Localization of the Cytochrome cd_1 and Copper Nitrite Reductases in Denitrifying Bacteria†

MARK S. COYNE,[1] ALAHARI ARUNAKUMARI,[1,2] H. STUART PANKRATZ,[2] AND JAMES M. TIEDJE[1,2]‡*

Departments of Crop and Soil Sciences[1] and of Microbiology and Public Health,[2] Michigan State University, East Lansing, Michigan 48824

The locations of cytochrome cd_1 nitrite reductases in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and copper nitrite reductases in *Achromobacter cycloclastes* and *Achromobacter xylosoxidans* were identified. Immunogold labeling with colloidal-gold probes showed that the nitrite reductases were synthesized exclusively in anaerobically grown (denitrifying) cells. Little immunogold label occurred in the cytoplasm of these four strains; most was found in the periplasmic space or was associated with cell membranes. Immunogold labeling of thin sections was superior to fractionation by osmotic shock for locating nitrite reductases. The results support models of denitrification energetics that require a periplasmic, not a cytoplasmic, location for nitrite reductases.

Denitrification is a respiratory process in which nitrogenous oxides serve as terminal electron acceptors in the absence of O_2 (19). During the reduction of NO_2^- to N_2O, energy is conserved by oxidative phosphorylation (13). Consequently, nitrite reductases (dNirs) in denitrifying bacteria have been perceived as membrane-associated components of energy generation. Understanding this role requires information about their cellular localization.

Three approaches have been used: immunochemical detection, proton translocation measurements, and cell fractionation. Ferritin-conjugated antibody was used by Saraste and Kuronen (20) to locate dNir in *Pseudomonas aeruginosa* at the inner face of the cytoplasmic membrane. On the basis of proton translocation studies, dNir from *Paracoccus denitrificans* was placed on the inner face of the cytoplasmic membrane in one study (14) and in the periplasmic space in another (5).

Cell fractionation approaches have also given conflicting results. dNir has been identified in the cytoplasm (15, 26) and on the cytoplasmic membrane (15, 22, 26), where it is either tightly (15) or loosely (20, 26) bound. It has been found in the periplasm after spheroplast formation (2, 6, 24), although some activity remains associated with the spheroplast membranes (2), and as a soluble periplasmic protein (Mingawa and Zumft, 1988 [cited in reference 10]). All of these cultures contained cytochrome cd_1-type dNir. Among organisms with a Cu-type dNir, only *Rhodobacter sphaeroides* has been examined for the cellular location of its dNir, and there it was found in the periplasm by osmotic-shock procedures (21).

Although the evidence for the location of dNirs is not always consistent, the periplasmic location has been gaining favor. Our objective was to resolve the localization of dNirs in two strains that contain the cytochrome cd_1-type enzyme and two strains that contain the Cu-type enzyme, as this

latter class has not been well studied. *Pseudomonas fluorescens* was among the cytochrome cd_1-containing strains chosen for study, since it is the predominant denitrifier in nature and has not been previously examined. We attempted to visualize dNir location by immunogold labeling with colloidal-gold probes and to confirm this location by cell fractionation instead of relying solely on the cell fractionation or proton translocation procedures used in previous studies.

MATERIALS AND METHODS

Bacterial strains and growth. *Achromobacter cycloclastes* ATCC 21921, *P. aeruginosa* ATCC 10145, and *P. fluorescens* ATCC 33512 were obtained from the American Type Culture Collection. *Achromobacter xylosoxidans* NCIB 11015 was obtained from H. Iwasaki.

Strains were grown in 15 g of tryptic soy broth (Difco) per liter with 1 μM $CuSO_4 \cdot 7H_2O$ and 10 mM KNO_3. Plates and slants for routine cultures contained the same medium with 1.5% agar. For cell fractionation studies, strains were grown in sealed 500-ml sidearm flasks with 250 ml of medium. The headspace was made anaerobic by repeated flushing with sterile argon, and the flasks were inoculated with 12.5 ml of a late-log-phase culture of anaerobically grown cells. Growth was monitored by determining the optical density at 660 nm during incubation at 30°C. For immunogold labeling, cells were taken from freshly grown aerobic slants or plates, from aerobic broth cultures, or from anaerobic broth cultures which had dissimilated all NO_3^- in the medium.

Preparation of antisera. Antisera against *A. cycloclastes* Cu dNir and *P. aeruginosa* cd_1 dNir were raised in rabbits as described in a previous paper (8). Western blot (immunoblot) immunoassays showed no cross-reactivity of antisera to nonhomologous dNir antigen, and the antisera were used without further purification.

Immunogold labeling and electron microscopy. Cells from denitrifying broth cultures and aerobic plate or broth cultures were pelleted and suspended in 1% glutaraldehyde–0.1 M sodium cacodylate (pH 7.2) for 40 min at 4°C and then washed three times in 0.1 M sodium cacodylate for a total of 45 min. The cells were dehydrated in a graded series of

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ethanol washes before being embedded in Lowacryl K4M resin overnight (3). The samples were polymerized at 24°C under a UV lamp for 1.5 days. After polymerization, thin sections were cut with a diamond knife, collected onto nickel grids, and stored at 22°C until use.

Grids were placed specimen side down on a drop of TBST buffer (10 mM Tris [pH 7.4], 0.5% Tween 80, 2.5% NaCl) containing 1% bovine serum albumin. 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 for 10 min three times in TBST and incubated for 60 min with a 1:250 dilution of goat anti-rabbit antibody–15-nm colloidal-gold particles (Auroprobe EM GAR15; Janssen Life Science Products) (4). The grids were then washed for 10 min three times with TBST before receiving a final counterstain with uranyl acetate and lead citrate. Grids were examined with a Philips CM-10 electron microscope.

Cell fractionation and enzyme assays. The osmotic-shock method of Yamamoto et al. (25) was used to release periplasmic proteins. The periplasmic fraction was defined as those proteins released into the supernatant after osmotic shock. The shocked pelleted cells were suspended, sonicated, and centrifuged at 12,000 × g for 20 min, and the supernatant was used to assay components in this largely cytoplasmic fraction.

Enzymatic NO_2^- reduction was measured by the evolution of N_2O, as previously described (8). Acid phosphatase and glucose 6-phosphate dehydrogenase activity were measured as described by Absolom (1) and Maurer et al. (16), respectively.

RESULTS

Immunogold labeling. Immunocytochemical detection with colloidal-gold probes was used as the primary method to determine the localization of dNirs in denitrifying cells. Four strains containing either Cu (*A. cycloclastes, A. xylosoxidans*) or cd_1 (*P. aeruginosa, P. fluorescens*) dNirs were used. When colloidal-gold probes were used alone or in conjunction with preimmune serum, there were no gold particles associated with anaerobically grown (denitrifying) cells (not shown). Likewise, when aerobically grown (non-denitrifying) cells were incubated with primary antiserum and colloidal-gold probes, few gold particles were observed (Fig. 1A through 4A).

When anaerobically grown *A. cycloclastes* and *A. xylosoxidans* were incubated with Cu dNir antiserum and colloidal-gold probes, many gold particles were associated with the periplasmic spaces (Fig. 1B) or cell membranes (Fig. 2B) of these strains. This was also the case with anaerobically grown *P. aeruginosa* (Fig. 3B) and *P. fluorescens* (Fig. 4B) incubated with cytochrome cd_1 dNir antiserum and colloidal-gold probes. Autolysed *P. aeruginosa* cells in particular retained gold particles in association with membrane components (Fig. 3B), while gold particles accumulated in the periplasmic space of *P. fluorescens* (Fig. 4B).

Few gold particles were found in the cytoplasm, indicating that dNirs of both types were not soluble cytoplasmic enzymes in these denitrifying bacteria. This was not an artifact of the fixation treatments. Approximately the same fixation and incubation conditions were used to establish the cytoplasmic location of the urease enzyme in *Klebsiella aerogenes* (17), indicating that cytoplasmic enzymes are not lost by these procedures.

Cell fractionation. The periplasmic and cytoplasmic components of denitrifying cells were not successfully separated into discrete fractions by the osmotic-shock procedure. However, the periplasmic location of dNirs was supported by finding some of the dNir activity in the periplasmic fraction, while the activity of a cytoplasmic enzyme, glucose 6-phosphate dehydrogenase, was found in the cytoplasm and not in the periplasmic fraction. The mild osmotic shock did not completely release periplasmic proteins in these strains. Acid phosphatase activity, a marker enzyme for periplasmic proteins, and dNir activity were both found among cytoplasmic components derived from the osmotically shocked and sonicated cells.

DISCUSSION

Two independent methods were used to locate dNirs in denitrifying bacteria: immunogold labeling and cell fractionation. Immunochemical labeling has been previously used to locate nitrate reductase (9) and dNir (20), but immunogold labeling has not been previously used to examine denitrifying systems. Most studies with cytochrome cd_1 dNirs have been confined to *Paracoccus denitrificans* and *P. aeruginosa*. With the exception of those in *R. sphaeroides*, the locations of Cu dNirs have not been determined.

Our results indicate a specific association of gold particles only with those cells in which dNir was induced by denitrifying conditions. This binding was not associated with the cytoplasm. The immunogold localization suggests a periplasmic location for the dNirs of *A. cycloclastes, P. fluorescens, A. xylosoxidans*, and *P. aeruginosa*, although in the latter two bacteria (Fig. 2B and 3B), more gold particles were associated with membranes than with the periplasmic spaces.

In cells in which plasmolysis occurred, gold particles distinctly appeared between outer and cytoplasmic membranes in two of the four bacteria (Fig. 1B and 4B). In *P. aeruginosa* (Fig. 3B), it is not apparent whether gold particles attached to the cytoplasmic membrane are on the cytoplasmic or, as we suggest, the periplasmic side. However, recent studies with the sequenced cytochrome cd_1 dNir of *P. aeruginosa* indicate that the mature protein is preceded by a residue with features typical of a signal peptide, which suggests that the enzyme is secreted into the periplasm (23). Furthermore, 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 antibody–gold complexes may be as much as 20 nm away from the actual locations of the dNirs (12).

Immunogold labeling was clearly superior to cell fractionation procedures for dNir localization. After mild osmotic shock, most of the dNir and acid phosphatase activities remained with the cytoplasmic fraction, which contained cytoplasm, cell membranes, and periplasmic proteins from cells not fully disrupted by the osmotic-shock treatment. This was predictable, since periplasmic proteins will be lost only if the cell wall is grossly disrupted (2). An alternative to the osmotic-shock procedure was fractionation by spheroplast formation, but this has also given equivocal results. Spheroplast preparations from *R. sphaeroides* indicated that spheroplast supernatants (i.e., the periplasmic fraction) contained 90% of the dNir activity (21). However, in *Paracoccus denitrificans*, only 53% of the total dNir activity was released into spheroplast supernatants; the rest remained associated with cell membrane (2).
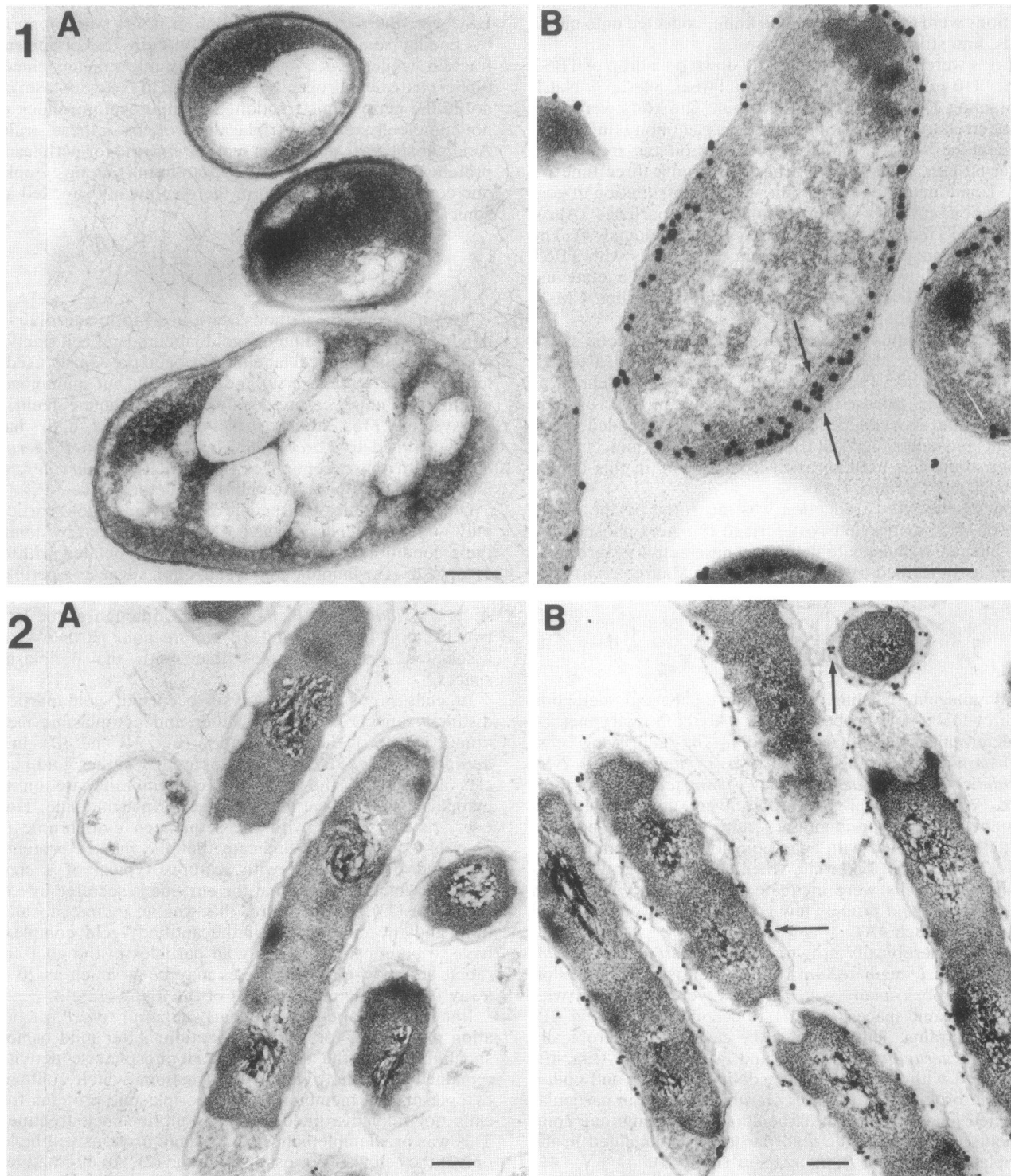
FIG. 1. Thin-section electron micrograph of *A. cycloclastes* incubated with Cu dNir antiserum and 15-nm colloidal-gold probes. Bars, 0.2 μm. Panels: A, aerobically grown; B, anaerobically grown. Arrows in panel B point to inner and outer membranes. Note the localization of the gold particles in the periplasmic space.

FIG. 2. Thin-section electron micrograph of *A. xylosoxidans* incubated with Cu dNir antiserum and 15-nm colloidal-gold probes. Bars, 0.2 μm. Panels: A, aerobically grown; B, anaerobically grown. Arrows in panel B point to association of the gold particles with the outer membrane.
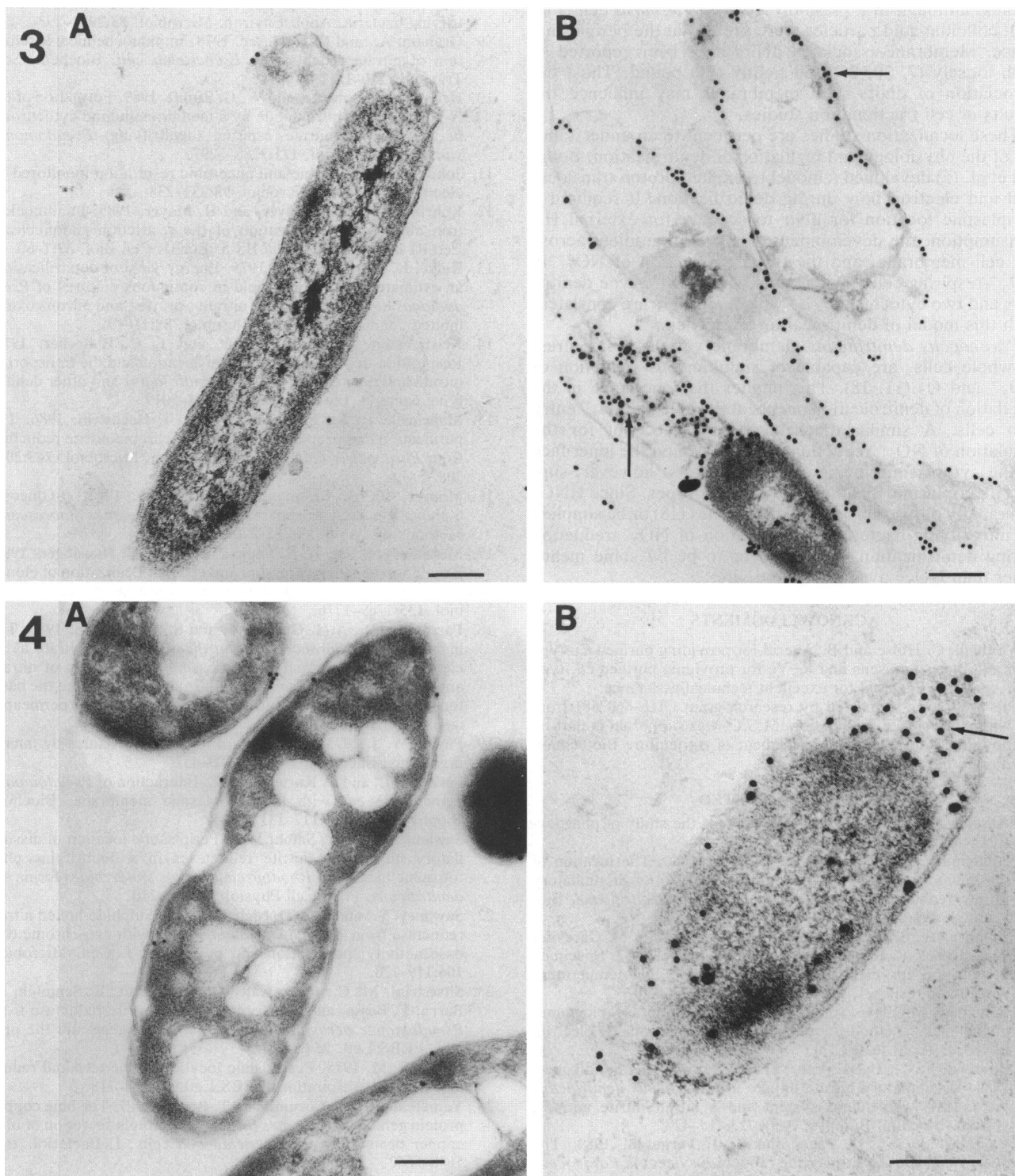
FIG. 3. Thin-section electron micrograph of *P. aeruginosa* incubated with cd_1 dNir antiserum and 15-nm colloidal-gold probes. Bars, 0.2 μm. Panels: A, aerobically grown; B, anaerobically grown. Autolysis of the denitrifying cells occurred. Arrows in panel B indicate where gold particles have remained associated with cell membranes and where they have accumulated in the periplasmic space of a plasmolyzed cell.

FIG. 4. Thin-section electron micrograph of *P. fluorescens* incubated with cd_1 dNir antiserum and 15-nm colloidal-gold probes. Bars, 0.2 μm. Panels: A, aerobically grown; B, anaerobically grown. Arrow in panel B points to localization of the gold particles in the periplasmic space.

Immunogold labeling suggested greater association of dNirs in *A. xylosoxidans* and *P. aeruginosa* with cell membranes, although in a plasmolyzed *P. aeruginosa* cell (Fig. 3B), colloidal-gold particles were present in the periplasmic space. Membrane-associated dNirs have been reported as both loosely (7, 20, 26) and tightly (15) bound. Thus, the association of dNirs with membranes may influence the results of cell fractionation studies.

These localization studies are pertinent to an understanding of the physiology and regulation of denitrification. Boogerd et al. (5) developed a model to explain proton translocation and electron flow during denitrification. It required a periplasmic location for dNir to account for external H^+ consumption, the development of a proton gradient across the cell membrane, and the further reduction of NO_2^- in NO_3^--respiring cells. Our results with two Cu-type denitrifiers and two cytochrome cd_1-type denitrifiers are consistent with this model of denitrification energetics.

Paracoccus denitrificans membrane vesicles, in contrast to whole cells, are capable of simultaneous reduction of NO_3^- and O_2 (11, 18). This implies that one stage in the regulation of denitrification occurs at the level of NO_3^- entry into cells. A similar mechanism could account for the regulation of NO_2^- reduction if it was also on the inner face of the cytoplasmic membrane. Our results, however, support a periplasmic location for both dNir types. Since HNO_2 can rapidly diffuse across cell membranes (18) or be supplied by nitrosifying bacteria, the regulation of NO_2^- reduction during denitrification would appear to be by some means other than NO_2^- availability to dNirs.

ACKNOWLEDGMENTS

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