Criteria and Methodology for Identifying Respiratory Denitrifiers

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Respiratory denitrification is not always adequately established when bacteria are characterized. We have tested a simple method that allows one to evaluate whether the two necessary criteria to claim denitrification have been met, namely, that N₂ or N₂O is produced from nitrate or nitrite and that this reduction is coupled to a growth yield increase. Microorganisms were cultured in sealed tubes under a helium headspace and in the presence of 0, 2, 4, 7, and 10 mM nitrate or nitrite. After growth had ceased, N₂ and N₂O were quantified by gas chromatography and the final protein concentration was measured. Net protein production was linearly related to nitrate concentration for all denitrifiers tested and ranged from 2 to 6 g of protein per mol of electron equivalent reduced. Nitrogen recovery as N₂ plus N₂O from nitrate and nitrite transformed exceeded 80% for all denitrifiers. We also suggest that a rate of N gas production of >10 µmol/min/g of protein can be used as an additional characteristic definitive of denitrification since this process produces gas more rapidly than other processes. These characteristics were established after evaluation of a variety of well-characterized respiratory denitrifiers and other N₂O-producing nitrate reducers. Several poorly characterized denitrifiers were also tested and confirmed as respiratory denitrifiers, including *Aquaspirillum itersonii*, *Aquaspirillum fasciculus*, *Bacillus azotoformans*, and *Corynebacterium nephridii*. These criteria distinguished respiratory denitrifiers from other groups that reduce nitrate or produce N₂O. Furthermore, they correctly identified respiratory denitrification in weak denitrifiers, a group in which the existence of this process may be overlooked.

Environmental scientists use the term denitrification to describe any process in which nitrate or nitrite is converted to nitrogen gases (19). Processes such as nitrification, dissimilatory nitrate reduction to ammonium, chemodenitrification, and other nonrespiratory N₂O production processes are examples of additional sources of N₂O, NO, and N₂ (2, 3, 26, 29). Microbiologists typically restrict the term denitrification to a bacterial respiratory process in which the sequential reduction of nitrogenous oxides is coupled to electron transport phosphorylation (19, 27, 34). Both definitions have merit. The environmental scientist is usually interested in the quantities of nitrogen in different pools and flux rates between those pools, with less concern about mechanisms causing those fluxes (29). The microbiologist is more interested in how the process benefits the microorganism and in understanding the unique features that are responsible for the process (12, 19, 27). We suggest that the term respiratory denitrification be used when this mechanism is important to identify or when misunderstanding may occur. Respiratory denitrification more correctly describes the process microbiologists are usually referring to when they describe denitrification (29, 34).

Many organisms that have been at some time termed denitrifiers have not actually been confirmed as respiratory denitrifiers (13, 19). This is because the simple test methods in common use do not necessarily establish respiratory denitrification or detect all denitrifiers (1, 29). The most common quick method is to test for nitrate removal from the medium (23). This has incorrectly led some to the assumption that the organism that removes nitrate must be a denitrifier. Now that dissimilatory nitrate reduction to ammonium is better known

(6, 20, 29), it can be understood how complete consumption of both nitrate and nitrite can lead to a product other than nitrogen gases. Other simple methods that have been used are not always specific for respiratory denitrifiers, i.e., bubble production in inverted (Durham) tubes (23), the increase in pH detected by an indicator dye in the medium (30), and the production of N₂O (18). These methods will detect many respiratory denitrifiers, but gases other than N₂ can form bubbles, processes other than nitrate removal can alter pH, and microbial reactions other than denitrification can produce N₂O (29). What is needed is a convenient method that will conclusively establish if an organism is a respiratory denitrifier and correctly detect even those organisms that are weak respiratory denitrifiers.

In this paper, we describe two criteria that must be met to claim respiratory denitrification and report typical values for these plus another characteristic, the rate of gas production, that is usually indicative of respiratory denitrification. We tested these methods with several well-studied denitrifier strains and evaluated other strains for which denitrification has not been confirmed or that are not particularly well studied. All of these organisms were confirmed to be respiratory denitrifiers and could be readily distinguished from nitrate-respiring ammonium producers, the group of organisms most likely to be misidentified as denitrifiers.

MATERIALS AND METHODS

Organisms. Bacterial strains *Achromobacter cycloclastes* ATCC 21921, *Aquaspirillum fasciculus* ATCC 27740, *Aquaspirillum itersonii* ATCC 12639, *Azospirillum brasilense* ATCC 29145, *Azospirillum lipoferum* ATCC 29707, *Bacillus azotoformans* ATCC 27988, *Corynebacterium nephridii* ATCC 11425, and *Bradyrhizobium japonicum* ATCC 10324 were obtained from the American Type Culture Collection, Rockville, Md. *Alcaligenes* sp. strain NCIB 11015 was obtained from the National Collection of Industrial Bacteria. *Pseudomonas stutzeri* JM 300 (4) was provided by J. Ingraham, University of California, Davis. The *Flavobacterium* sp. was isolated in this laboratory (9) and deposited with the American Type Culture Collection under accession number 33514. *Escherichia coli* K-12 ESH-10, *Enterobacter aerogenes* ENT-5-9, and *Serratia marcescens*

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SER-1 originated from the Department of Microbiology Culture Collection, Michigan State University. Stock cultures were kept on slants of tryptic soy agar (Difco) plus 0.1% KNO_3 and stored at 4°C.

Measurement of growth yield and quantity of N gases produced. Bacteria were grown in 26-ml anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.) containing 15 ml of tryptic soy broth for all strains except *Bradyrhizobium japonicum*, which was grown in standard yeast extract-mannitol medium. In order to determine nitrate- or nitrite-dependent growth yields, media were supplemented with 0, 2, 4, 7, and 10 mM KNO_3 or NaNO_2 . Tubes were capped with butyl rubber stoppers and aluminum crimp seals and were sterilized by autoclaving for 10 min at 121°C.

Media were inoculated with 0.3 ml of a culture previously grown anaerobically in medium of the same composition but supplemented with 4 mM nitrate. The headspace atmosphere of the inoculated tube was replaced with filter-sterilized helium by evacuating for 3 min and refilling the tubes three times. The tubes were then filled with He to a slight overpressure (~ 0.08 atm [~ 8.1 kPa]), the stopper was covered with silicone rubber to minimize leakage, and the tube was incubated in an inverted position to exclude direct contact of the gas phase with the stopper. Cultures were incubated at 28 or 35°C, depending on the requirements of the bacterial strain.

Growth of bacteria was monitored by measuring the optical density at 660 nm. When the culture entered the stationary growth phase, the gas pressure was measured by a pressure transducer (Uni Measure, Inc., Grants Pass, Oreg.) attached to a needle (17). A gas sample (0.2 ml) was taken through the rubber stopper with a gas-tight syringe (tuberculin type) flushed with helium and fitted with a Mininert valve. The gas sample was equilibrated to 1 atm (ca. 100 kPa) and immediately analyzed for N_2O and N_2 by gas chromatography. Gas production was calculated on the basis of measured headspace concentration; the volume increase was calculated from the pressure measurement and corrected for dissolved gas, using the Bunsen coefficient (28, 31). Immediately after the gas was sampled, 5- to 10-ml samples of liquid phase were taken to determine cell biomass. Cells were harvested by centrifuging the samples for 10 min at $10,000 \times g$ and washing them three times with 50 mM phosphate buffer by resuspending and centrifuging each time. The final cell suspension was analyzed by the method of Lowry et al. for protein content after alkaline digestion of the cells (10). Bovine serum albumin was used as the protein standard.

Protein was measured in media without nitrate or nitrite to correct for cells carried over from the inoculum, any increased growth due to fermentative metabolism, and possible residual traces of oxygen in the tubes. Molar growth yields (Y) were calculated from the linear relationship between net protein produced and moles of electron (e^-) equivalent consumed. The latter was determined after correcting for any residual NO_3^- or NO_2^- and the distribution of N_2O and N_2 in the products.

Rate of N_2O production by resting cells. Bacterial strains were grown anaerobically in tryptic soy broth medium supplemented with 10 mM KNO_3 . After nitrate and nitrite were used, cells were harvested by centrifuging for 10 min at $10,000 \times g$ and washed three times with 50 mM phosphate buffer (pH 7.0) containing chloramphenicol (200 $\mu\text{g}/\text{ml}$) (16). Cells were then resuspended in the same buffer. Washed cells were stored at 4°C until use but not longer than 24 h. Cell biomass was quantified by protein determination (9).

The assay mixture was prepared in 6-ml serum bottles capped with butyl rubber stoppers and contained cells (0.1 to 3.0 mg of protein), 4 μmol of KNO_3 or NaNO_2 , and 400 μg of chloramphenicol in 2 ml of tryptic soy broth medium buffered with 50 mM phosphate, pH 7.0 (16). The atmosphere in the assay bottle was replaced with a gas mixture of 90% He and 10% acetylene, and the bottle was held for 10 min at 35°C to equilibrate the temperature to the incubation condition. After the addition of KNO_3 or NaNO_2 , samples of headspace gas (0.2 ml) were removed at 5- to 10-min intervals. The N_2O produced was corrected for changes in volume resulting from removal of gas samples and for dissolved N_2O .

Analytical methods. Nitrate and nitrite concentrations were measured with a Technicon Autoanalyser II equipped with a Cd reduction column.

N_2 and N_2O in headspace samples were quantified on a gas chromatograph equipped with a microthermistor detector (Carle model 8515). Two columns (1.8 m by 3.2 mm each), one filled with Porapak Q and the other filled with molecular sieve 5A, were connected in series and bypass configurations by a six-port switching valve to provide separation of both N_2 and N_2O (25). The carrier gas was He at a flow rate 30 ml/min; the column temperature was 50°C.

RESULTS

Energy conservation efficiency. A linear relationship between biomass produced and N-anion concentration was observed for all denitrifiers grown on NO_3^- (Fig. 1A) and NO_2^- (Fig. 1B), except the strains for which NO_2^- appeared to be toxic (representative examples shown in Fig. 1B). Nitrite at concentrations of >4 mM was inhibitory to *P. stutzeri*, *Azospirillum lipoferum*, *Alcaligenes* sp., and *Achromobacter cycloclastes*, and at concentrations of >2 mM it was inhibitory for *Bradyrhizobium japonicum*, *C. nephridii*, and the two *Aquaspi-*

FIG. 1. Growth yields of strains on nitrate and nitrite that illustrate different patterns. (A) Nitrate: Δ , *E. coli*; $\circ$, *Achromobacter cycloclastes*; $\bullet$, *Bacillus azotoformans*; $\blacksquare$, *Azospirillum lipoferum*; and $\square$, *Aquaspirillum itersonii*. (B) Nitrite: Δ , *E. coli*; $\circ$, *Flavobacterium* sp.; $\bullet$, *Bacillus azotoformans*; $\square$, *P. stutzeri*; and $\blacksquare$, *Bradyrhizobium japonicum*. Data are not corrected for any incomplete reduction of nitrate or nitrite. The standard errors of the mean are shown.

illum strains. This interpretation was supported by finding residual NO_2^- present only in the tubes which did not show a linear increase in biomass with NO_2^- concentration. The large difference in efficiency of energy conservation among the various strains was surprising (Table 1). This comparison can be made because the medium and procedure were the same for all strains except *B. japonicum*. The most efficient strain was *Flavobacterium* sp., with $Y_{\text{NO}_3^-/e^-}$ of 6.12 and $Y_{\text{NO}_2^-/e^-}$ 5.97, which correspond to 29.2 g of protein per mol of NO_3^- and 17.5 g of protein per mol of NO_2^- , respectively. Other efficient strains were *Alcaligenes* sp., *Achromobacter cycloclastes*, and *Azospirillum brasilense*, all with growth yields of greater than 20 g of cell protein per mol of nitrate. In contrast, the two *Aquaspirillum* strains and *B. japonicum* were very inefficient, producing less than 10 g of protein per mol of NO_3^- (adjusted for the amount of electrons needed to convert NO_3^- to N_2).

When nitrite was the sole external electron acceptor, the growth yields of four strains were markedly less than those found with nitrate as the electron acceptor. For two strains the growth yield was greater on nitrite than on nitrate (Table 1).

A different relationship between the growth yield and the

TABLE 1. Molar growth yields under anaerobic conditions of selected denitrifiers and nitrate respirers grown on limiting nitrate or nitrite

Bacterial species	Molar growth yield (Y) (g of protein/mol of e ⁻ equivalent) ^a	
	NO ₃ ⁻	NO ₂ ⁻
<i>Flavobacterium</i> sp.	6.12 ± 0.41 (12)	5.97 ± 0.27 (12)
<i>Alcaligenes</i> sp.	4.69 ± 0.31 (11)	3.11 ± 0.55 (6)
<i>Achromobacter cycloclastes</i>	4.67 ± 0.15 (8)	2.36 ± 0.67 (4)
<i>Azospirillum brasilense</i>	3.96 ± 0.29 (6)	3.40 ± 0.41 (8)
<i>Corynebacterium nephridii</i>	3.61 ± 0.17 (12)	4.63 ± 0.33 (8)
<i>Bacillus azotoformans</i>	3.32 ± 0.12 (12)	3.01 ± 0.39 (6)
<i>Pseudomonas stutzeri</i>	3.12 ± 0.23 (8)	1.90 ± 0.28 (2)
<i>Aquaspirillum itersonii</i>	2.64 ± 0.08 (12)	1.24 ± 0.19 (4)
<i>Aquaspirillum fasciculus</i>	2.01 ± 0.46 (6)	2.32 ± 0.21 (2)
<i>Azospirillum lipoferum</i>	1.79 ± 0.57 (6)	2.37 ± 0.36 (2)
<i>Bradyrhizobium japonicum</i>	1.48 ± 0.28 (8)	1.55 ± 0.27 (2)
<i>Escherichia coli</i> ^b	1.23 ± 0.03–3.66 ± 0.40 (3)	1.58 ± 0.02–4.77 ± 0.83 (3)
<i>Serratia marcescens</i> ^b	1.33 ± 0.30–4.59 ± 0.35 (2)	ND ^c
<i>Enterobacter aerogenes</i>	0.95 ± 0.25 (2)	ND

^a Values are means of the indicated number of measurements (given in parentheses) ± standard errors. Values are corrected for any remaining unreduced N species.

^b A range of values is reported since the yield was not proportional to NO₂⁻ consumed.

^c ND, not determined.

amount of nitrate and nitrite consumed was found for the fermentative nitrate respirers, as shown in Fig. 1 for *E. coli*. Nitrate and nitrite stimulated growth yield when present in low concentrations, but the growth response was not proportional to the amount of the N anion consumed. A nonlinear response was also observed for *S. marcescens* (Table 1).

Azospirillum lipoferum also grew to a limited extent by fermentation since some growth occurred in the absence of electron acceptor (Fig. 1). The growth yield remained linearly related to electron acceptor concentration but passed through the ordinate at a biomass value corresponding to the fermentative portion of growth.

Stoichiometry of nitrate and nitrite conversion to gaseous N products. The sum of N₂O and N₂ produced by all denitrifier strains averaged 105% of the NO₃⁻ and NO₂⁻ N used and in all cases was greater than 80% of the N reduced (Fig. 2). N₂ was the only detected gaseous nitrogen product of NO₃⁻ and NO₂⁻ during growth of *P. stutzeri*, *Bacillus azotoformans*, *Azospirillum brasilense*, and *Bradyrhizobium japonicum*. N₂O was

the only gaseous product from *Aquaspirillum fasciculus*, *Aquaspirillum itersonii*, and *C. nephridii*. *Alcaligenes* sp., *Flavobacterium* sp., *Azospirillum lipoferum*, and *Achromobacter cycloclastes* produced a mixture of N₂O and N₂. For these strains, the proportion of each gas in the mixture depended on the initial concentration of NO₃⁻ and NO₂⁻. At 2 mM, only N₂ was detected, but the proportion of N₂O produced increased as NO₂⁻ and NO₃⁻ increased (Fig. 3). When the initial concentration of nitrite exceeded 4 mM, cells of *Azospirillum lipoferum* failed to grow and growth of *Achromobacter cycloclastes* was retarded. Nitrite remained in the medium in both cases. Despite the differences in product distribution, the growth yield of the denitrifier strains remained proportional to the amount of nitrate or nitrite consumed (Fig. 1), and the proportion of N anions converted to N gases was always greater than 80% (Fig. 2).

When nitrate respirers grew anaerobically in the presence of nitrate or nitrite, they also produced N₂O, but the conversion of the N ions to gas was less than 50% (Fig. 2) and was

FIG. 2. Percent nitrate- and nitrite-N recovered as N gases (N₂O plus N₂) during anaerobic growth with 4 mM nitrate (□) and nitrite (■). The standard errors of the mean are shown. Ps, *P. stutzeri*; Bo, *Bacillus azotoformans*; Ab, *Azospirillum brasilense*; Rj, *Bradyrhizobium japonicum*; Cn, *C. nephridii*; As, *Alcaligenes* sp.; Fs, *Flavobacterium* sp.; Ai, *Aquaspirillum itersonii*; Af, *Aquaspirillum fasciculus*; Ac, *Achromobacter cycloclastes*; Al, *Azospirillum lipoferum*; Ec, *E. coli*; Sm, *S. marcescens*; Ea, *Enterobacter aerogenes*.

FIG. 3. Recovery of different products of nitrate (a) and nitrite (i) reduction by *Flavobacterium sp.* and *Achromobacter cycloclastes* as a function of electron acceptor concentration. The products are N_2 (hatched), N_2O (solid), and NO_2^- (clear).

proportional to the initial concentration of the electron acceptor.

We used a wider range of electron acceptor concentrations than typically used in denitrification studies so that we could test the linearity of the growth response and could evaluate the concentration of nitrate or nitrite necessary to measure accurately the amount of N_2 produced. Because there is some residual N_2 in control tubes without NO_3^- or NO_2^- added (1 to 2% [vol/vol] in headspace), we recommend a minimum of 4 mM electron acceptor for accurate measurement of N_2 produced. This quantity of NO_3^- yields approximately 7% (vol/vol) of N_2 in the headspace of this test system if all of the electron acceptor is converted to N_2 .

Rate of N_2O production. The rate of N gas production by resting cells was evaluated in the presence of acetylene so that N_2O reduction was prevented (19, 25). This was done because N_2O is more easily quantified than N_2 due to the difficulty of avoiding contamination from atmospheric N_2 . The pattern of N_2O production from nitrate and nitrite was linear, as illustrated for one of the denitrifiers (Fig. 4), until nitrate and nitrite were consumed. The rate of N_2O production calculated

FIG. 4. N_2O production from NO_2^- and NO_3^- by resting cells of *Flavobacterium sp.* in the presence of acetylene. The reaction mixture contained 2 mM electron acceptor and 0.26 mg of cell protein. Data are means and standard deviations of duplicate assay vials.

TABLE 2. Specific N_2O production rates by resting cells of different bacteria and recovery of NO_3^- -N or NO_2^- -N as N_2O in the presence of acetylene^a

Bacteria	N ₂ O production rate (μmol/min/g of protein)		% N recovered as N ₂ O from:	
	NO ₃ ⁻	NO ₂ ⁻	NO ₃ ⁻	NO ₂ ⁻
<i>Flavobacterium sp.</i>	133 (12.0)	232 (6.4)	88 (2.5)	93 (6.7)
<i>Pseudomonas stutzeri</i>	128 (20.4)	253 (24.0)	95 (8.1)	113 (3.4)
<i>Alcaligenes sp.</i> (NCIB 11015)	77.4 (1.1)	90.4 (9.3)	90 (5.3)	114 (3.5)
<i>Aquaspirillum itersonii</i>	47.1 (3.0)	37.2 (2.5)	92 (2.5)	94 (3.5)
<i>Azospirillum brasilense</i>	24.0 (1.7)	18.6 (0.4)	98 (0.4)	97 (0.4)
<i>Achromobacter cycloclastes</i>	9.7 (2.3)	32.8 (2.7)	ND ^b	110 (4.2)
<i>Enterobacter aerogenes</i>	ND	0.16	ND	7

^a Values are means of duplicates; standard errors are given in parentheses.

^b ND, not determined.

from the linear production phase for other organisms was over 1 order of magnitude (Table 2). *Flavobacterium sp.* and *P. stutzeri* were the most potent N_2O producers. The least active strain, *Achromobacter cycloclastes*, still produced N_2O from nitrate at a rate of 9.7 $\mu\text{mol}/\text{min}/\text{g}$ of cell protein. This rate was still 2 orders of magnitude higher than the rate of N_2O evolution from an enteric organism (*Enterobacter aerogenes*), typically the most active N_2O producer from among the nonrespiratory denitrifiers (Table 2).

Four of the six denitrifying species tested produced N_2O at a greater rate from nitrite than from nitrate under the same conditions. Only *Aquaspirillum itersonii* and *Azospirillum brasilense* produced N_2O faster from nitrate than from nitrite (Table 2).

The acetylene block was effective in these strains since 88% or more of the nitrate or nitrite N was recovered as N_2O (Table 2). The conversion of only 7% of the nitrite N to N_2O was found for the nonrespiratory denitrifier *Enterobacter aerogenes*. This quantity is in the same range as was found for this bacterium in the experiment with growing cells (Fig. 2).

DISCUSSION

The characteristic feature of respiratory denitrification is that the reduction of nitrogenous oxide to N gases is coupled to electron transport phosphorylation (19, 27, 34). Therefore, there are two criteria that must be met for an organism to be called a respiratory denitrifier: (i) nitrogen gases, principally N_2 and N_2O , are products of nitrite or nitrate reduction, and (ii) the criterion described above is coupled to a growth yield increase that is greater than when the nitrogenous oxide simply served as an electron sink. The described method of quantifying the cell yield (Fig. 1) and the gaseous products (Fig. 2) as a function of nitrate or nitrite concentration appears to be the most straightforward and simplest way to establish these two criteria unequivocally.

Enhanced growth yield due to NO_3^- or NO_2^- reduction would be expected for any microbial reduction process that was coupled to electron transport phosphorylation, such as denitrification, nitrate respiration, and nitrite (to ammonium) respiration (27). Denitrifiers and nitrate respirers both showed enhanced growth from N-anion reduction, although $Y_{\text{NO}_3^-/\text{e}^-}$ was generally lower for the nitrate respirers studied. The greater distinction between respiratory denitrifiers and the latter group came from the fact that growth yield was not proportional to NO_3^- or NO_2^- concentration for the nitrate

respirers, but it was for denitrifiers, especially when they were grown on NO_3^- . Growth enhancement due to reduction serving as an electron sink often is not detectable or is less than the Y/e^- found here (5, 6, 13).

In this study, 1 of the 11 denitrifiers, *Azospirillum lipoferum*, had weak fermentative growth. The amount of growth by fermentation by this organism was an order of magnitude less than the growth of *E. coli* by fermentation. This is consistent with the general observation that fermentative organisms do not denitrify and those few that do are only weak fermenters (8, 29).

The growth yields of denitrifiers tested varied by a factor of 4, which is broader than we expected for a process thought to have basically the same biochemistry (12, 32, 35). The denitrifying bacteria could be divided into three subgroups on the basis of their growth yields on nitrate. The first group was most effective in energy conservation and consisted of organisms that had produced ≥ 4.5 g of protein per mol of e^- . These would usually be referred to as vigorous denitrifiers. There was also a group of five organisms that were moderate denitrifiers, yielding 2.5 to 4.0 g of protein per mol of e^- . Well-recognized denitrifiers are also in this group. The third group consists of four organisms in which the yield was ≤ 2 g of protein per mol of e^- . Members of the last group grew more slowly and may not often be recognized as being capable of respiratory denitrification. The differences in yield must derive from differences in reaction rates or energy coupling. Growth yields measured in batch systems, such as what we used, are not as accurate as those measured in continuous culture, but for the purposes of claiming whether respiratory denitrification exists, the batch method is adequate. These yields are comparable to or slightly higher than those Koike and Hattori (15) measured for *Pseudomonas denitrificans* in continuous culture (5.7 g [dry weight]/mol of e^-) and in batch culture (3.6 g [dry weight]/mol of e^-) (14). Differences in growth yields for two more active denitrifiers, *P. stutzeri* and *Paracoccus denitrificans*, were also observed in continuous culture, 15.8 and 32.3 g (dry weight) of NO_3^- per mol, respectively (20).

When determining growth yield, it is important to use data for only the concentrations of nitrate and nitrite which show a linear response and are not influenced by apparent toxicity of nitrite. Of the 11 strains we tested, 8 showed inhibition of growth yield in the presence of nitrite at concentrations greater than 4 mM and 3 of these strains showed no growth beyond 2 mM nitrite. An abbreviated linear response due to nitrite toxicity was less of a problem with nitrate as the electron acceptor. Because of this, we favor use of nitrate over nitrite in the test for respiratory denitrification.

The second criterion to be met before an organism is called a respiratory denitrifier is that the N_2O and/or N_2 is a major product of nitrogenous oxide reduction. It is important to measure the product and not rely on the classical method of nitrate and nitrite disappearance, because it has been this approach that has led to uncertainty about which organisms are truly respiratory denitrifiers (1). Since N_2 is the most common product of denitrification, we sought a method that would allow identification and quantitation of this product as well as of N_2O . We chose gas chromatographic analysis, since this is now rather commonly available, and devised a preparation scheme (especially three headspace flushes with He) and incubation and analysis protocol that would provide sufficiently accurate mass balances of nitrogen. To measure the stoichiometry of gas production, we used tubes sealed with a butyl rubber stopper for incubation with an He headspace so that we could quantify N_2 as well as N_2O . Standard errors of these measurements were low given the difficulty of making precise

measurements, especially of N_2 . Our average recovery of N gases was 105%, which is slightly more than expected. Assimilation of some of the nitrate or nitrite N is not expected under these incubation conditions because nutrient broth contains sufficient ammonium and organic N to repress assimilatory nitrite reduction (21). Thus, approximately 100% recovery of N as gases would be expected. The method was equally satisfactory for N_2O and N_2 as sole products or as mixtures. Since all of our incubations produced at least 80% N gases and this stoichiometry is sufficient to claim respiratory denitrification, we suggest that at least 80% of the nitrate or nitrite N transformed should be found as N_2O plus N_2 to establish respiratory denitrification. Nitrate respirers and other nonrespiring N_2O producers never produce this proportion of gas, with a few notable exceptions. Kaspar (13) reported 100% conversion of nitrate to N_2O by a *Propionibacterium* sp., and Shoun and Tanimoto (22) reported 100% conversion of nitrate to N_2O by *Fusarium oxysporum*. In both of these cases, however, the nitrogenous oxide reduction did not result in growth enhancement; thus, the first criterion was not met.

Scientists who have worked with respiratory denitrifiers know that there are two characteristics other than the above diagnostic criteria that are typical only of denitrifiers: (i) the rate of gas production by respiratory denitrifiers is higher than that by other mechanisms (2), and (ii) respiratory denitrifiers contain a unique suite of enzymes and cytochromes that are involved in this process (11, 19, 32, 35). In this paper, we quantified and evaluated the first characteristic to see if it could be used to help establish whether an organism is a respiratory denitrifier. We measured this rate in resting cells induced for denitrification and found a wide range of rates, from 233 to 9.7 μmol of N gas per min per g of protein. This rate was still 2 orders of magnitude greater than the rate of N_2O production by the nitrate respirers tested. Hence, we suggest that another characteristic of respiratory denitrification is that the rate of gas production is 10 $\mu\text{mol}/\text{min}/\text{g}$ of protein or greater. We measured this rate by quantifying N_2O production in the presence of acetylene, because this was easier to quantify in our circumstances, but the same approach and threshold should apply when N_2 production is measured.

The second character that usually is associated with respiratory denitrification is the presence of nitrite, nitric oxide, or nitrous oxide reductases, unique cytochromes associated with this chain, and the gene sequences coding for these proteins and electron carriers (35). This laboratory has found that these proteins can be recognized in many denitrifiers by using Western blots (immunoblots) (7) and that the gene sequences coding for these proteins can be detected by Cu and heme *nir* reductase probes (24, 33). While these molecular methods do identify the presence of denitrifier proteins or genes, they do not reveal if the genes are expressed or if the proteins actually function in a respiratory manner. Therefore, it is more important to use the criteria described in this paper to establish if the organism can actually function as a respiratory denitrifier before claiming that the organism is a denitrifier.

In this paper, we selected several well-characterized denitrifier strains as well as some additional strains in which respiratory denitrification has not been adequately established (1, 8, 29). All 11 strains met the criteria for growth by respiratory denitrification, and all could be distinguished from the three nitrate-respiring strains. Several strains were weak respiratory denitrifiers, and the ability to detect this process in such strains is equally important given the importance of this trait in taxonomy.

In conclusion, we have presented a simple method to determine whether a microorganism meets the two necessary crite-

ria for respiratory denitrification. We hope that this information will help other investigators clarify what organisms are truly respiratory denitrifiers and also help to identify organisms in which some of the features may deviate from standard denitrifier characteristics, thus making them candidates for more study.

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