suggesting that single copies of Tn*5* had been inserted into different positions in the genome (data not shown). One of the *Eco*RI and *Bam*HI fragments of each mutant strain with the intact *neo* gene of Tn*5* was subcloned in pUC19 by selection for resistance to kanamycin. Subclones from YT2511, YT2471, and YT3221 hybridized to a common 12-kb *Eco*RI DNA fragment, which also hybridized to structural genes of nitrite reductases from *P. aeruginosa* (21) and *P. stutzeri* JM300 (23), suggesting that all three Tn*5* insertion sites were clustered in this *nir* operon. By obtaining DNA sequences from regions flanking the Tn*5* insert in pUC19 subclones and comparing them with published *nir* operon sequences (15, 21, 23), we found that Tn*5* was inserted near the middle of the nitrite reductase gene in YT2511 and near the carboxyl terminus in YT2471. In YT3221, Tn*5* was located near the heme binding site in *nirC*, the third gene in the operon. Subclone fragments from mutants YT4221 and YT31 did not hybridize to the 12-kb *Eco*RI genomic DNA fragment containing the nitrite reductase gene and had no homology with each other.

Biochemical characterization of Nir^- mutants. Crude cell extracts of all Nir^- mutants showed no nitrite reductase activity (Table 2). When a Western blot was developed with antibodies against the nitrite reductase, mutants YT4221,

TABLE 2. Rates of NO_2^- and NO reduction and extents of ^{18}O exchange by wild-type and mutant strains of *P. fluorescens* AK-15[a]

Strain	nmol of N mg^{-1} min^{-1}			^{18}O Exchange[b] (%)
	Crude extract		Cells (+NO)	
	$+NO_2^-$	+NO		
YT101	110	17.4	38.2	25.5 ± 1.3
YT2511	0[c]	6.4	10.1	1.3 ± 0.4
YT2471	0	8.2	8.0	1.5 ± 0.5
YT3221	0	7.1	12.8	1.3 ± 1.3
YT31	0	7.7	10.4	1.2
YT4221	0	8.4	17.6	3.9 ± 2.5
YT25	ND[d]	ND	37.6	23.3 ± 6.4
YT15	ND	ND	39.4	23.7 ± 5.0

[a] Data are means of duplicate samples for crude extracts and triplicate samples for whole cells. The nitrite reductase activity assay mixture had about 0.04 mg of protein and 1 μmol of nitrite. The NO reductase assay contained 0.08 mg of protein and 2 μmol of NO. The same amount of NO was used in the whole-cell assay.

[b] Reaction mixtures contained 4 mg of whole-cell protein, 5.14% $H_2{}^{18}O$, and 1.5 μmol of ^{15}NO in an 8-ml serum bottle with 1 ml of TSB solution. The reaction was stopped with 0.1 ml of NaOH (10 N) after 40 min. The data are obtained from triplicate samples.

[c] The detection limit for specific activity was about 0.13 nmol mg^{-1} min^{-1}.

[d] ND, not determined.

YT31, and YT3221 showed a positive band corresponding in size to the wild-type nitrite reductase protein, indicating that defective proteins were made (Fig. 1). However, mutants YT2511 and YT2471 showed no such band. This was consistent with the physical characterization in that these two mutants had Tn*5* inserted inside *nirS*. The fact that a defective protein was made by YT3221 suggested that *nirC*

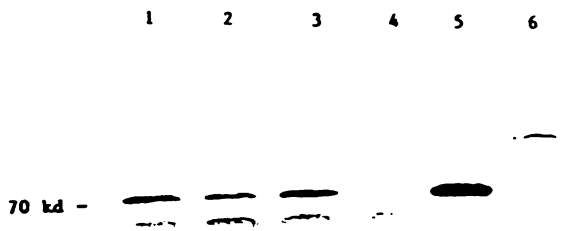

FIG. 1. Western blot of proteins from wild-type and mutant strains. The blot was developed with polyclonal antibodies raised against the nitrite reductase purified from *P. aeruginosa*. Samples of 5 μg of protein were used per blot for all strains. Lanes 1 through 6 contained strains YT4221, YT31, YT3221, YT2511, YT101, and YT2471, respectively.

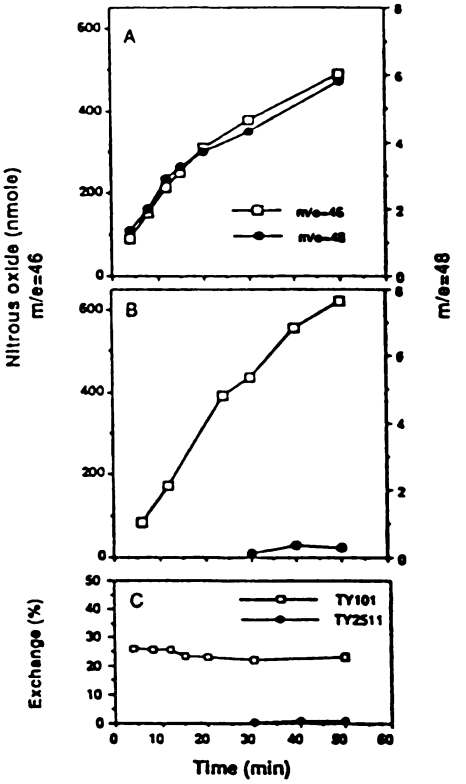

FIG. 2. The time course of ^{18}O exchange in resting cells of the YT101 (A) and YT2511 (B) mutant strains. The extent of exchange (C) was calculated based on the data in panels A and B. Data are means of duplicate samples. The amounts of cells were equivalent to 1 mg of protein for the wild type and 4 mg of protein for YT2511. In YT2511, the $^{15,15}N_2{}^{18}O$ species could not be detected until after 20 min.

or a gene(s) further downstream is essential for the production of active nitrite reductase.

The NO reduction activity in resting cells in Nir^- mutants of *P. fluorescens* AK-15 was reduced by about three- to fourfold relative to that of the Rif^r wild-type strain YT101. When crude cell extracts were assayed in the presence of artificial electron donors (PMS and NADH), the NO reduction activity was reduced by about two- to threefold (Table 2). The NO reduction activity in Nos^- mutants was not altered. Thus, there is a consistent reduction in NO reduction activity only in Nir^- strains.

^{18}O-exchange studies to probe the mechanism of NO reduction. To study whether mutations in *nir* genes also affected the mechanism of nitric oxide reduction, the extent of oxygen atom exchange during the reduction of ^{15}NO in resting cells was measured. The ratio of $^{15,15}N_2{}^{18}O$ (*m/e*, 48) to the total amount of nitrous oxide produced reflects the extent of exchange (27); this was about 25% in the YT101 and Nos^- mutants (YT25 and YT15) (Table 2). However, in Nir^- mutants the extent of exchange was reduced to background levels except in YT4221, which was slightly above the background. The percentage of exchange remained constant at 25% throughout the course of reduction of NO to N_2O, indicating that the hydration-dehydration reaction rap-

idly reached equilibrium (Fig. 2). Since Nir$^-$ mutants had a reduced rate of NO reduction, four times more cells from mutant YT2511 were used in the time course study. Only a very small amount of $^{15,15}N_2{}^{18}O$ was observed, suggesting that the decreased ^{18}O exchange for YT2511 (Table 2) reflects an intrinsic difference in mechanism rather than simply a difference in the rate at which isotopic equilibrium was reached.

DISCUSSION

Among the five characterized Nir$^-$ mutants, three had Tn*5* inserted in the *nir* operon containing the nitrite reductase structural gene (*nirS*). Insertion of Tn*5* into the *nirS* genes in YT2511 and YT2471 resulted in no nitrite reductase activity and the absence of identifiable nitrite reductase protein in a Western blot (Fig. 1). Insertions in the *nirC* genes and two other regions, however, produced defective nitrite reductase proteins, suggesting that three or more genes are involved in the production of an intact nitrite reductase. These may include genes involved in the synthesis of the unique heme d_1 chromophore or for assembling or processing of the polypeptide.

All five Nir$^-$ mutants isolated from this strain of *P. fluorescens* showed a decrease in the rate of NO reduction and a lack of ^{18}O exchange with $H_2{}^{18}O$ during reduction of NO (Table 2). The decrease in NO reduction rate was about 3- to 4-fold in whole cells, but all mutants except YT4221 showed at least a 10-fold reduction in ^{18}O exchange, suggesting that there is a genetic and/or mechanistic relationship between the dissimilatory reduction of NO_2^- and that of NO. This relationship cannot, however, be due simply to mutations in the regulatory region of the genome: YT2511 and YT2471 had Tn*5* inserted inside the nitrite reductase structural gene, yet they had the same decrease in NO reduction and ^{18}O exchange as the others. Further, Tn*5* mutants deficient in N_2O reduction (Nos$^-$) exhibited normal rates of NO reduction and ^{18}O exchange, indicating that the Tn*5* insertion event per se does not result in the observed effects on NO reduction.

Possible explanations for these results include the following. (i) In normal cells in vivo, a substantial portion of the NO to N_2O flux is catalyzed by the nitrite reductase, such that Nir$^-$ mutants exhibit reduced NO reduction levels with different ^{18}O exchange characteristics due to the functional NO reductase. Although definitive data with *P. fluorescens* have not yet been obtained, studies on *P. stutzeri* have shown that purified nitrite reductase is incapable of reducing NO either by itself or in the presence of NO_2^- (17). Furthermore, quantitative studies of NO concentrations during reduction of NO_2^- are consistent with the bulk of the nitrogen flux of denitrification occurring via NO (28). Thus, this explanation seems highly improbable. (ii) Production of N_2O from NO_2^- is a two-step process involving NO production by the nitrite reductase and subsequent NO reduction to N_2O by NO reductase. These two enzymes may associate with each other in vivo to channel products from one to another. Loss of functional nitrite reductase may lead to disruption of this association, resulting in some loss of NO reductase activity and the absence of ^{18}O exchange. (iii) A third possibility is that a functional nitrite reductase or functional nitrite reduction system is necessary for full expression of NO reductase activity at the functional or genetic level. (iv) Similarly, a functional nitrite reductase might be necessary for the synthesis of one or more electron transfer proteins, such as cytochromes, that are specific electron donors for the NO reductase.

The results of Zumft et al. (29) with Tn*5*-induced mutants of *P. stutzeri* Zobell may be consistent with the last interpretation. Although they found no significant changes in NO reduction activity, they did find two Nir$^-$ mutants that had significant changes in the amount of cytochrome c_{552} and/or alpha-peak *c*-type cytochrome, and they suggested that these cytochromes exhibited a functional and/or regulatory interdependence. However, *P. fluorescens* AK-15 and *P. stutzeri* Zobell differ both physiologically and genetically. For example, the former does not grow efficiently on N_2O alone. This phenomenon is often observed in *P. aeruginosa* (14). The structure of the *nir* operon in *P. fluorescens* AK-15 consists of *nirS* followed immediately by *nirM* and *nirC*, similiar to the *nir* operon in *P. aeruginosa* (unpublished results). The data, however, do not allow us to distinguish explanations ii, iii, and iv.

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ADDENDUM

During the reviewing process for this paper, a Nor$^-$ mutant of another species was isolated by gene replacement (3). This mutant blocks the denitrification pathway at nitric oxide, indicating that N_2O cannot be formed from nitrite directly via the nitrite reductase and thus providing more convincing evidence to reject explanation i. The Nor$^-$ mutant isolated is conditionally lethal, which explains why no Nor$^-$ mutants were found in this study.

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Chapter Five

Characterization of Tn*5* Mutants Deficient in Dissimilatory Nitrite Reduction in *Pseudomonas* sp. strain G-179 with a Copper Nitrite Reductase

Abstract. Tn*5* was used to generate mutants that were deficient in dissimilatory reduction of nitrite in *Pseudomonas* sp. strain G-179, which contains a copper nitrite reductase. Three types of mutants were isolated. The first type showed a lack of growth on nitrate, nitrite, and nitrous oxide. The second type grew on nitrate and nitrous oxide, but not on nitrite (Nir^-). The two mutants isolated in this group accumulated nitrite, showed no nitrite reductase activity, and had no detectable nitrite reductase protein bands in a Western blot. Tn*5* insertions in these two mutants were clustered in the same region and were within the structural gene for the nitrite reductase. The third type of mutant grew on nitrate, but not on nitrite or nitrous oxide (N_2O). It accumulated significant amounts of nitrite, NO, and N_2O during anaerobic growth on nitrate and showed a slower growth rate than the wild type. Diethyldithiocarbamic acid (DDC), which inhibited nitrite reductase activity in the wild type, did not affect NO reductase activity, indicating that nitrite reductase did not participate in NO reduction. NO reductase activity in Nir^- mutants was lower than the wild type when they were grown on nitrate, but was the same as the wild type when grown on nitrous oxide. These results suggest that the reduction of NO and N_2O were carried out by two distinct processes and that mutants in nitrite reduction reduced NO reductase activity following anaerobic growth with nitrate.

Introduction

Denitrification is the dissimilatory reduction of nitrate to nitrogen gases by the pathway of: NO_3^- --> NO_2^- -->NO --> N_2O -->N_2. The key step in denitrification is the reduction of nitrite by nitrite reductases, since it is the point of divergence from assimilation. Based on the characteristics of active sites, two major types of dissimilatory nitrite reductases are found: those containing the cytochrome *c* and *d1* and those containing copper (17). Cu-type nitrite reductases from many denitrifiers have homology to each other as shown by cross-reactivity in immunoblots (8, 24). Studies of the Cu-type nitrite reductase from *Achromobacter cycloclastes* reveals that the enzyme is a trimer with three type I and three type II coppers (10,13). Type I copper is bound within a single monomer, while type II copper is held by residues from each of two monomers of the trimer. Evidence from the crystal structure suggests that nitrite is bound to the type-II copper site (13). Pseudoazurin is the physiological electron donor (20, 21, 35).

Recently, convincing evidence has accumulated to support the hypothesis that NO is a free and obligate intermediate at least in denitrifiers containing cytochrome *cd1* nitrite reductases. Purified enzymes produce nitric oxide as the major product and can not convert NO to N_2O (5, 22, 31). Isotope and kinetics experiments indicate that NO is kinetically competent and obligatory for N_2O formation (3, 11, 14, 15). An insertional mutation in the structural gene of NO reductase in *P. stutzeri* Zobell strain yielded NO as the only detectable product of NO_2^- reduction, thus showing that there is no other physiological alternative for N_2O

formation from nitrite (4). Furthermore, NO reductases have been purified from two organisms, *P. stutzeri* Zobell and *Paracoccus denitrificans* (6, 9 ,16). Some uncertainty, however, still exists as to whether there might also be a direct NO_2^- to N_2O pathway for denitrifiers containing copper nitrite reductases. It has been found that small amount of N_2O can be formed from NO via a "NO-rebound" pathway by the purified copper nitrite reductase from *Achromobacter cycloclastes* (18), and ^{18}O isotope studies on *A. cycloclastes* and *P. aureofaciens* showed lower level of ^{18}O exchange occurred with NO_2^- as substrate versus NO (33).

All genetic information on denitrifiers comes from strains that contain the heme cd_1 nitrite reductase and none from strains with the copper enzyme. Because strains containing the copper type make up over one-third of isolates, represent a wider phylogenetic distribution(8), and because the enzyme has different mechanistic features than the heme type, it is worthwhile to obtain and compare genotypic and phenotypic information on this group of organisms as well. For the heme type strains, some genes involved in nitrite reduction have been identified. In *P. stutzeri* (19) one operon contains the structural gene for nitrite reductase (*nirS*), a tetraheme protein (*nirT*), cytochrome c_{552} (*nirM*), cytochrome C_{551} (*nirB*) and another monoheme protein (*nirC*); while in both *P. fluorescens* AK-15 (34) and *P. aeruginosa* (1), this operon contains *nirS*, *nirB* and *nirC*, without *nirT* and *nirM*. Nir^- mutants from *P. stutzeri* showed a normal NO reductase activity (36). In contrast, similar mutants isolated from *P. fluorescens* AK-15 showed not only a loss of nitrite reductase activity, but also a reduction in NO reductase activity and ^{18}O exchange in the NO to N_2O step (34).

In this paper, we report the isolation and characterization of three groups of mutants deficient in dissimilatory reduction of nitrite in a strain containing a Cu-type nitrite reductase, as well as the effects of Nir$^-$ mutants on NO reduction.

Materials and Methods

Bacterial strains. *Pseudomonas* sp. strain G-179 was from Gamble's collection (12). It was isolated by this lab from a pampa agricultural soil from the Parana Experimental Station, Argentina. This strain contains a Cu-type nitrite reductase as demonstrated by diethyldithiocarbamic acid (DDC) inhibition and immuno-reaction to polyclonal antibodies against the nitrite reductase from *A. cycloclastes* (8). This strain was selected because it was Kan^s, could produce exconjugants at a reasonable frequency, and formed distinct colonies on NO_2^- plates in an anaerobic glove box. A rifampin-resistant clone, RTC01, was obtained from a spontaneous mutation.

Conjugation and isolation of mutants. Transposon mutagenesis by conjugation was carried out by using the rifampin-resistant strain, RTC01, of wild type *Pseudomonas* sp. G-179, as the recipient; and *Escherichia coli* S-17, carrying the pSUP2021 plasmid containing Tn*5* , as the donor (33). Mating was carried out at 25^{0}C overnight. Exconjugants were selected on tryptic soy broth (TSB, Difco Laboratories, Detroit, Mich) agar plates supplemented with rifampin (50μg/ml) and kanamycin (50 μg/ml).

Colonies were replica plated onto TSB agar plates with 10 mM KNO_2 and grown overnight in an anaerobic glovebox. Those clones that showed no growth were selected from the master plates and further tested for their ability to grow anaerobically on broth supplemented with nitrate, nitrite, or nitrous oxide as the terminal electron acceptors.

Growth conditions. The growth medium contained TSB (30 g /L), 2 μM of $CuSO_4$, and 30 mM of potassium nitrate, if used. For growth on nitrous oxide, TSB was saturated with nitrous oxide before inoculation and then the gas phase was again filled with nitrous oxide. Strain RTC01 was grown in the presence of rifampin (50 μg/ml) and mutant strains were grown in the presence of both rifampin (50 μg/ml) and kanamycin (50 μg/ml). An inoculum from fresh plates was first transferred to 10 ml of growth medium and grown anaerobically overnight; this was used to inoculate a sealed bottle containing 120 ml growth medium. Cells were harvested after 27 h of anaerobic growth at 25°C with constant shaking (100 rpm). Cells were washed several times with 50 mM HEPES buffer (pH 7.0) until no nitrite was present. Cells not immediately used were stored at -70°C.

Measurement of nitrate, nitrate, NO and N_2O. Nitrate and nitrite were determined by HPLC using an anion exchange column (7). NO and N_2O were measured with gas chromatography (34).

Crude extract preparation and activity assays. Cells were disrupted by sonication. The supernatant after centrifugation (13,000 x g for 30 min) was assayed for NO_2^- and NO reductase activities. Assays for nitrite and

nitric oxide reductase were carried out in 8-ml serum bottles with 1 ml solution as described earlier (8, 34). For the nitrite reductase assay, the rate of evolution of both NO and N_2O was measured. For the nitric oxide reductase assay, the rate of N_2O evolution was measured. For rate of nitrous oxide reduction in whole cells, the rate of N_2O disappearance was measured.

Western and Southern blots. Western blots were developed with antibodies raised against the nitrite reductase from *A. cycloclastes* as described previously (8). Genomic DNA isolation, restriction enzyme digestion, electrophoresis, and Southern blotting were done as described by Maniatis, *et al* (23). Tn*5* was labelled with a random primer kit (Boehringer Mannheim, Germany).

Results

Isolation of mutants deficient in nitrite reduction. Transconjugants that did not grow anaerobically on TSB agar plates supplemented with potassium nitrite were screened and further tested for growth and bubble formation in tubes with TSB medium containing potassium nitrate or nitrous oxide. Three types of mutants were isolated. The first group of mutants, as represented by RTC07 and RTC14, showed a lack of growth not only on nitrite, but also on nitrate and N_2O (Table 1). The second type of mutants (RTC22 and RTC23) were deficient only in nitrite reduction and grew under anaerobic conditions with nitrate and nitrous oxide as the terminal electron acceptor (Table 1). These two Nir^- mutants accumulated nitrite (Table 2). Mutant strain RTC03 represented the third type of mutant; it grew on nitrate, but not on nitrite or nitrous oxide. It accumulated nitrite after overnight growth (Table 2).

Physical characterization of Tn*5* mutants. To detect the presence of Tn*5*, genomic DNA from mutant strains of the first group of mutants and RTC03 was digested with *Eco*RI and *Bam*HI, which has a recognition site inside Tn*5*. The Southern blot was probed with labelled Tn*5*. Bands from these different mutant strains varied in size, suggesting different locations of Tn*5* (data not shown).

Genomic DNA digested with *Eco*RI from both Nir^- mutant strains, RTC22 and RTC23, showed a band equal in size in a Southern blot probed with Tn*5* (Fig. 1). *Bam*HI digestion resulted in two bands and the combined

size of these two bands was also the same between these two mutants, indicating that the two Tn*5* insertions were clustered in the same region. The *Eco*RI and *Bam*HI DNA fragment containing the *neo* gene of the Tn*5* and a flanking genomic region (Fig.1, lanes 5 and 6) was subcloned in pUC19 by screening for kan^r and used as a probe to isolate the corresponding wild type *Eco*RI and *Bam*HI fragment. The resulting clone, pYTC18, contains a 1.9 kb fragment, in which both Tn*5* insertion sites were located by mapping (Fig.2). Sequencing results indicated that this 1.9 kb fragment contains the structural gene of the copper nitrite reductase (32).

Biochemical characterization of Nir^- Tn*5* mutants. The wild type strain had nitrite reductase activity that was abolished upon the addition of the copper chelator DDC (Table 3), which is characteristic of Cu-type nitrite reductases. Both Nir^- mutants lacked nitrite reductase activity. Mutant strain RTC03 had a slightly higher nitrite reductase activity than did the wild type. In a Western immuno-blot developed with polyclonal antibodies against the Cu-type nitrite reductase, both wild type clone (RTC01) and RTC03 showed positive nitrite reductase bands, while no such bands were found in RTC22 and RTC23 (Fig.3). Presence of two bands in wild type and RTC03 may be due to degradation, two different start sites or two different processing sites for signal peptide.

Addition of DDC did not affect NO reductase activity in the wild type, suggesting that nitrite reductase did not participate in the NO reduction (Table 3). However, the NO reductase activities in Nir^- strains RTC22 and RTC23 were lower relative to the wild type when cells were grown on nitrate. When the cells were grown on N_2O, this difference was not

detected, although the NO reductase activity of all strains, including wild type, was lower than that of the nitrate grown cells. In mutant strain RTC03, the NO reductase activity was similar to that found in the wild type. There was essentially no difference in the ability to convert N_2O to N_2 among wild type, RTC22, and RTC23. However, this ability was lost in mutant RTC03, which is consistant with the result that significant amounts of N_2O accumulated during anaerobic growth on nitrate (Fig. 4).

The wild type strain, RCT01, produced very low levels of NO during anaerobic growth on nitrate (Fig. 4). In contrast, much higher levels of NO as well as N_2O were observed at late stages of growth in mutant strain RTC03. This mutant also showed a much slower growth rate and lower yield.

Discussion:

Both Nir$^-$ mutants RTC22 and RTC23 showed a lack of nitrite reductase protein bands on a Western blot (Fig. 3), suggesting that Tn*5* insertions in the Nir$^-$ mutants were located in the operon containing the nitrite reductase structural gene. We sequenced the 1.9 kb DNA fragment that contained both Tn*5* insertion sites (32). This fragment revealed an open reading frame which has 80% identity and 89% similarity in amino acid sequence to the nitrite reductase isolated from *Achromobacter cycloclastes* (10). Amino acid residues responsible for type I and type II copper binding were also conserved (32). When the 1.9 kb fragment was used as a probe, it hybridized to most other denitrifiers with Cu-type nitrite reductases (32).

We have previously suggested that NO is an intermediate of denitrification in some denitrifiers containing copper nitrite reductases (33). We also suggested that there is a possible alternative route for N_2O formation without the production of NO for *P. aureofaciens* and *Achromobacter cycloclastes*, since ^{18}O exchange occurred during reduction of NO to N_2O, but not of NO_2^- to NO (33). This alternative, if it exists, appears to be a minor one in *Pseudomonas* sp. strain G-179. Reduction of nitrite and nitric oxide were carried out by two different processes, and nitrite reductase does not seem to participate in the reduction of NO, since inhibition of nitrite reductase by DDC had no effect on NO reduction (Table 3). This is consistant with the results previously obtained in other denitrifiers (28,29). Furthermore, when cells were grown

on N_2O, the rates of NO reduction were similar between the wild type and mutants (Table 3). As a result, there is no evidence to support that nitrite reductase can directly reduce NO_2^- to N_2O without formation of NO in this strain.

The lower NO reductase activity in Nir^- mutants (RTC22 and RTC23) relative to the wild type when grown on nitrate may be due to the level of expression. This explanation is supported by the results that NO reductase activity from these mutant cells was similar to the wild type when grown on N_2O as the only terminal electron acceptor. When cells were grown under denitrifying conditions with nitrate, most nitrogen was converted to N_2 via NO and therefore, NO reductase activity should be fully expressed. In contrast, the availability of NO was very limited in the two Nir^- mutants. Other explanations we provided (34), such as close functional and/or genetic relationship between NO_2^- and NO reduction, can not be excluded. Cells grown on N_2O from both wild type and Nir^- mutant strains have lower NO reductase activity as compared to those grown on nitrate (Table 3). This suggests that the level of expression of NO reductase is higher when grown on nitrate than on N_2O. We also observed a lower rate of NO reduction in Nir^- mutant relative to the wild type of *P. fluorescens* AK-15, which contains a cytochrome *c,d1* nitrite reductase (34). However, *P. fluorescens* AK-15 did not grow well on N_2O alone. Thus, we do not know whether the explanation provided here can apply to this organism.

NO is normally found in very low concentrations during denitrification (3,14). The wild type strain of *Pseudomonas* sp. G-179 also produced a typical low level of NO (Fig. 4). In contrast, mutant RTC03

generated a larger amount of NO at late stages of growth. This mutant also accumulated nitrite (Table 2) and N_2O and had a slower growth rate (Fig.4), even though it had the capacity to convert NO_2^- to NO and NO to N_2O (Table 3). One possible explanation is that the coupling process between energy conservation and the NO_2^- or NO reduction steps was blocked, resulting in slower growth rate and slower conversion of these two intermediates (Table 2). Precedent for such a lesion in energy coupling may occur in *Chromobacterium violaceum*, since growth yield results indicate that denitrification is not coupled to growth (2). The accumulation of intermediates in this mutant could be due to several factors including a kinetic effect resulting from a general inhibition (reduced growth in this case) to sequential Michaelis-Menten reactions (which has been modeled and verifed for denitrifiers (3)) and a partial inhibition of NO reductase by nitrite, which has been shown in membrane fractions (29). A deficiency in N_2O reduction (Table 3) led to the accumulation of N_2O. It is also possible that the mutation may affect a factor common to several steps, such as the involvement of copper.

The group I mutants (such as RCT07 and RTC14) that were deficient in growth on all intermediates in the denitrification pathway may have resulted from Tn*5* insertion into regions responsible for gene regulation or electron transport chain components unique to denitrification (Table 1). Tn*5* insertion occurred in different sites in 10 of these mutants (data not shown) and this class was obtained at a high frequency. This suggests that there are many factors other than those involved in the direct catalytic steps of the pathway that are essential to the process of denitrification.

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Table 1. Growth characteristics of Tn*5* mutants of *Pseudomonas* sp. strain G-179 deficient in denitrification.[a]

Strains	+ NO_3^-	+ NO_2^-	+ N_2O
RTC01	+	+	+
RTC07 [b]	-	-	-
RTC14	-	-	-
RTC03 [c]	+	-	-
RTC22	+	-	+
RTC23	+	-	+

[a] Growth on nitrate and nitrite was tested on plates and in broth under anaerobic conditions. Growth on nitrous oxide was tested in tubes with 10-ml TSB and 1 atmosphere nitrous oxide.

[b] Nine additional mutants of this type were isolated.

[c] One additional mutant of this type was isolated.

Table 2. Remaining nitrate and accumulation of nitrite following anaerobic growth of Tn*5* mutants of *Pseudomonas* sp. strain G-179 on nitrate medium.[a]

Strains	Nitrite (mM)	Nitrate (mM)
RTC01	ND[b]	0.8
RTC03	12.5±0.4	15.7±1.0
RTC22	25.7±3.7	3.2±1.4
RTC23	26.9±2.6	2.0±2.4

[a] Cells were grown in a sealed serum bottle with 120 ml TSB supplemented with 30 mM potassium nitrate. Cultures were shaken constantly and harvested after 27 h of growth. Data are the averages and standard deviation from triplicates.

b ND=not detectable. The detection limit was 1 uM.

Table 3. Activities of NO_2^-, NO, and N_2O reduction in wild type and mutant strains of *Pseudomonas* sp. G-179.[a]

	nmol produced or consumed · min^{-1} · mg $protein^{-1}$			
Strains	NO_2^- ->NO[b]	NO ->N_2O + NO_3^- [b]	NO ->N_2O + N_2O [c]	N_2O->N_2 [b]
RTC01	82±3.5	28.2±2.2	5.7±0.6	44.8±0.9
RTC01 + DDC	0	26.3±1.6	ND [d]	ND
RTC03	118±1.2	24.8±0.9	ND	0.3±0.3
RTC22	0	15.7±0.5	6.0±0.4	36.7±1.4
RTC23	0	13.8±1.7	7.6±0.9	37.7±0.6

[a] Crude extracts were used to assay the appearance of product(s) of nitrite and NO reductases. Whole cells were used to assay for nitrous oxide disappearance of nitrous oxide was measured. Initial substrate amounts were 5 μmole nitrite and 2 μmole NO. EDTA(5mM) or DDC(5mM) was added in assays for NO reduction.

[b] Anaerobic growth on nitrate.

[c] Anaerobic growth on nitrous oxide.

[d] ND = Not determined

Figure legends

FIG.1 Southern analyses of Tn*5* insertions from Nir$^-$ mutant strains. The blot was probed with ^{32}P labeled *Hpa*I fragment of Tn*5*. Lane 1, wild type DNA digested with *Eco*RI and *Bam*HI. DNA in lanes 2 (RTC23) and 3 (RTC22) were digested with *Eco*RI, 4 (RTC23) and 5 (RTC22) with *Bam*HI, 6 (RTC23) and 7 (RTC22) with both *Eco*RI and *Bam*HI.

FIG. 2. Physical mapping of Tn*5* insertion sites of mutant strains RTC22 and RTC23 in the 1.9 kb *Eco*RI and *Bam*HI fragment. A, *Acc*I; E, *Eco*RI; X, *Xmn*I; B, *Bam*HI; S, *Sal*I. Triangle represents Tn*5*. Arrows indicate the orientation of Tn*5* with respect to its *Sal*I and *Bam*HI sites.

FIG. 3. Western blot of proteins from wild-type and mutant strains. The blot was developed with polyclonal antibodies raised against the nitrite reductase purified from *A. cycloclastes*. Lanes 1 through 4 were wild type, RTC22, RTC23 and RTC03, respectively.

FIG. 4. Growth curve and evolution of N_2O and NO during anaerobic growth on nitrate in wild type RTC01 and mutant strain RTC03. Optical density was measured at 600 nm. Cells were grown under anaerobic conditions in sealed 160-ml serum bottles with 100 ml TSB and 30mM of KNO_3. The gas phase was argon. Data are averages of triplicates.

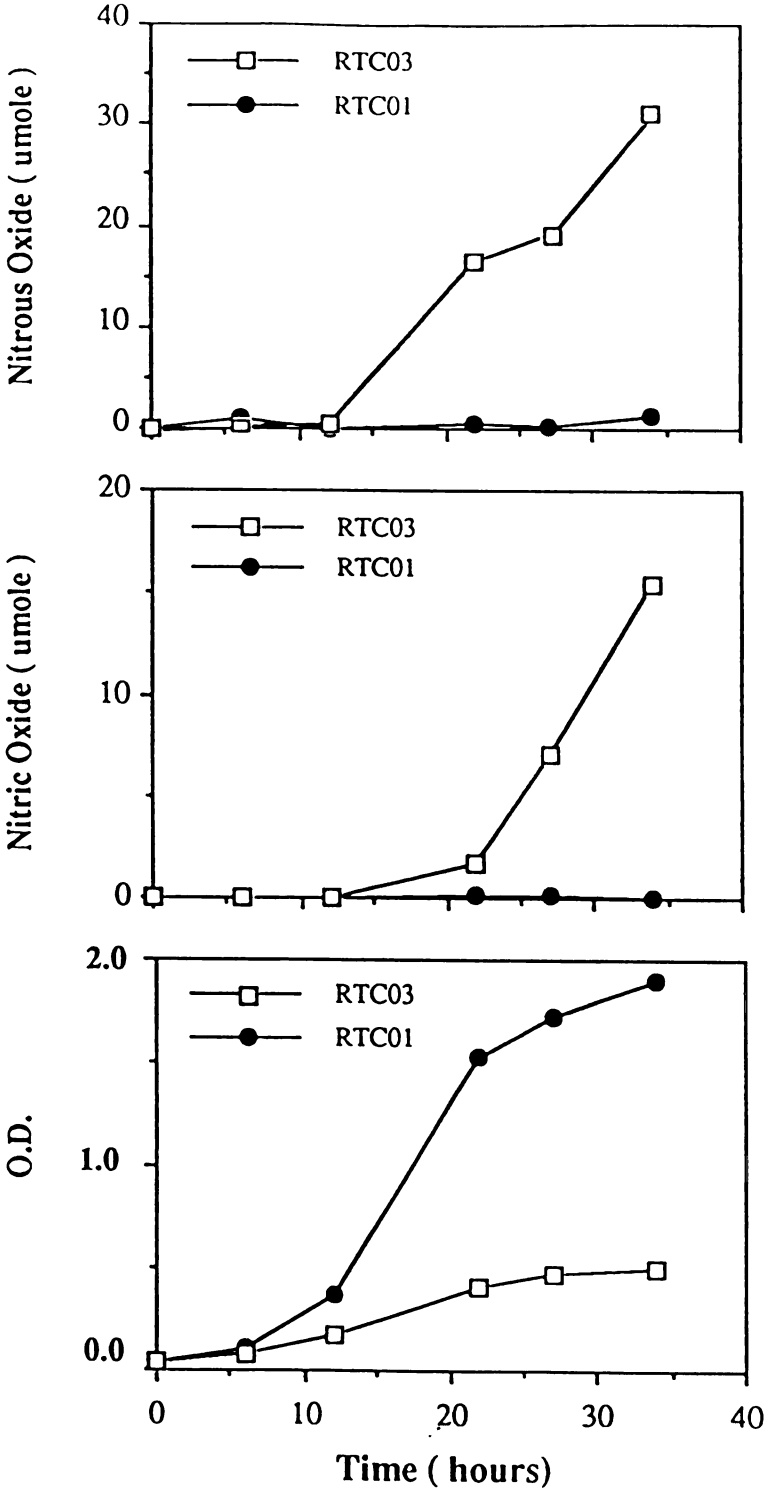
Nitrous Oxide (umole)
40
30
20
10
0
RTC03
RTC01
Nitric Oxide (umole)
20
10
0
RTC03
RTC01
O.D.
2.0
1.0
0.0
RTC03
RTC01
0
10
20
30
40
Time (hours)

Figure 4

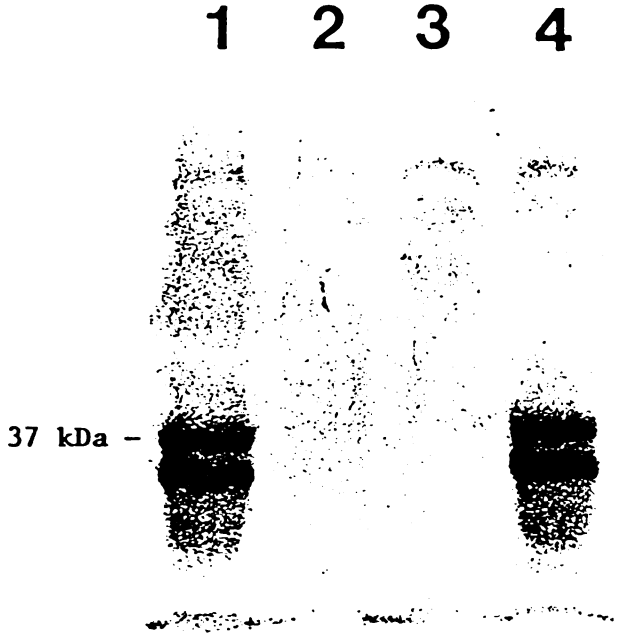

Fig. 3

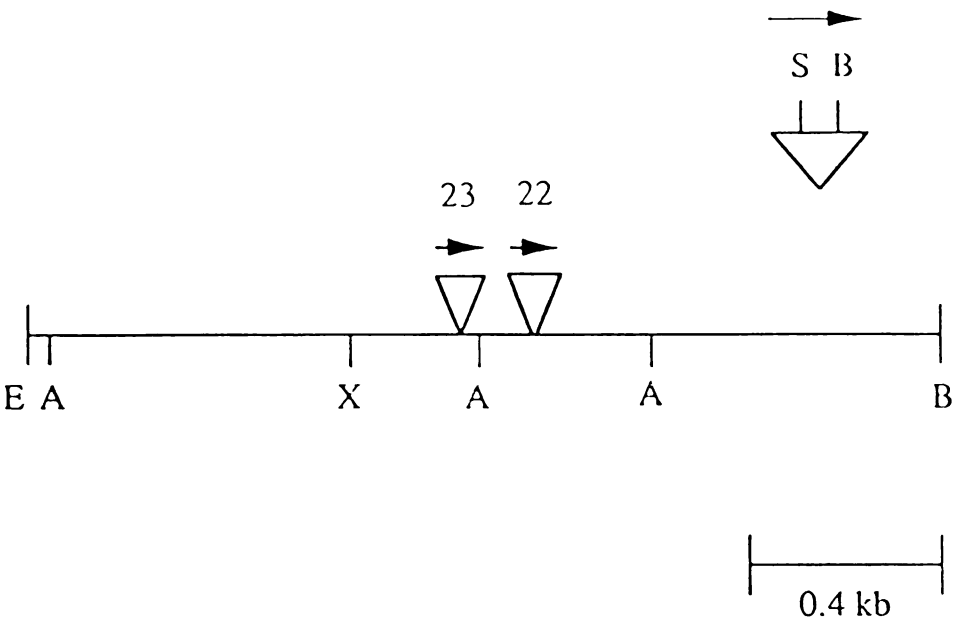

Fig. 2

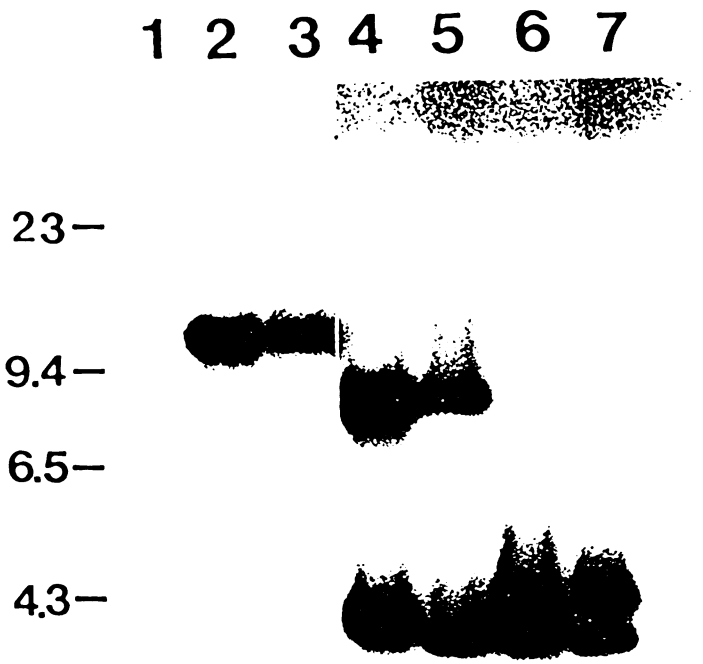

Figure 1

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Chapter Six

Characterization of the Structural Gene for a Copper Containing Nitrite Reductase and its Homology to Other Denitrifiers

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Abstract. A copper-containing nitrite reductase gene (*nirA*) from *Pseudomonas* sp. G-179 was found in a 1.9 kb *Eco*RI and *Bam*HI DNA fragment. The coding region contained information for a polypeptide of 379 amino acids. The encoded protein had 78% identity and 88% similarity in amino acid sequence to the nitrite reductase purified from *Achromobacter cycloclastes* . Ligands for type 1 and type 2 copper binding sites were conserved. Analysis of the promoter region revealed two tandem putative *ntrA* boxes, suggesting that a σ^{54}-like factor is needed for its expression. Upstream from the promoter, one putative *fnr* box was found, indicating that a FNR-like protein may be involved in regulation of the nitrite reductase gene under anaerobic conditions. When the structural gene was used to study the homology among different denitrifiers, it showed positive signals with DNA from17 out of 18 gram-negative denitrifiers containing copper nitrite reductases. Except for *Pseudomonas stutzeri* JM300, all denitrifiers that contain heme c,d_1 nitrite reductases showed no or weak signals with this probe. Thus this structural gene may be useful as a probe to detect denitrifiers with copper nitrite reductases .

Introduction

Denitrification is the dissimilatory reduction of nitrate to nitrogen gases. The key environmental step in denitrification is the reduction of nitrite by nitrite reductases since this enzyme converts a mineral form of nitrogen to a gaseous form, NO, which can not be assimilated by the biosphere. Based on the characteristics of active sites, two major types of dissimilatory nitrite reductases are known (9,22): those containing the cytochrome *c* and d_1 (*c*,d_1 -dNirs) and those containing copper (Cu-dNirs). Cu-dNirs from many denitrifiers exhibit homology to each other as shown by cross-reactivity in immunoblots (2, 18). Studies of the Cu-dNir from *Achromobacter cycloclastes* have revealed that the enzyme is a trimer with three type 1 and three type 2 coppers (5,8). Type 1 copper is bound within a single monomer, while type 2 copper is held by residues from each of two monomers of the trimer. Evidence from the crystal structure shows that nitrite is bound to the type 2 copper site (5), implying that type 2 copper is essential for enzyme activity. Another copper containing protein, pseudoazurin, is the physiological electron donor for the Cu-nitrite reductase in vitro (12,13, 30).

Denitrifiers grow using aerobic respiration in aerobic environments but can shift to using nitrogen oxides as electron acceptors under anaerobic conditions. How these genes involved in denitrification are regulated is poorly understood. In *Escherichia coli* and other enteric bacteria, the *fnr* (fumarate and nitrate reductases) gene is a positive regulator for the expression of genes involved in fumarate and nitrate respiration as well as

other anaerobic processes (see reviews, ref. 11 and 25). It is generally accepted that under anaerobic conditions, the FNR protein acts as a transcriptional activator by binding to its binding site, the FNR box, upstream of the promoter. FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa* has been demonstrated (6). In *P. stutzeri* JM300, two FNR boxes have been found upstream of the promoter in the operon containing the nitrite reductase structural gene, suggesting the existence of a similar type of regulation (23). Another regulator, the *ntrA* gene product σ^{54}, is involved in diverse physiological processes (14). Both *fnr* and *ntrA* boxes have been found in the upstream region of the promoter of azurin and pseudoazurin genes in several denitrifiers (10) and upstream of the *nifA* promoter of *Azorhizobium caulinodans* (19).

Recently, research efforts have been devoted to isolating denitrifiers that degrade environmental contaminants and to studying the ecology of denitrifiers in soil. The use of gene probes to identify and monitor populations of denitrifiers in natural environments and enrichments offers several advantages over the conventional culture-dependent methods (20,23). The majority of the available genetic information on denitrifiers comes from strains that contain the cd_1 -dNirs. Because strains containing the copper type make up over one-third of the studied isolates and represent a wider phylogenetic distribution (2), and because the enzyme has different mechanistic features than does the c,d_1-dNir, it is important to obtain genotypic, phenotypic, and ecological information on this group of organisms as well. Probes for c,d_1-dNirs alone will not meet the needs for

population studies. Hence, it is important to evaluate whether there are suitable probes for Cu-dNir containing denitrifiers.

In this paper, we report the primary structure of a Cu-dNir structural gene. The upstream and the promoter region have been analyzed for possible regulatory mechanisms. We also report on the homology of this gene to the DNA of other denitrifiers and find it specific for most of Cu-type denitrifiers.

Materials and Methods

Strains and growth conditions. *Pseudomonas* sp. strain G-179 was isolated from a pampa agricultural soil from the Parana Experimental Station, Argentina (7). This strain contains a Cu-type nitrite reductase as demonstrated by diethyldithiocarbamic acid (DDC) inhibition and immuno-reaction to the polyclonal antibodies raised against the Cu-type nitrite reductase from *A. cycloclastes* (2). The growth medium contained half-strength tryptic soy broth (TSB), 2 μM of $CuSO_4$, and 5 mM of potassium nitrate, if used. Cells not immediately used were stored at -70°C.

Southern blots and sequencing. Genomic DNA isolation, restriction enzyme digestion, electrophoresis, and Southern blotting were done as described by Maniatis, *et al* (16). DNA fragments were isolated from agrose gel with a Geneclean kit (Bio 101, La Jola, California) and was labelled with ^{32}P with a random primer kit (Boehringer Mannheim, Germany). After hybridization, Southern blots were washed three times at room temperature with following solution: 2 x SSC/0.1% SDS, 0.5 x SSC/0.1 % SDS

and 0.1 x SSC/0.1%. The final wash was carried out at 50°C with 0.1% SSC/1% SDS. All DNA sequencing reactions were carried out by the di-deoxy method using the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). Both strands were sequenced. Analysis of DNA sequence was performed with the GCG sequence analysis program (4)

Results

Isolation of the nitrite reductase gene. We have previously found that two Tn*5* Nir$^-$ mutants of *Pseudomonas* sp. G-179, RTC22 and RTC23, had no nitrite reductase activity and showed no nitrite reductase protein band in Western blots (29). The Tn*5* insertion sites in these two mutants were clustered in the same *Eco*RI and *Bam*HI fragment. Genomic DNA from these two mutants was digested with *Eco*RI and *Bam*HI and the DNA fragments containing the *neo* gene of the Tn*5* plus the flanking genomic region were subcloned in pUC19 by selection on kanamycin plates (50 ug/ml). Subclones were then used as probes to isolate the corresponding wild type *Eco*RI and *Bam*HI fragment. Several positive clones were obtained and they all possessed a 1.9 kb *EcoR*I and *BamH*I fragment as shown in Fig. 1. Results from DNA sequencing revealed an open reading frame corresponding to a copper nitrite reductase transcribed in the direction of *Eco*RI to *Bam*HI (Fig. 2). The Tn*5* in both mutants was found inside the structural gene (29). The sequence has been placed in Genebank with the accession No.M97294.

Properties of the derived protein product. When the deduced amino acid sequence was compared to the amino acid sequence of the Cu-dNir

from *Achromobacter cycloclastes* (5), 78% identity and 88 % of similarity were found (Fig.3). Studies on the crystal structure and amino acid sequence of the dNir from *Achromobacter cycloclastes*, have revealed both type 1 and type 2 copper binding sites (5,8). Amino acids responsible for copper binding in the Cu-dNir from *A. cycloclastes* and the corresponding region of the *Pseudomonas* sp. G-179 sequence are compared in Fig. 4. All of the ligands to both copper sites are strictly conserved and the amino acid sequences in these regions exhibit a very high degree of homology. This suggests that the Cu-dNir from *Pseudomonas* sp. G-179 contains both type 1 and type 2 copper.

Due to the presence of two methionines among the first 12 amino acids, there are two possible start sites . The first 32 amino acids showed strong hydrophobicity (Fig. 5), a feature of typical signal peptides. It has been shown by immunogold labelling and electron microscopy that nitrite reductases from two Cu-dNir containing organisms and two c,d_1-dNir containing organisms are located in the periplasmic space in gram-negative bacteria (3). Presence of signal peptide is consistant with this observation. The first amino acid of the mature protein is likely to be Glu-32, since it is proceeded by an Ala-X-Ala processing site, another feature of signal peptides. The mature protein had low hydrophobicity, suggesting that the enzyme is soluble (Fig.5)

Characterization of the promoter region. The protein FNR has been found to be an important activator for anaerobic metabolism in *E.coli* and other organisms. The consensus sequence to which the FNR protein binds is TTGATN$_4$ATCAA (10,19,24). The five nucleotides in the first and second

halves and the spacing of four nucleotides between are highly conserved. Such an FNR box, is found upstream of the promoter in the copper nitrite reductase gene of *Pseudomonas* sp G-179; it is located 364 bp from the assumed start codon. The sequence was (289)TTGATGAAAATCAA (303) (Fig. 2).

The *ntrA* gene, which encodes σ^{54}, has been found to be a regulatory factor in nitrogen metabolism and many other physiological processes (14). The *ntrA* box has the consensus sequence of CTGGYAYRN4TTGCA (1). The highly conserved features include GG and GC doublets separated by 10 bp. Two such consensus sequences were found, (580) TTGGAGCAAACATGCT (595) and (623)GTGGAGCCGAGGTTGCT(639) (Fig. 2) . In the first *ntrA*-like box the GG and GC doublet was separated by 9 bp instead of 10. Two *ntrA*-like boxes can also be seen in the operon containing the *c,d*$_1$ -dNir gene from *Pseudomonas aeruginosa* and upstream of genes involved in denitrification (Table 1).

Homology of the Cu-nitrite reductase gene to DNA from other denitrifiers. To test the homology of the nitrite reductase gene with DNA from other denitrifiers and evaluate its potential as a probe for denitrifiers, the 1.2 kb *Xmn* I and *Bam*HI fragment was used. Southern blots containing DNA from different strains of denitrifiers were probed with this labelled fragment. Among all 18 Cu-dNir-containing and gram-negative denitrifiers, 17 have moderate to strong homology with this probe and only *Alcaligenes eutrophus* gave a weak signal (Table 2). Among the *c,d*$_1$ -dNir-containing and gram-negative denitrifiers, only *Pseudomonas stutzeri* JM 300 showed a strong band with the Cu probe. This band did not correspond

to the band hybridized by the c,d_1 -dNir probe isolated from this strain (23). Two the c,d_1-dNir containing denitrifiers had weak hybridization with the Cu gene probe and six had no signal. A gram-positive denitrifier, *Bacillus azotoformans* , showed strong hybridization to this probe, but a second strain of *Bacillus* gave no hybridization. Overall, the results obtained with Southern blots were consistant with the results obtained with Western blots (Table 2).

Discussion:

The amino acid sequence of the copper nitrite reductase from *Achromobacter cycloclastes* determined by Fenderson, *et al* (5), was highly homologous with the derived sequence we found for *Pseudomonas* sp. G-179. The ligands responsible for binding both types of copper present in the former are conserved (Fig. 3), indicating that the copper nitrite reductase from *Pseudomonas* sp. G-179 also contains both type 1 and type 2 copper. As a result, a similar model of protein structure for this Cu-dNir can be proposed. The type 1 Cu would be coordinated by four ligands from domain I of the same monomer: C-175, H-184, M-189, and H-134. The type 2 Cu would be coordinated by H-139 and H-174 from domain I of one molecule and H-345 from domain II of the second (Fig. 4).

Cu-dNirs isolated from *Alcaligenes* sp. and *P. aureofaciens* have been reported to contain only type 1 copper based on their EPR spectra (17,30). Therefore it has been suggested that type 1 copper is the active center and that the type 2 copper may not be required for enzymatic activity. However, recent protein crystal structure studies suggest that type 2 copper is the binding site for the substrate, nitrite (8). It has also been observed

that type 2 copper can be removed from the enzyme, and when reconstituted with copper the enzymatic activity is restored proportional to the amount of type 2 copper added (E. Libby and B.A. Averill, submitted for publication). These results indicate that the type 2 copper is essential for the enzymatic activity, but that it may be easily lost during the purification process.

The gene product of *ntrA*, σ^{54}, confers specificity on core polymerase for diverse physiological functions, including nitrogen fixation, catabolism of toluene and xylene, and nitrate and nitrite assimilation (14). Analysis of DNA sequence in the promoter regions of some genes involved in denitrification or anaerobic metabolism reveals the existence of putative *ntrA* boxes (Table 2). This box was found upstream of the N_2O reductase gene from *P. stutzeri* Zobell (26), the *cd*$_1$-dNir gene from *P. aeruginosa* (21) and the pseudo-azurin gene from *Alcaligenes faecalis* S-6 (27), which is the physiological electron donor for Cu-type nitrite reductase *in vitro* (12). In the copper-nitrite reductase gene from *Pseudomonas* sp. G-179, we noted two tandem *ntrA*-like boxes were found (Fig. 2 and Table 2). These results suggest that the *ntrA* protein may involved in the regulation of promoters involved in denitrification under anaerobic conditions. However, involvement of σ^{54} has not been tested in NtrA$^-$ mutants.

Upstream of the promoter region in *Pseudomonas.* sp. G-179, an *fnr* box was also found, suggesting an FNR-analog in this organism may play the role of activator (Fig. 2 and Table 1). It has been shown that a mutation in a *fnr*-like gene results in loss of nitrate and nitrite dissimilation in *P. aeruginosa*, which contains a *c,d*$_1$-dNir (6). Two *fnr* boxes were found upstream of the promoter of the nitrite reductase gene in *P. stutzeri* (23).

The *fnr* box is also found upstream of the azurin and pseudoazurin genes (Table 1). Preliminary experiments with an FNR$^-$(ANR$^-$) mutant of *P. aeruginosa* PAO1, provided by Dieter Haas (6) suggest the involvement of this regulatory mechanism in denitrification (unpublished data). As a result, it is very likely that an FNR-like protein (ANR) plays an global role in regulation of denitrification under anaerobic conditions. One hypothetical model for the regulation of Cu-nitrite reductase from *Pseudomonas* sp. G-179 is that under anaerobic conditions, an FNR-like protein activates transcription by contacting the RNA polymerase-promoter complexes. This process could be mediated by formation of a DNA loop since the *fnr* binding site is more than 300 bp from the promoter. Such a loop formation mechanism has been found in regulation of the *glnA* promoter, which is controlled by NtrC and σ^{54} (28). There are two putative *ntrA* boxes in the promoter region and this may explain the existence of two protein bands with slightly different molecular weights that we observed in Western blots (29). Obviously, mutants that are deficient in FNR and NtrA needs to be further characterized. Further analysis of the promoter region is also required to elucidate the mode of regulation.

When the 1.2 kb *Xmn*I and *Bam*HI fragment containing the nitrite reductase was used as a probe, it hybridized to most of the gram-negative, Cu-dNir containing denitrifiers tested and not to those containing cd_1 dNir except for *P. stutzeri* JM300. As a result, this probe may be useful in identifying this group of denitrifiers. The c,d_1 -dNir genes have been used as probes (15,23), but they are primarily limited in c,d_1 -dNir containing denitrifiers. The suite of both Cu- and heme-type nitrite reductases gene

probes may be useful to reveal ecological features of these two groups of organisms.

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Table 1. Putative regulatory sequences from denitrifying bacteria.[a]

Strains	Gene	*fnr* box	*ntrA* box
P. sp. strain G-179	Cu-*nir*	TTGAT....ATCAA	TTGGAGCAAAC ATGCT GTGGAGCCGAGGTTGCT
P. aeruginosa (21)[b]	*cd1 -nir*	UN[c]	CGGGAGTTCCCGACGCA AAGGGAGCGCC TCGCA
P. stutzeri JM300 (23)	*cd1 -nir*	TTGAT....GTCAA TTGAC....ATCAA	 UN
P. stutzeri Zobell (23)	*cd1 -nir*	TTGAT....ATCAA TTGAT....GTCAA	 UN
P. stutzeri Zobell (26)	*nos*	UN	GTGGAACCCTGAGCGCG
P. aeruginosa (10)	*azu*	TTGAC....ATCAG	GCGGCACATCT GTGCT
Alcaligenes denitrificans (10)	*azu*	TTGAT....GTCAA	CAGGCATGTGCCTGGCG
Alcaligenes faecalis S-6 (27)	Peudo-*azu*	TTGAT....ATCAA	GTGGCGTGTTGAGGCC

[a] References are given in the parenthesis following the strain names. Abbreviations are *nir*: nitrite reductase gene; *nos*: nitrous oxide reductase gene; *azu*: azurin gene.

[b] The presence of possible regulatory regions were identified in this work based on published results.

[c] UN=Unknown or inadequate information

Table 2. A summary of hybridization results by Western and Southern blots of different denitrifiers.

Strains	nir type	Antibody[a]		*nirA*[b] (Cu)
		cd$_1$-	Cu	
Ps. sp. G-179	Cu	-	+	+
Achr cycloclastes ATCC 2192	Cu	-	+	+
Ps. aureofaciens ATCC 13985	Cu	-	+	+
Alcal. xyolsoxidans NCIB 11015	Cu	-	+	+
Alcal. eutrophus ATCC 17699	Cu	-	ND	+/-
Rhodops. sphaeroides f. sp denitrificans	Cu	-	+	+
Corynebacterium nephridii ATCC11425	Cu	-	+	+
Bacillus azotoformans ATCC 29788	Cu	-	+	+
Ps. chlororaphis ATCC 43928	Cu	-	ND	+
Ps. fluorescens ATCC 17575	Cu	ND	ND	+
Agrobacterium tumefaciens A.348	Cu	-	ND	+
Ps. type 11 G-107	Cu	-	+	+
Ps. type 11 G-163	Cu	-	+	+
Ps. type 11 G-188	Cu	-	+	+
Alcal. faecalis G-191	Cu	-	+	+
Alcal. faecalis G-41	Cu	-	+	+
Alcal. faecalis G-65	Cu	-	+	+
Bacillus sp. G-193	Cu	-	-	-
Ps. picketii PK01	Cu	ND	ND	+
Ps. aeruginosa PAO1	heme	+	-	-
Ps. aeruginosa ATCC 10145	heme	+	-	-

Ps. aeruginosa ATCC19429	heme	ND	ND	+/-
Ps. aeruginosa ATCC 15692	?			+/-
Ps. fluorescens ATCC 33512	heme	+	-	-
Ps. fluorescens AK-15	heme	+	-	-
Ps. stutzeri JM 300	heme	+	-	+
Ps. stutzeri ATCC 11607	heme	+	-	+/-
Ps. stutzeri ATCC 11405	heme	+	ND	-
Pa. halodenitrificans				+/-
Ps. type 2 G-83	ND	-	-	-
Tol 4	heme	ND	ND	-
Ps. sp KC	ND	ND	ND	-
Non-denitrifiers:				
Agrobacterium tumefaciens A.60				-
Ps. mendocina				-

[a] Results of Coyne, et al (2). Some nir types were also determined by inhibition of activity after adding the Cu chelator, DDC.

[b] The extent of hybridization in Southern blots with the 1.2 kb fragment containing the Cu-dNir gene is indicated by plus (+) or negative (-) signs.

Figure legends

FIG. 1. Physical map of the 1.9 kb *Eco*RI and *Bam*HI fragment. The open reading frame region for *nirA* is shaded and the arrow indicates the direction of transcription.

FIG.2. Nucleotide sequence of the 1.9 kb *Eco*RI and *Bam*HI fragment and the predicted amino acid sequence of the Cu-type nitrite reductase. Numbers given on the right are for nucleotides and on the left for amino acids. Possible regulatory regions (*fnr* box and *ntr*A box) and the putative ribosome-binding site (Shine-Dalgarno sequence, SD) are underlined.

FIG. 3. Comparison of the amino acid sequences of Cu-type nitrite reductases from *Pseudomonas* sp. G-179 and *Achromobacter cycloclastes* (AC).

FIG. 4. Comparison of the copper binding region for Cu-dNirs from *Pseudomonas*. sp. G-179 and *Achromobacter cycloclastes* (8). The ligands responsible for Cu are shown in bold and the number underneath indicates the type of Cu it binds.

FIG.5 . Hydropathy index of the encoded nitrite reductase by the method of Goldman *et al* (——) or Kyte and Doolittle (-------) (4). The analyses were performed with the GCG program. The hyrophobic regions are above the horizontal zero line, whereas the regions of relatively hydrophilic nature are below the zero line. The amino acid numbers are the same as in Fig.2.

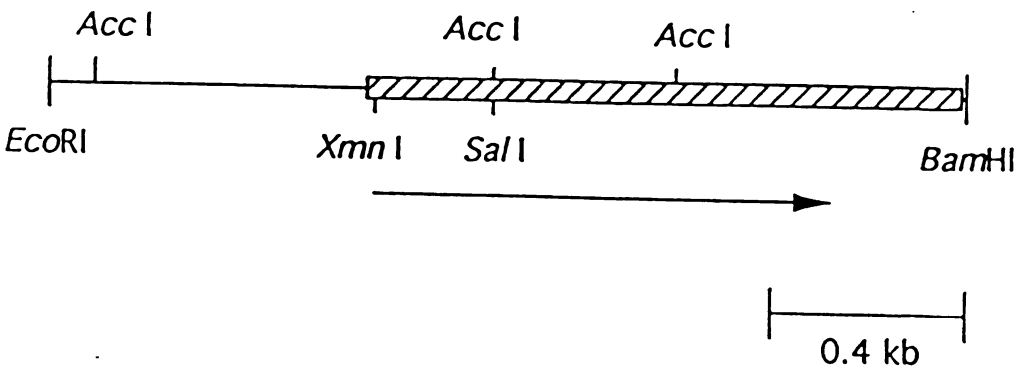
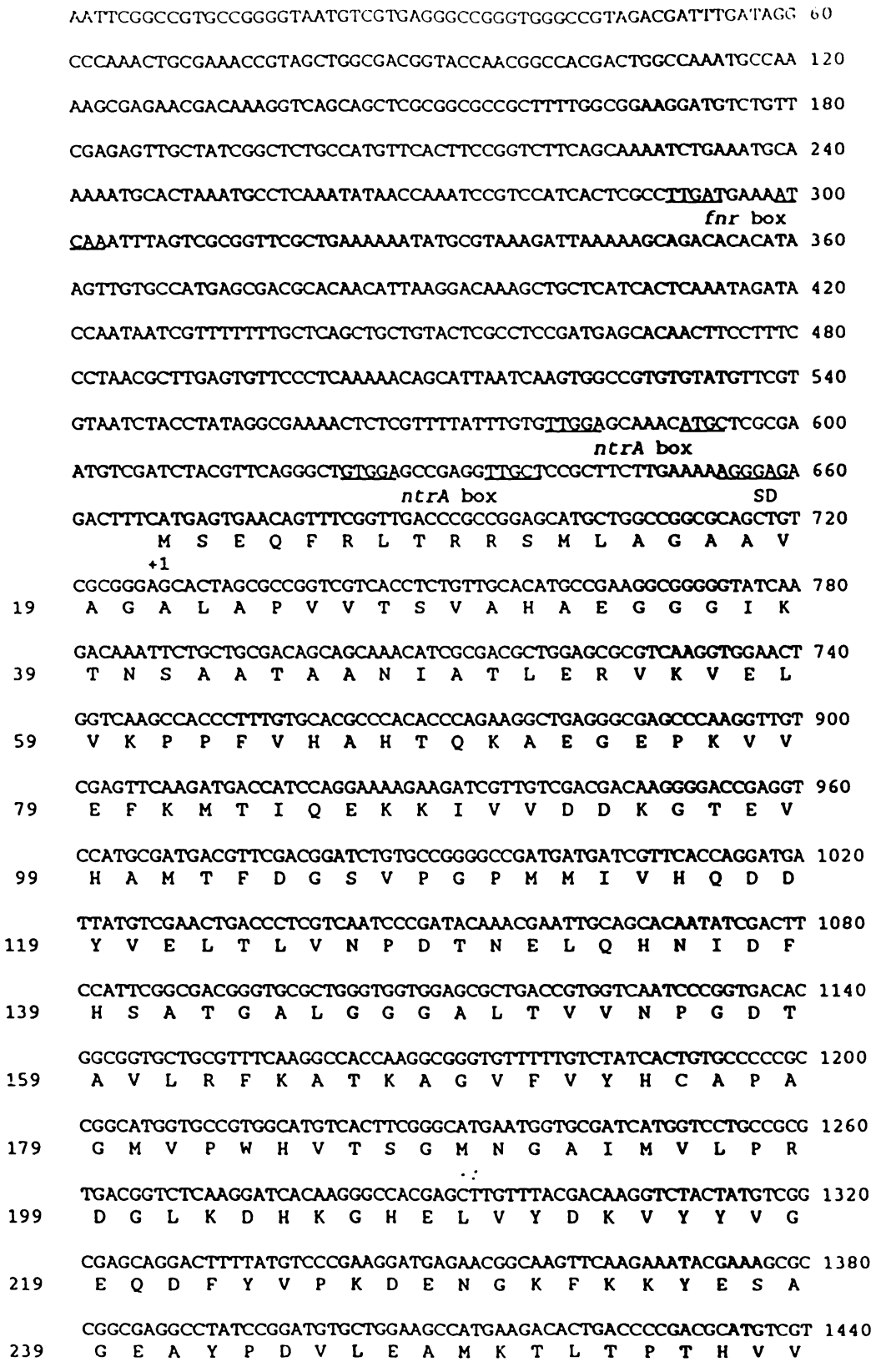


Figure 1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CGAGAGTTGCTATCGGCTCTGCCATGTTCACTTCCGGTCTTCAGCAAAATCTGAAATGCA 240
AAAATGCACTAAATGCCTCAAATATAACCAAATCCGTCCATCACTCGCCTTGATGAAAAT 300
fnr box
CAAATTTAGTCGCGGTTCGCTGAAAAAATATGCGTAAAGATTAAAAAGCAGACACACATA 360
AGTTGTGCCATGAGCGACGCACAACATTAAGGACAAAGCTGCTCATCACTCAAATAGATA 420
CCAATAATCGTTTTTTTGCTCAGCTGCTGTACTCGCCTCCGATGAGCACAACTTCCTTTC 480
CCTAACGCTTGAGTGTTCCCTCAAAAACAGCATTAATCAAGTGGCCGTGTGTATGTTCGT 540
GTAATCTACCTATAGGCGAAAACTCTCGTTTTATTTGTGTTGGAGCAAACATGCTCGCGA 600
ntrA box
ATGTCGATCTACGTTCAGGGCTGTGGAGCCGAGGTTGCTCCGCTTCTTGAAAAAGGGAGA 660
ntrA box
SD
GACTTTCATGAGTGAACAGTTTCGGTTGACCCGCCGGAGCATGCTGGCCGGCGCAGCTGT 720
M S E Q F R L T R R S M L A G A A V
+1
CGCGGGAGCACTAGCGCCGGTCGTCACCTCTGTTGCACATGCCGAAGGCGGGGGTATCAA 780
19 A G A L A P V V T S V A H A E G G G I K
GACAAATTCTGCTGCGACAGCAGCAAACATCGCGACGCTGGAGCGCGTCAAGGTGGAACT 740
39 T N S A A T A A N I A T L E R V K V E L
GGTCAAGCCACCCTTTGTGCACGCCCACACCCAGAAGGCTGAGGGCGAGCCCAAGGTTGT 900
59 V K P P F V H A H T Q K A E G E P K V V
CGAGTTCAAGATGACCATCCAGGAAAAGAAGATCGTTGTCGACGACAAGGGGACCGAGGT 960
79 E F K M T I Q E K K I V V D D K G T E V
CCATGCGATGACGTTCGACGGATCTGTGCCGGGGCCGATGATGATCGTTCACCAGGATGA 1020
99 H A M T F D G S V P G P M M I V H Q D D
TTATGTCGAACTGACCCTCGTCAATCCCGATACAAACGAATTGCAGCACAATATCGACTT 1080
119 Y V E L T L V N P D T N E L Q H N I D F
CCATTCGGCGACGGGTGCGCTGGGTGGTGGAGCGCTGACCGTGGTCAATCCCGGTGACAC 1140
139 H S A T G A L G G G A L T V V N P G D T
GGCGGTGCTGCGTTTCAAGGCCACCAAGGCGGGTGTTTTTGTCTATCACTGTGCCCCCGC 1200
159 A V L R F K A T K A G V F V Y H C A P A
CGGCATGGTGCCGTGGCATGTCACTTCGGGCATGAATGGTGCGATCATGGTCCTGCCGCG 1260
179 G M V P W H V T S G M N G A I M V L P R
TGACGGTCTCAAGGATCACAAGGGCCACGAGCTTGTTTACGACAAGGTCTACTATGTCGG 1320
199 D G L K D H K G H E L V Y D K V Y Y V G
CGAGCAGGACTTTTATGTCCCGAAGGATGAGAACGGCAAGTTCAAGAAATACGAAAGCGC 1380
219 E Q D F Y V P K D E N G K F K K Y E S A
CGGCGAGGCCTATCCGGATGTGCTGGAAGCCATGAAGACACTGACCCCGACGCATGTCGT 1440
239 G E A Y P D V L E A M K T L T P T H V V

Figure 2

```
     GTTCAACGGGGCGGTCGGCGCCCTGACGGGCGATAACGCCTTGCAGGCAAAGGTGGGCGA 1500
259   F  N  G  A  V  G  A  L  T  G  D  N  A  L  Q  A  K  V  G  D

     TCGTGTCCTGATTCTTCATTCGCAAGCCAACAGGGATACGCGCCCGCACCTGATCGGGGG 1560
279   R  V  L  I  L  H  S  Q  A  N  R  D  T  R  P  H  L  I  G  G

     GCATGGCGATTATGTCTGGGCTACCGGCAAGTTCGCCAACCCGCCGGAACTCGATCAGGA 1620
299   H  G  D  Y  V  W  A  T  G  K  F  A  N  P  P  E  L  D  Q  E

     AACCTGGTTCATTCCCGGAGGTGCTGCCGGGGCGGCTTACTACACGTTCCAGCAGCCCGG 1680
319   T  W  F  I  P  G  G  A  A  G  A  A  Y  Y  T  F  Q  Q  P  G

     TATCTATGCGTATGTAAACCACAATCTGATCGAGGCGTTCGAACTCGGCGCGGCCGGCCA 1740
339   I  Y  A  Y  V  N  H  N  L  I  E  A  F  E  L  G  A  A  G  H

     CTTCAAGGTGACGGGCGACTGGAACGACGACCTGATGACAGCCGTGGTTTCGCCGACCTC 1800
359   F  K  V  T  G  D  W  N  D  D  L  M  T  A  V  V  S  P  T  S

     GGGTTGACGGTGTCGGCCCGGCGCCGGATCAGGCGCCGGGCCACATCCTTATGCCGGCCG 1860
379   G  *

     GGGGAGGCCGGAAGGATCGGAGGATC 1886
```

Nir-179 x Nir-AC

Percent Similarity: 88.496 Percent Identity: 78.466

```
                    MSEQFRLTRRSMLAGAAVAGALAPVVTSVAHAEGGGIKT
                    .         .         .         .         .
G179   40 NSAATAANIATLERVKVELVKPPFVHAHTQKAEGEPKVVEFKMTIQEKKI 89
          ..:|.:.:|.||.||||:||||||||||.| |..:|:||||.|||:|||:
AC      1 aagaapvDISTLPRVKVDLVKPPFVHAHDQVAKTGPRVVEFTMTIEEKKL 50
                    .         .         .         .         .
       90 VVDDKGTEVHAMTFDGSVPGPMMIVHQDDYVELTLVNPDTNELQHNIDFH 139
          |:| .|||:|||||:||||||:|:||::||||| |:||||||.| |||||| 
       51 VIDREGTEIHAMTFNGSVPGPLMVVHENDYVELRLINPDTNTLLHNIDFH 100
                    .         .         .         .         .
      140 SATGALGGGALTVVNPGDTAVLRFKATKAGVFVYHCAPAGMVPWHVTSGM 189
          .|||||||||||  ||||:...|||||||:|||||||||.||||||||||| 
      101 AATGALGGGALTQVNPGEETTLRFKATKPGVFVYHCAPEGMVPWHVTSGM 150
                    .         .         .         .         .
      190 NGAIMVLPRDGLKDHKGHELVYDKVYYVGEQDFYVPKDENGKFKKYESAG 239
          |||||||||||||.||:.|.|||:|||||||||||||.|.:||||.:| 
      151 NGAIMVLPRDGLKDEKGQPLTYDKIYYVGEQDFYVPKDEAGNYKKYETPG 200
                    .         .         .         .         .
      240 EAYPDVLEAMKTLTPTHVVFNGAVGALTGDNALQAKVGDRVLILHSQANR 289
          |||.|.:.||:||||||:||||||||||:|| | ||:|||::|||||| 
      201 EAYEDAVKAMRTLTPTHIVFNGAVGALTGDHALTAAVGERVLVVHSQANR 250
                    .         .         .         .         .
      290 DTRPHLIGGHGDYVWATGKFANPPELDQETWFIPGGAAGAAYYTFQQPGI 339
          |||||||||||||||||| |||:||||||:||||.||||:|||.|||: 
      251 DTRPHLIGGHGDYVWATGKFRNPPDLDQETWLIPGGTAGAAFYTFRQPGV 300
                    .         .         .
      340 YAYVNHNLIEAFELGAAGHFKVTGDWNDDLMTAVVSPTS 378
          ||||||||||||||||||||||||:|||||||.||.|.| 
      301 YAYVNHNLIEAFELGAAGHFKVTGEWNDDLMTSVVKPAS 339
```

Figure 3

G-179	D1	132	LQHNIDFHSA	168	AGVFVYHCAPAGMVPWHVTSGMNGA
AC	D1	93	LLHNIDFHAA	129	PGVFVYHCAPEGMVPWHVTSGMNGA
			1 2		2 1 1 1
G-179	D2	337			PGIYAYVNHNLI
AC	D2	298			PGVYAYVNHNLI
					2

Figure 4

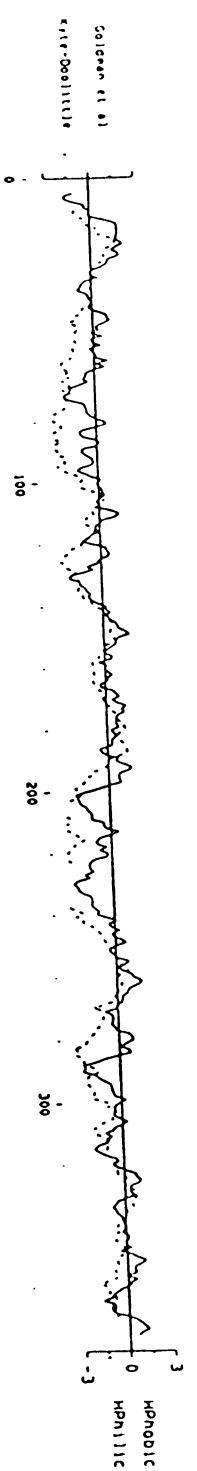

Figure 5

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Contributions by Other Authors

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Dr. Alahari Arunakumari isolated *Pseudomonas fluorescens* AK-15 and initiated the work on isolation of Tn5 mutants. The mutants represented in this thesis were ones that I isolated.

Mr. Marcos R. Fries provided the information on the homology of Cu-type nitrite reductase gene to other organisms. We collaborated on this work.


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