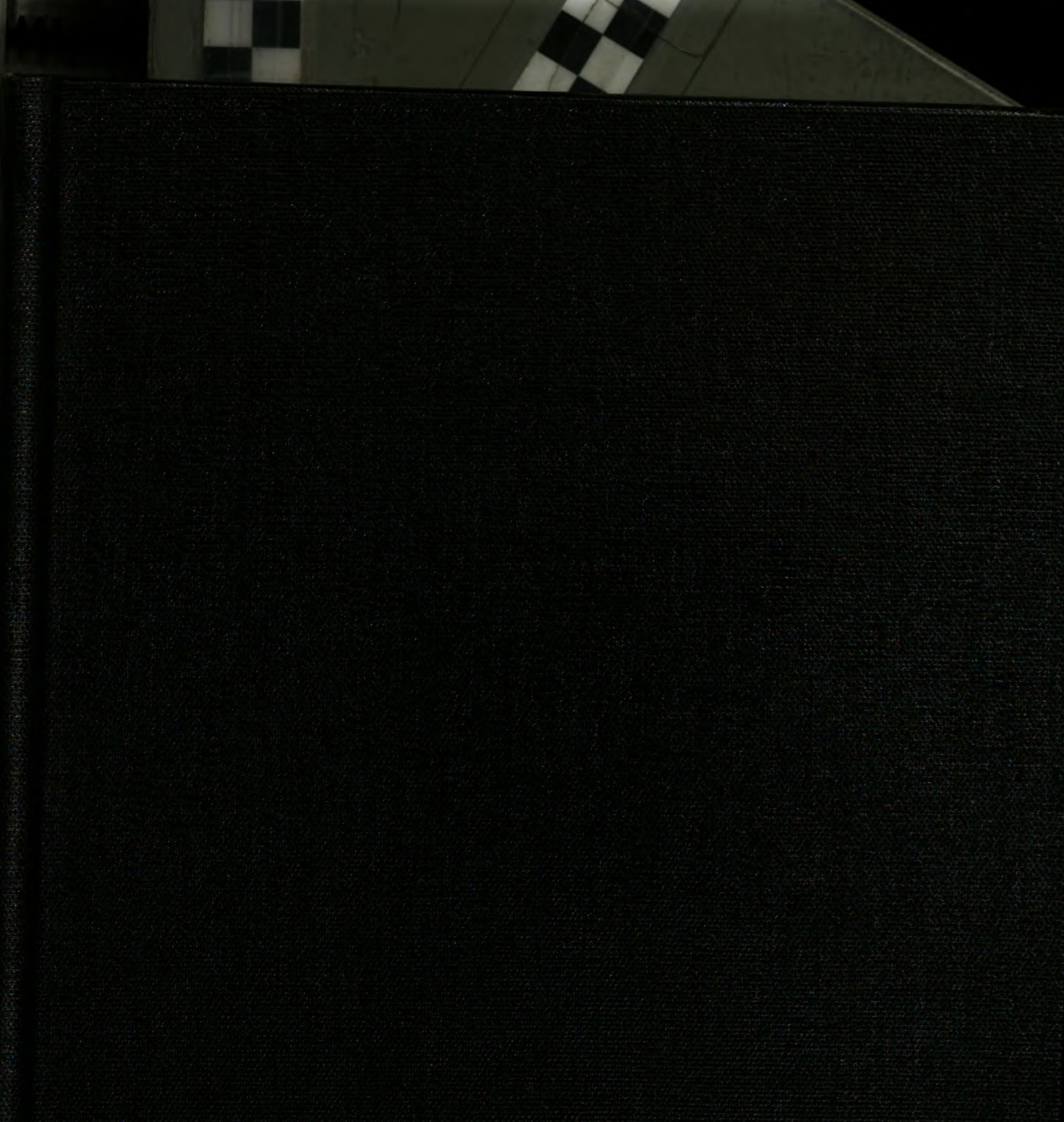

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Mark Steven Coyne

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Ph.D. degree in Crop and Soil Sciences

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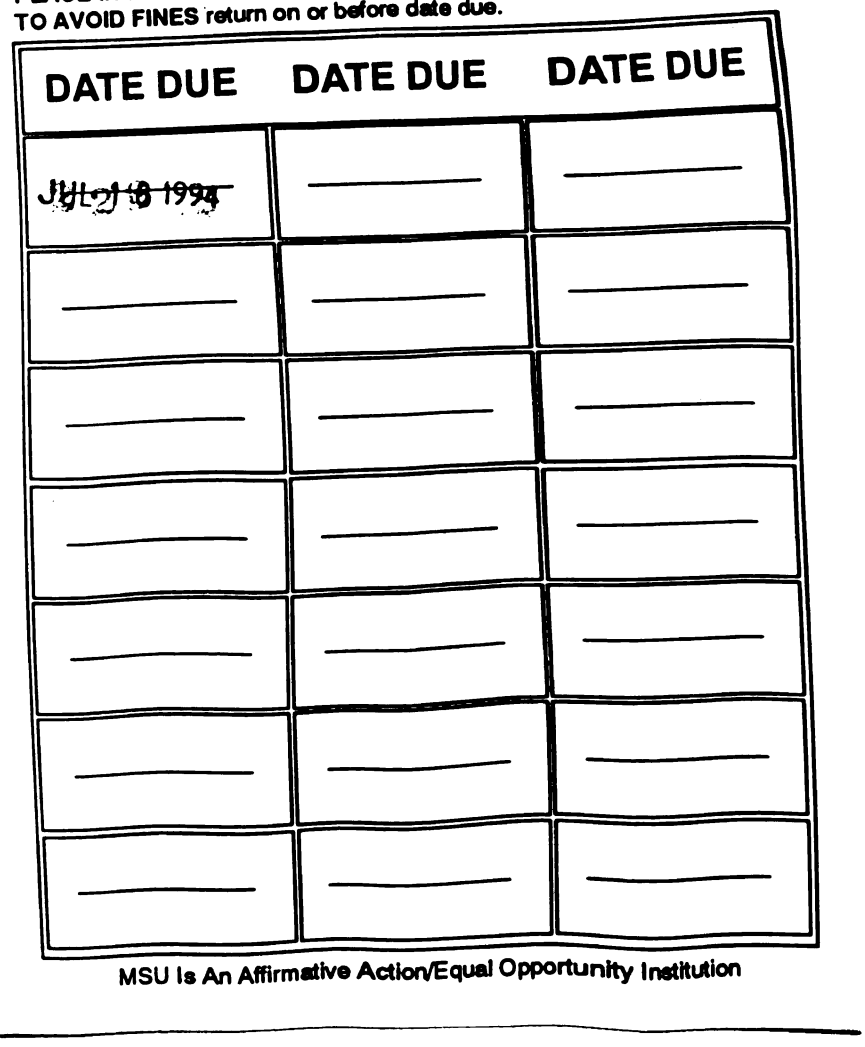

IMMUNOLOGICAL STUDIES ON DISSIMILATORY NITRITE REDUCTASES IN DENITRIFYING BACTERIA

By

Mark Steven Coyne

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ABSTRACT

IMMUNOLOGICAL STUDIES ON DISSIMILATORY
NITRITE REDUCTASES IN DENITRIFYING BACTERIA

By

Mark Steven Coyne

The distribution, divergence, localization, and induction of dissimilatory nitrite reductases were studied in denitrifying bacteria. Polyclonal antibodies were raised against heme $\underline{cd}_1$ nitrite reductase from *Pseudomonas aeruginosa* and from *Pseudomonas stutzeri*, and against Cu nitrite reductase from *Achromobacter cycloclastes*. Nitrite reductases were identified by Western immunoblot. Diethyldithiocarbamate, which specifically inhibits Cu nitrite reductases, was used to confirm the immunological characterization and determine which type was present in those strains which were nonimmunoreactive.

Nitrite reductases were characterized in the following groups: 23 taxonomically diverse denitrifiers from culture collections, 100 numerically dominant denitrifiers from geographically diverse environments, and 51 denitrifiers from a culture collection not selected for denitrification. Heme $\underline{cd}_1$ nitrite reductases were the most common type found in environmental samples, and appeared more conserved if judged by similiarity in molecular weights and reaction with antisera.

The divergence among heme $\underline{cd}_1$ nitrite reductases and Cu nitrite reductases was examined by immunodiffusion and immunoblotting techniques. Two groups of heme $\underline{cd}_1$ type nitrite reductases, exemplified by *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*, were observed. *Achromobacter cycloclastes* Cu nitrite reductase appeared distantly related to the other Cu type nitrite reductases tested.

Nitrite reductases were localized in cd_1- and Cu-type denitrifiers by immunogold labeling. In *Achromobacter cycloclastes* and *Achromobacter xylosoxidans* the Cu nitrite reductase was found in the periplasmic space as was the cd_1 nitrite reductase of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*.

The induction of denitrifying enzyme synthesis and activity was studied in *Achromobacter cycloclastes* continuous cultures gradually deprived of oxygen. Nitrate and nitrous oxide reductases were synthesized at all oxygen concentrations but full induction only occurred at 2 uM oxygen. Nitrite reductase was neither synthesized nor active until dissolved oxygen was below 2 uM.

DEDICATION

This is for Sharon, Pepe, and Dad

ACKOWLEDGEMENTS

I would like to thank my advisor Dr. Jim Tiedje and all the members of my graduate committee for their guidance and patience during the past five years. I would especially like to thank all the members of the MSU community: students, graduate students, post docs, professors, and research technicians, who assisted me with my program. I may have done most of the work, but you told me what it meant.

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INTRODUCTION

Denitrification is the respiratory reduction of nitrogenous oxides to gaseous products and can be functionally defined by these criterion (Tiedje, 1988):

(1) Sensitivity to O_2 inhibition;

(2) Growth yield proportional to the amount of nitrogenous oxide present;

(3) Accumulation of 80% or more of respired NO_3^- and NO_2^- as N_2O or N_2;

(4) Rapid NO_3^- or NO_2^- dissimilation without NH_4^+ accumulation;

(5) Presence of one of two types of dissimilatory NO_2^- reductase - heme $\underline{cd}_1$ or nonheme Cu.

Denitrification is characterized by irreversible NO_2^- reduction to NO and N_2O by the enzyme dissimilatory NO_2^-reductase (dNir). This enzyme is often confused with assimilatory NO_2^- reductase (Nir) which, in contrast to dNir, is regulated by NH_4^+ and organic N concentration, and is unaffected by O_2 (Cole, 1987). Dissimilatory NO_2^-reductase is also confused with a dissimilatory NO_2^-reductase which leads to NH_4^+ formation (Cole, 1987). A fourth NO_2^- reductase, identified in *Propionibacteria* by Kaspar (1982), apparently lacks the physiological roles of dissimilatory NO_2^- reductase, and may simply be a detoxification enzyme. Hereafter, by NO_2^- reductase, I mean

dissimilatory NO_2^- reductase (dNir) not assimilatory NO_2^- reductase or dissimilatory NO_2^- reduction to NH_4^+.

Some of the most interesting questions in microbial ecology concern mechanisms, like NO_2^- reduction, which enable bacteria to exploit novel environmental niches. Global aspects of denitrification are well studied. Less is known about dNirs in terms of distribution, variability, regulation, and genetics. This thesis addresses those questions.

Distribution

Denitrification is an ubiquitous environmental process, and its reviews legion (Firestone, 1982; Focht, 1981; Focht and Verstraete, 1977; Hochstein and Tomlinson, 1988; Kuenen and Robertson, 1987; Knowles, 1982; Payne, 1973; Payne, 1981; Stouthammer, 1976, 1988; Tiedje, 1988; Zumft et al., 1987). It is easier to note the groups in which dNir is absent, than those in which it is present (Tiedje, 1988):

(1) Obligate anaerobes;

(2) Gram-positive organisms other than *Bacillus*;

(3) The Enterobacteriaceae.

Nitrite reductase is found in organisms which cover the gamut of physiological groups: general aerobic organotrophs, oligocarbophiles, fermentors, halophiles, thermophiles, sporeformers, magnetotactic bacteria, dinitrogen-fixers (free and symbiotic), plant and animal pathogens, phototrophs, hydrogen oxdizers, sulfur oxidizers, ammonium oxidizers (Tiedje, 1988).

The strains for which dNir type is known are few, and fewer still the bacteria from which dNirs have been purified. Two basic types have

been found: one contains Cu centers (Cu-dNir), the other hemes c and d_1 (cd_1-dNir).

Heme cd_1-dNirs have been purified from *Alcaligenes faecalis* (Iwasaki and Matsubara, 1971), *Paracoccus* (*Micrococcus*) *denitrificans* (Newton, 1969), *P. aeruginosa* (Gudat et al., 1973; Yamanaka and Okunuki, 1963), *P. stutzeri* (formerly *P. perfectomarina*)(Liu et al., 1983), and *Thiobacillus denitrificans* (Sawhney and Nicholas, 1978). The enzyme has been detected in *Azospirillum brasilense* (Lalande and Knowles, 1987), *P. halodenitrificans* (Grant and Hochstein, 1984), and an *Erythrobacter* strain (Shioi et al., 1988).

Nonheme Cu-dNirs have been purified from *Achromobacter cycloclastes* (Iwasaki et al., 1975; Hulse and Averill, 1988; Kashem et al., 1987), *A. xylosoxidans* (synonymous with *Alcaligenes* sp. NCIB 11015, *A. denitrificans* ss *xylosoxidans*, and *P. denitrificans*)(Iwasaki et al., 1963), *A. faecalis* strain S6 (Kakutani et al., 1981), *Nitrosomonas europaea* (Dispirito et al., 1985; Ritchie and Nicholas, 1974; Miller and Wood, 1983), *P. aureofaciens* (Zumft et al., 1987), *Rhodobacter* (*Rhodopseudomonas*) *sphaeroides* f.sp. *denitrificans* (Michalski and Nicholas, 1985, 1988a). It has further been identified in *P. denitrificans* ATCC 13867 (distinct from *A. xylosoxidans*)(Radcliffe and Nicholas, 1968; Michalski and Nicholas, 1988b) and *Thiosphaera pantotropha* (L.A. Robertson, Ph.D. thesis, University of Delft, 1988).

To reiterate an observation from Gamble et al. (1977), it is unfortunate that most physiological and genetic studies on denitrification (particularly dNir) have been done with only a few strains - *P. aeruginosa* and *P. denitrificans* to name two - which inaccurately reflect true microbial populations. Nitrite reductase has

yet to be purified from *P. fluorescens*, the numerically dominant denitrifier in soils, sediments, and sludges (Gamble et al., 1977).

Heme $\underline{cd}_1$-dNirs are assumed to be the most common type (Shapleigh and Payne, 1985). This may reflect biased isolation procedures, since the numerically dominant denitrifiers in readily cultured environmental samples are typically pseudomonads (Gamble et al., 1977). In one study, *Bacillus* sp. were the dominant denitrifiers from soil samples (Abd-el-Malek et al., 1974). Since environmental samples have usually been from agricultural sites, questions remain about soil effects on dNir types.

Polyclonal antibodies raised against specific dNir types, could facilitate comparisons of dNirs between strains and between genera. Qualitative immunoblotting procedures don't replace protein purification, but do provide rapid information about the relatedness of dNirs and, in conjunction with SDS-Page, useful information about subunit structure. Körner et al. (1987) used polyclonal antibodies to look at the relatedness of N_2O reductases and NO_2^- reductase in *P. perfectomarina* (now *P. stutzeri*) isolates. Nitrite reductases from $\underline{cd}_1$-type denitrifiers were much less related than N_2O reductases.

Michalski and Nicholas (1988b) immunologically compared NO_3^-, NO_2^-, and N_2O reductases in NO_3^- respiring nondenitrifiers, and denitrifiers which were predominantly of the Cu-dNir type. Cu-dNirs, in the organisms they tested, were very similar in terms of molecular mass and antibody affinity. There was no immunological similarity between $\underline{cd}_1$- and Cu-dNirs.

These studies were limited by a narrow focus towards only a few organisms (mostly well-characterized laboratory strains) or one dNir type. Alternately, Shapleigh and Payne (1985) demonstrated that the

chelating agent diethyldithiocarbamate (DDC), specifically inhibited Cu-dNir during *in vitro* dNir activity assays. They have suggested that dNir types could be characterized by differential DDC inhibition of cd_1- and Cu-dNirs.

Characteristics

The main differences between Cu- and cd_1-dNirs are in molecular mass and metal content. Heme cd_1-dNirs have similar molecular weights, typically 120,000. The enzymes are dimers composed of identical subunits. Each subunit contains one heme *c* and one heme d_1. Other molecular weights have been reported, ranging from 90,000 to 140,000 (Hochstein and Tomlinson, 1988).

All cd_1-dNirs have cytochrome oxidase activity. This is presumably not their main physiological role. Their affinity for NO_2^- is greater than for O_2, their K_m for O_2 is high, and other oxidases are better competitors for available electrons (Hochstein and Tomlinson, 1988; Sawhney and Nicholas, 1978). The cd_1-dNirs isolated from *P. stutzeri* JM300 (E. Weeg-Aerssens, Ph.D. thesis, Michigan State University, 1987) has a pI of 5.4. The pI of *P. aeruginosa* cd_1-dNir was reported as 6.9 (Barber et al., 1976) and from *P. denitrificans* as 3.85 (Newton, 1969).

There are two forms of Cu-dNir, distinguished by characteristic EPR signals from their Cu centers. One form has type I and type II copper centers, the other form has only type I copper centers. Cu-dNirs are heterogeneous with respect to other parameters: molecular mass, subunit composition, and pI (Table 1).

Table 1. Selected physical characteristics of Cu-dNirs

Enzyme source	Mass (kDa)	Subunit Structure	Cu types	pI	Reference
A. cycloclastes	69	36.8 x 2	I&II	nd	Iwasaki et al., 1972 Liu et al., 1986
A. xylosoxidans	70	37.0 x 2	I	8.4	Iwasaki et al., 1963 Masuko et al., 1984
A. faecalis S6	110	30.0 x 4	I&II	4.5	Kakutani et al., 1981
N. europaea	120	40.0 x 4	I&II	4.63	Miller & Wood, 1983 Dispirito et al., 1985
P. aureofaciens	85	40.0 x 2	I	6.05	Zumft et al., 1987
R. sphaeroides	80	37.5 x 1 39.5 x 1	I II	nd	Sawada et al., 1970 Michalski & Nicholas, 1985

The only Cu-dNir to date with a basic pI is from *A. xylosoxidans*. Like $\underline{cd_1}$-dNirs, Cu-dNirs have cytochrome oxidase activity, with the exception of Cu-dNir from *P. aureofaciens* (Zumft et al., 1987).

It isn't clear what importance can be associated with having both type I and type II Cu centers. There is no obvious immunological difference (Michalski and Nicholas, 1988b). Type I Cu is presumably the catalytic site. Type II Cu may be an artifact. It tends to disappear with enzyme purification. *Achromobacter cycloclastes* Cu-dNir was recently crystallized by Turley et al. (1988). In contrast to earlier reports from their lab and others, they found only two Cu atoms/protein, and only one type of Cu (Type I) which existed in identical environments. If *A. cycloclastes* Cu-dNir, the most studied type, serves as a model, one would suspect that the enzyme contains identical subunits each with a Type I Cu center (C. Hulse, Ph.D. thesis, University of Virginia, 1989).

Nitrite reductase types are presumably associated with the cytoplasmic membrane in accordance with their role in generating energy through electron transport phosphorylation (Koike and Hattori, 1975a,b). Their exact location, however, is not known. Cu-dNir in *R. sphaeroides* was localized in the periplasm (Sawada and Satoh, 1980). No other Cu-dNirs have been similarly examined.

Heme $\underline{cd_1}$-dNirs may be loosely bound to the cytoplasmic membrane (Sawhney and Nicholas, 1978), they may be soluble (Mancinelli et al., 1986), or both (Zumft et al., 1979). If membrane bound, they have been reported as cytoplasmically (Saraste and Kuronen, 1978) or periplasmically (Wood, 1978) oriented.

Divergence

Cu-dNirs appear to be more diverse than cd_1-dNirs. DNA sequence comparison of the dNir genes would be the ultimate measure of divergence. Alternately, protein mapping could reveal protein sequence differences in purified proteins (Cleveland et al., 1977). However, without suitably cloned genes, or purified proteins, alternate methods are needed to screen divergence among dNir types.

Immunoblot analysis is a quantitative measure of immunological similarity between proteins based on antibody binding (Howe and Hershey, 1981). With saturating concentrations of antibodies, the degree of antibody binding is a quantitative measure of similarity based on common epitopes. Immunoblot distance is a reflection of that similarity. It is the ratio of antibody binding to type strains vs. antibody binding to test strains (Howe and Hershey, 1984). The divergence of dNirs from type strains can then be directly measured in relatively crude protein preparations to determine whether Cu-dNirs are, indeed, more immunologically diverse than cd_1-dNirs; whether there are differences in cd_1-dNir antibody binding based on the particular cd_1-dNir used as an antigen; whether the divergence among cd_1- and Cu-dNirs is at the same rate.

In the same functional class of proteins, sequence evolution goes on at approximately the same rate (Wilson et al., 1977). In a given protein, evolution will depend on the compatibility of sequence change with biochemical function and the likelihood that the organism will survive without that biochemical function. That Cu-dNirs appear to diverge more than heme types may not reflect a longer evolutionary history, only a greater capacity of nonheme Cu-dNirs to accept sequence

changes without losing function compared to $\underline{cd_1}$-dNirs. There are no obligate denitrifiers, and in the presence of O_2, dNir synthesis and activity is inhibited (Tiedje, 1988). Consequently, loss of protein activity due to sequence substitution is not a lethal change.

The presence of Cu-dNirs in Pseudomonads such as *P. aureofaciens* and *P. denitrificans* is interesting since most *Pseudomonas* spp. examined so far contain a $\underline{cd_1}$-dNirs. Bacterial phylogenies based on 5S rRNA (Ohkubo et al., 1986), 16S rRNA (Fox et al., 1980), RNA homology (De Vos and De Ley, 1983) or DNA homology (Palleroni et al., 1973) do not predict whether an organism will have Cu- or $\underline{cd_1}$-dNir. This may indicate insufficient phylogenetic data or perhaps is an indication that mechanisms exist in nature for bacteria to acquire dNir if they have lost this function, and there is a strong selection for denitrification.

There is no clear answer for why two enzymes have evolved. Nor is there a clear answer for the existance of both Cu- and $\underline{cd_1}$-dNir in the same genus or in closely related genera (Ohkubo et al., 1986). One possiblity is horizontal gene transfer via a plasmid. Nitrite reductase may be on a plasmid in one *A. eutrophus* strain (Romermann and Friedrich, 1985). In *Pseudomonas* sp., therefore, strains which have lost dNir activity - and truncated denitrifiers are common (Jeter and Ingraham, 1981) - may have regained it by plasmid acquisition. Albeit, the dNir they recovered was not the same dNir type they originally possessed.

Aerobic Denitrification

Perhaps there is still a positive selection for dNir despite the current O_2 rich environment. Denitrification is second only to respiration in terms of the number of gene copies present (J.M. Tiedje,

S. Simkins, and P. Groffman, Plant and Soil, in press). Not only do dNirs persist in organisms from anaerobic NO_3^- free environments (K.S. Jorgenson and J.M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, N87, p. 299), but they also persist in organisms from long term, air-dried soils (Smith and Parsons, 1985). It has been suggested that copies of mRNA for synthesis of NO_3^- and NO_2^- reductases are 'long lived' in cells (Michalski and Nicholas, 1985). Smith and Parsons (1988) theorized that the enzymes were stabilized in cells during O_2 exposure. Certainly, organisms in transiently anaerobic zones could benefit from denitrification.

There are reports of N_2O evolution from seemingly well-aerated soils (Bremner and Blackmer, 1978; Smith and Tiedje, 1979). This has been partitioned into three processes based on differential acetylene (Berg et al., 1982) and O_2 inhibition (Robertson and Tiedje, 1987):

(1) Denitrification;

(2) Nitrification;

(3) Other.

Denitrification is thought to occur in anaerobic microsites. These develop either from physical restraints on O_2 diffusion (Sexstone et al., 1985), or because localized concentrations of available carbon, cause respiratory depletion of O_2, and concurrent CO_2 evolution (Parkin, 1987; Rice et al., 1988).

Autotrophic and heterotrophic nitrification have been implicated as another aerobic N_2O source. The most commonly cited autotroph, *N. europaea*, may evolve N_2O via true dissimilatory NO_2^- reduction (Poth and Focht, 1985). An alternate mechanism postulates that an intermediate from hydroxylamine oxidation dimerizes to hyponitrous acid, and rapidly

decomposes to form N_2O (Bremner and Blackmer, 1978; Yoshida and Alexander, 1970).

A heterotrophic, nitrifying, *Alcaligenes* sp., which also denitrified, produced equimolar amounts of NH_4^+ and N_2O when switched from denitrifying growth to aerobic growth with hydroxylamine (Castignetti and Hollocher, 1982). Nitrous oxide was assumed to formed via a nitroxyl which dimerized to N_2O. Cu-dNir from *A. cycloclastes* catalyzes N_2O formation from hydroxylamine and NO_2^- (Iwasaki and Matsubara, 1972). Likewise, *P. stutzeri* $\underline{cd}_1$-dNir catalyzes nitrosation reactions between hydroxylamine and NO_2^- (Aerssens et al., 1986). The enzyme bound nitrosyl is presumably the reactive species. This reaction, however, was inhibited by 0.1 mM NO_2^-.

Other aerobic sources of N_2O remain inexplicable. Organisms which dissimilate NO_3^- to NH_4^+, for example, also release some N_2O (Bleakley and Tiedje, 1982; Yoshida and Alexander, 1970). This is apparently the result of NO_3^- reductase catalyzing the reduction of NO_2^- to N_2O (Cole, 1987). Nitrous oxide is also formed by unknown mechanisms of NO_3^- assimilation by fungi (Yoshida and Alexander, 1970).

At least two denitrifying organisms, magnetic *Spirillum* (*Aquaspirillum magnetotacticum* and strain MS1) and *N. europaea*, are obligate aerobes (Bazylinski and Blakemore, 1983; Escalante-Semerena et al., 1980; Poth and Focht, 1985). Periodic reports of aerobic denitrification not only challenge the dictum that denitrification is a strictly anaerobic process (Payne, 1973), but suggest alternate mechanisms for N_2O evolution in aerobic soils. Many studies simply reflect inadequate O_2 supply in solution (Hochstein et al., 1984). Denitrification in the two most frequently cited organisms, an

Alcaligenes sp. from activated sludge (Krul, 1976), and *Hyphomicrobium* X (Meiberg et al., 1980) consisted solely of NO_3^- reduction. The implication, at least with the *Alcaligenes* sp., was that dissimilatory NO_3^- reductase was constitutive. It was still partially repressed by O_2, since NO_3^- reduction was slow until measurable dissolved O_2 was gone.

Thiosphaera pantotropha, a facultatively anaerobic, facultatively autotrophic, sulfur bacterium isolated from a desulphurizing, denitrifying, effluent treatment system, was reported to denitrify at 90% air saturation (Robertson and Kuenen, 1983). Part of the evidence for this phenomenon was increased growth in chemostat culture with both NO_3^- and O_2, and instantaneous N_2 evolution in anaerobic manometric experiments (Robertson and Kuenen, 1984a). It was proposed that electron flow through the electron transport chain was restricted by a bottleneck which could be overcome by allowing electrons to flow to N-oxide reductases as well as oxygen (Robertson and Kuenen, 1984b). The regulation of NO_3^- reduction in *Alcaligenes* sp. was partly attributed to inhibition of O_2 respiration by NO (Krul, 1976). Parsonage et al. (1985) also noted that NO, because of its affinity for hemes, inhibited O_2 respiration, and allowed NO_2^- reduction in the presence of O_2.

Genetics

The genetics of dNirs are still largely unknown, as is the regulation of their synthesis. Dissimilatory NO_2^- reductase mutants (nirA, D, and E) were isolated from *P. aeruginosa* by van Hartingsveldt et al. (1971). They were distinct from assimilatory NO_2^- reductases, and mapped at three separate loci on the chromosome. While attempting to isolate N_2O reductase mutants from *P. stutzeri* by transposon Tn5 mutagenesis, Zumft et al. (1985) also found mutants defective in NO_3^-

and NO_2^- respiration. Three representative nir mutants were selected and studied further (Zumft et al., 1988). One mutant, MK201, overproduced cytochrome $\underline{c}_{552}$ but had an *in vitro* functional $\underline{cd}_1$-dNir. Mutant MK 202 lacked cytochrome $\underline{cd}_1$-dNir entirely, and had low amounts of cytochrome $\underline{c}_{552}$. The last mutant, MK203, synthesized $\underline{cd}_1$-dNir defective in the heme $\underline{d}_1$ prosthetic group. All could reduce NO. No similar research has been done with organisms which contain an identified Cu-dNir.

The only other organism in which denitrification genes have been studied is *A. eutrophus*. Hogrefe et al. (1984), while looking for hydrogenase genes, observed mutants with pleiotropic phenotypes which were unable to grow with hydrogen (Hox^-) and were also unable to either assimilate or dissimilate NO_3^- (Nit^-). These mutations mapped on the chromosome, and were given the overall designation Hno^-. Later, loss of a megaplasmid, pHG, was correlated with loss of denitrifying ability (Romermann and Friedrich, 1985). Plasmid-free mutants converted some NO_3^- to NO_2^-, but could not metabolize NO_2^- anaerobically. Both lithoautotrophy and denitrification could be transferred by plasmid pHG to strains which lacked these functions (Schneider et al., 1988). The chromosomal mutation, with the Hno^- phenotype (hydrogen oxidation and denitrification deficient), was cloned from a genomic library of *A. eutrophus* and used to find the wild-type gene (Romermann et al., 1989). The resulting plasmid, incorporating the wild-type Hno gene, was able to complement Hno^- mutants and relieve glutamine auxotrophy in $NtrA^-$ mutants of enteric bacteria. This suggested that the hno gene product was functionally similar to the NtrA protein which is a novel sigma factor of RNA polymerase. The hno gene product did not complement Fnr^- mutants.

It would be tempting to adopt, as a paradigm for denitrifiers, the genetics and regulation associated with anaerobic NO_3^- metabolism in *E. coli*. With respect to NO_3^- reductase, this may be possible, but until further work with the genetics of other N-oxide reductases is done, this paradigm must be viewed with caution.

The focus of this thesis eventually became an examination of NO_2^- reductase diversity. The first objective was to examine the distribution of dNirs by immunological and chemical means. The second objective was to compare dNirs of the same type, from among different organisms, to begin identifying physical, and hopefully evolutionary differences. The third objective, was to utilize dNir-specific polyclonal antibodies, and localize dNirs in denitrifiers. The last objective was to examine O_2 regulation in the Cu-type denitrifier *A. cycloclastes*, in the context of aerobic denitrification.

SECTION 1

IMMUNOLOGICAL IDENTIFICATION, DISTRIBUTION, AND DIVERGENCE OF DISSIMILATORY HEME cd_1 AND NONHEME CU NITRITE REDUCTASES IN DENITRIFYING BACTERIA

INTRODUCTION

Dissimilatory NO_2^- reductases (dNirs) are pivotal to the fate of combined nitrogen in the environment. They are the point where nitrogen is routed to dissimilation instead of assimilation (Payne, 1981). Two distinct types of dNirs are known: one contains a Cu center (Cu-dNir), the other hemes c and d_1 (cd_1-dNir). Both seem to carry out the same physiological reaction. NO is typically produced from NO_2^- during *in vitro* enzyme assays, but under some conditions, N_2O is also produced (Hochstein and Tomlinson, 1988; Wharton and Weintraub, 1980).

Cytochrome cd_1-dNir has been identified in *Alcaligenes faecalis* (Matsubara and Iwasaki, 1971), *Paracoccus* (formerly *Micrococcus*) *denitrificans* (Newton, 1969), *P. halodenitrificans* (Grant and Hochstein, 1984), *Pseudomonas aeruginosa* (Yamanaka and Okunuki, 1963), *P. stutzeri* (Korner et al., 1987), and *Thiobacillus denitrificans* (LeGall et al., 1979).

Nonheme Cu-dNirs have been isolated from *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972; Hulse et al., 1988), *A. xylosoxidans* (synonymous with *Alcaligenes* sp. NCIB 11015, *A. denitrificans* ss. *xylosoxidans*)(Masuko et al., 1984), *A. faecalis* strain S6 (Kakutani et al., 1981), *Nitrosomonas europaea* (Ritchie and Nicholas, 1974), *P. aureofaciens* (Zumft et al., 1987), *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides* f. sp. *denitrificans* (Michalski and Nicholas, 1985; Sawada et al., 1978), and *Thiosphaera pantotropha* (L.A. Robertson, Ph.D. Thesis, University of Delft, 1988). Two forms of Cu-

dNir which have different EPR spectra have been observed (Michalski and Nicholas, 1988b).

The distribution of the two dNir types in the environment is unknown. Strains which are typically studied in the laboratory, do not reflect the dominant denitrifying populations in nature (Gamble et al., 1977). One way to screen denitrifiers for dNir type is to distinguish between Cu and heme components. Shapleigh and Payne (1985) used diethyldithiocarbamate (DDC), a Cu chelator, to identify Cu-dNirs. Another approach is to characterize the proteins with polyclonal antibodies specific for each dNir type.

Polyclonal antibodies raised against both heme $\underline{cd}_1$- and nonheme Cu-dNirs have facilitated their comparison. Körner and Zumft (1987) found that $\underline{cd}_1$-dNirs from *P. aeruginosa* and *P. fluorescens* were not recognized by *P. perfectomarina* (now *P. stutzeri* strain Zobell) $\underline{cd}_1$-dNir antiserum, which did recognize $\underline{cd}_1$-dNir from a *P. stutzeri* isolate. Michalski and Nicholas (1988a) showed that polyclonal antibodies specific to Cu-dNir from *R. sphaeroides* f. sp. *denitrificans* cross-reacted with Cu-dNirs in two other denitrifying *R. sphaeroides* strains. They also cross-reacted with Cu-dNirs from *A. cycloclastes*, *A. denitrificans* and two *Pseudomonas* sp. (Michalski and Nicholas, 1988b). This confirmed that dNir type differed among closely related *Pseudomonas* spp., since Zumft et al. (1987) had already found Cu-dNir in *P. aureofaciens*.

Bacterial phylogenies based on 16S rRNA sequencing have largely supplanted phylogenies developed from comparing enzyme sequence differences (Woese, 1987). Sequence difference information, based on immunological differences, still reveals diversity and evolutionary

change within classes of proteins as was demonstrated for DNA polymerase I (Tafler et al., 1973), glutamine synthetases (Baumann and Baumann, 1970; Tronick et al., 1973), alkaline phosphatases (Cocks and Wilson, 1972), and hydrogenases (Kovacs et al., 1989).

Howe and Hershey (1981) have described a method of immunoblotting analysis, by which antibodies can be used to quantitate specific proteins in cell lysates. They used this technique to explore evolutionary divergence of initiation factors in species other than *Escherichia coli*. The immunological relationships between the species they studied and *E. coli* were similar to other previously studied proteins (Howe and Hershey, 1984).

Immunoblotting analysis may help examine relationships among denitrifiers based on the dNir type they possess, without resorting to individual protein purifications. Rather, a first approximation of diversity can be derived. Because it can be quantitatively employed, a measure of the divergence among different dNir types can be calculated. This would focus attention on the most interesting dNirs to study further.

The following research was a joint effort. Collaborators at the University of Virginia, Chuck Hulse and Bruce Averill, provided the purified Cu NO_2^- reductase. Purified $\underline{cd}_1$ NO_2^- reductases from *P. aeruginosa* and *P. stutzeri* were provided by Els Weeg-Aerssens and Rick Ye. Dr. Alahari Arunakumari was involved in every step of antisera production and was responsible for much of the immunoscreening for $\underline{cd}_1$-dNirs. My contribution to the research included antisera production, verification of antisera specificity, all immunoscreening for Cu-dNirs, all rescreening of initially nonimmunogenic strains (for both heme $\underline{cd}_1$-

and Cu-dNirs), identification of denitrifying strains from isolates in the aerobic 2,4-D degrading enrichments, all DDC inhibition assays, and the immunoblot analysis.

This section presents the use of polyclonal antibodies in Western immunoblots to identify the distribution and immunological relatedness of $\underline{cd}_1$- and Cu-dNirs in taxonomically diverse denitrifiers, and to determine the predominant dNir type in numerically dominant denitrifiers from the environment. Diethyldithiocarbamate (DDC) was used to confirm the immunological identification and to characterize the dNir type in denitrifiers which did not react with antisera. Immunoblot analyses were used to examine the divergence of Cu- and heme $\underline{cd}_1$-dNirs.

METHODS AND MATERIALS

Bacterial strains

Denitrifying strains from culture collections, and their sources, are listed in Table 1. Numerically dominant denitrifiers of geographically diverse origin were previously collected and characterized by Gamble et al. (1977). For immunoblot analysis, *A. eutrophus* #154, *A. faecalis* #40, and *A. faecalis* #43 were obtained from this collection. Strains isolated by W. Holben from aerobic 2,4-dichlorophenoxyacetic acid (2,4-D) degrading soil enrichments were used to examine cultures not selected on the basis of their denitrifying capacity. Of the 250 isolates, 51 were respiratory denitrifiers based on diagnostic criteria summarized by Tiedje (1988) and were tested for dNir type. These isolates were from soils of two Michigan sites (East Lansing and Kellogg Biological Stations), Kansas (Konza Prairie), and

Saskatchewan, Canada (W. Holben, personal communication). Isolates 1.6.L and 2.4.E were from this collection.

Media and growth conditions

For immunological screening, strains were grown in 16 by 125 mm test tubes sealed with butyl rubber septa. The tubes contained 10 ml medium, an inverted Durham tube, and an initially aerobic headspace. Most strains were grown in tryptic soy broth (TSB)(Difco, Detroit, MI) supplemented with 1.0 g KNO_3 or 0.4 g KNO_2 per liter. *Rhizobium* spp. and *Bradyrhizobium japonicum* were grown in yeast extract mannitol broth (YEMB)(Zablotowicz and Focht, 1981) supplemented with 1.0 g KNO_3 per liter. *Halobacterium* species were grown in HYH-2 medium (per liter: 5.0 g yeast-extract, 2.0 g casein, 176.0 g NaCl, 5 g KNO_3, 20.0 g $MgCl_2 \cdot 6H_2O$, 2.0 g KCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 13.0 g HEPES, pH 6.7, L. Hochstein, personal communication). All cultures were incubated at 24 °C and maintained for routine studies on slants or plates of the same medium amended with 1.5% agar.

Cultures harvested for enzyme assays were grown in 150 ml TSB in 250 ml Erlenmeyer flasks sealed with rubber stoppers. Bacterial growth depleted headspace O_2 sufficiently to induce denitrification, and all cultures were producing N_2O at harvest. Poorly growing strains were first cultured aerobically in 500 ml sidearm flasks containing 300 ml TSB until stationary phase was reached. The flasks were then sealed with rubber stoppers, flushed with sterile argon, and incubated 24 h at 30 °C before harvest to induce denitrifying enzymes. *Rhizobium* spp. and *B. japonicum* were cultured aerobically in 2 l flasks with 1 l YEMB until turbid, and were then shifted to anaerobic conditions as described above.

Cultures used for immunoblot analysis were grown in 15 g l^{-1} Tryptic soy broth (Difco, Detroit, MI) supplemented to 10 mM KNO_3 and 1 uM $CuSO_4 \cdot 7H_2O$. Growth was at 24 °C in sealed 500 ml sidearm flasks filled with 250 ml of media. The headspace was made anaerobic by repeated flushing with sterile argon from a gassing manifold.

Immune sera and immunological techniques

Polyclonal antibodies against dNirs were raised in New Zealand white rabbits. Purified Cu-dNir from *A. cycloclastes* ATCC 21921 was provided by Hulse et al. (1988). Purified cd_1-dNirs from *P. aeruginosa* ATCC 10145 and from *P. stutzeri* JM300 was provided by E. Weeg-Aerssens and R. Ye. The enzymes were emulsified in 1 ml of Freund's incomplete adjuvant and injected subcutaneously. Booster injections of enzyme in Freund's incomplete adjuvant were made at 8 weeks and 16 weeks. Blood samples were taken at 2 weeks and on a weekly basis thereafter. Immunization was followed by Ouchterlony double immunodiffusion (Jurd, 1981).

Cultures which met one of the following criteria for denitrification were harvested for immunoscreening:

(1) Bubble formation in the inverted Durham tube;

(2) Accumulation of N_2O;

(3) Removal of NO_3^- and NO_2^- from the media as detected by the diphenylamine test (Tiedje, 1982).

An aliquot of the culture was centrifuged, the pellet resuspended in sample buffer (0.0625M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue), and the cells then lysed in a boiling water bath (Laemmli, 1970). Strains were extracted by phenol (Carlson and Vidaver, 1982) if they lysed poorly under this protocol, or

if proteins were not immunologically recognized by antiserum to either dNir type. Proteins from the crude lysates were separated by SDS-PAGE on 10% gels and transferred by electrophoresis onto nitrocellulose (Towbin et al., 1979).

All dilutions and incubations for dNir detection were in TBST buffer: 10 mM Tris-HCL (pH 8.0), 150 mM NaCl, 0.05% Tween 20 (Promega Western Blot AP System). The nitrocellulose filters were incubated 1 h with 1% BSA to block nonspecific binding, then incubated 1 h with a 1:1000 dilution of antiserum. The filters were rinsed in TBST three times for a total of 15 min then incubated with a 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit goat IgG (Sigma). The filters were rinsed in TBST as before, and the dNirs were visualized by development of purple color following incubation with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) substrate (Sigma).

The criteria for a positive immunological response were:

(1) Recognition (color development) of proteins at approximately the same molecular weight as simultaneously recognized purified dNirs, or proteins from denitrifiers of a known dNir type;

(2) No recognition when subsequently screened with antiserum from the opposite dNir type.

Preparation of crude extracts

For gas chromatography assays and native gel analysis, denitrifying cultures were harvested by centrifugation at 4 °C and 10,000 rpm for 10 min. Cells were washed with 50 mM HEPES buffer (pH 7.3), centrifuged as before, and resuspended in 5 ml of the same buffer.

Cultures not immediately used were stored at -70 °C. Resuspended or thawed cultures were passed three times through a French press at 15,000 psi, divided into aliquots and stored at -70 °C. Prior to use, the crude extracts were spun 5 min at 4 °C in a microfuge to remove debris; this supernatant was used in enzyme assays. The protein content in crude extracts was measured by the Lowry method using BSA as a standard (Lowry et al., 1951).

For immunoblot analysis, bacteria were grown until all NO_3^- was removed from the media as judged by the colorimetric diphenylamine assay (Tiedje, 1982). The cells were pelleted at 4 °C for 10 min at 10,000 rpm, washed in 50 mM HEPES buffer pH 7.3, pelleted as before, and resuspended in 5 ml of the HEPES buffer. The resuspended cells were disrupted by sonication, cell debris pelleted by centrifugation at 10,000 rpm for 30 min at 4 °C and the supernatant (crude extract) used in all further assays. Protein concentration was determined by Bicinchoninic acid protein assay reagent (BCA - Pierce) using BSA as a standard.

In vitro activity stain

Denitrifying cells were ruptured by French press, the cells removed by centrifugation, and the proteins separated by nondenaturing 10% PAGE at 4 °C and 100 V for 20 h. Sites with NO_2^- reducing activity were located by the method of Zumft et al. (1987). Gels were stained 5 min with 10 mM methyl viologen in 50 mM HEPES buffer (pH 7.3) with 2 mg ml^{-1} sodium dithionite. The gels were then immersed in 1 M $NaNO_2$ (in HEPES buffer) which caused rapid bleaching where dNirs were located. DDC (10 mM) was incorporated in some of the activity stains to preferentially inhibit Cu-dNir activity (Shapleigh and Payne, 1985).

Inhibition of nitrite reductase activity by antisera

Purified Cu-dNir and extracts prepared by French press of denitrifying *A. cycloclastes* ATCC 21921 and *P. aeruginosa* ATCC 10145 were incubated 24 h at 4 °C with an equal volume of either buffer, preimmune serum, Cu-dNir rabbit antiserum, or cd_1-dNir rabbit antiserum. Precipitated proteins were removed by centrifugation and the soluble proteins were separated by nondenaturing 10% PAGE at 4 °C and 100 V for 20 h. The gels were stained for dNir activity and dNirs were identified by Western immunoblot.

Gas chromatography

Enzymatic NO_2^- reduction was measured as NO and N_2O evolution from crude extracts using NADH/phenazine methosulfate (PMS) as the electron donating system. Assays contained 1 mM NO_2^-, 0.12 mM PMS, 2 mM NADH, and crude extract (generally 200 ul) in a total volume of 1 ml. All stock solutions were made in HEPES buffer (50 mM, pH 7.3) and the NADH stock was prepared daily (E. Weeg-Aerssens, Ph.D. thesis, Michigan State University, 1987). Assays were started by adding NO_2^- to 8 ml serum bottles containing the other combined ingredients. The bottles were previously made anaerobic by repeated flushing of the headspace with argon. When DDC was used as an inhibitor, it was added to a concentration of 12.5 mM 20 min prior to the NO_2^- addition and the bottle was incubated on ice (Shapleigh and Payne, 1985). All enzyme assays were at 24 °C.

Gas formation was monitored on a Perkin-Elmer 910 gas chromatograph equipped with dual columns and ^{63}Ni electron capture detectors for simultaneous analysis of two samples (Kaspar and Tiedje, 1980). Carrier gas was 95% argon, 5% methane. Temperatures were:

detector 300 °C, injector 70 °C, and column 30 °C. The columns were 2 m x 2 mm i.d. stainless steel filled with Porapak Q. Carrier gas flow was adjusted to maximize NO and O_2 separation. Nitrite reductase activity was measured in the presence and absence of DDC by monitoring the initial rate of NO and N_2O evolution over 15 min. Each assay was done in duplicate. No NO or N_2O was evolved in the absence of crude extract or in controls with autoclaved crude extract.

Immunodiffusion analysis

Ouchterlony immunodiffusion was done according to Howe and Hershey (1984). Agar gel plates containing 500 mM KCl, 1% agar, and 0.01% sodium azide in 20 mM phosphate buffer pH 6.8 were prepared. Fifty microliters of undiluted antiserum or crude extracts were added where indicated in Figure 6. The plates were allowed to develop at 30 °C for 48 h before examination.

Immunoblot analysis

Nitrite reductases in crude extracts were separated from other cell proteins by 10% SDS-PAGE. The crude extracts were prepared for electrophoresis as previously noted. The crude extract added to each well was adjusted so that the amount of dNir activity per sample was equivalent (Table 5). Samples were separated on a Hoefer SE 250 minigel for 90 min at 25 mAmp constant current then transferred by electrophoresis to nitrocellulose filters (Towbin et al., 1979).

Filters were incubated overnight at 4 °C in TBST buffer (10 mM Tris pH 8.0; 150 mM NaCl; 0.05% Tween 20) containing 5% w/v nonfat dry milk. The filters were then incubated 30 min with a 1:1000 dilution of specific antiserum to Cu-dNir, or to cd_1-dNir from either *P. aeruginosa* or *P. stutzeri*. The filters were washed 3 x 5 min in TBST buffer and

incubated 1 h in TBST buffer containing a 1:7500 dilution of goat anti rabbit IgG conjugated to alkaline phosphatase (Promega).

Nitrite reductases were detected by color development on the filters following incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) substrate (Sigma). The relative levels of antibody binding to the various dNirs were quantified by whole band analysis using a Visage 110 Image Analyzer. In cases where a doublet of immunologically recognized proteins was present, both bands were quantified, and the sum of those values used in further analysis.

RESULTS

Specificity of antisera

Silver stains of the dNirs used for immunization demonstrated their purity (Figure 1). Immunodiffusion assays indicated the antisera were specific for the antigens against which they were raised (not shown). Neither type of $\underline{cd}_1$-dNir antisera crossreacted with crude extracts containing Cu-dNir, nor did they crossreact with crude extracts containing nonhomologous $\underline{cd}_1$-dNir.

In activity-stained, non-denaturing PAGE, reductase activity in crude extracts of *A. cycloclastes* (Cu-dNir) and *P. aeruginosa* ($\underline{cd}_1$-dNir) (Figure 2A) comigrated with immunologically recognized proteins in immunoblots of the same gels (Figure 2B). Other proteins with reductase activity were not immunologically recognized. *Achromobacter cycloclastes* Cu-dNir migrated as two distinct immunologically recognized bands only one of which had reductase activity. Possibly, the more rapidly migrating band represents a degradation product.

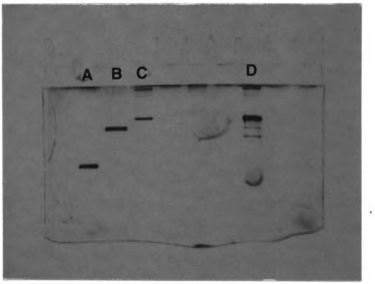

Figure 1. Silver stain of proteins used in rabbit immunizations. Lane A, *A. cycloclastes* Cu-dNir; B, *P. stutzeri* $\underline{cd_1}$-dNir; C, *A. cycloclastes* N_2O reductase; D, *P. stutzeri* N_2O reductase.

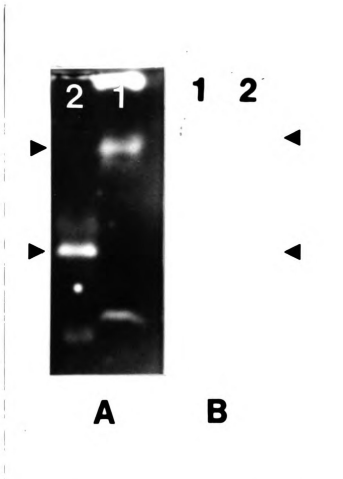

Figure 2. Comigration of dNir activity with immunologically recognized proteins in denitrifier crude extracts. A. Nitrite reductase activity stain; B. Western immunoblot with Cu- and $\underline{cd_1}$-dNir antisera. Lane 1, *A. cycloclastes* ATCC 21921; 2, *P. aeruginosa* ATCC 10145. Arrows indicate the location of comigrating proteins.

Bleached regions with reductase activity cut from a duplicate gel of *A. cycloclastes* and *P. aeruginosa* crude extracts (indicated by the arrows in Figure 2A) had enzymatic NO_2^- reducing activity and produced 222 and 261 umol N-gas (as a mixture of NO and N_2O), respectively, after 90 min incubation in sealed vials. A control from a nonbleached portion of the gel had no enzymatic activity. If DDC, a specific inhibitor of Cu-dNir, was incorporated into activity stains Cu-dNir, but not $\underline{cd_1}$-dNir activity, was inhibited (not shown).

Finally, Cu- and $\underline{cd_1}$-dNirs were not precipitated by preimmune serum, but activity stains of nondenaturing gels showed specific loss of Cu-dNir activity following incubation with Cu-dNir antiserum (Figure 3). Specific loss of $\underline{cd_1}$-dNir activity after incubation with $\underline{cd_1}$-dNir antiserum was observed in similar gels (not shown).

Immunoscreen of denitrifying strains

Twenty-three strains from culture collections representing 17 taxonomically diverse denitrifying bacteria were screened by Western immunoblot for immmunologic recognition of Cu- and $\underline{cd_1}$-dNirs (Table 1). The criteria for a positive result were immunological recognition (as described in Methods) after incubation with rabbit antiserum, and nonrecognition following incubation with antiserum from the opposite dNir type (Figure 4A-B). For these strains, antiserum to *P. stutzeri* $\underline{cd_1}$-dNir gave similar results to antiserum against *P. aeruginosa* $\underline{cd_1}$-dNir.

The dNir type was identified for several strains in which dNir had not previously been characterized. *Aquaspirillum itersonii*, *Flavobacterium* sp., and *P. fluorescens* contained $\underline{cd_1}$-dNir. *Bacillus azotoformans*, and *C. nephridii* contained Cu-dNir. Of the 23 strains

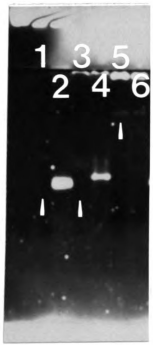

Figure 3. Removal of dNir activity by Cu-dNir antisera. Samples in lanes 1-6 were preincubated with Cu-dNir antisera (1,3,5) or buffer (2,4,6). Lanes 1,2, *A. cycloclastes* Cu-dNir (1 ug); 3,4, *A. cycloclastes* ATCC 21921 crude extract; 5,6, *P. aeruginosa* ATCC 10145 crude extract. Arrows indicate where Cu-dNir but not $\underline{cd}_1$-dNir has been lost after preincubation with Cu-dNir antisera.

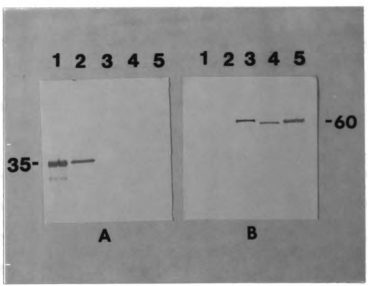

Figure 4. Western immunoblots of denitrifier crude extracts separated on 10% SDS-polyacrylamide gels. A. Immunoblotted with *A. cycloclastes* ATCC 21921 Cu-dNir antisera; B. Immunoblotted with *P. aeruginosa* ATCC 10145 $\underline{cd_1}$-dNir antisera. Lane 1, *A. cycloclastes* ATCC 21921 purified Cu-dNir (1 ug); 2, *A. cycloclastes* ATCC 21921 crude extract; 3, *P. aeruginosa* ATCC 10145 crude extract; 4. *P. stutzeri* JM300 crude extract; 5, *P. stutzeri* JM300 purified $\underline{cd_1}$-dNir (1 ug). Molecular mass indicators are in kDa.

(tested by immunological recognition only), 10 contained $\underline{cd_1}$-dNir and 6 contained Cu-dNir. The dNirs of *B. japonicum*, *Halobacterium* spp., *Rhizobium* spp., and *R. sphaeroides* were not recognized by the antisera (Table 1) even though the *Halobacterium spp.* (L. Hochstein, personal communication) and *R. sphaeroides* (Michalski and Nicholas, 1985) are known to contain Cu-dNirs.

Distribution of nitrite reductase types in naturally occurring denitrifiers

One hundred denitrifying isolates from geographically diverse environmental samples were also screened in Western immunoblots to determine the predominant dNir type in nature. Data are presented in Table 2.

Ninety-one isolates gave a positive immunological response to at least one of the antisera used in the analysis. Most strains (63/100) contained $\underline{cd_1}$-dNir. The $\underline{cd_1}$-type was present among *P. fluorescens* and among the denitrifiers most closely related to this species. This group comprises the numerically dominant denitrifiers in soil (Gamble et al., 1977). In cases where isolates were screened with both types of $\underline{cd_1}$-type dNir antisera, immunological recognition did not always coincide.

Table 1. Western immunoblot response of denitrifiers to polyclonal antibodies against Cu- and cd_1-dNirs.

Strain	Source	NO_2^- reductase	
		cd_1	Cu
A. cycloclastes	ATCC 21921	-	+
A. xylosoxidans NCIB 11015	H. Iwasaki	-	+
A. itersonii	ATCC 11331	+	nd
A. lipoferum	ATCC 29707	+	-
B. azotoformans	ATCC 29788	nd	+
B. japonicum	ATCC 10324	-	-
C. nephridii	ATCC 11425	nd	+
Flavobacterium sp.	ATCC 33514	+	nd
H. denitrificans S1	L. Hochstein	-	-
Halobacterium sp. D4	L. Hochstein	-	-
Halobacterium sp. Baja 12	L. Hochstein	-	-
P. denitrificans	ATCC 19367	+	-
P. aeruginosa	ATCC 10145	+	-
P. aureofaciens	ATCC 13985	-	+
P. denitrificans	ATCC 13867	nd	+
P. fluorescens	ATCC 17822	+	-
P. fluorescens	ATCC 33512	+	-
P. stutzeri	ATCC 11607	+	-
P. stutzeri JM300	J. Ingraham	+	-
P. stutzeri	ATCC 14405	+	nd
Rhizobium sp. 8A55	D. Focht	-	-
Rhizobium sp. 32H1	D. Focht	-	-
R. sphaeroides f.sp. *denitrificans* I1106	T. Satoh	-	-

\+ Positive immunologic response
\- Negative immunologic response
nd Not determined

Tab

Iso
Sc

13
42
63
205
62
70
72
47
64
44
53
45
73
79
84
68
66
62
105
49
67
79
8
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5
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7
7
11
8
18
18
1

Table 2. Immunochemical classification of nitrite reductase type in denitrifying isolates from sediments, sludges, and the soils of six countries.

Isolate No.[a]	Similarity matrix Group[a]	Species or Group[a]	Immunoblot reaction[b] Cu	cd_1 Pa.	cd_1 Ps.	DDC Inhib[c]
13	1A	*P. fluorescens* II	-	+	nd	-
42	1A	*P. fluorescens* II	-	+	-	nd
63	1A	*P. fluorescens* II	-	+	nd	nd
205	1A	*P. fluorescens* II	-	+	-	nd
62	1A	*P. aureofaciens*	-	+	nd	nd
70	1A	*P. fluorescens* II	-	+	+	nd
72	1A	*P. fluorescens* II	-	+	+	nd
47	1A	*P. fluorescens* II	-	+	+	nd
64	1A	*P. fluorescens* II	-	+	+	nd
44	1A	*P. fluorescens* II	-	+	+	nd
53	1A	*P. fluorescens* II	-	+	nd	nd
45	1A	*P. fluorescens* II	-	+	+	nd
73	1A	*P. fluorescens* II	-	+	-	nd
79	1A	*P. fluorescens* II	-	+	+	nd
84	1A	P. type 6	-	+	+	nd
68	1A	*P. fluorescens* II	-	+	+	nd
66	1A	*P. fluorescens* II	-	+	+	nd
61	1A	*P. fluorescens* II	-	+	+	nd
105	1A	*P. fluorescens* II	-	+	nd	nd
49	1A	*P. fluorescens* II	-	+	+	nd
67	1A	*P. fluorescens* II	-	+	+	nd
75	1A	*P. fluorescens* II	-	+	+	nd
89	1A	*P. fluorescens* II	-	+	nd	nd
82	1A	P. type 5	-	+	+	nd
52	1A	*P. fluorescens* II	-	+	+	nd
69	1A	P. type 5	-	+	+	nd
71	1A	P. type 5	-	+	+	nd
74	1A	P. type 5	-	+	+	nd
111	1A	*P. fluorescens*	-	+	+	-
85	1B	P. type 7	-	+	+	-
183	1B	*P. fluorescens* IV	-	+	-	nd
185	1B-C	*P. fluorescens* IV	-	nd	+	-
15	1B-C	*P. fluorescens*	-	+	nd	nd

Table 2 (cont'd)

Isolate No.[a]	Similarity matrix Group[a]	Species or Group[a]	Immunoblot reaction[b] Cu	Cd1 Pa.	Cd1 Ps.	DDC Inhib[c]
12	1C	P. type 2	-	+	-	-
54	1C	P. type 2	-	+	+	nd
83	1C	P. type 2	-	-	-	-
81	1C-D	P. type 2	-	+	nd	nd
110	1C-D	P. type 12	-	+	+	nd
55	1C-D	P. type 4	-	+	nd	-
78	1C-D	P. type 6	-	+	+	nd
58	1C-D	*P. fluorescens* II	-	+	+	nd
80	1D	*P. fluorescens* II	-	+	nd	-
190	1	*P. fluorescens*	-	+	+	nd
206	2	*P. fluorescens* IV	-	+	-	nd
107	2	P. type 11	+	-	-	nd
163	2	P. type 11	+	-	-	+
188	2	P. type 11	+	-	nd	+
143	2	P. type 11	+	nd	-	+
234	2	P. type 11	+	-	-	+
149	2-3	P. type 11	+	-	-	+
98	2	*P. fluorescens* II	+	-	-	nd
133	3	P. type 18	+	nd	-	nd
135	3	P. type 18	+	nd	-	nd
118[1]	3	P. type 16	+	-	nd	nd
141	3	P. type 16	+	-	-	nd
172	3-4	P. type 11	+	-	-	+
14	3	P. type 1	-	+	nd	nd
156	4	*P. aeruginosa*	-	+	nd	nd
87	4-5	P. type 8	-	-	-	+
103	5A	P. type 10	-	-	-	nd
18	5A	*A. faecalis*	-	+	nd	nd
20	5A	*A. faecalis*	-	+	nd	nd
4	5A	*A. faecalis*	-	+	nd	nd
30	5A	*A. faecalis*	-	+	nd	nd
43	5A-B	*A. faecalis*	+	-	-	+
99	5B	*A. faecalis*	-	+	-	nd
90	5B	*A. faecalis*	-	+	nd	nd

Table 2 (cont'd)

Isolate No.[a]	Similarity matrix Group[a]	Species or Group[a]	Immunoblot reaction[b] Cu	cd1 Pa.	cd1 Ps.	DDC Inhib[c]
104	5C	*A. faecalis*	-	+	-	nd
144	5C	*A. faecalis*	+	-	-	+
191	5C	*A. faecalis*	+	-	nd	nd
102	5	*A. faecalis*	-	+	nd	nd
40	5	*A. faecalis*	+	-	-	+
28	5	*A. faecalis*	+	-	nd	nd
31	5	*A. faecalis*	-	+	nd	nd
41	5	*A. faecalis*	+	-	-	nd
65	5-6	*A. faecalis*	+	-	+	+
36	6	Unknown type 3	-	-	+	nd
151	6	P. type 19	+	-	-	+
153	6	P. type 19	+	-	-	+
179	6-7	P. type 19	+	-	-	+
148	7	*A. faecalis*	+	-	nd	nd
232	7	P. type 25	+	-	-	nd
39	7-8	Unknown type 3	-	-	-	+
221	9	*P. stutzeri*	-	+	nd	nd
224	9-10	*P. stutzeri*	-	+	-	nd
231[2]	9-10	*P. stutzeri*	-	+	+	-
195	9-10	*P. stutzeri*	-	+	+	nd
108	9-10	Unknown type 3	-	-	-	nd
154	9-10	*A. eutrophus*	+	-	-	+
106	9-10	*A. faecalis*	-	+	-	nd
97	10	P. type 9	-	-	-	nd
177	10	*Flavobacterium*	-	+	+	nd
46	10	*Flavobacterium*	-	+	nd	nd
192	>10	*Bacillus*	-	-	-	+
193	>10	*Bacillus*	-	-	-	+
199	>10	Unknown type 22	+	-	nd	nd
189	>10	Unknown type 21	+	-	-	+
204	>10	Unknown type 24	-	+	nd	nd
115	>10	Unknown type 15	-	-	nd	nd
171	>10	*A. faecalis*	+	-	-	+

Table 2 (

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Table 2 (cont'd)

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- Negative immunologic response
nd Not Determined

[a] Species identification and similarity matrix grouping are from Gamble, et al. (1977). Approximately one-third of the isolates collected were not identified to the species level or beyond. These isolates, based on the taxonomic properties tested, were given "type" numbers in the order in which they were isolated.

[b] Cu - antibodies raised against *A. cycloclastes* ATCC 21921 Cu-dNir
Pa.- antibodies raised against *P. aeruginosa* ATCC 10145 $\underline{cd}_1$-dNir
Ps.- antibodies raised against *P. stutzeri* JM300 $\underline{cd}_1$-dNir

[c] DDC Inhib: +/- indicates whether inhibition of dNir activity occurred after preincubation with 12.5 mM DDC for 20 min.

There were 28 denitrifiers with Cu-dNir among the immunologically reactive isolates. These strains were predominantly *A. faecalis* and *Pseudomonas* strains which Gamble et al. (1977) could not easily characterize to species level (Table 2). One isolate (#65, an *Alcaligenes* sp.) had a positive immunological response to antisera against Cu-dNir and *P. stutzeri* $\underline{cd_1}$-dNir, but not *P. aeruginosa* $\underline{cd_1}$-dNir. Only Cu-dNir was detected by DDC analysis (Table 2, Table 3). The response to this particular $\underline{cd_1}$-dNir antiserum was probably nonspecific. DDC inhibited dNir activity in all denitrifiers determined by immunoblotting to have Cu-dNir (Table 2). Of the nine isolates which gave no immunological response to any of the antisera, DDC analysis indicated that four contained Cu-dNir (#39, #87, #192, #193) and one contained $\underline{cd_1}$-dNir (#83). The data for some of these strains are included in Table 4. The remaining four strains (#97, #103, #108, #115) could not be checked by DDC analysis because of poor growth under denitrifying conditions. In no case were $\underline{cd_1}$- and Cu-dNir both present in the same isolate.

Matrix group was not an adequate basis on which to predict the dNir type type of an isolate. Seemingly closely related strains within the same matrix group had different types, particularly *A. faecalis* isolates.

The subunit molecular weight of $\underline{cd_1}$-dNirs varied less than those of Cu-dNirs. At least four distinct Cu-dNirs were recognized after SDS-PAGE, with molecular weight ranging from 35,000 to 91,000 (Figure 5).

Fifty-one denitrifying isolates from aerobic 2,4-D degrading soil enrichments were also used for immunological screening. Forty-seven of these isolates contained $\underline{cd_1}$-dNir. There was no apparent correlation

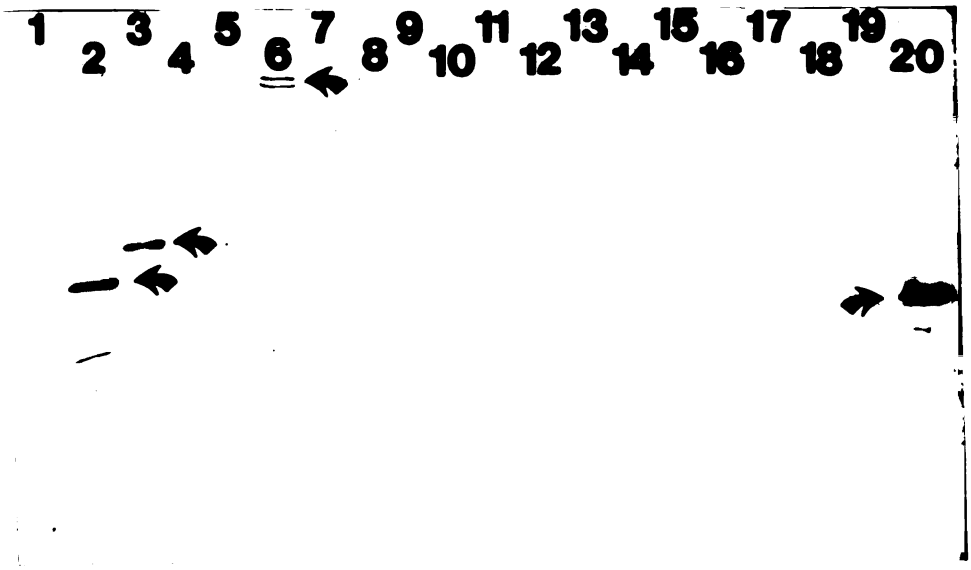

Figure 5. Western immunoblot of denitrifier crude extracts separated on 10% SDS-polyacrylamide gels and immunoblotted with Cu-dNir antisera. Lane 1, *P. aeruginosa* ATCC 10145;
2, #41 *A. faecalis*; 3, #40 *A. faecalis*; 4, #15 *P. fluorescens*;
5, #39 Unknown type 3; 6, #171 *A. faecalis*;
7, #31 *A. faecalis*; 8, #3 *A. faecalis*; 9, #43 *A. faecalis*;
10, #36 Unknown type 3; 11, #144 *A. faecalis*;
12, #224 *P. stutzeri*; 13, #192 *Bacillus* sp.;
14, #106 *A. faecalis*; 15, #177 *A. faecalis*;
16, #191 *A. faecalis*; 17, #224 *P. stutzeri*;
18, #167 *P. aeruginosa*; 19, #62 *P. aureofaciens*;
20, *A. cycloclastes* ATCC 21921. Arrows show the location of Cu-dNirs. Strain numbers (#) are from Gamble et al. (1977).

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Chemical inhibition assays with DDC

The immunological results were confirmed by DDC inhibition assays. Nitrite reductase activity (measured as NO and N_2O evolution) in denitrifiers with cd_1-dNirs was only inhibited 0 to 14% by preincubation with DDC but was almost completely inhibited in denitrifiers with Cu-dNirs (Table 3). The exception was P. aeruginosa (cd_1-dNir) in which NO evolution was inhibited. This may be an artifact of the low rate of NO evolution. Nitrite reduction to N_2O was completely inhibited by DDC. There was complete agreement between the results of DDC inhibition and immunological characterizations.

DDC inhibition was also used to differentiate between the two known dNir types in strains which gave no immunological response to the antisera. With the exception of a pseudomonad type (#83), DDC almost completely inhibited dNir activity in crude extracts of these nonimmunoreactive strains (Table 4). Strains containing Cu-dNir included a *Bacillus* sp., *B. japonicum*, and two *Rhizobium* species. NO but not N_2O was evolved by *B. japonicum* and *Rhizobium* strain 32H1. Since N_2O reductase activity is readily lost after cell disruption, the production of NO but not N_2O indicates that Fe catalyzed conversion of NO to N_2O was not a factor in these assays. The complete inhibition of NO production by DDC indicates that the members tested in these two genera have Cu-dNir.

Table 3.

Strain/	
cd_1-typ	
P. typ	
P. aer	
ATCC	
P. flu	
ATCC	
P. flu	
(#111	
P. stu	
P. stu	
(#231	
Cu-typ	
A. cy	
ATCC	
A. xy	
NCIB	
A. fa	
A. fa	
A. fa	
A. fa	
B. az	
ATCC	
C. ne	
ATCC	
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P. ty	
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Table 3. Effect of DDC on nitric and nitrous oxide evolution by denitrifier crude extracts for which nitrite reductase type was classified by immunoblots.

	Activity (nmol min^{-1} mg $protein^{-1}$)					
	NO			N_2O		
	DDC		%	DDC		%
Strain/type	-	+	Inhib	-	+	Inhib
cd_1-type						
P. type 2 (#12)	0	0.2	0	1.9	2.4	0
P. aeruginosa ATCC 10145	0.4	0	100	2.2	1.9	14
P. fluorescens ATCC 33512	0.7	1.5	0	0	0	-
P. fluorescens (#111)	0	0	-	2.0	2.3	0
P. stutzeri JM300	11.4	9.6	16	2.0	1.9	5
P. stutzeri (#231[2])	2.2	2.4	0	3.2	3.1	3
Cu-type						
A. cycloclastes ATCC 21921	2.9	0	100	3.3	0	100
A. xylosoxidans NCIB 11015	9.6	1.8	81	5.0	0.4	92
A. faecalis (#40)	1.5	0	100	2.6	0	100
A. faecalis (#43)	0.3	0	100	4.2	0	100
A. faecalis (#65)	0	0	-	2.2	0.1	95
A. faecalis (#171)	1.3	0.1	92	0.1	0	92
B. azotoformans ATCC 29786	3.9	0.9	77	1.4	0.2	86
C. nephridii ATCC 11425	3.2	0	100	2.2	0.1	95
P. type 19 (#151)	1.1	0	100	4.7	0	100
P. type 19 (#153)	0.5	0	100	1.5	0.1	93
Unknown type 21 (#189)	0.5	0	100	0.7	0	100

Table 4

Strain

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Table 4. Effect of DDC on nitric and nitrous oxide evolution by denitrifier crude extracts unreactive to antisera against Cu- or $\underline{cd}_1$-dNirs.

	Activity (nmol min^{-1} mg $protein^{-1}$)					
	NO			N_2O		
	DDC		%	DDC		%
Strain	-	+	Inhib	-	+	Inhib
Bacillus sp. (#192)	0	0	-	0.6	0	100
B. japonicum ATCC 10324	0.8	0	100	0	0	-
Rhizobium sp. 8A55	1.5	0	100	0.2	0	100
Rhizobium sp. 32H1	2.9	0	100	0	0	-
R. sphaeroides f. sp. *denitrificans*	3.6	0.2	94	0.2	0	100
Unknown type 3 (#39)	0.3	0	100	0.2	0	100
P. type 2 (#83)	0	0	-	0.2	0.5	0
P. type 8 (#87)	0.7	0	100	0	0	-

Immunodiffusion analysis

Immunodiffusion analysis of the crude extracts from *A. cycloclastes*, *A. xylosoxidans*, *A. faecalis*, *A. eutrophus*, and *P. aureofaciens* was done with antiserum against *A. cycloclastes* Cu-dNir. Immunodiffusion analysis of the crude extracts from *P. aeruginosa*, *P. fluorescens*, *P. stutzeri* and two denitrifying soil isolates 1.6.L. and 2.4.E, was done with antiserum against *P. aeruginosa* $\underline{cd}_1$-dNir.

The immunodiffusion analysis for *P. aeruginosa* $\underline{cd}_1$-dNir is shown in Figure 6. Precipitin lines which formed in the analysis with Cu-dNir antiserum were too faint to interpret, presumably because of dissimilarities in the Cu-dNirs. When *P. aeruginosa* $\underline{cd}_1$-dNir antiserum was used, precipitin lines formed with *P. aeruginosa*, *P. fluorescens*, and isolate 1.6.L. Spur formation suggested partial identity of *P.*

Fig

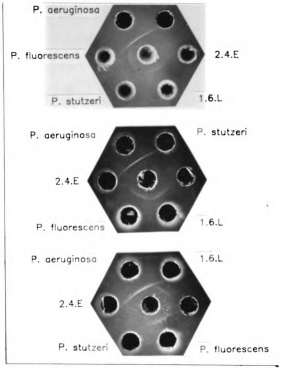

Figure 6. Immunodiffusion analysis with *P. aeruginosa* $\underline{cd_1}$-dNir antiserum. Center wells contain undiluted *P. aeruginosa* $\underline{cd_1}$-dNir antiserum. Outer wells contain crude extract from each of the $\underline{cd_1}$-type strains as indicated in the figure.

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fluorescens and 1.6.L with *P. aeruginosa* cd_1-dNir. The precipitin lines of *P. fluorescens* and 1.6.L. fused, suggesting identity of the cd_1-dNir antigens. Neither *P. stutzeri*, nor isolate 2.4.E, formed precipitin lines. The pattern of antigenic similarity based on response to *P. aeruginosa* cd_1-dNir was *P. aeruginosa* > *P. fluorescens* = 1.6.L > 2.4.E = *P. stutzeri*.

Immunoblot analysis

Immunoblot analyses were done on bacterial crude extracts with the antiserum used in the immunodiffusion assays and with *P. stutzeri* cd_1-dNir antiserum. Equivalent amounts of dNir protein were analyzed based on dNir activity in crude extracts of each strain (Table 5). Cu-dNir antiserum had less affinity for Cu-dNirs other than *A. cycloclastes* Cu-dNir (Figure 7). As had been previously noted (Figure 5), the molecular mass of Cu-dNir subunits from the different bacteria varied. The subunits ranged from 36 kDa for *A. cycloclastes* to 42 kDa for *A. faecalis* #40.

All cd_1-dNirs in crude extracts were recognized by antiserum raised against *P. aeruginosa* cd_1-dNir (Figure 8). The recognition by antiserum for cd_1-dNir other than *P. aeruginosa* cd_1-dNir varied. Subunit molecular masses were similar, but a characteristic doublet of immunologically recognized proteins in crude extracts from *P. fluorescens* and isolate 1.6.L. were observed (Figure 8). When antiserum raised against *P. stutzeri* cd_1-dNir was used, the recognition pattern was different (Figure 9). *Pseudomonas stutzeri* cd_1-dNir was recognized while the other cd_1-dNirs were not.

Table 5. In vitro activity of nitrite reductases in denitrifying bacteria used for immunoblot analysis.

Strain	Cu types present[a]	In vitro activity (nKat mg^{-1} protein)[b]
cd_1 type		
P. aeruginosa	na	1272
P. fluorescens	na	1500
P. stutzeri	na	3897
Isolate 1.6.L.	na	2105
Isolate 2.4.E	na	2825
Cu-type		
A. cycloclastes	I & II	2036
A. xylosoxidans	I	2720
A. faecalis #43	nd	947
A. faecalis #43	nd	2158
A. eutrophus #154	nd	2450
P. aureofaciens	I	2814

[a] na = not applicable
nd = not determined

[b] Enzyme activity is expressed in units of Katals (Kat), the conversion of one mol of substrate per sec. One nKat = 10^{-9} Kat.

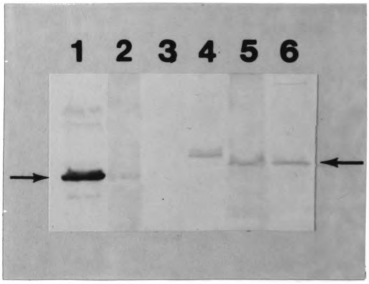

Figure 7. Western immunoblot of denitrifier crude extracts containing Cu-dNir incubated with *A. cycloclastes* Cu-dNir antiserum. Lane 1, *A. cycloclastes* ATCC 21921; 2, *A. xylosoxidans* NCIB 11015; 3, *P. aureofaciens* ATCC 13985; 4, *A. faecalis* #40; 5, *A. faecalis* #43; 6, *A. eutrophus*, #154. Arrows point to the location of Cu-dNirs.

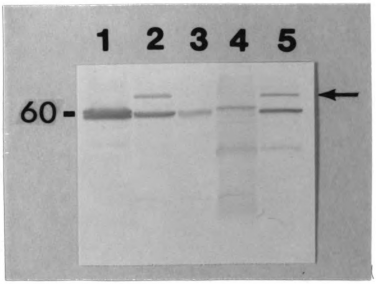

Figure 8. Western immunoblot of denitrifier crude extracts containing $\underline{cd_1}$-dNirs incubated with *P. aeruginosa* $\underline{cd_1}$-dNir antiserum. Lane 1, *P. aeruginosa* ATCC 10145; 2, *P. fluorescens* ATCC 33512; 3, *P. stutzeri* JM300; 4, Isolate 2.4.E; 5, Isolate 1.6.L. The arrow points to a characteristic protein recognized in *P. fluorescens* and Isolate 1.6.L. Molecular mass indicator is in kDa.

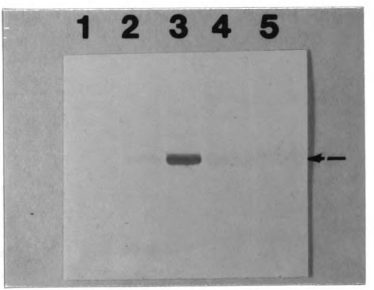

Figure 9. Western immunoblot of denitrifier crude extracts containing $\underline{cd_1}$-dNirs incubated with *P. stutzeri* $\underline{cd_1}$-dNir antiserum. Lane 1, *P. aeruginosa* ATCC 10145; 2, *P. fluorescens* ATCC 33512; 3, *P. stutzeri* JM300; 4, Isolate 2.4.E; 5, Isolate 1.6.L. The arrow points to the location of *P. stutzeri* $\underline{cd_1}$-dNir.

Immunoblot distance

The immunoblotting technique is quantitative, since the degree of antibody binding to immunologically recognized proteins is related to the intensity of alkaline phosphatase activity at those proteins. So divergence among dNir types was measured by densitometric scanning of the immunologically recognized bands corresponding to dNirs (Figures 7,8, and 9). When the intensity of antibody binding to antigens against which the anitisera were raised were set to 100%, the values for other immunologically recognized proteins in the crude extracts (with the exception of *P. fluorescens*) were less (Table 6, Table 7) because they have fewer cross reacting determinants.

Table 6. Measurement of antibody binding to Cu-dNirs of denitrifying cell lysates.

Strain	Area Value (%)[a]
A. cycloclastes	100
A. faecalis #43	15
A. faecalis #40	11
A. xylosoxidans	10
A. eutrophus #154	2
P. aureofaciens	0

[a] Antiserum were raised against *A. cycloclastes* Cu-dNir. The degree of antibody binding at positions corresponding to Cu-dNirs was measured as described in Methods and Materials. The area value for each strain is shown as a percentage of the *A. cycloclastes* value which was placed at 100%.

Table 7. Measurement of antibody binding to $\underline{cd_1}$-dNirs of denitrifying cell lysates.

Strain	Area Value (%)[a]
P. aeruginosa	100
P. fluorescens	138
Isolate 1.6.L	91
Isolate 2.4.E	48
P. stutzeri	48

[a] Antiserum were raised against *P. aeruginosa* $\underline{cd_1}$-dNir. The degree of antibody at positions corresponding to $\underline{cd_1}$-dNirs was measured as described in Methods and Materials. The area value for each strain is shown as a percentage of the *P. aeruginosa* value which was placed at 100%.

The evolutionary distance from the type strains, *A. cycloclastes*, *P. aeruginosa*, and *P. stutzeri*, respectively, can be designated as the immunoblot distance (Hershey and Howe, 1984):

$$\text{Immunoblot distance} = 100 \times \log \frac{\text{(Intensity of Type Strain)}}{\text{(Intensity of Species)}}$$

The immunoblot distance of crude extracts with Cu-dNir is shown in Figure 10. *Achromobacter cycloclastes* Cu-dNir shares some common epitopes with the other Cu-dNirs examined, but there are clear differences in the amount of antibody binding. Cu-dNirs in three of the strains shared similar numbers of common epitopes to *A. cycloclastes* Cu-dNir. A fourth strain, *A. eutrophus*, shared even fewer common epitopes.

Two patterns appeared when looking at immunoblot distances of crude extracts tested with *P. aeruginosa* $\underline{cd_1}$-dNir antiserum (Figure 11). *Pseudomonas aeruginosa* and *P. fluorescens* $\underline{cd_1}$-dNirs shared many common epitopes, as did isolate 1.6.L $\underline{cd_1}$-dNir. A second group contained

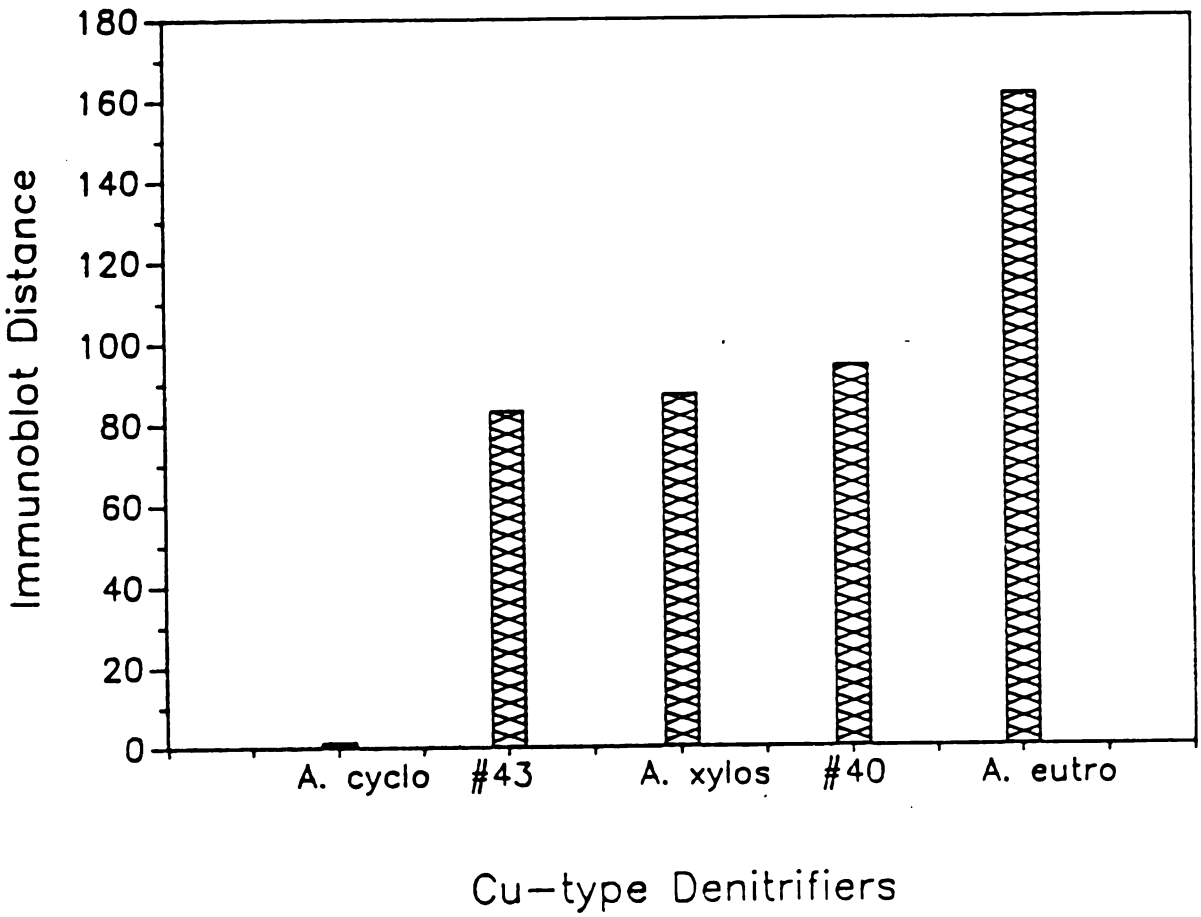

Figure 10. Phylogeny of bacteria which crossreact with antiserum to *A. cycloclastes* Cu-dNir.

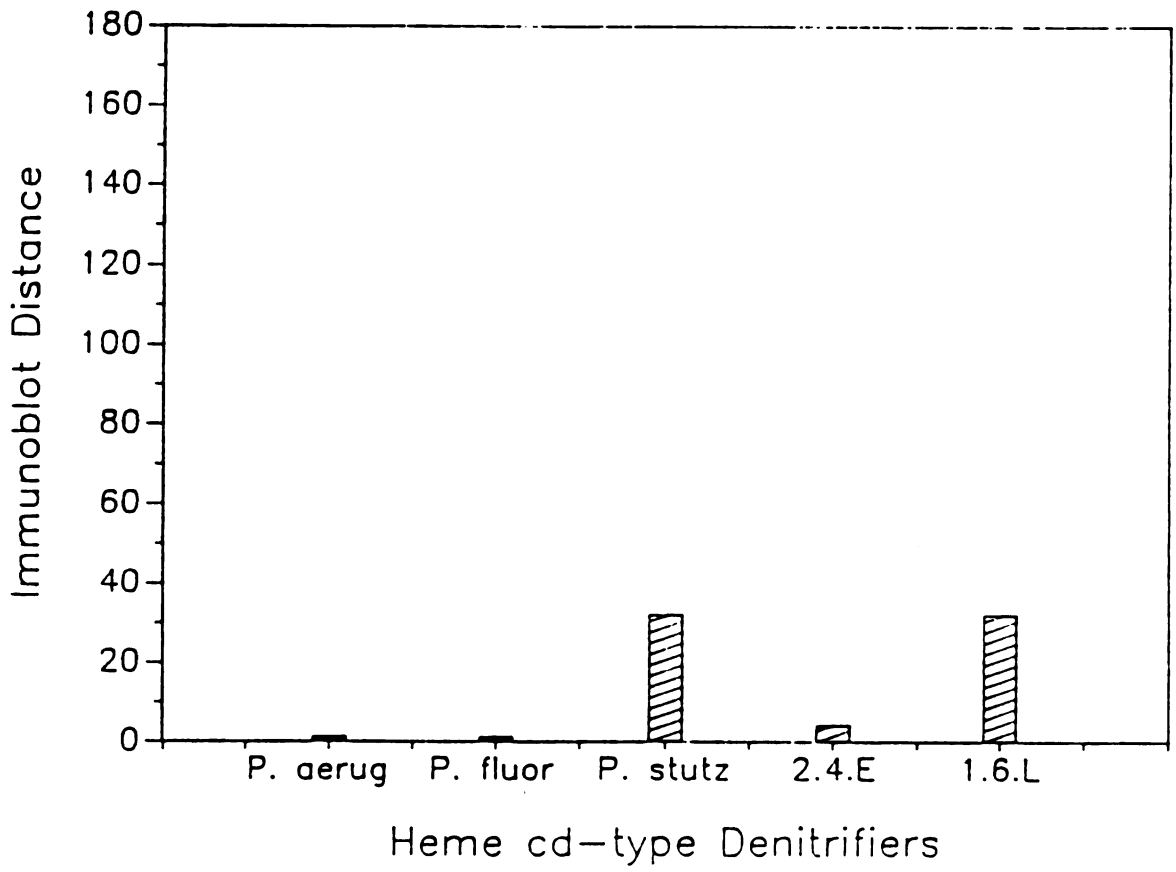

Figure 11. **Phylogeny of bacteria which crossreact with antiserum to *P. aeruginosa* cd_1-dNir.**

P. stutzeri and isolate 2.4.E cd_1-dNirs, which shared fewer common epitopes with *P. aeruginosa* cd_1-dNir. The analysis could not be performed with *P. stutzeri* antiserum because recognition of cd_1-dNirs other than *P. stutzeri* cd_1-dNir by this antiserum was weak.

DISCUSSION

We identified the dNir type in over 90% of more than 150 strains for which dNirs were uncharacterized, by using immunoreaction to at least two antisera, and in many cases, also by DDC inhibition assays. Nitrite reductases which were not recognized by antisera were characterized with DDC inhibition assays. The heme cd_1-dNir predominated in the environmental isolates. Data for the three culture groups studied: culture collections, numerically dominant denitrifiers, and aerobic soil isolates are summarized in Table 8.

Table 8. Distribution of Cu- and heme cd_1-dNirs.

Culture group	Number examined	NO_2^- reductase type		
		heme cd_1[a]	Cu[a]	Unknown[b]
Culture collections	23	44%	56%	0%
Numerically dominant isolates	100	64%	32%	4%
Aerobic soil enrichments[c]	51	92%	8%	0%

[a] NO_2^- reductase type based on immunological reactivity and/or response to DDC.
[b] Tested by immunological response only.
[c] Isolates not originally selected for denitrification ability.

There are two forms of Cu-dNir which have different EPR spectra depending on the type of Cu they contain, either Type I Cu alone or both Type I and II Cu. Immunochemical recognition of Cu-dNirs was independent of whether the enzymes had Type I or Type II Cu, since enzymes containing both types have been characterized in strains recognized by our antiserum (Michalski and Nicholas, 1988b; Ohkubo et al, 1986; Zumft et al., 1987). *Achromobacter xylosoxidans* and *P. aureofaciens* Cu-dNirs contain only Type I Cu. The former was recognized by *A. cycloclastes* (Type I and II) Cu-dNir antiserum (Figure 7) while the latter was poorly recognized not under the conditions employed in this study (Figure 7). This implies that the type of Cu in Cu-dNirs is largely irrelevant to immunological characterization as has been previously noted (Michalski and Nicholas, 1988b). This would not be surprising if the Cu-containing sites are sequestered in dNirs and have no antigenic role.

Antiserum against Cu-dNir from *R. sphaeroides* prepared by Michalski and Nicholas (1988b) recognized Cu-dNirs in the eight denitrifiers they tested, including *A. cycloclastes*. Although *A. cycloclastes* Cu-dNir shares common epitopes with other Cu-dNirs, the number of common epitopes between *R. sphaeroides* Cu-dNir and other denitrifers may be greater than for *A. cycloclastes*. This may explain why some Cu-dNirs were not recognized by our antiserum. Another possibility is that these unrecognized strains contain a non Cu, non $\underline{cd}_1$-type dNir heretofore uncharacterized.

The strains we used represented frequently studied laboratory cultures as well as the predominant denitrifiers from soils of four continents, sediments, and sludges (Gamble et al., 1977). *Pseudomonas*

spp. are the most prevalent denitrifiers in these environments (Tiedje, 1988) and 73% of the denitrifying *Pseudomonas* or *Pseudomonas* type isolates (Tables 1 and 2) contained $\underline{cd}_1$-dNirs. This suggests that $\underline{cd}_1$-dNirs predominate in nature. They were also the most prevalent dNir among isolates from our study regardless of the geographic origin or environment from which the isolates were collected.

This could be an inevitable affect of the isolation methods used to recover the denitrifiers. *Bacillus* spp., for example, were the predominant population in one study of denitrifying soil isolates from undefined sites (Abd-el-Malek et al., 1974). However, even when selection was not originally made for denitrification, eg. the isolates from 2,4-D degrading soil enrichments, the denitrifiers among these isolates predominantly contained $\underline{cd}_1$-dNirs.

Cu-dNirs, nevertheless, may be significant in denitrifying ecosystems. Cu-dNir has been found in more physiological groups and ecological niches than the $\underline{cd}_1$-dNir. These groups include chemolithotrophic nitrifiers (Ritchie and Nicholas, 1974), heterotrophic nitrifiers (Kuenen and Robertson, 1987), extreme halophiles (L. Hochstein, personal communication), and phototrophic bacteria (Sawada et al., 1978) as well as pseudomonads (Michalski and Nicholas, 1988b; Zumft et al., 1987). In addition to these groups we observed Cu-dNir in hydrogen oxidizers (*A. eutrophus*), symbiotic N_2-fixers (*B. japonicum*), and gram positive spore formers (*B. azotoformans*). As more genera are examined, $\underline{cd}_1$-dNirs appear in other taxonomic groups besides *Pseudomonas*. In addition to *P. fluorescens*, we observed $\underline{cd}_1$-dNir in *Aquaspirillum*, *Azospirillum*, and *Flavobacterium*.

Cu-dNirs were more heterogeneous than $\underline{cd_1}$-dNirs. Subunit molecular weights of Cu-dNirs were similar to some, but not all, of those which have been reported: 39,000 (Liu et al., 1986); 37,000 and 39,500 (Michalski and Nicholas, 1985); 40,000 (Zumft et al., 1987). Most were larger (around 40,000) than the molecular weight of the *A. cycloclastes* standard (35,000)(Hulse et al., 1988). The molecular weight of an immunoreactive protein in *Alcaligenes* isolate, #171, was much greater than other Cu-dNirs (Figure 5). Cu-dNir from *Alcaligenes* strain S6 is a tetramer of molecular weight 120,000 which required extensive denaturing at 50 °C before the various oligomers were separated (Kakutani et al., 1981). Isolate #171 may contain a similar enzyme. The variability of Cu-dNirs among denitrifiers may reflect significant differences in Cu-types (Masuko et al., 1984), or simple differences in processing of the enzyme. It may also explain why we were unable to recognize all Cu-dNirs with our antiserum.

We showed the utility of using DDC inhibition assays to identify dNir type in a variety of different physiological groups as had been proposed by Shapleigh and Payne (1985). In all cases, DDC inhibited dNir activity in denitrifiers with Cu- but not $\underline{cd_1}$-dNirs. This verified the results of the immunoscreening. For the nonimmunoreactive isolates, the assays demonstrated, with one exception, that all contained Cu-dNir. The dNir type in virtually all strains used in this study could therefore be identified using a combination of these two methods. With the exception of *A. itersonii*, all of the genera which were assigned a dNir type were examined with at least two independent tests (immunological or chemical) to establish that designation.

DDC inhibition assays and immunoscreening are complementary methods of characterizing dNir types in denitrifying populations. The DDC inhibition assay unambiguously distinguishes Cu- from $\underline{cd}_1$-dNir. The method is rapid and the analysis simple to perform, but it does not reveal immunological relatedness and heterogeneity among dNirs as antibodies do for the dNirs they recognize. For simple identification of dNir type in a denitrifier, the DDC inhibition assay is preferred because it is independent of dNir recognition. If many isolates are screened, raising antibodies against specific dNirs becomes a reasonable alternative because multiple isolates can be examined simultaneously.

The intensity of immunological recognition varies in immunoscreened denitrifier crude extracts. This is undoubtedly due to differences in the amount of dNir present, and the similarity of dNirs in the crude extracts to dNirs against which antiserum were raised. Nonspecific binding may occur, but this can be minimized by adjusting the incubation time and antiserum dilution.

Nitrite reductase type may differ among closely related strains - notably *Pseudomonas* spp. (Table 1)(Körner et al., 1987; Michalski and Nicholas, 1988b; Zumft et al., 1987). Among *A. faecalis* isolates, 47% contained Cu-dNir and 53% contained $\underline{cd}_1$-dNir (Table 2). While dNir types do not appear to be absolutely conserved within a species, they do appear to be incompatible within the same host. No isolate had more than one type present.

One can only speculate about evolutionary mechanisms behind the distribution of $\underline{cd}_1$- and Cu-dNirs among strains. Bacterial phylogenies show that dNir types are widely distributed among many different genera (Woese, 1987) and many different physiological groups (Tiedje, 1988).

Denitrification may have risen in an ancestral photodenitrifier, and like photosynthesis in numerous branches of the purple bacteria, been lost in some strains over time (Betlach, 1982). This would lead to closely related denitrifying (i.e. dNir-containing) and nondenitrifying species. Examples of truncated denitrifiers are numerous (Tiedje, 1988).

Differences of dNir type among *Pseudomonas* and *Alcaligenes* spp. might be explained by horizontal gene transfer. Nitrite reductase may be plasmid borne in *A. eutrophus* (Romermann and Friedrich, 1985). Gene transfer provides a plausible route by which dNirs could have spread among pseudomonads and other groups. Since Cu- and $\underline{cd}_1$-dNirs were never observed in the same strain, this could involve dNir acquisition by truncated denitrifiers or replacement of a preexisting dNirs.

It is also possible that the two dNir types originated at very different times: the Cu-type very early with the variation resulting from divergence, and the heme $\underline{cd}_1$-type much later as a variation of oxygen respiration. The immunoblot method was used to quantify divergence among dNirs in denitrifying bacteria. This method has the advantage of sensitivity compared to immunodiffusion assays, and simplicity compared to microcomplement fixation techniques (Howe and Hershey, 1981; 1984).

The validity of the results rests on a key assumption that the dNir added from each sample was similar. If the specific activity of dNirs differed greatly among strains, the results would be confounded. Assuming that specific activities are similar, if not identical in some instances, immunoblot analysis is a rapid method to visually inspect differences in dNirs from different species. The Cu-dNirs examined differed in terms of molecular mass and antigenicity. *Achromobacter*

cycloclastes Cu-dNir was not closely related to any of the other Cu-dNirs based on antibody affinity. *Achromobacter xylosoxidans* and two *A. faecalis* species had similar antibody affinities. *Alcaligenes eutrophus* shared the fewest common epitopes with *A. cycloclastes* Cu-dNir. Although Michalski and Nicholas (1988b) reported recognition of all Cu-dNirs they used by *R. sphaeroides* Cu-dNir antiserum, they did not quantify the degree of recognition. Our results suggest that the Cu-dNirs against which antisera are raised have a profound effect on recognition.

It was noted (Table 2) that the immunologic response of $\underline{cd}_1$-dNirs sometimes differed depending on what type of $\underline{cd}_1$-dNir the antisera were raised against, i.e. *P. aeruginosa* vs. *P. stutzeri*. This suggested greater variability in $\underline{cd}_1$-dNirs than was indicated by comparisons of molecular mass. Antiserum raised against *P. aeruginosa* $\underline{cd}_1$-dNir recognized the $\underline{cd}_1$-dNirs of all strains examined by immunoblot analysis. That *P. fluorescens* has 138% of the binding of *P. aeruginosa*, probably reflects slight differences in the amount of enzyme loaded. Three strains, *P. aeruginosa*, *P. fluorescens*, and isolate 1.6.L had similar antibody affinity, while the affinities of two other strains, isolate 2.4.E and *P. stutzeri* were closer to each other than to the first group. *Pseudomonas aeruginosa*, *P. fluorescens*, and *P. stutzeri* are in the same DNA homology group as defined by Palleroni et al. (1973). *Pseudomonas aeruginosa* and *P. stutzeri* also share the same RNA homology group. Consequently, it's surprising that the $\underline{cd}_1$-dNir they possess seem to differ more than *P. aeruginosa* and *P. fluorescens* $\underline{cd}_1$-dNir. The results confirm earlier observations during screening of many denitrifiers with $\underline{cd}_1$-dNirs, that the $\underline{cd}_1$-dNirs also have diverse antibody recognition.

There was a characteristic doublet of strongly antigenic bands in *P. fluorescens* and isolate 1.6.L, which suggests that they are related. Purified $\underline{cd}_1$-dNirs have all been dimeric, and composed of identical subunits. Michalski and Nicholas (1985) reported that *R. sphaeroides* Cu-dNir was a dimer composed of non identical subunits. *Pseudomonas fluorescens* $\underline{cd}_1$-dNir may also have nonidentical subunits, but has yet to be purified. The higher molecular mass band may represent unprocessed $\underline{cd}_1$-dNir still attached to a leader region required for its transport and insertion into the cell membrane. The DNA sequence of another denitrifying enzyme, N_2O reductase, is consistant with a leader region of approximately 35 amino acid residues (Viebrock and Zumft, 1988).

The difference in antigenicity between the two groups of $\underline{cd}_1$-dNirs in this study - exemplified by *P. aeruginosa* and *P. stutzeri*, respectively - was further revealed when the same $\underline{cd}_1$-dNirs were probed with antiserum to *P. stutzeri* $\underline{cd}_1$-dNir (Figure 9). Only *P. stutzeri* $\underline{cd}_1$-dNir was strongly recognized under these conditions. Although proteins were recognized in the other strains, only those in isolate 1.6.L and *P. fluorescens* could be quantified. The immunogenically recognized high molecular mass protein in both of these strains, which had previously been recognized by *P. aeruginosa* $\underline{cd}_1$-dNir antiserum, was not recognized by *P. stutzeri* $\underline{cd}_1$-dNir antiserum. Körner and Zumft (1987) noted in immunodiffusion assays, that the $\underline{cd}_1$-dNir of a *P. perfectomarina* (now *P. stutzeri* strain Zobell) only cross reacted with one other $\underline{cd}_1$-dNir type denitrifier - also *P. stutzeri*. It appears that there is a suite of epitopes common to *P. stutzeri* type $\underline{cd}_1$-dNir that is not found in the other $\underline{cd}_1$-dNirs.

Proteins which differ by more than 40% in amino acid sequence generally fail to show cross reactivity (Cocks and Wilson, 1972). Based on protein sequence differences derived from immunoblot distance (Cocks and Wilson, 1972) other Cu-dNirs differed from *A. cycloclastes* Cu-dNir by some 20-30 % of the protein sequence. The difference between $\underline{cd}_1$-dNirs is less than 10%. To what extent these differences reflect the time for divergence, the conservation of structure, or the diversity of organisms is unclear given the few dNirs which have been examined.

In the same functional class of proteins, sequence evolution goes on at approximately the same rate (Wilson et al., 1977). In a given protein, evolution will depend on the compatibility of sequence change with biochemical function and the likelihood of organism survival without that biochemical function (Wilson et al., 1977). That Cu-dNirs appear to diverge more than $\underline{cd}_1$-dNirs may reflect a greater capacity of nonheme Cu-dNirs to accept sequence changes without losing function compared to heme $\underline{cd}_1$-dNirs.

Since the basic criterion for the selection of these organisms was their ability to denitrify, it might be more productive to study dNir divergence by looking for dNirs in organisms which have lost the ability to denitrify, NO_3^- respirers for example, on the assumption that these organisms may represent truncated denitrifiers which have lost the capacity to make functional dNirs though not the enzymes themselves.

SECTION 2

LOCALIZATION OF CYTOCHROME $\underline{cd}_1$ AND CU NITRITE REDUCTASES IN DENITRIFYING BACTERIA

INTRODUCTION

Denitrification is a respiratory process in which nitrogenous oxides serve as terminal electron acceptors in the absence of O_2 (Firestone, 1982). During the reduction of NO_2^- to N_2O, energy is conserved by oxidative phosphorylation (Koike and Hattori, 1975a,b). Consequently, NO_2^- reductases (dNirs) in denitrifying bacteria have been perceived as membrane-bound components of energy generation. Understanding this role requires information about their cellular localization.

Three approaches have been used: immunochemical detection, measurement of proton translocation, and cellular fractionation. Ferritin-conjugated antibody was used by Saraste and Kuronen (1978) to localize dNir in *Pseudomonas aeruginosa* at the inner face of the cytoplasmic membrane. Boogerd et al. (1981) placed dNir from *Paracoccus denitrificans* in the periplasmic space based on proton translocation studies. This was in contrast to similar studies on the same organism by Kristjansson et al. (1978) which gave dNir a cytoplasmic orientation.

Cellular fractionation approaches have proved more confusing. In *P. aeruginosa*, dNir was periplasmic, in contrast to the immunochemical results (Wood, 1978). Nitrite reductase was found in the periplasm of *P. denitrificans* after sphaeroplast formation (Alefounder and Ferguson, 1980), but some dNir activity remained bound to the sphaeroplast membranes. Mancinelli et al. (1986) found dNir in cytoplasmic and membrane fractions of *P. halodenitrificans*. Nitrite reductase in the

membrane fraction was tightly bound. It was found on the cytoplasmic membrane of *Thiobacillus denitrificans* (Sawhney and Nicholas, 1978), and both the cytoplasmic membrane and cytoplasm of *P. perfectomarina* (now *P. stutzeri* strain Zobell)(Zumft and Vega, 1979). Its presence in the cytoplasm was credited to loose binding to the cytoplasmic membrane. A more recent report placed it as a soluble periplasmic protein in *P. stutzeri* (Mingawa and Zumft, 1988 cited in Heiss et al., 1989).

All of these cultures contained $\underline{cd}_1$-type dNir. Among organisms with a Cu-type dNir, only *Rhodobacter sphaeroides* has been examined for its cellular location, and there it was found in the periplasm (Sawada and Satoh, 1980).

We recently raised specific antibodies to *P. aeruginosa* $\underline{cd}_1$-dNir and *Achromobacter cycloclastes* Cu-dNir. As an alternative to cellular fractionation or proton translocation studies, we attempted to indirectly visualize dNir location by immunocytochemical localization using colloidal gold probes. This method has been used successfully to study other systems including: restriction endonucleases in *Escherichia coli* (Kohring et al., 1985), component C of the methyl reductase system in methanogens (Ossmer et al., 1986), methyl-coenzyme M reductase in *Methanobacterium thermoautotrophicum* (Aldrich et al., 1987), and APS reductase as well as bisulfite reductase in *Desulfovibrio* spp. (Kremer et al., 1988).

In the following study, the localization of $\underline{cd}_1$- and Cu-dNirs in denitrifying bacteria are reported based on cellular fractionation and immunocytochemical localization. Immunocytochemical localizations were performed with goat anti rabbit-gold complexes (Auroprobe EM GAR15) on

resin embedded cells grown either aerobically, or anaerobically with KNO_3 as a terminal electron acceptor.

The portions of the study for which I was solely responsible were the cellular fractionation assays. Otherwise, the study represents a joint effort. Purified Cu-dNir was provided by C. Hulse of the University of Virginia. Purified cd_1-dNir was provided by E. Weeg-Aerssens of Michigan State University. Antibody production and cell growth were a joint effort between myself and Dr. Alahari Arunakumari. Electron microscopy and sample preparation were done by Stu Pankratz, Department of Microbiology and Public Health, Michigan State University.

METHODS AND MATERIALS

Bacterial strains and growth

A. cycloclastes ATCC 21921, *P. aeruginosa* ATCC 10145, and *P. fluorescens* ATCC 33512 were obtained from the American Type Culture Collection. *Achromobacter xylosoxidans* NCIB 11015 and *P. stutzeri* JM300 were obtained from H. Iwasaki and J. Ingraham, respectively.

Strains were grown in 15 g l^{-1} TSB supplemented to 1 uM $CuSO_4 \cdot 7H_2O$ and 10 mM KNO_3. Plates and slants for routine culture contained the same media with 1.5% agar. For cell fractionation, bacterial strains were grown in sealed 500 ml sidearm flasks with 250 ml of media. The headspace was made anaerobic by repeated flushing with sterile argon at a gassing manifold, and the flasks were inoculated with 12.5 ml of a late log phase culture of anaerobically-grown cells. Growth was monitored by the optical density at 660 nm during incubation at 30 °C. For immunogold localization, cells were either taken from aerobic slants

or plates, or from anaerobic broth cultures which had dissimilated all NO_3^- in the media.

Cell fractionation

The osmotic shock method of Yamamoto et al. (1987) was used to release periplasmic proteins. Cells in early stationary phase were harvested and washed twice with 10 mM Tris pH 7.5 containing 25% sucrose. Washed cells were resuspended in the same buffer containing 1 mM EDTA and incubated with shaking for 10 min at 24 °C. The cells were then centrifuged at 7000 rpm for 10 min, the supernatant decanted, and the cells quickly resuspended in ice-cold 10 mM Tris pH 7.5. The suspension was incubated on ice with periodic shaking for 10 min and afterwards centrifuged at 9000 rpm for 10 min. The supernatant was decanted and saved. The pelleted cells were resuspended in 10 mM Tris pH 7.5 and sonicated. The periplasmic fraction was defined as those proteins released into the supernatant after treatment with the cold buffer. The cytoplasmic fraction, containing cytoplasm and cell membranes, was defined as those proteins found in the supernatant after sonication of the pelleted cells. Protein concentrations in both fractions were determined by Bicinchoninic acid protein assay reagent (BCA - Pierce) using BSA as a standard, after pelleting cell debris.

Enzyme assays

Nitrite reductase activity was measured by gas chromatography as described in Section 1.

Acid phosphatase activity was measured by the increase in absorbance at 405 nm in a 2 ml reaction volume containing 0.05% Triton X100, 0.1 ml cell extract, and 4.5 mM p-nitrophenol phosphate in 0.1 M acetic acid buffer pH 4.5 (Absolom, 1986).

Glucose-6-phosphate (G-6-P) dehydrogenase activity was measured by the increase in absorbance at 340 nm. Stock solutions in 50 mM Tris buffer pH 8.9 were prepared for NAD^+ (30 mM), ($NADP^+$ 30 mM), and D-glucose-6-phosphate (200 mM). The reaction consisted of 0.2 ml of NAD^+ or $NADP^+$ stock solution, 0.2 ml of G-6-P, cell extract (between 0.05 and 0.2 ml) and Tris buffer to a final volume of 2 ml (Maurer et al., 1982).

Preparation of antisera

Antisera against *A. cycloclastes* Cu-dNir and *P. aeruginosa* cd_1-dNir were prepared as described in Section 1.

Immunogold labeling and electron microscopy

Cells from denitrifying broth cultures and aerobic plate cultures were pelleted and resuspended in 1% glutaraldehyde, 0.1 M sodium cacodylate pH 7.2 for 40 min then washed three times in 0.1 M sodium cacodylate for a total of 45 min. The cells were dehydrated in an increasing graded series of ethanol washes before embedding in Lowacryl K4M resin overnight. The samples were polymerized at 24 °C under a UV lamp for one and a half days. After polymerization, thin sections were cut from the resin with a diamond knife, collected on water, then onto nickel grids and stored at room temperature until use.

Grids with resin embedded cells were incubated in TBST buffer (10 mM Tris pH 7.4, 0.5% Tween 80, 2.5% NaCl) containing 1% BSA. The grids were then transferred to 1:200 dilutions of primary antibody (in TBST), preimmune sera, or buffer (depending on the treatment). After 60 min, grids were washed 3 X 10 min in TBST and incubated 60 min with a 1:250 dilution of goat anti-rabbit 15 nm colloidal gold particles (Auroprobe EM GAR15 - Janssen Life Science Products). The grids were then washed 3 X 10 min with TBST before a final counterstain with uranyl acetate.

Grids were examined and photographed on a Philips CM-10 electron microscope.

RESULTS

Mild osmotic shock did not separate periplasmic and cytoplasmic components of denitrifying cells into discrete fractions. In four of the five strains, most dNir activity was in the cytoplasmic fraction after osmotic shock (Table 1), as was true of acid phosphatase activity, a marker enzyme for periplasmic proteins.

The distribution of dNir activity in cytoplasmic and periplasmic fractions did not always match the distribution of acid phosphatase activity between the same two fractions, although, it was close in *P. fluorescens* and *P. stutzeri* (Table 1). The recovery of total dNir and acid phosphatase activities in the periplasmic fractions were *A. cycloclastes* 27% vs. 3%, *A. xylosoxidans* 1%< vs. 3%, *P. aeruginosa* 3% vs. 31%, *P. fluorescens* 27% vs. 18%, and *P. stutzeri* 88% vs. 83%.

These results indicate that mild osmotic shock did not completely release the periplasmic proteins in all strains, as might be expected for different bacterial species. However, in all strains, a cytoplasmic enzyme such as glucose-6-phosphate dehydrogenase was not found in the periplasmic fraction. This indicates that the integrity of cytoplasmic membranes was maintained in those cells which did respond to the osmotic shock (Table 1). These data suggest a periplasmic location for both dNir types.

Table 1. Cellular distribution of enzyme activities.

Strain	Enzyme type	Fraction	NO_2^- Reductase[a]	Acid Phosphatase[b]	G-6-P Dehydrogenase[c]	Total Protein (mg ml^{-1})
A. cycloclastes ATCC 21921	Cu	Cyto	2562	223	115	1.40
		Peri	924	6	0	0.15
A. xylosoxidans NCIB 11015	Cu	Cyto	5904	250	13	1.60
		Peri	14	10	0	0.12
P. aeruginosa ATCC 10145	$\underline{cd}_1$	Cyto	8795	91	13	1.65
		Peri	231	40	0	0.22
P. fluorescens ATCC 33512	$\underline{cd}_1$	Cyto	74	141	44	1.85
		Peri	28	30	0	0.20
P. stutzeri JM300	$\underline{cd}_1$	Cyto	54	10	31	0.90
		Peri	394	50	0	0.39

[a] Activity measured as nmol N_2O min^{-1} mg^{-1}
[b] Activity measured as umol phosphate min^{-1} mg^{-1}.
[c] Activity measured as nmol NAD^+ or $NADP^+$ reduced min^{-1} mg^{-1}.

Cytoplasmic (cyto) and periplasmic (peri) fractions are as defined in the Methods and Materials.

Immunocytochemical detection of dNirs using colloidal gold probes was used as a second, indirect, approach to determine their localization in denitrifying cells. Four strains were used, containing either Cu (*A. cycloclastes*. *A. xylosoxidans*) or $\underline{cd}_1$ (*P. aeruginosa*, *P. fluorescens*) dNirs. The specificity of the primary antiserum for each dNir type was previously established by Western immunoblot assay (see Section 1). When colloidal gold probes were used alone, or in conjunction with preimmune sera, there was no binding of gold particles to anaerobically-grown (induced) cells. Likewise, when aerobically-grown (uninduced) cells were

incubated with primary antisera and colloidal gold probes, there was little or no binding of gold particles (Figures 1,4,6, and 8).

An anaerobically-grown, Cu-type denitrifier, *A. cycloclastes*, incubated with $\underline{cd}_1$-dNir antiserum and colloidal gold probes, had some binding of gold particles, but these mostly appeared on the cell wall and were assumed to be nonspecific (Figure 3). In contrast, when anaerobically-grown *A. cycloclastes* and *A. xylosoxidans* were incubated with the Cu-dNir antiserum, followed by colloidal gold probes, numerous gold particles were associated with the periplasmic space of both strains (Figures 2 and 5). This was also the case with anaerobically-grown *P. aeruginosa* and *P. fluorescens* incubated with cd_1-dNir antiserum and colloidal gold probes (Figures 7 and 9). Few gold particles were found in the cytoplasm indicating that dNirs of both type were not soluble cytoplasmic enzymes in these four strains.

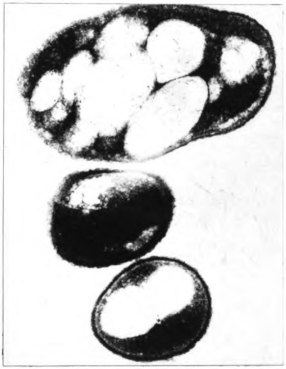

Figure 1. Electron micrograph of aerobically-grown *A. cycloclastes* incubated with Cu-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X).

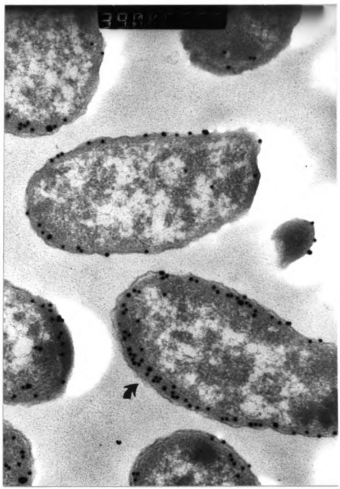

Figure 2. Electron micrograph of anaerobically-grown *A. cycloclastes* incubated with Cu-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 66,300X). Arrow points to periplasmic location of the gold particles.

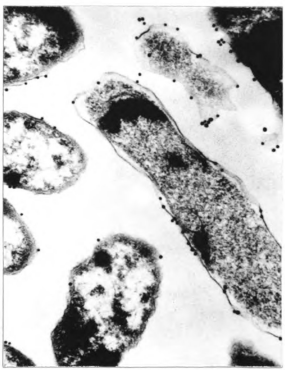

Figure 3. Electron micrograph of anaerobically-grown *A. cycloclastes* incubated with $\underline{cd_1}$-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X).

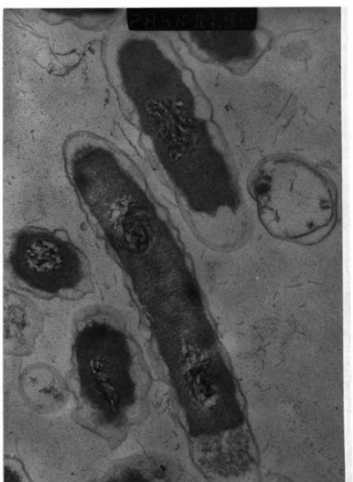

Figure 4. Electron micrograph of aerobically-grown *A. xylosoxidans* incubated with Cu-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X).

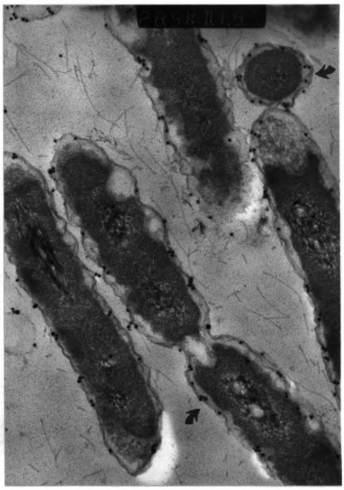

Figure 5. Electron micrograph of anaerobically-grown *A. xylosoxidans* incubated with Cu-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X). Arrow points to localization of the gold particles in the periplasmic space.

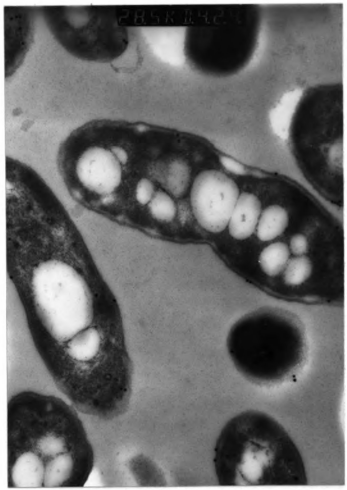

Figure 6. Electron micrograph of aerobically-grown *P. aeruginosa* incubated with $\underline{cd_1}$-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X).

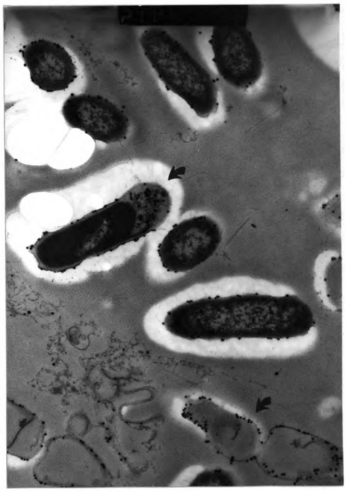

Figure 7. Electron micrograph of anaerobically-grown *P. aeruginosa* incubated with $\underline{cd_1}$-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X). Arrow points to localization of the gold particles around the cytoplasmic membrane.

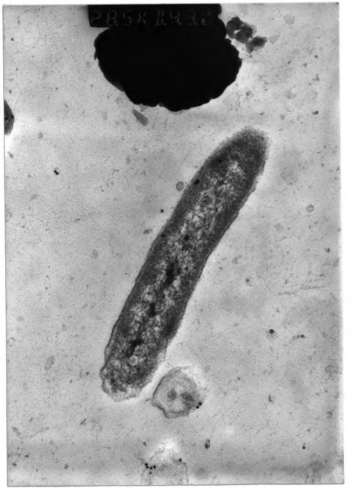

Figure 8. Electron micrograph of aerobically-grown *P. fluorescens* incubated with $\underline{cd_1}$-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 48,450X).

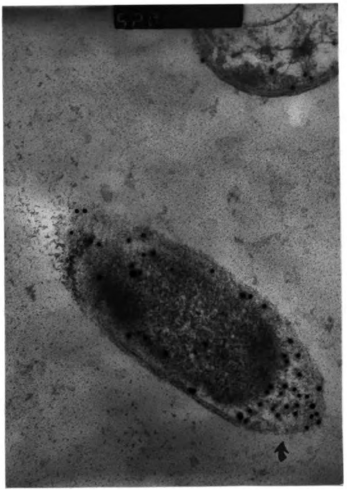

Figure 9. Electron micrograph of anaerobically-grown *P. fluorescens* incubated with $\underline{cd_1}$-dNir antiserum and treated with 15 nm colloidal gold probes (magnification 88,400X). Arrow points to localization of gold particles in the periplasmic space.

DISCUSSION

Two independent means were used to localize dNirs in denitrifying bacteria: cell fractionation and immunogold labeling. Mild osmotic shock expelled periplasmic proteins such as acid phosphatase without the concomitant expulsion of cytoplasmic proteins such as glucose-6-phosphate dehydrogenase. Nitrite reductases of both $\underline{cd}_1$- and Cu-type were expelled along with the acid phosphatase.

Most of the dNir and acid phosphatase activity remained with the cytoplasmic fraction, which contained cytoplasm and cell membranes. This was expected, since periplasmic proteins will only be lost if the cell wall is grossly disrupted (Alefounder and Ferguson, 1980). Only 57% of the total dNir activity in osmotically shocked *R. sphaeroides* was recovered in the periplasmic fraction by Sawada and Satoh (1980). Likewise, only 55% of a periplasmic blue Cu protein was recovered from *A. faecalis* strain S6 in the osmotic shock procedure used by Yamamoto et al. (1987).

An alternative to the osmotic shock procedure was fractionation by sphaeroplast formation. Sphaeroplasts and sphaeroplast supernatants contained 53% and 47%, respectively, of total NO_2^- reductase activity in preparations of *P. denitrificans* made by Alefounder and Ferguson (1980). A sphaeroplast preparation of *R. sphaeroides* made by Sawada and Satoh (1980) indicated that sphaeroplast supernatants (i.e. the periplasmic fraction) contained 90% of the dNir activity. However, the sphaeroplast procedures of Sawada and Satoh (1980) and Maagd and Lugtenberg (1986) gave no better results than osmotic shock in preparations of *A. cycloclastes* which were done as preliminary experiments. A third alternative, expulsion of periplasmic proteins by chloroform treatment

(Ames et al., 1984) also gave no better results than the osmotic shock procedure. So, osmotic shock was chosen since it was the easiest method for all strains.

It was interesting that the magnitude of dNir and acid phosphatase release into the periplasmic fraction did not always coincide, as might be expected if both are periplasmic proteins. This could reflect differences in the degree of binding between dNir and the cytoplasmic membrane. In *P. aeruginosa* (Saraste and Kuronen, 1978) and *P. perfectomarinus* (*P. stutzeri*) (Cox and Payne, 1973; Zumft and Vega, 1979) the dNirs were described as loosely bound in contrast to *P. halodenitrificans* in which membrane associated dNir was tightly bound (Mancinelli et al., 1986). With respect to *A. cycloclastes*, *P. fluorescens*, and *P. stutzeri*, the dNirs were more readily expelled from cells by osmotic shock than were acid phosphatases (Table 1). Nitrite reductases in *A. xylosoxidans* and *P. aeruginosa* were not expelled as well as acid phosphatases, perhaps indicating tighter association of these dNirs to the cytoplasmic membrane.

Immunochemical labelling has been previously used to localize denitrifying enzymes. Most studies with $\underline{cd}_1$-dNirs have been confined to *P. denitrificans* and *P. aeruginosa*. With the exception of *R. sphaeroides*, Cu-dNirs have not been localized. Our results indicate a specific binding of gold particles only to those cells in which dNir was induced. This binding was not associated with the cytoplasm, but was associated with the cytoplasmic membrane and/or periplasmic space in all the strains examined.

These results differ from studies with *P. halodenitrificans* in which 99% of the dNir activity was associated with the cytoplasm

(Mancinelli et al., 1986), but do agree with other studies which associate dNirs with the cytoplasmic membrane or periplasmic space (Alefounder and Ferguson, 1980; Sawada and Satoh, 1980; Wood, 1978; Zumft and Vega, 1979).

The immunogold localization indicates a periplasmic for the Cu-dNirs of *A. cycloclastes*, *A. xylosoxidans*, *P. aeruginosa*, and *P. fluorescens*. This was an indirect localization and the dimensions of the antibody-gold complexes have to be considered. The gold particles in the goat, anti rabbit-gold complexes may be up to 20 nm away from the actual location of the dNirs (Kohring et al., 1985). In cells where plasmolysis has occurred, there is a distinct localization of gold particles between cell wall and cytoplasmic membrane in all four strains.

Boogerd et al. (1981) developed a model based on indirect evidence from alkalinization of external media, to explain proton translocation and electron flow during denitrification. This model required a periplasmic location for dNir to permit external H^+ consumption (Figure 10). The results from our immunogold localization with two Cu-type denitrifiers and two $\underline{cd}_1$-type denitrifiers support this model and a periplasmic location for both dNir types.

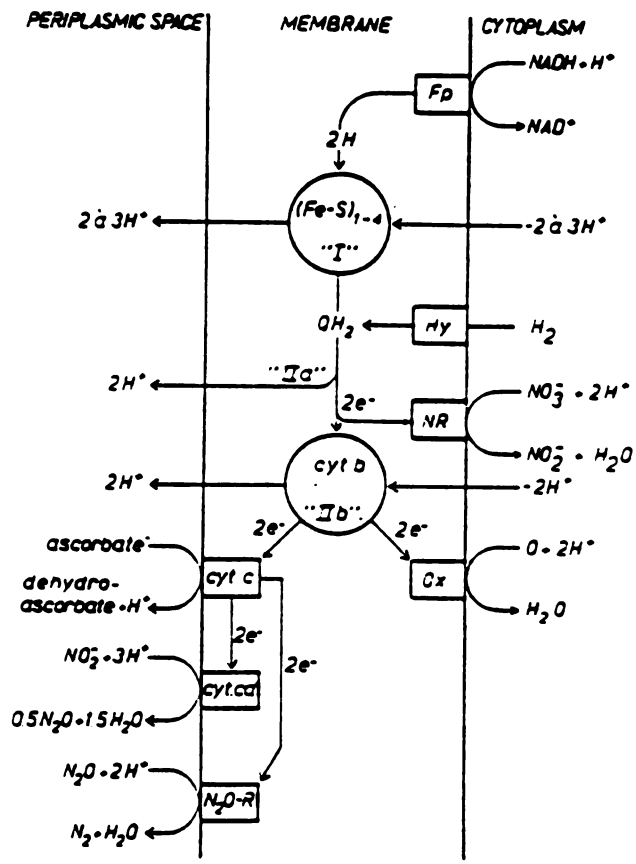

Figure 10. Anaerobic respiratory chain of *P. denitrificans* and its proton-translocating properties. Fp, flavoprotein; Fe-S, iron sulfur center; QH_2, ubiquinol; Hy, hydrogenase; NR, nitrate reductase; cyt., cytochrome; N_2O-R, nitrous oxide reductase; Ox, alternate oxidase. I and IIa + IIb are the traditional sites of energy conservation (From F.C. Boogerd, Ph.D. Thesis, Free University of Amsterdam, 1984).

SECTION 3

INDUCTION OF N-OXIDE REDUCTASES IN OXYGEN-LIMITED CHEMOSTAT CULTURES OF *ACHROMOBACTER CYCLOCLASTES*

INTRODUCTION

Oxygen represses the synthesis and activity of N-oxide reductases (Payne, 1973). In environmental samples, denitrification generally begins between 3 and 12 uM O_2 (Tiedje, 1988), however, some well-aerated environments evolve N_2O (Smith and Tiedje, 1979). One frequently invoked source is anaerobic microsites (Parkin, 1987), although, other explanations include chemodenitrification, autotrophic nitrification (Bremner and Blackmer, 1978), heterotrophic nitrification (Castignetti and Hollocher, 1984), and fungal N-metabolism (Yoshida and Alexander, 1970).

It is possible, though not yet demonstrated, that N_2O evolution from soils comes from aerobic denitrification. Denitrification in *Thiosphaera pantotropha* was reported at greater than 90% air saturation (Robertson and Kuenen, 1984a). N-oxide reductases have reportedly been synthesized at relatively high O_2 concentrations in *Hyphomicrobium* X (Meiberg et al., 1980). A magnetic *Spirillum*, MS-1, has an absolute requirement for O_2 even during denitrifying growth (Escalante-Semerena et al., 1980).

Ecologically-oriented denitrification studies typically focus on rates of the entire process, spatial changes in zones supporting denitrification, or temporal changes in pathway intermediates (Firestone and Tiedje, 1979; Rice et al., 1988; Smith and Tiedje, 1979). Few studies have looked for the relationship between specific N-oxide

reductases and discrete O_2 concentrations which either induce their synthesis or repress their activity. This would be particularly important knowledge for the management of denitrification in any commercial process which seeks to employ denitrifiers as alternatives to obligate aerobes.

Nitrate and nitrite reductase were derepressed at 15 and 91 uM O_2, respectively, in *Thiobacillus denitrificans* continuous cultures undergoing a gradual shift to anaerobiosis (Justin and Kelly, 1978). The denitrification products in *Paracoccus halodenitrificans* continuous cultures undergoing a gradual shift from anaerobiosis to aerobic growth were, sequentially, N_2, N_2O, NO_2^-, and NO_3^- (Hochstein et al., 1984). This was interpreted as N-oxide reductase inhibition at different O_2 levels in the growing cells. Nitrite still comprised 74% of the N-oxide present when dissolved O_2 concentrations were below the detection level of the O_2 probe (3 uM). Körner and Zumft (1989) found discrete O_2 levels at which NO_3^-, NO_2^-, and N_2O reductases were derepressed in continuous culture of *P. stutzeri* strain Zobell. N_2O reductase was constitutively expressed, while NO_3^- and NO_2^- reductases were synthesized at 5.0 and 2.5 mg O_2 l^{-1} (156 and 78 uM O_2), respectively.

These strains contain $\underline{cd}_1$-type NO_2^- reductase ($\underline{cd}_1$-dNir)(Grant and Hochstein, 1984; Körner et al., 1987; LeGall et al., 1979). No similar studies have been done with organisms containing Cu-type NO_2^- reductase. The purpose of this study was to examine the conditions required for Cu-type NO_2^- reductase synthesis in *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972). and to determine the dissolved O_2 concentration at which derepression of NO_2^- reductase and other N-oxide reductase synthesis and activity occurs.

METHODS AND MATERIALS

Organism and culture media

Achromobacter cycloclastes ATCC 21921 was grown in TSB media (Difco, Detroit MI), 15 g l^{-1} TSB, 1 uM $CuSO_4 \cdot 7H_2O$, 10 mM KNO_3, for routine culture and maintenance. Slants and plates contained 1.5 % agar. Batch culture experiments contained 6 g l^{-1} TSB. Chemostat media consisted of 20 mM phosphate buffer pH 6.5 containing: 1 g l^{-1} TSB, 1 g l^{-1} NH_4Cl, 0.1 g l^{-1} KNO_3, and 1 uM $CuSO_4 \cdot 7H_2O$.

Chemostat growth

Continuous culture was in a one liter LH 500 Series fermenter with media outlet set for a 500 ml working volume. An Ingold polarographic O_2 electrode, pH electrode, thermistor, heating and cooling rods, sampling column, and the gas in- and outlet tubes penetrated the lid of the chemostat. Temperature was maintained at 30 °C and stirring speed at 1000 rpm. The high stirring rate and low carbon levels used in the chemostat were intended to insure low cell density and optimal exposure to the measured O_2 concentration.

Two point calibrations at 0 and 100% air-saturation, were performed by immersing the O_2 electrode in water saturated with N_2 and atmospheric air, respectively. Gas flow was regulated by flow controllers and adjusted to give the desired air-saturation as recorded by the O_2 electrode. Gas flow through the media was 100 ml min^{-1} and was filter sterilized through a Millex-GS 0.22 uM filter unit (Millipore Corp., Bedford, MA). The dilution rate (D) was 0.05 hr^{-1}.

Chemostat experiments were begun by inoculating air-sparged chemostats with 5 ml of late log phase cells grown aerobically in chemostat media which lacked KNO_3. Media flow was begun immediately

after inoculation, and sampling started when optical density measurements (at 660 nm) were stable.

Gas chromatography

Nitrous oxide formation during batch culture experiments was monitored on a Shimadzu GC-mini 2 gas chromatograph equipped with a ^{63}Ni electron capture detector. Carrier gas was 95% argon, 5% methane. The temperatures were: detector 300 °C, injector and column 30 °C. The column was 2 m x 2 mm i.d. stainless steel filled with Porapak Q (Waters Assoc. Inc., Framingham, MA). Gas flow was 46 ml min^{-1}.

Nitrous oxide formation and disappearance in continuous culture was monitored on a Perkin-Elmer 910 gas chromatograph equipped with dual columns and ^{63}Ni electron capture detectors for simultaneous analysis of two samples (Kaspar and Tiedje, 1980). Conditions were as above except that carrier gas flow was 15 ml min^{-1} which was adequate to separate NO and O_2 peaks. Nitric oxide was obtained from Matheson Corp., as were N_2O gas standards.

Western immunoblots

Procedures for sample preparation and immunoblotting are given in Section 1. Purified N_2O reductase from *A. cycloclastes* was obtained from C. Hulse (C. Hulse, Ph.D. thesis, University of Virginia, 1989). Silver staining indicated that the protein was electrophoretically pure (see Figure 1, Section 1). The emulsified protein was used to immunize a New Zealand white rabbit according to the protocol outlined in Section 1. The antiserum obtained was used without further purification.

Batch culture 1 - Conditions for nitrite reductase synthesis

Twenty ml of *A. cycloclastes* was removed from an aerobic batch culture growing without N-oxides and 0.5 ml aliquots were dispensed into

replicate 3.0 ml Venoject tubes (Terumo Medical Corp., Elkton, MD). Treatments were: 10 mM Na_2WO_4 + 1 mM KNO_3; 1 mM KNO_3; 1 mM Diethyldithiocarbamate (DDC) + 1 mM $NaNO_2$; 1 mM $NaNO_2$; 0.5 ml of 100% N_2O; no amendment. The final volume was adjusted to one ml in all cases with sterile batch culture media. The tubes were stoppered and incubated at 30 °C for 24 h without evacuating the headspace. After 24 h, the cells in one tube from each treatment were pelleted and washed in 50 mM HEPES buffer pH 7.3. Protein was determined in these samples by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard (Sigma). The cells in the remaining tube from each treatment were used for Western immunoblot analysis. Based on the protein analysis, 50 ug total protein from each treatment were loaded into the sample wells for SDS-PAGE.

Continuous culture - N-oxide reductase induction with decreasing air saturation

Growth was in continuous culture at D = 0.05 h^{-1}. After one volume change at each air saturation level, the OD (660 nm) was measured, the chemostat headspace was sampled, and a sample of growth medium was removed. The aqueous sample was centrifuged and the supernatant analyzed for NO_3^- by absorbance at 210 nm following HPLC separation (Christensen and Tiedje, 1988) and NO_2^- by absorbance at 543 nm following diazotization (Hanson and Phillips, 1981).

To detect the induction of N-oxide reductases, whole cell assays were used. Approximately 40 ml of growth medium was removed, cells harvested by centrifugation at 10,000 rpm for 5 min at 4 °C, washed twice in 20 mM phosphate buffer pH 6.5 containing 10 mM glucose and 1 mg ml^{-1} chloramphenicol, and resuspended in 10 ml of the wash medium. The

washed cells were kept on ice for the duration of sampling and used within 4 hours.

To detect the activity of N-oxide reductases these protocols were used: NO_3^- reductase - 0.5 ml aliquots of the washed cells were dispensed into replicate serum vials, amended with 0.5 ml of 1 mM KNO_3^-, sealed, flushed with argon, and incubated at 25 °C for 1 h. At 10 min intervals, a vial was removed and frozen at -70 °C until NO_2^- accumulation or NO_3^- disappearance could be measured as noted previously; NO_2^- reductase - 0.5 ml aliquots were dispensed into two replicate 15 ml serum vials, amended with 0.5 ml of 1 mM $NaNO_2$, sealed, flushed with argon, amended with 0.1 ml of C_2H_2 to block N_2O reduction (Yoshinari and Knowles, 1976)(prepared by hydrating calcium carbide in an argon atmosphere), and incubated at 25 °C. At 10 min intervals the vials were shaken and the headspace of each vial was sampled by gas chromatography for N_2O production; NO reductase - 0.5 ml aliquots were dispensed into two replicate 15 ml serum vials, amended with 0.5 ml of wash medium, sealed, flushed with argon, amended with 0.1 ml of 100% NO and 0.1 ml of C_2H_2, and incubated at 25 °C. At 10 min intervals N_2O evolution was monitored by gas chromatography; N_2O reductase - 0.5 ml aliquots were dispensed into two replicate 15 ml serum vials, amended with 0.5 ml wash medium, sealed, flushed with argon, and flushed 1 min with a 30 ppm N_2O gas standard. The vials were incubated at 25 °C and at 10 min intervals N_2O disappearance was monitored by gas chromatography. Boiled controls were used for all treatments. Additional 0.5 ml aliquots of the washed cell suspensions were removed and stored at -70 °C for analysis of protein and for immunoblotting.

RESULTS

Conditions for nitrite reductase synthesis

NO_2^- reductase synthesis was detected by Western immunoblot. Synthesis occurred in all treatments except when N-oxides were absent or when DDC was present (Figure 1). Synthesis appeared greatest when NO_3^- was present. Presumably, NO_2^- reductase was not synthesized when DDC was present because the cells had little or no growth. One hundred micromolar DDC severely retarded aerobic growth of *A. cycloclastes* in a preliminary experiment. Total protein yield in the DDC amended treatment was approximately half that of the other treatments (Table 1).

Table 1. Effect of different substrates and inhibitors on the protein yield of semianaerobically incubated *A. cycloclastes*.

Incubation Treatment	Final Protein Yield (ug ml^{-1})
10 mM WO_4^{2-} + 1 mM NO_3^-	344
1 mM NO_3^-	469
1 mM DDC + 1 mM NO_2^-	211
1 mM NO_2^-	406
0.5 ml N_2O	469
No N-oxide	359

Nitrite reductase was synthesized in the presence of 10 mM WO_4^{2-} which prevents formation of functional NO_3^- reductases by competitively inhibiting Mo incorporation into molybdoproteins (Scott et al., 1979). Assimilatory NO_3^- reductases should also have been inhibited by the protein-rich TSB media as well as by the WO_4^-. So, NO_2^- reductase synthesis does not require prior synthesis of functional NO_3^-

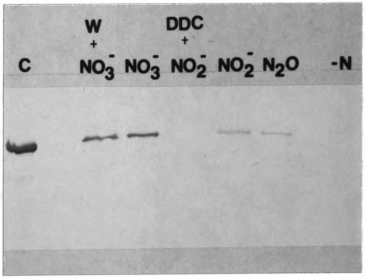

Figure 1. Synthesis of *A. cycloclastes* Cu-dNir in the indicated treatments. C = Control, 1 ug of *A. cycloclastes* Cu-dNir; W = Na_2WO_4; DDC = Diethyldithiocarbamate; -N = No N-oxide terminal electron acceptors.

reductases. Without NO_3^- reductase, anaerobic growth would be limited as the results in Table 1 indicate. Apparently, sufficient growth occurred on residual O_2 in the tubes to permit NO_2^- synthesis (since there was more protein in this treatment than the treatment with DDC, which inhibits growth), or NO_3^- reductase was present in the batch cultures which circumvented WO_4^{2-} inhibition.

N-oxide reductase induction at decreasing dissolved oxygen concentrations

The O_2 concentrations at which N-oxide reductase proteins were synthesized and became active was determined. Dissolved O_2 concentrations ranged from approximately 230 to 0 uM. The lowest O_2 concentration above 0 (N_2-sparged chemostat) that could reliably be measured by the electrode was 2 uM. The carbon content of the medium (1 g l^{-1} TSB) allowed a maximum optical density of 0.19 (Table 2). The optical density in fully anaerobic growth was 55% that of fully aerobic growth. This is consistant with observations that denitrifying growth yield is approximately half that of O_2 respiration (Koike and Hattori, 1975a).

Trace N_2O was present at all O_2 concentrations throughout the continuous culture. It was not considered physiologically significant. Trace NO_2^- was also present at all O_2 concentrations and could have been due to low constitutive levels of NO_3^- reductase activity. Full derepression of NO_3^- and NO_2^- reductases was not apparent until the measured O_2 concentration was 2 uM (Table 2).

Table 2. Denitrification by *A. cycloclastes* during continuous culture.

% Air saturation	O_2 (uM)	OD (660 nm)	NO_3^-[a] (uM)	NO_2^-[a] (uM)	Denitrification[b] flux (umol N $l^{-1}h^{-1}$)
100	228	0.19	1290	4	nd
50	114	0.20	1190	4	2.5
20	46	0.19	1150	4	3.4
10	23	0.18	990	8	7.4
4	9	0.16	830	4	11.5
1	2	0.14	310	0	24.6
0	0	0.10	0	0	32.4

[a] Concentration in the chemostat medium.

[b] After one volume change at the various air saturation levels, the effluent gas phase and the growth medium were measured for the indicated NO_3^-, NO_2^-, NO, and N_2O. No NO and only trace N_2O were observed in the chemostat headspace. The denitrification flux is based on the difference between the total NO_3^--N in the resevoir medium and sum of NO_3^-- and NO_2^--N in the chemostat medium.

The activities of individual N-oxide reductases were measured in washed cell suspensions removed from continuous culture at each dissolved O_2 concentration (Table 3). Nitrate reductase activity was present at all O_2 concentrations from complete air-saturation to complete anaerobiosis. Further enzymatic reduction of NO_2^- was expected at the lowest O_2 concentrations, since NO_2^- reductase was active under these conditions. So, for the lowest O_2 concentrations, NO_3^- reductase activity was measured by NO_3^- disappearance. Nitrite reductase was stringently regulated by O_2. No activity was present above 2 uM O_2, nor was any enzyme synthesized until 2 uM O_2 (as detected by Western immunoblot) (Figure 2). Full induction did not occur until complete anaerobiosis was reached. For comparison, under optimal denitrifying growth conditions in rich medium (15 g l^{-1} TSB, 10 mM KNO_3), NO_3-

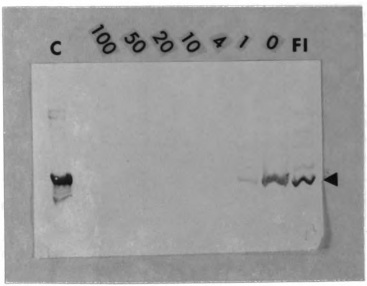

Figure 2. Western immunoblot of Cu-dNir synthesis in *A. cycloclastes* continuous cultures during increasing anaerobiosis. C = Control, 1 ug *A. cycloclastes* Cu-dNir; FI = Fully induced culture. Values indicate % air saturation. Corresponding dissolved oxygen concentrations are given in Table 3. Marker indicates *A. cycloclastes* Cu-dNir.

reductase activity was 3.5 umol-N min^{-1} mg^{-1} while NO_2^- reductase activity was 5.4 umol-N min^{-1} mg^{-1}.

Table 3. In vivo activity of N-oxide reductases in *A. cycloclastes* washed cells removed from continuous culture at varying air saturations.

% Air Saturation	uM O_2	N-oxide reductase activity (nmol-N min^{-1} mg^{-1})			
		NO_3^- ->NO_2^-	NO_2^- ->N_2O	NO ->N_2O	N_2O ->N_2
100	228	6 ± 0[a]	0	0	0
50	114	10 ± 0[a]	0	0	0
20	46	32 ± 1[a]	0	0	0
10	23	67 ± 2[a]	0	0	27 ± 9
4	9	220[b]	0	0	94 ± 20
1	2	326[b]	973[c]	2235 ± 333	151 ± 97
0	0	581[b]	1917 ± 190	1784 ± 91	31 ± 12

[a] NO_3^- reductase activity measured by NO_2^- accumulation.

[b] NO_3^- reductase activity measured by NO_3^- disappearance. One replicate only.

[c] Replicate lost.

Nitric oxide reducing activity was also stringently controlled by dissolved O_2 concentrations. No activity was observed until the dissolved O_2 concentration was 2 uM. In contrast, low levels of N_2O reductase protein were synthesized at all dissolved O_2 concentrations (Figure 3) but activity was not detected until the O_2 concentration fell to 23 uM.

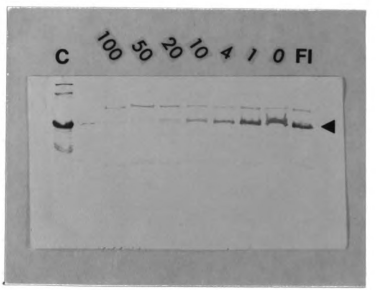

Figure 3. Western immunoblot of N_2O reductase synthesis in *A. cycloclastes* continuous cultures during increasing anaerobiosis. C = Control, 1 ug *A. cycloclastes* N_2O reductase; FI = Fully induced culture. Values indicate % air saturation. Corresponding dissolved oxygen concentrations are given in Table 3. Marker indicates N_2O reductase.

DISCUSSION

These results demonstrated the conditions in which NO_2^- reductase was synthesized in *A. cycloclastes*. They also demonstrated the O_2 concentrations at which N-oxide reductases were synthesized or derepressed in *A. cycloclastes* continuous cultures.

Anaerobiosis alone was not sufficient for the synthesis of NO_2^- reductase. The complementary presence of an N-oxide was also required. Nitrate had the greatest effect on enzyme induction. When tungsten was incorporated into one induction treatment to block formation of functional NO_3^- reductase, it had no apparent effect on NO_2^- reductase synthesis. Subsequent experiments demonstrated NO_3^- reductase activity in aerobic cells, so presynthesized NO_3^- reductase in these treatments probably provided NO_2^- for enzyme induction. Korner and Zumft (1989) found that NO_3^- had the greatest effect on overall enzyme induction in *P. stutzeri* strain Zobell, but enzyme induction for each terminal reductase was greatest when its specific N-oxide was present. The effect of NO_3^- on N-oxide reductases other than NO_3^- reductases was explained as the result of further reduction of NO_3^- to NO_2^- and N_2O.

Pseudomonas denitrificans (Payne, 1973) and *Rhodobacter sphaeroides* f. sp. *denitrificans* (Michalski and Nicholas, 1984) reportedly synthesize Cu-type NO_2^- reductase in anaerobic conditions alone. In *R. sphaeroides*, induction was greater with a terminal N-oxide. The requirement of a complementary N-oxide for enzyme synthesis may be strain specific. Maintenance of some N-oxide reductases anaerobically in the absence of N-oxides could be a competitive advantage. Isolates from NO_3^--free freshwater sediments and digested sludge had no lag in

denitrification activity when amended with NO_3^- (K.S. Jorgensen and J.M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989. N87, p. 299).

Nitrite reductase was the most stringently regulated N-oxide reductase. No enzyme was synthesized at detectable limits above 2 uM O_2, nor was enzyme activity noted above this level. Körner and Zumft (1989) found NO_2^- reductase expression at $\leq$ 2.5 mg O_2 l^{-1} (78 uM). The discrepency between these results could reflect true differences between the strains studied, or differences in O_2 experienced by the cells caused by different cell densities, stirring speeds, and gas sparging rates. Christensen and Tiedje (1988) noted that efficient mixing of O_2 in solution was a prerequisite for establishing meaningful denitrification thresholds. Thus, with high culture density, and uneven mixing, anaerobic zones resulting from respiration could develop around bacteria or clumps of bacteria, causing them to sense a greater O_2 deficit than probes indicate.

Nitrite was present at all O_2 levels and NO_3^- reductase activity were observed in washed cells from this study and by Körner and Zumft (1989). They attributed this to assimilatory NO_3^- reductase activity even in the presence of ammonium, because their antibody to dissimilatory NO_3^- reductase did not detect the presence of this protein, and the antiserum did not cross react with assimilatory NO_3^- reductase. I believe this represents low levels of constitutive dissimilatory NO_3^- reductase activity since, in my study, the activity increased continuously as O_2 concentration decreased, and the immunological methods used by Korner and Zumft were insensitive to low protein concentrations. Krul (1976) reported that NO_3^- reductase in a denitrifying *Alcaligenes* sp. was constitutive. A heterotrophic,

nitrifying *Alcaligenes* sp. also synthesized NO_3^- reductase in nitrifying, aerobic conditions (Castignetti and Hollocher, 1982).

Nitrate reductase activity was affected by growth rate in *Hyphomicrobium* X (Meiberg et al., 1980). At D = 0.15 h^{-1} NO_3^- reductase activity was significant only at low O_2 (9.3 uM), while at D = 0.01 h^{-1} NO_3^- reductase was present at relatively high O_2 (78 uM). In contrast, at D = 0.12 h^{-1} (Korner and Zumft, 1989) expression of NO_3^- reductase was at 169 uM O_2 while I observed NO_3^- reductase expression at 228 uM O_2 with D = 0.05 h^{-1}.

Trace N_2O was evolved at all O_2 concentrations during continuous culture. This was not considered physiologically important, but possible sources other than denitrification are: NO_3^- reductase acting on NO_2^- (Cole, 1987), an uncharacterized NO_3^- assimilation pathway (Bleakley and Tiedje, 1982; Yoshida and Alexander, 1970), or heterotrophic nitrification (Castignetti and Hollocher, 1982, 1984; Eylar and Schmidt, 1959; Obaton et al., 1968; Robertson and Kuenen, 1988; Verstraete and Alexander, 1972a,b;).

Nitrous oxide reductase was synthesized at all dissolved O_2 concentrations from air-saturation to full anaerobiosis (Figure 3). However, no activity was apparent until dissolved O_2 concentration fell below 23 uM. This may represent synthesis of precuser forms of the enzyme which do not have activity until inserted into the membrane (Cole, 1987). This is consistant with Körner and Zumft's (1989) results in which *P. stutzeri* N_2O reductase was constitutively synthesized, and for which expression was increased when O_2 fell below a discrete level (119 uM O_2).

Nitrous oxide reductase activity in washed cell assays was approximately 10 times less than NO_2^- reductase activity (Table 3). In direct measurements of the chemostat headspace N_2O did not accumulate, despite a rapid flux of NO_3^- through the system (Table 2). This illustrates a phenomenon noted with other denitrifiers, that exogenously supplied N_2O is not reduced as rapidly as internal N_2O derived from NO_2^- reduction. Some *P. aeruginosa* strains are unable to grow well on exogenously supplied N_2O as a direct consequence of turnover dependent inactivation of N_2O reductase (Snyder et al., 1987).

Nitric oxide was never detected during direct measurement of the air-sparged chemostat. If there are separate NO_2^- and NO reductases, they would appear to be similarly regulated by O_2. Nitric oxide reducing activity was only observed when O_2 concentrations fell to 2 uM. Zafiriou et al. (1989) found NO evolution from sparged, *P. perfectomarina* anaerobic batch cultures. As sparging rate increased, the proportion of gaseous N-oxide recovered as NO increased.

Nitrite reductase had the highest activity of any of the N-oxide reductases in fully anaerobic conditions (Table 3). Since only low levels of NO_3^- reductase are synthesized before NO_2^- reductase is induced, it is unlikely that *A. cycloclastes* faces situations in which NO_2^- accumulates to toxic levels during denitrification.

What regulatory model explains differential induction of N-oxide reductases? The rationale is simple, formation of a given electron acceptor is prevented when an alternate terminal electron acceptor that would give a higher energy yield is present (Stouthammer, 1988). While this explains why O_2 respiration is preferable to NO_3^- respiration, it doesn't adequately explain why NO_3^- respiration should be preferred to

NO_2^-, NO, or N_2O respiration, since oxidative phosphorylation occurs to a similar extent with each reductive step (Garber et al., 1982; Koike and Hattori, 1975b). Nor does it explain why N_2O reductase in *P. stutzeri* and *A. cycloclastes* is constituitively synthesized.

Oxygen controls denitrification at several levels, transcription, translation, and at least with N_2O reductase, direct inactivation (Stouthammer, 1988). There is no direct effect of O_2 on NO_3^- reductase. Membrane vesicles from *Pa. denitrificans* reduced O_2 and NO_3^- simultaneously (John, 1977; Parsonage et al., 1985). Both $\underline{cd}_1$- and Cu-type NO_2^- reductases have oxidase activity (Zumft et al., 1987). Each N-oxide reductase could control its own synthesis by autogenous regulation (Stouthammer, 1988). In this model, N-oxide reductase binding with the appropriate N-oxide results in conformational changes that prevent the protein from inhibiting transcription.

In the redox control model, the oxidation reduction status of the electron transport chain components, or the terminal N-reductase, regulates synthesis (Robertson and Kuenen, 1984b). The redox state of the electron transport chain could also regulate the assembly of the N-oxide reductases into active, membrane bound forms, thus explaining how synthesis of N-oxide reductases and activity of N-oxide reductases can be temporally separated (Stouthammer, 1988). Terminal electron acceptors compete for available e^-. In *Thiosphaera pantotropha*, aerobic denitrification is explained, in part, by suggesting that e^- flow to oxygen as the terminal electron carrier is impeded, thus shunting e^- to alternate terminal reductases (Robertson and Kuenen, 1984b). These models still require rigorous proof.

APPENDIX

APPENDIX A

PRELIMINARY EFFORTS TO CLONE CU-TYPE NITRITE REDUCTASE

Studies on the physiological genetics of denitrification invariably employ mutants in the reductive steps. We attempted to clone the structural gene for Cu-type NO_2^- reductase (Cu-dNir) with the goal of creating defined mutations in this enzyme. Three basic approaches were used:

(1) Probing with synthetic oligonucleotides derived from the N-terminal amino acid sequence of *Achromobacter cycloclastes* Cu-dNir.

(2) Creating expression libraries in the phage vector lambda gt11 followed by immunoscreening for expression of Cu-dNir.

(3) Generating DNA probes for Cu-dNir using mixed primer Polymerase Chain Reaction (PCR).

With respect to the first approach, a cosmid library was constructed in the cosmid vector pWH4 (a gift from Peter Wolk, DOE Plant Research Lab, Michigan State University). Total genomic DNA from *A. cycloclastes* ATCC 21921, *A. xylosoxidans* NCIB 11015, *P. aeruginosa* ATCC 10145, and *P. stutzeri* JM300 were partially digested with Sau 3A and

individually ligated into Bam HI digested, alkaline phosphatase treated, pWH4 using methods outlined by Maniatis et al. (1982). The concatamers formed, were used as substrates for in vitro packaging of bacteriophage particles, which were then introduced into *E. coli* strain HB101. Based on plasmid minipreps of 10 random kanamycin resistant colonies from each genomic digest, approximately 60% of the clones contained an insert. The clones were stored enmass in 50% glycerol at -70 °C.

Purified *A. cycloclastes* Cu-dNir was obtained from C. Hulse (Hulse et al., 1988) and the N-terminal amino acid sequence determined at the MSU macromolecular sequencing facility. The first 30 amino acids were obtained (Figure 1) and two synthetic oligonucleotides, each 17 bases long, were constructed by the sequencing facility from nonoverlapping sections of the N-terminus (Figure 2). Both probes were mixed. Probe #1 contained 256 different species, while Probe #2 contained 1024 different species.

1	2	3	4	5	6	7	8	9	10
ALA	PRO	VAL	ASP	ILE	SER	THR	LEU	PRO	ARG
11	12	13	14	15	16	17	18	19	20
VAL	LYS	VAL	ASP	LEU	VAL	LYS	PRO	PRO	PHE
21	22	23	24	25	26	27	28	29	30
VAL	ALA	ALA	ALA	ASP	GLN	VAL	ALA	LYS	THR

Figure 1. N-terminal sequence of Cu-dNir from *A. cycloclastes* ATCC 21921.

Peptide sequence: MET ALA PRO VAL ASP ILE

Probe #1: 5'- ATNTCNACNGGNGCCAT - 3'

Peptide sequence: ASP GLN VAL ALA LYS THR

Probe #2: 5' - GTNTTNGCNACNTGNTC - 3'

Figure 2. Synthetic oligonucleotides derived from the N-terminal amino acid sequence of *A. cycloclastes* Cu-dNir. N = A+G+C+T.

The oligonucleotides were detritylated, 5' end-labeled with ^{32}P-ATP, and used as radioactive probes for colony hybridizations, slot blots, and Southern blots. Although putative hybridization signals were obtained in some colonies, none could be authenticated in subsequent hybridizations. Slot blots were also unsuccessful because of nonspecific hybridization.

The apparent lack of sensitivity, and specificity, of the oligonucleotide probes, led to the second approach used in the cloning experiments. An expression library was prepared by Dr. A. Arunakumari from total Eco RI digests of *A. cycloclastes*. These digests were ligated to the phosphatase treated arms of the phage vector lambda gt11 (Promega Corp.) which was packaged, and used to infect *E. coli* strain Y1090r$^-$ (Huynh et al., 1985). Production of B-galactosidase fusion proteins was induced by the addition of IPTG, and could then be immunologically screened by Cu-dNir specific antiserum.

Self-ligated lambda gt11 was identified by blue plaque formation with X-gal, an indicator of functional B-galactosidase activity. Plaques carrying inserts putatively associated with Cu-dNir, were identified by immunoscreening with Cu-dNir antiserum after proteins were

adsorbed to nitrocellulose. Immunoscreening protocols and verification of antibody specificity are given in Section 1.

Eight immunoreactive plaques were obtained and passed through rescreening. Phage DNA was obtained using Promega lambda-sorb phage adsorbant and DNA isolation protocols. The DNA was exhaustively digested with Eco RI, but no inserts were recovered. The restriction pattern of Bam HI digested phage DNA, compared to the Bam HI restriction pattern of unligated lambda gt11, suggested that phage DNA contained inserts. Subsequent restriction digests with other endonucleases demonstrated that there were at least four unique inserts among the eight phage isolates.

None of the isolated phage, which were tested for immunologically recognized B-galactosidase fusion proteins in Western blots, gave a protein of higher molecular mass than B-galactosidase. Immunologically recognized proteins with a slightly higher molecular mass than *A. cycloclastes* Cu-dNir were observed in some of the phage lysates. The possibility that these represented authentic degradation fragments of B-galactosidase fusion protein bearing Cu-dNir epitopes was raised, but not explored further.

The final experimental cloning approach which has been used, is generation of DNA probes using mixed primer polymerase chain reaction (PCR) (Girgis et al., 1988). PCR reactions have been a collaborative effort between myself and Dr. Geoffrey Smith, with assistance provided by Dr. Jim Cole and Dr. W. Holben.

An attempt to clone the heme $\underline{cd}_1$-dNir was included in the PCR approach. The N-terminal amino acid sequence of *P. stutzeri* JM300 $\underline{cd}_1$-

dNir was previously determined by MSU's macromolecular sequencing facility for R. Ye, and is given in Figure 3.

1	2	3	4	5	6	7	8	9
ALA	ALA	PRO	ASP	MET	XXX	ALA	GLU	GLU
10	11	12	13	14	15	16	17	18
LYS	ALA	ALA	LYS	LYS	ILE	TYR	PHE	GLU
19	20	21	22	23	24	25	26	27
ARG	XXX	ALA	GLY	XXX	HIS	GLY	VAL	LEU
28	29	30	31	32	33	34	35	
LEU	LYS	GLY	ALA	THR	GLY	LYS	ASN	

Figure 3. The N-terminal amino acid sequence of heme cd_1-dNir from *P. stutzeri* JM300. XXX indicates that the amino acid is not known.

A search for sequence data on NO_2^- reductase using Sequence Analysis Software (SAS, Genetics Computer Group, Madison, WI) was unsuccessful. No information was available in any of the data bases searched, including: GenBank, EMBL, VecBase, NBRF-Nucleic, and NBRF-Protein. The structural gene for N_2O reductase from *P. stutzeri* has been cloned and sequenced by Viebrock and Zumft (1988) and the codon usage identified. We used this codon usage as a basis on which to generate the PCR primers. In cases where the codon usage was redundant, the most frequently used bases at each wobble position were employed. This reflected a bias towards G and C at the wobble position (Viebrock and Zumft, 1988).

Primers were prepared by Genetic Designs Inc. (Houston, TX) and were used without further purification or verification that they represented the desired sequences. Each primer was 5′ to 3′, 15 bases long, and from nonoverlapping regions of the N-terminus. Heme A

represents the codon choices for amino acids 1-5 (Figure 4). Heme B represents the codon choices for amino acids 13-17 (Figure 4). The total length of the anticipated amplified gene product was 51 base pairs.

Heme A

5′ - GCSGCSCCGGAYATG - 3′

Heme B

5′ - GAAGTAGATTCTTCTT - 3′

Figure 4. PCR oligonuleotide primers derived from the N-terminal amino acid sequence of *P. stutzeri* $\underline{cd}_1$-dNir. S = G+C; Y = T+C.

The codon usage of *P. stutzeri* N_2O reductase was also used to generate primers for Cu-dNir since no information on any proteins from *A. cycloclastes* were available in the data bases searched. Each primer was 5′ to 3′, 15 bases long, and from a nonoverlapping region of the N-terminus of *A. cycloclastes* Cu-dNir. These primers were also synthesized by Genetic Designs Inc. Cu A represents the codon choices for amino acids 5-9 (Figure 5) and Cu B represents the codon choices for amino acids 25-30 (Figure 5). The total length of the anticipated amplification product was 75 base pairs.

Cu A

5′ - ATCTCCACCCTSCCG - 3′

Cu B

5′ - GGTCTTSGCSACCTG - 3′

Figure 5. PCR oligonucleotide primers derived from the N-terminal amino acid sequence of *A. cycloclastes* Cu-dNir. S = G+C.

PCR was done using the Gene Amp Kit from Perkin Elmer Cetus and thermostable Taq Polymerase in the standard Cetus PCR experimental protocol. Annealing temperatures, and extension time were varied, but no amplified gene products corresponding to the anticipated product length were obtained.

It was thought that perhaps the codon choice had made the primers nonspecific by overemphasizing G and C in the wobble position. New primers for *P. stutzeri* cd_1-dNir were obtained from Genetic Designs Inc., which were completely mixed. CD1 was 15 bases long, and represented codon choices for amino acids 31-35 (Figure 6). CD2 was 15 bases long, and represented codon choices for amino acids 13-17 (Figure 6). The anticipated amplification product length was 69 base pairs.

CD 1

5' - RTTYTTNCCVGTNGC - 3'

CD 2

5' - AARAARATHTAYGAR - 3'

Figure 6. PCR oligonucleotides derived from the N-terminal amino acid sequence of *P. stutzeri* cd_1-dNir. H = A+C+T; N = A+G+C+T; R = G+A; V = A+G+C; Y = T+C.

This ended my actual contribution to the cloning work. Subsequent PCR with the new primers has also failed to give amplified gene products in the desired size range.

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