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Immunological Identification and Distribution of Dissimilatory Heme cd_1 and Nonheme Copper Nitrite Reductases in Denitrifying Bacteria†

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Polyclonal antibodies were used to identify heme or copper nitrite reductases 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. Antisera were raised against heme nitrite reductases from *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* and against copper nitrite reductase from *Achromobacter cycloclastes*. Nitrite reductases were identified by Western immunoblot. Diethyldithiocarbamate, which specifically inhibits copper nitrite reductases, was used to confirm the immunological characterization and determine which type was present in strains nonreactive with any antiserum. For groups in which the type of nitrite reductase has not been previously described, we found that *Alcaligenes eutrophus*, *Bacillus azotoformans*, *Bradyrhizobium japonicum*, *Corynebacterium nephridii*, and *Rhizobium* spp. contained copper nitrite reductase, while *Aquaspirillum itersonii*, *Flavobacterium* spp., and *Pseudomonas fluorescens* contained heme nitrite reductase. Heme nitrite reductases dominated, regardless of soil type or geographic origin. They occurred in 64 and 92%, respectively, of denitrifiers in the numerically dominant and nonselected collections. The two nitrite reductase types were mutually exclusive in individual bacteria, but both appeared in different strains from the *Alcaligenes* and *Pseudomonas* genera. The heme type predominated in *Pseudomonas* strains. The heme-type nitrite reductase appeared more conserved if judged by similarities in molecular weights and immunological reactions. The Cu type was found in more taxonomically unrelated strains and varied in molecular weight and antiserum recognition.

Dissimilatory nitrite reductases (dNirs) are pivotal to the fate of combined nitrogen in the environment. They determine the point at which nitrogen is dissimilated instead of assimilated (24). Two distinct types of nitrite reductase are known: one contains a Cu center, and the other contains hemes *c* and d_1. 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 (6, 32).

Cytochrome cd_1 dNir has been identified in *Alcaligenes faecalis* (18), *Micrococcus* (*Paracoccus*) *denitrificans* (22), *Paracoccus halodenitrificans* (5), *Pseudomonas aeruginosa* (34), *Pseudomonas stutzeri* (12), and *Thiobacillus denitrificans* (14). Nonheme Cu dNir has been found in *Achromobacter cycloclastes* (8), *Alcaligenes* sp. (*Achromobacter xylosoxidans*) (17), *Alcaligenes faecalis* S6 (10), *Nitrosomonas europaea* (25), and *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* (*Rhodobacter sphaeroides*) (27).

The distribution of the two dNir types in the environment is unknown, although cd_1 dNir has been identified most often in denitrifying isolates. These strains, which are typically studied in the laboratory, do not reflect the dominant denitrifying populations in nature (4). One way to screen denitrifiers for dNir type is to distinguish between Cu and heme components. Shapleigh and Payne used diethyldithiocarbamate (DDC), a Cu chelator, to identify Cu dNir (28). Another approach is to characterize the proteins with polyclonal antibodies specific for each dNir type.

Körner et al. (12) showed limited cross-reactivity in immunodiffusion assays with cd_1 dNirs from pseudomonads. Michalski and Nicholas demonstrated that polyclonal antibodies specific to Cu-type dNir from *R. sphaeroides* forma sp. *denitrificans* cross-reacted with Cu dNir in two other denitrifying *R. sphaeroides* strains (20). They also cross-reacted with Cu dNir from *A. cycloclastes*, *Alcaligenes denitrificans*, and two *Pseudomonas* spp. (21). This confirmed that the dNir type differed among closely related *Pseudomonas* spp., since Zumft et al. (36) had already found Cu dNir in *Pseudomonas aureofaciens*.

In this article, we report our use of polyclonal antibodies in Western immunoblots to identify the distribution and immunological relatedness of cd_1- and Cu-type dNirs in taxonomically diverse denitrifiers and to determine the predominant type of dNir found in numerically dominant denitrifiers from the environment. We also used DDC to confirm the immunological identification and to characterize the dNir type in denitrifiers which did not react with antisera against either dNir type.

MATERIALS AND METHODS

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. (4). To examine strains not selected on the basis of denitrifying capacity, we used a collection isolated by W. Holben from aerobic 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading

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

Strain	Source	Response[a]	
		cd_1 dNir	Cu dNir
Achromobacter cycloclastes	ATCC 21921	−	+
A. xylosoxidans NCIB 11015	H. Iwasaki	−	+
Aquaspirillum itersonii	ATCC 11331	+	ND
Azospirillum lipoferum	ATCC 29707	+	−
Bacillus azotoformans	ATCC 29788	ND	+
Bradyrhizobium japonicum	ATCC 10324	−	−
Corynebacterium nephridii	ATCC 11425	ND	+
Flavobacterium sp.	ATCC 33514	+	ND
Halobacterium denitrificans S1	L. Hochstein	−	−
Halobacterium sp. D4	L. Hochstein	−	−
Halobacterium sp. Baja 12	L. Hochstein	−	−
Paracoccus denitrificans	ATCC 19367	+	−
Pseudomonas 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	−	−
Rhodopseudomonas sphaeroides f.sp. *denitrificans* I1106	T. Satoh	−	−

[a] +, Positive immunological response; −, negative immunological response; ND, not determined.

soil enrichments. Of the 250 isolates, 51 were respiratory denitrifiers, on the basis of diagnostic criteria summarized by Tiedje (30), 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).

Media and growth conditions. For immunological screening, strains were grown in test tubes (16 by 125 mm) sealed with butyl rubber septa. The tubes contained 10 ml of medium, an inverted Durham tube, and an initially aerobic headspace. Most strains were grown in tryptic soy broth (Difco Laboratories) supplemented with 1.0 g of KNO_3 or 0.4 g of KNO_2 per liter. *Rhizobium* spp. and *Bradyrhizobium japonicum* were grown in yeast extract mannitol broth (YEMB) (35) supplemented with 1.0 g of KNO_3 per liter. *Halobacterium* species were grown in HYH-2 medium (containing the following per liter: 5.0 g of yeast extract, 2.0 g of casein, 176.0 g of NaCl, 5 g of KNO_3, 20.0 g of $MgCl_2 \cdot 6H_2O$, 2.0 g of KCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 13.0 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [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 of tryptic soy broth in 250-ml Erlenmeyer flasks sealed with rubber stoppers. Bacterial growth depleted headspace oxygen 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 of tryptic soy broth until stationary phase was reached. The flasks were then sealed with rubber stoppers, flushed with sterile argon, and incubated for 24 h at 30°C before harvest to induce denitrifying enzymes. *Rhizobium* spp. and *B. japonicum* were cultured aerobically in 2-liter flasks with 1 liter of YEMB until turbid and were then shifted to anaerobic conditions as described above.

Immune sera and immunological techniques. Polyclonal antibodies against dNir were raised in New Zealand White rabbits. Purified Cu-type dNir from *A. cycloclastes* ATCC 21921 was provided by Hulse et al. (7). Purified cd_1-type dNir from *P. aeruginosa* ATCC 10145 and *P. stutzeri* JM300 was provided by E. Weeg-Aerssens. The enzymes were emulsified in 1 ml of Freund incomplete adjuvant and injected subcutaneously. Booster injections of enzyme in Freund incomplete adjuvant were given at 8 and 16 weeks. Blood samples were taken at 2 weeks and on a weekly basis thereafter. Immunization was followed by Ouchterlony double immunodiffusion (9), and antisera were used without further purification.

Cultures which met one of the following criteria for denitrification were harvested for immunoscreening: (i) bubble formation in the inverted Durham tube; (ii) accumulation of N_2O; or (iii) removal of NO_3^- and NO_2^- from the medium, as detected by the diphenylamine test (29). A sample of the culture was centrifuged, the pellet was suspended in sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue), and the cells were then lysed in a boiling water bath (13). Strains were extracted by phenol (3) 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-polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels and transferred by electrophoresis onto nitrocellulose (31).

All dilutions and incubations for dNir detection were in TBST buffer (10 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 0.05% Tween 20) (Western blot AP system; Promega Biotec). The nitrocellulose filters were incubated for 1 h with 1% bovine serum albumin to block nonspecific binding and then incubated for 1 h with a 1:1,000 dilution of antiserum. The filters were rinsed in TBST three times for a total of 15 min and then incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated anti-rabbit goat immunoglobulin G (Sigma Chemical Co.). The filters were rinsed in TBST as before, and the dNirs were visualized by development of purple color following incubation with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) substrate (Sigma).

The criteria for a positive immunological response were (i) recognition (color development) of proteins of approximately the same molecular weights as simultaneously recognized purified dNirs or proteins from denitrifiers of a known dNir type and (ii) 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 8,000 × *g* 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 lb/in^2, divided into aliquots, and stored at −70°C. Prior to use, the crude extracts were spun for 5 min at 4°C in a Microfuge to remove debris; this supernatant was used in all enzyme assays. Protein content in crude extracts was measured by the method of Lowry et al., using bovine serum albumin as a standard (16).

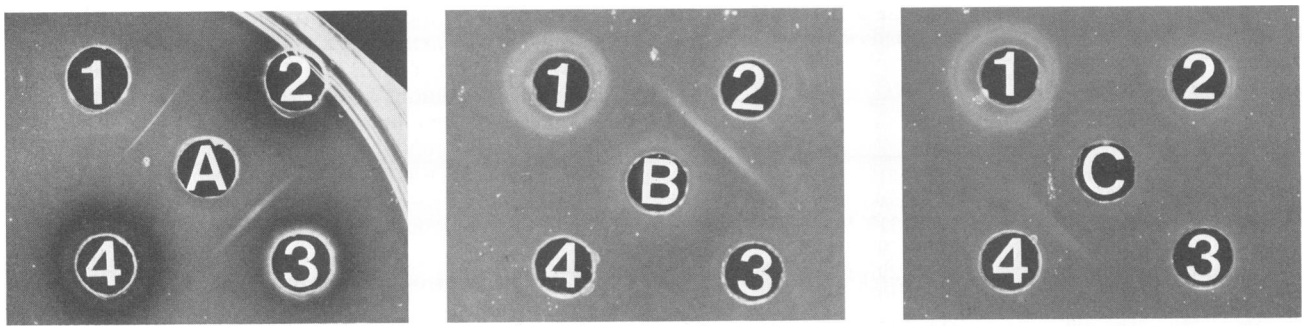

FIG. 1. Ouchterlony immunodiffusion of Cu and cd_1 dNir antisera. (A to C) Center wells contain antiserum to *A. cycloclastes* ATCC 21921 Cu dNir, *P. aeruginosa* ATCC 10145 cd_1 dNir, and *P. stutzeri* JM300 cd_1 dNir, respectively. Outer wells: 1, *A. cycloclastes* ATCC 21921 crude extract; 2, *P. aeruginosa* ATCC 10145 crude extract; 3, *Achromobacter xylosoxidans* NCIB 11015 crude extract; 4, *P. stutzeri* JM300 crude extract.

In vitro activity stain. Denitrifying cells were ruptured by French press, the cells were removed by centrifugation, and the proteins were separated by nondenaturing 10% PAGE at 4°C and 100 V for 20 h. Sites with nitrite-reducing activity were located by the method of Zumft et al. (36). Gels were stained for 5 min with 10 mM methyl viologen in 50 mM HEPES buffer (pH 7.3) with 2 mg of sodium dithionite per ml. The gels were then immersed in 1 M $NaNO_2$ (in HEPES buffer), which caused rapid bleaching where dNir was located. DDC (10 mM) was incorporated in some of the activity stains to preferentially inhibit Cu dNir activity (28).

Inhibition of dNir activity by antisera. Purified Cu dNir and extracts prepared by French press of *A. cycloclastes* ATCC 21921 and *P. aeruginosa* ATCC 10145 were incubated for 24 h at 4°C with an equal volume of buffer, preimmune serum, Cu dNir rabbit antiserum, or cd_1 dNir rabbit antiserum. Precipitated proteins were removed by centrifugation, and 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.

Assay for NO and N_2O production. Enzymatic nitrite reduction was measured as NO and N_2O evolution from crude extracts by using NADH and phenazine methosulfate as the electron-donating system. Assays contained 1 mM NO_2^-, 0.12 mM phenazine methosulfate, 2 mM NADH, and crude extract (generally 200 μl) 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, East Lansing, 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 (28). All enzyme assays were at 24°C.

Gas formation was monitored on a 910 gas chromatograph (The Perkin-Elmer Corp.) equipped with dual columns and ^{63}Ni electron capture detectors for simultaneous analysis of two samples (11). The carrier gas was 95% argon–5% methane. The temperatures were as follows: detector, 300°C; injector, 70°C; and column, 30°C. The columns (2 m by 2 mm, inside diameter) were stainless steel filled with Porapak Q. The carrier gas flow was adjusted to 15 ml/min to maximize NO and O_2 separation. Nitrite reductase activity was measured in the presence and absence of DDC by monitoring the initial rates of NO and N_2O evolution over 15 min. Each assay was done in duplicate. No NO or N_2O evolved in the absence of crude extract or in controls with autoclaved crude extract.

RESULTS

Specificity of antisera. Immunodiffusion assays indicated that the antisera were specific for the antigens against which they were raised. Neither type of cd_1 dNir antiserum cross-reacted with crude extracts containing Cu dNir, nor did they cross-react with crude extracts from the other cd_1-type denitrifier (Fig. 1A to C).

In activity-stained, nondenaturing PAGE, proteins with reductase activity in crude extracts of *A. cycloclastes* (Cu dNir) and *P. aeruginosa* (cd_1 dNir) (Fig. 2A) comigrated with immunologically recognized proteins in immunoblots of the same gels (Fig. 2B). Other proteins with reductase activity were not immunologically recognized. *A. 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.

Bleached regions with reductase activity cut from a duplicate gel of *A. cycloclastes* and *P. aeruginosa* crude extracts (indicated by the arrows in Fig. 2A) had enzymatic nitrite-reducing activities and produced 222 and 261 μmol of N gas (as a mixture of NO and N_2O), respectively, after 90 min of incubation in sealed vials. A control from a nonbleached

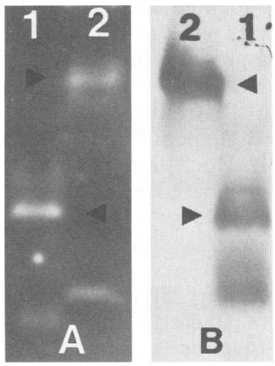

FIG. 2. Comigration of dNir activity with immunologically recognized proteins in denitrifier crude extracts. (A) dNir activity stain; (B) Western immunoblot with Cu and cd_1 dNir antisera. Lanes: 1, *A. cycloclastes* ATCC 21921; 2, *P. aeruginosa* ATCC 10145. Arrows indicate the location of comigrating proteins and dNir activity.

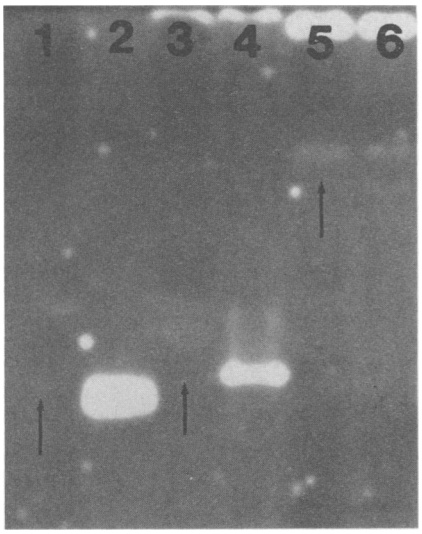

FIG. 3. Inhibition of Cu dNir activity by Cu dNir antiserum. Samples were preincubated with Cu dNir antiserum (lanes 1, 3, and 5) or buffer (lanes 2, 4, and 6). Lanes: 1 and 2, *A. cycloclastes* Cu dNir (1 μg); 3 and 4, *A. cycloclastes* ATCC 21921 crude extract; 5 and 6, *P. aeruginosa* ATCC 10145 crude extract. Arrows indicate where Cu dNir activity but not cd_1 dNir activity has been lost after preincubation with Cu dNir antiserum.

portion of the gel had no enzymatic activity. If DDC, a specific inhibitor of Cu dNir, was incorporated into activity stains, Cu but not cd_1 dNir activity was inhibited (data not shown).

Finally, Cu and cd_1 dNirs were not precipitated by preimmune serum, but activity stains of nondenaturing gels showed a specific loss of Cu dNir activity following incubation with Cu dNir antiserum (Fig. 3). The specific loss of cd_1 dNir activity after incubation with cd_1 dNir antiserum was observed in similar gels (data not shown).

Immunoscreen of denitrifying strains. A total of 23 strains from culture collections representing 17 taxonomically diverse denitrifying bacteria were screened by Western immunoblot for immunological recognition of Cu and cd_1 dNirs (Table 1). The criteria for a positive result were immunological recognition (as described in Materials and Methods) after incubation with rabbit antiserum and nonrecognition following incubation with antiserum from the opposite dNir type (Fig. 4A and B). Antiserum to *P. stutzeri* cd_1 dNir gave results similar to those for antiserum against *P. aeruginosa* cd_1 dNir.

The dNir type was identified for several strains in which dNir had not previously been characterized. *Aquaspirillum itersonii*, *Flavobacterium* spp., and *P. fluorescens* contained cd_1 dNir. *Bacillus azotoformans* and *Corynebacterium nephridii* contained Cu dNir. Of the 23 strains (tested by immunological recognition only), 10 contained cd_1 dNir and six 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* (19) are known to contain Cu dNir.

Distribution of dNir type in naturally occurring denitrifiers. One hundred denitrifying isolates from geographically diverse environmental samples were also screened in Western

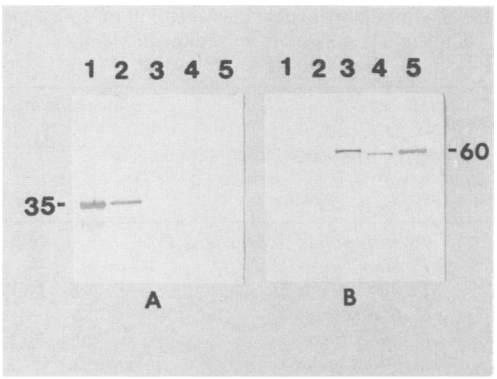

FIG. 4. Western immunoblots of denitrifier crude extracts separated on 10% SDS-polyacrylamide gels. (A) Immunoblot with *A. cycloclastes* ATCC 21921 Cu dNir antiserum; (B) immunoblot with *P. aeruginosa* ATCC 10145 cd_1 dNir antiserum. Lanes: 1, *A. cycloclastes* ATCC 21921 purified Cu dNir (1 μg); 2, *A. cycloclastes* ATCC 21921 crude extract; 3, *P. aeruginosa* ATCC 10145 crude extract; 4, *P. stutzeri* JM300 crude extract; 5, *P. stutzeri* JM300 purified cd_1 dNir (1 μg). Molecular mass indicators are given in kilodaltons.

immunoblots to determine the predominant dNir type in nature (Table 2).

Ninety-one isolates gave positive immunological responses to at least one of the antisera used in the analysis. Most strains (63 of 100) contained cd_1 dNir. The 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 (4). In cases in which isolates were screened with both types of cd_1 dNir antiserum, immunological recognition did not always coincide.

There were 28 Cu dNir-containing denitrifiers among the immunologically reactive isolates. These strains were predominantly *Alcaligenes faecalis* and *Pseudomonas* strains which Gamble et al. (4) could not easily characterize to the species level (Table 2). One isolate (isolate 65, an *Alcaligenes* sp.) had a positive immunological response to antisera against Cu dNir and *P. stutzeri* cd_1 dNir but not to antiserum against *P. aeruginosa* cd_1 dNir. Only Cu dNir was detected by DDC analysis (Table 2). The response to this particular cd_1 dNir antiserum was probably nonspecific. DDC inhibited dNir activity in all denitrifiers determined to be Cu type by immunoblotting (Table 2).

Of the nine isolates which gave no immunological response to any of the antisera, DDC analysis indicated that four contained Cu dNir (isolates 39, 87, 192, and 193) and one contained cd_1 dNir (isolate 83). The data for some of these strains are included in Table 3. The remaining four strains (strains 97, 103, 108, and 115) could not be checked by DDC analysis because of poor growth under denitrifying conditions. In no case were cd_1 and Cu dNirs both present in the same isolate.

The matrix group was not an adequate basis on which to predict the dNir type of an isolate. Seemingly closely related strains within the same matrix group differed in dNir type, particularly *Alcaligenes faecalis* isolates.

The subunit molecular weights of cd_1 dNirs varied little compared with those of Cu dNirs. At least four distinct Cu dNirs were recognized after SDS-PAGE, with molecular weights ranging from 35,000 to 91,000 (Fig. 5).

Fifty-one denitrifying isolates from aerobic 2,4-D-degrading soil enrichments were also used for immunological

TABLE 2. Immunochemical classification of dNir type in denitrifying isolates from sediments, sludges, and soils of six countries

Isolate no.	Similarity matrix group[a]	Species or group[a]	Immunoblot reaction[b]			DDC inhibition[c]
			Cu	cd_1		
				Pa	Ps	
13	1A	*Pseudomonas 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	*Pseudomonas* 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	*Pseudomonas* type 5	−	+	+	ND
52	1A	*P. fluorescens* II	−	+	+	ND
69	1A	*Pseudomonas* type 5	−	+	+	ND
71	1A	*Pseudomonas* type 5	−	+	+	ND
74	1A	*Pseudomonas* type 5	−	+	+	ND
111	1A	*P. fluorescens*	−	+	+	−
85	1B	*Pseudomonas* type 7	−	+	+	−
183	1B	*P. fluorescens* IV	−	+	−	ND
185	1B, 1C	*P. fluorescens* IV	−	ND	+	−
15	1B, 1C	*P. fluorescens*	−	+	ND	ND
12	1C	*Pseudomonas* type 2	−	+	−	−
54	1C	*Pseudomonas* type 2	−	+	+	ND
83	1C	*Pseudomonas* type 2	−	−	−	−
81	1C, 1D	*Pseudomonas* type 2	−	+	ND	ND
110	1C, 1D	*Pseudomonas* type 12	−	+	+	ND
55	1C, 1D	*Pseudomonas* type 4	−	+	ND	−
78	1C, 1D	*Pseudomonas* type 6	−	+	+	ND
58	1C, 1D	*P. fluorescens* II	−	+	+	ND
80	1D	*P. fluorescens* II	−	+	ND	−
190	1	*P. fluorescens*	−	+	+	ND
206	2	*P. fluorescens* IV	−	+	−	ND
107	2	*Pseudomonas* type 11	+	−	−	ND
163	2	*Pseudomonas* type 11	+	−	−	+
188	2	*Pseudomonas* type 11	+	−	ND	+
143	2	*Pseudomonas* type 11	+	ND	−	+
234	2	*Pseudomonas* type 11	+	−	−	+
149	2, 3	*Pseudomonas* type 11	+	−	−	+
98	2	*P. fluorescens* II	+	−	−	ND
133	3	*Pseudomonas* type 18	+	ND	−	ND
135	3	*Pseudomonas* type 18	+	ND	−	ND
1181	3	*Pseudomonas* type 16	+	−	ND	ND
141	3	*Pseudomonas* type 16	+	−	−	ND
172	3, 4	*Pseudomonas* type 11	+	−	−	+
14	3	*Pseudomonas* type 1	−	+	ND	ND
156	4	*P. aeruginosa*	−	+	ND	ND
87	4, 5	*Pseudomonas* type 8	−	−	−	+
103	5A	*Pseudomonas* type 10	−	−	−	ND
18	5A	*Alcaligenes faecalis*	−	+	ND	ND
20	5A	*A. faecalis*	−	+	ND	ND
4	5A	*A. faecalis*	−	+	ND	ND
30	5A	*A. faecalis*	−	+	ND	ND
43	5A, 5B	*A. faecalis*	+	−	−	+
99	5B	*A. faecalis*	−	+	−	ND
90	5B	*A. faecalis*	−	+	ND	ND
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	*Pseudomonas* type 19	+	−	−	+
153	6	*Pseudomonas* type 19	+	−	−	+
179	6, 7	*Pseudomonas* type 19	+	−	−	+
148	7	*Alcaligenes faecalis*	+	−	ND	ND
232	7	*Pseudomonas* type 25	+	−	−	ND
39	7, 8	Unknown type 3	−	−	−	+
221	9	*Pseudomonas stutzeri*	−	+	ND	ND
224	9, 10	*P. stutzeri*	−	+	−	ND
2312	9, 10	*P. stutzeri*	−	+	+	−
195	9, 10	*P. stutzeri*	−	+	+	ND
108	9, 10	Unknown type 3	−	−	−	ND
154	9, 10	*Alcaligenes eutrophus*	+	−	−	+
106	9, 10	*A. faecalis*	−	+	−	ND
97	10	*Pseudomonas* type 9	−	−	−	ND
177	10	*Flavobacterium* sp.	−	+	+	ND
46	10	*Flavobacterium* sp.	−	+	ND	ND
192	>10	*Bacillus* sp.	−	−	−	+
193	>10	*Bacillus* sp.	−	−	−	+
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	*Alcaligenes faecalis*	+	−	−	+

[a] Species identification and similarity matrix grouping are from Gamble et al. (4). 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, Antiserum raised against *A. cycloclastes* ATCC 21921 Cu dNir; Pa, antiserum raised against *P. aeruginosa* ATCC 10145 cd_1 dNir; Ps, antiserum raised against *P. stutzeri* JM300 cd_1 dNir; +, positive immunological response; −, negative immunological response; ND, Not determined.

[c] + or − indicates whether inhibition of dNir activity occurred after preincubation with 12.5 mM DDC for 20 min.

screening. Forty-seven of these isolates contained cd_1 dNir. There was no apparent correlation between the abilities to denitrify and to degrade 2,4-D or between dNir type and 2,4-D degradation.

Chemical inhibition assays with DDC. The immunological results were confirmed by DDC inhibition assays. Nitrite reductase activity in cd_1 dNir denitrifiers (measured as NO and N_2O evolution) was inhibited only 0 to 14% by preincubation with DDC but was almost completely inhibited in Cu dNir denitrifiers (Table 4). 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. NO_2^- reduction to N_2O by Cu dNir denitrifiers was completely inhibited by DDC. There was complete agreement between

TABLE 3. Effect of DDC on nitric and nitrous oxide evolution by denitrifier crude extracts that showed no reaction with antisera against Cu or cd_1 dNirs

Strain (isolate no.)	Activity (nmol/min per mg of protein)					
	NO			N_2O		
	− DDC	+ DDC	% Inhibition	− DDC	+ DDC	% Inhibition
Bacillus sp. (192)	0	0		0.6	0	100
Bradyrhizobium 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	
Rhodopseudomonas sphaeroides forma sp. *denitrificans*	3.6	0.2	94	0.2	0	100
Unknown type 3 (39)	0.3	0	100	0.2	0	100
Pseudomonas type 2 (83)	0	0		0.2	0.5	0
Pseudomonas type 8 (87)	0.7	0	100	0	0	

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 (isolate 83), DDC almost completely inhibited dNir activity in crude extracts of these nonimmunoreactive strains (Table 3). Cu dNir-containing strains included a *Bacillus* sp., *B. japonicum*, and two *Rhizobium* species. NO but not N_2O was evolved by *B. japonicum* and *Rhizobium* sp. strain 32H1. Since nitrous oxide 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 of these two genera have a Cu-type dNir.

TABLE 4. Effect of DDC on nitric and nitrous oxide evolution by denitrifier crude extracts for which dNir type was classified by immunoblots

Strain and dNir type (isolate no.)	Activity (nmol/min per mg of protein)					
	NO			N_2O		
	− DDC	+ DDC	% Inhibition	− DDC	+ DDC	% Inhibition
cd_1						
Pseudomonas type 2 (12)	0	0.2	0	1.9	2.4	0
Pseudomonas 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						
Achromobacter 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
Alcaligenes 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
Bacillus azotoformans ATCC 29786	3.9	0.9	77	1.4	0.2	86
Corynebacterium nephridii ATCC 11425	3.2	0	100	2.2	0.1	95
Pseudomonas type 19 (151)	1.1	0	100	4.7	0	100
Pseudomonas type 19 (153)	0.5	0	100	1.5	0.1	93
Unknown type 21 (189)	0.5	0	100	0.7	0	100

DISCUSSION

We identified the dNir type in more than 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 using DDC inhibition assays. dNirs which were not recognized by antisera were characterized with DDC inhibition assays. The heme-type dNir predominated in the environmental isolates. The data for the three culture groups studied (culture collections, numerically dominant denitrifiers, and aerobic soil isolates) are summarized in Table 5.

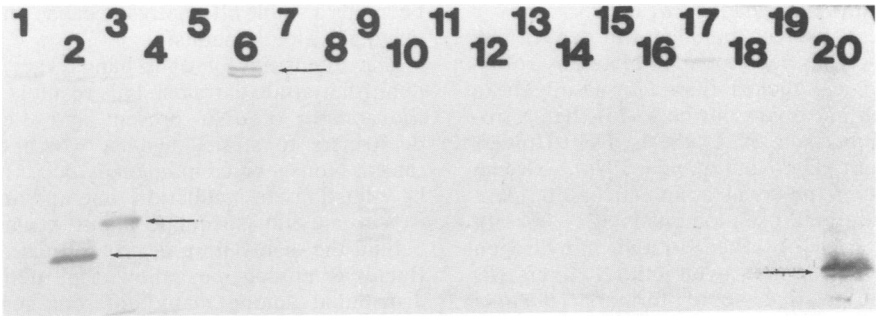

FIG. 5. Western immunoblot of denitrifier crude extracts separated on 10% SDS-polyacrylamide gels and immunoblotted with Cu dNir antiserum. Lanes: 1, *P. aeruginosa* ATCC 10145; 2, *Alcaligenes faecalis* isolate 41; 3, *Alcaligenes faecalis* isolate 40; 4, *P. fluorescens* isolate 15; 5, unknown type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

TABLE 5. Distribution of Cu- and heme cd_1-type dNirs

Culture group	No. examined	% dNir 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] Type based on immunological reactivity and/or response to DDC.
[b] Tested by immunological response only.
[c] Isolates not originally selected for denitrification ability.

Immunochemical recognition of Cu dNir was independent of whether the enzyme had type I or II Cu, since enzymes containing both types have been characterized (21, 23, 36) in strains recognized by our antiserum. Antiserum against Cu dNir from *R. sphaeroides* prepared by Michalski and Nicholas recognized Cu dNir in the eight denitrifiers they tested, including *A. cycloclastes* (21). Although Cu dNir from *A. cycloclastes* shares common epitopes with other Cu dNirs, the number of epitopes common between Cu dNir from *R. sphaeroides* and other denitrifiers may be greater than for *A. cycloclastes*. This may explain why some Cu dNirs were not recognized by our antisera.

We also noted (Table 2) that the immunological responses of cd_1-type dNirs sometimes differed, depending on what type cd_1 dNir antigen the antisera were raised against, i.e., *P. aeruginosa* versus *P. stutzeri*. This suggests greater variability in cd_1-type dNirs than is indicated by comparisons of molecular masses.

Our strains represented frequently studied laboratory cultures as well as the predominant denitrifiers from soils of four continents, sediments, and sludges (4). *Pseudomonas* spp. are the most prevalent denitrifiers in these environments (30), and 73% of the denitrifying *Pseudomonas* or *Pseudomonas*-type isolates (Tables 1 and 2) contained cd_1 dNir. This suggests that cd_1-type dNir predominates in nature. It was also the most prevalent nitrite reductase among isolates from our study, regardless of the geographic origin or environment from which the isolates were collected.

Isolation methods inevitably affect the type of denitrifiers recovered. *Bacillus* spp., for example, were the predominant population in one study of denitrifying soil isolates from undefined sites (1). However, even when selection was not originally made for denitrification (e.g., the isolates from 2,4-D-degrading soil enrichments), the denitrifiers among these isolates contained predominantly cd_1 dNir.

Cu dNir, nevertheless, may be significant in denitrifying ecosystems. The Cu type has been found in more physiological groups and ecological niches than cd_1 dNir. These groups include chemolithotrophic nitrifiers (25), heterotrophic nitrifiers (L. A. Robertson, Ph.D. thesis, Delft University of Technology, Delft, The Netherlands, 1988), extreme halophiles (L. Hochstein, personal communication), phototrophic bacteria (27), and pseudomonads (21, 36). In addition to these groups, we observed Cu dNir in hydrogen oxidizers (*Alcaligenes eutrophus*), symbiotic N_2 fixers (*B. japonicum*), and gram-positive spore formers (*Bacillus azotoformans*). As more genera are examined, however, cd_1 dNir appears in other taxonomic groups besides the genus *Pseudomonas*. We observed cd_1 dNir in *Aquaspirillum*, *Azospirillum*, and *Flavobacterium* species, in addition to *P. fluorescens*.

The nitrite reductase type may differ among closely related strains, notably *Pseudomonas* spp. (Table 1) (12, 21, 36). Of our *Alcaligenes faecalis* isolates, 47% contained Cu dNir and 53% contained cd_1 dNir. 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 dNir type present.

The Cu dNirs we observed were more heterogeneous than cd_1 dNirs. cd_1 dNirs give similar immunological reactions, and the subunit molecular weights were approximately 60,000. The subunit molecular weights of Cu dNirs were similar to some, but not all, of those which have been previously reported: 36,800 (15), 37,000 and 39,500 (19), and 40,000 (36). Most were larger (around 40,000) than the molecular weight of the *A. cycloclastes* standard (35,000) (7). The molecular weight of an immunoreactive protein in *Alcaligenes* isolate 171 was much greater than that of other Cu dNir types (Fig. 5). Cu dNir from *Alcaligenes* sp. strain S6 is a tetramer of molecular weight 120,000 which required extensive denaturing at 50°C before the various oligomers were separated (10). Isolate 171 may contain a similar Cu dNir. The variability of Cu dNirs among denitrifiers may reflect significant differences in Cu dNir types (17) or simply in processing of the enzyme. It may also explain why we were unable to recognize all Cu dNirs with our antisera.

We showed the utility of using DDC inhibition assays to identify dNir types in different physiological groups, as had been proposed by Shapleigh and Payne (28). In all cases, DDC inhibited dNir activity in Cu-type, but not cd_1-type, dNir denitrifiers. This verified the results of the immunoscreening. For the nonimmunoreactive isolates, the assays demonstrated, with one exception, that all contained Cu-type dNir. The dNir type in virtually all strains used in this study could therefore be identified by using a combination of these two methods. With the exception of *Aquaspirillum itersonii*, all of the genera for which we have 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 distinguishes Cu from cd_1 dNirs unambiguously. The method is rapid, and the analysis is simple to perform. The DDC method does not, however, 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 the assay is independent of dNir recognition. If many isolates are screened, however, raising antibodies against specific dNirs becomes a viable alternative because multiple isolates can be examined simultaneously.

The intensities of dNir bands vary in immunoscreened denitrifier crude extracts. This result is due to differences in the amount of dNir present and the relatedness of the denitrifiers to strains against which dNir antiserum was raised. Nonspecific binding may occur but can be minimized by adjusting the incubation time and antiserum dilution.

We can only speculate about evolutionary mechanisms behind the distribution of cd_1 and Cu dNirs among strains. Bacterial phylogenies show that dNir types are widely distributed among many different genera (33) and many different physiological groups (30). Denitrification may have risen in an ancestral photodenitrifier and, like photosynthesis in numerous branches of the purple bacteria, may have been lost in some strains over time (2). This would lead to closely related denitrifying and nondenitrifying species. Examples of truncated denitrifiers are numerous (30).

Differences of dNir type among *Pseudomonas* and *Alcali-*

genes spp. might be explained by horizontal gene transfer. Cu dNir may be plasmid borne in *Alcaligenes eutrophus* (26). Gene transfer provides a plausible route by which Cu dNir could have spread among pseudomonads and other groups. Perhaps this also explains why there is greater variation among Cu dNirs than among cd_1 dNirs. Since Cu and cd_1 dNirs were never observed in the same strain, we do not know whether the absence of co-occurrence results from acquisition of dNir by a truncated denitrifier or replacement of a preexisting dNir. It is also possible that the two types of nitrite reductases originated at very different times, the Cu type very early, with the variation resulting from divergence, and the heme cd_1 type much later, as a variation of oxygen respiration.

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