APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1997, p. 2613–2618 Vol. 63, No. 7
0099-2240/97/$04.00+0

Physiology and Enzymology Involved in Denitrification by *Shewanella putrefaciens*

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Received 7 January 1997/Accepted 22 April 1997

Nitrate reduction to N_2O was investigated in batch cultures of *Shewanella putrefaciens* MR-1, MR-4, and MR-7. All three strains reduced nitrate to nitrite to N_2O, and this reduction was coupled to growth, whereas ammonium accumulation was very low (0 to 1 μmol liter^{-1}). All *S. putrefaciens* isolates were also capable of reducing nitrate aerobically; under anaerobic conditions, nitrite levels were three- to sixfold higher than those found under oxic conditions. Nitrate reductase activities (31 to 60 μmol of nitrite min^{-1} mg of protein^{-1}) detected in intact cells of *S. putrefaciens* were equal to or higher than those seen in *Escherichia coli* LE 392. K_m values for nitrate reduction ranged from 12 mM for MR-1 to 1.3 mM for MR-4 with benzyl viologen as an artifical electron donor. Nitrate and nitrite reductase activities in cell-free preparations were demonstrated in native gels by using reduced benzyl viologen. Detergent treatment of crude and membrane extracts suggested that the nitrate reductases of MR-1 and MR-4 are membrane bound. When the nitrate reductase in MR-1 was partially purified, three subunits (90, 70, and 55 kDa) were detected in denaturing gels. The nitrite reductase of MR-1 is also membrane bound and appeared as a 60-kDa band in sodium dodecyl sulfate-polyacrylamide gels after partial purification.

Biological denitrification is the reduction of nitrate or nitrite leading to the release of N_2O or N_2. An organism can be confirmed as a respiratory denitrifier when it is able to produce N_2O or N_2 and to couple this reduction to an increase in growth yield (27). Although denitrification is traditionally viewed as an anaerobic process, a number of facultative anaerobes are known to reduce nitrate in the presence of oxygen (1, 4, 5, 10, 40).

The denitrification pathway involves four terminal oxidases, including nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase, and nitrous oxide reductase. These four enzymes have been purified from several organisms and biochemically characterized (for reviews, see references 18, 42 and 46). Dissimilatory, oxygen-sensitive NaRs are membrane-bound enzymes and widespread among denitrifying bacteria. Many bacteria, however, have a second, oxygen-insensitive NaR located in the periplasm. Such NaRs have been identified in *Pseudomonas putida*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides* f. sp. *denitrificans*, *Rhodobacter capsulatus*, and *Alcaligenes eutrophus* (6, 28, 39, 40, 43).

Nitrite reduction is carried out by either a copper-containing or a heme-containing NiR (14, 42, 46). Immunological studies using polyclonal antibodies to identify the copper-containing or heme-containing NiR revealed that the latter was more common, occurring in 64 to 92% of all isolates tested (7). In addition to immunoscreening, molecular probes for both the copper-containing and heme-containing NiRs have been successfully used to detect denitrifying bacteria in the environment (24, 41, 45).

Shewanella putrefaciens, a facultative anaerobe, utilizes nitrate and a remarkable number of other electron acceptors for anaerobic respiration (21, 30, 32, 33). Although *S. putrefaciens* cannot ferment carbohydrates, the species is able to grow anaerobically in complex medium with amino acids (20, 21). In the presence of nitrate, Mn(IV), thiosulfate, and Fe(III) reduction in *S. putrefaciens* was inhibited, indicating that nitrate is a preferred electron acceptor for anaerobic respiration (11, 31).

Whether ammonium or a nitrogenous gas is the dominant end product of nitrate reduction in *S. putrefaciens* has been an issue of controversy. Nitrate reduction to N_2O has been reported for *S. putrefaciens* isolated from the water column of the Baltic Sea, where this group represented 77% of the total number of denitrifying bacteria isolated (3, 15). In earlier studies, a strain of *S. putrefaciens* was designated a dissimilatory ammonium producer capable of reducing nitrate to ammonium under some conditions (35–37). Samuelsson reported that the dissimilatory pathway to ammonia was dominant when cells were grown in medium with a high redox potential (36). However, the *S. putrefaciens* strain studied converted nitrate to N_2O and N_2 when the redox potential of the medium was lowered (36).

In this study, we investigated the respiratory denitrification in *S. putrefaciens* with a particular focus on the physiology of and the enzymes involved in this process. *S. putrefaciens* was tested for coupling of the reduction of nitrate and/or nitrite to growth and for its ability to reduce nitrate to N_2O. The ability of *S. putrefaciens* to respire nitrate under aerobic growth conditions was also examined. In addition to the physiological approach, the reductases catalyzing anaerobic nitrate and nitrite reduction were investigated. NaR and NiR activities in cell-free preparations were detected in nondenaturing polyacrylamide gels, and the corresponding proteins were further purified. Since different strains and species of denitrifying bacteria differ fundamentally in this pathway (46), one freshwater and two marine strains of *S. putrefaciens* were included in this study.

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MATERIALS AND METHODS

Organisms. *S. putrefaciens* MR-1 was isolated from Lake Oneida sediments (30). Marine strains MR-4 and MR-7 were isolated from the Black Sea by enrichment cultures (33).

Growth conditions and growth yield. All strains were grown in nutrient broth (pH 7.2) supplemented with 4 or 40 mM KNO_3. Cultures were incubated at 27°C for 4, 12, or 18 h in a Coy anaerobic chamber (10% H_2 and 90% N_2). For aerobic growth, cultures were kept on a shaker (200 rpm) at 30°C for 4 and 18 h. Growth was monitored as A_{500} with a DU-64 spectrophotometer (Beckman, Palo Alto, Calif.). Protein concentrations were determined by the Coomassie blue standard assay (Bio-Rad, Hercules, Calif.) after precipitation with 10% trichloroacetic acid.

Nitrate, nitrite, and ammonium analysis. Nitrate was measured with the flow injection method, an adaptation of the automated cadmium reduction method described previously (12). Nitrite was determined by the method of Daniels et al. (9) or the flow injection method (12). Ammonium was measured by using the indophenol blue method of Koroleff (19).

Determination of N_2O production. *S. putrefaciens* strains were grown aerobically in nutrient broth. One milliliter of these cultures was used to inoculate 150-ml vials containing 79 ml of nutrient broth supplemented with 4 mM nitrate. The atmosphere in the assay vials was replaced with 90% H_2 and 10% acetylene.

Gas samples (50 or 100 μl) were taken from the headspace with a gas-tight syringe after equilibration of the sample by vigorous shaking. N_2O was quantified with a gas chromatograph (Hewlett Packard 5890A) equipped with an electron capture detector operated at 300°C. Separation was performed by injecting gas samples in a Poropak Q 80/100 column (2 m by 0.3 cm) at 60°C. An Ar-CH_4 mixture (95:5, vol/vol) with a flow rate of 20 ml min^{-1} was used as the carrier gas. The headspace concentration of N_2O was obtained by using a 100-ppm N_2O standard (Scott Specialty Gases, Troy, Mich.). The aqueous concentration of N_2O was calculated by multiplying the headspace concentration with the Bunsen coefficient (44). Nitrate, nitrite, ammonia, and absorbance measurements were performed as described above, with 5-ml samples from each culture. These 5-ml samples were replaced with 5 ml of sterile nutrient broth to maintain a constant headspace volume.

NaR assay and determination of K_m. Cultures were grown anaerobically with 40 mM nitrate for approximately 14 h, harvested, and washed twice in 50 mM phosphate buffer (pH 7.0). Washed cells were resuspended in the same buffer to a concentration of 5 to 8 mg of protein per ml. NaR activity of freshly harvested cells (0.1 ml) was assayed under anaerobic conditions in a reaction mixture of 80 mM potassium phosphate (pH 7.0), 20 mM KNO_3, 0.2 mM benzyl viologen, and 10 mM sodium dithionite. The enzymatic reaction was stopped at several times by adding 2 ml of 95% ethanol to the mixture. The mixture was centrifuged at 2,000 × *g* for 15 min to remove cells. The presence of nitrite in the supernatant was determined colorometrically as described above. The same procedure was used to obtain K_m values for nitrate reduction by using different concentrations of nitrate (0 to 70 mM) in the reaction mixture.

Cell extracts. To prepare crude cell extracts, cells were lysed in the presence of 10-mg ml^{-1} lysozyme and 0.5-mg ml^{-1} EDTA and then incubated for 1 h at 37°C. Whole cells were removed by centrifugation at 3,440 × *g* for 15 min. The crude extract was centrifuged at 110,000 × *g* for 2 h. The resulting membrane fraction was resuspended in approximately 200 μl of 50 mM phosphate buffer (pH 7.0) and solubilized with Triton X-100 to a final concentration of 1%. Since the NaR activity appeared to be highly oxygen sensitive in cell-free preparations, all steps were performed under strictly anaerobic conditions.

Polyacrylamide gel electrophoresis (PAGE). NaR and NiR activities in nondenaturing gels were detected under anaerobic conditions as described by Lund and DeMoss (25). Native gels consisted of 5% polyacrylamide, 0.76 M Tris (pH 8.8), and 1% Triton X-100. Crude extracts and cytoplasmic and membrane fractions were used to detect NaR activity. Enzymatic activity of NaR appeared as a clear band of oxidized benzyl viologen after addition of nitrate to gels which had previously been stained with reduced benzyl viologen. A similar procedure was used to detect NiR activity, except that KNO_2 was substituted for KNO_3. To reveal the corresponding protein bands, native gels were also stained with 0.2% Coomassie blue or silver stain in accordance with the manufacturer's (Bio-Rad) protocol.

To determine the molecular masses of the NaR and NiR subunits, protein bands exhibiting NaR or NiR activity were cut out and incubated at 50°C for 30 min in 50 mM phosphate buffer (pH 7.0) containing 0.5% Triton X-100. The polyacrylamide gel pieces were homogenized on ice with a Teflon-coated pestle (Kontes, Vineland, N.J.), and samples were stored overnight at 37°C. To remove low-molecular-mass (<100-kDa) proteins and to concentrate proteins with molecular masses of more than 100 kDa, a Micropure separator combined with a Mirocon membrane unit (cutoff size, 100 kDa) was used in accordance with the manufacturer's (Amicon, Beverly, Mass.) protocol. Concentrated protein in the retentate was then applied to a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS). SDS-PAGE was carried out as described previously (25). Proteins in denaturing gels were stained as mentioned above. Molecular masses were determined by using standards ranging from 10 to 200 kDa (GIBCO BRL, Gaithersburg, Md.).

RESULTS

Consumption and growth on nitrate and nitrite. When *S. putrefaciens* MR-4 was grown anaerobically with 4 mM nitrate as the electron acceptor, nitrate was completely reduced to nitrite within the first 6 h (Fig. 1B). Between 6 and 9 h of incubation time, nitrite decreased rapidly to levels below 3 μmol $liter^{-1}$. N_2O concentrations reached a maximum of approximately 1.3 mmol $liter^{-1}$ after 9 h (Fig. 1B). The reduction of nitrate to nitrite and N_2O led to an increase in absorbance from 0 to 0.5 within the first 9 h (Fig. 1A). After this time, nitrite, N_2O, and absorbance levels remained constant. The same pattern of nitrate reduction to nitrite and N_2O coupled to growth was found in MR-1 cultures (data not shown). *S. putrefaciens* MR-7 produced nitrite faster from nitrate than did MR-1 and MR-4 (Fig. 1D). The nitrite formed (4 mmol $liter^{-1}$) was almost completely removed from the broth between 3 and 9 h of incubation time (3 μmol $liter^{-1}$). All three *S. putrefaciens* strains showed similar patterns of N_2O accumulation and increased absorbance (Fig. 1).

The addition of acetylene to cultures of MR-4 and MR-7 revealed a 1.8- to 2.4-fold accumulation of N_2O compared to batch cultures grown without acetylene (Table 1). In contrast to MR-4 and MR-7, MR-1 batch cultures produced less N_2O (2.01 and 2.34 mmol $liter^{-1}$) and accumulated less gas (1.2-fold increase in MR-4 and MR-7) in the presence of acetylene (Table 1). Rates of ammonium production were below 1 μmol $liter^{-1}$ in all of the *S. putrefaciens* cultures tested (data not shown).

Aerobic nitrate reduction. Surprisingly, all three isolates were able to respire nitrate under aerobic and anaerobic growth conditions (Table 2). Rates of nitrite produced at atmospheric levels of oxygen were (i) higher in MR-4 and MR-7 than in MR-1 and (ii) higher in cells harvested in the exponential phase than in those harvested in the stationary phase. The latter may be a function of nitrite accumulation in stationary-phase cultures, which may have been toxic for *S. putrefaciens* (see Discussion). In the absence of oxygen, rates of nitrite production were at least sixfold higher (4-h values) and threefold higher (18-h values) than those found under oxic conditions. *Escherichia coli* LE 392, which was used as a positive control, produced nitrite at lower rates under both aerobic and anaerobic growth conditions (Table 2).

Enzymatic activities and K_m values for nitrate reduction. The enzymatic activities of the NaRs in MR-1, MR-4, and MR-7 were measured with reduced benzyl viologen as the artifical electron donor. Whole-cell preparations of these strains showed specific NaR activities equal to or higher than those seen in preparations of *E. coli* LE 392 (Table 3). As a control, MR-1 cells were grown anaerobically in nutrient broth without an additional electron acceptor. Under these conditions, levels of NaR activity were below the detection threshold of the assay (<1.0 μM).

K_m values for NaR were determined in intact cells of all three *S. putrefaciens* strains by using reduced benzyl viologen as an artifical electron donor (Table 3). The K_m values for MR-4 and MR-7 were similar (1.32 ± 1.14 and 1.48 ± 0.06 mM, respectively) while the K_m for MR-1 was seven times as high. *E. coli* LE 392 showed a K_m value for NaR which was higher than those of MR-4 and MR-7 but lower than that of MR-1.

Localization of NaR and NiR. The NaRs of *S. putrefaciens* MR-1 and MR-4 were localized and characterized. NaR (Fig. 2A) and NiR (Fig. 3A) activities were detected in preparations of anaerobically grown cells by using nondenaturing polyacrylamide gels. Active NaR was found in crude extracts and membrane fractions of MR-1 and MR-4 after detergent treatment,

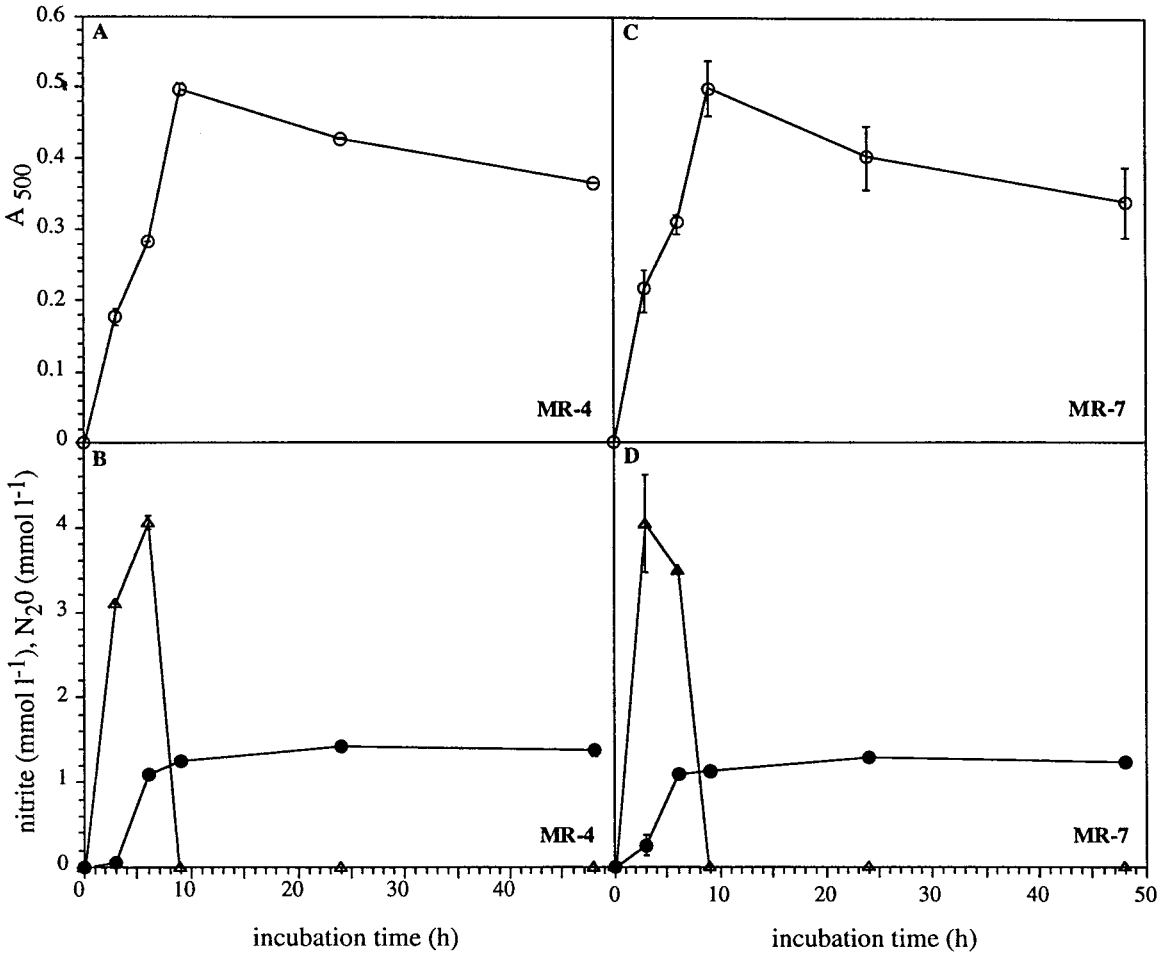

FIG. 1. Production of nitrite (△) and N_2O (●) in batch cultures of *S. putrefaciens* MR-4 (B) and MR-7 (D) grown anaerobically with 4 mM nitrate. Growth was measured as A_{500} (A and C). Nitrate reduction by *S. putrefaciens* MR-1 was similar to the results shown for MR-4 (data not shown). Data are means and standard deviations of duplicate assay vials.

suggesting that this enzyme is membrane bound (Fig. 2A). The protein revealing NaR activity in MR-1 was partially purified and appeared to consist of 90-, 70-, and 55-kDa subunits (Fig. 2B). Control assays with crude extracts from *E. coli* LE 392 revealed two bands of clearing in native gels due to the presence of two NaRs (16). In contrast to the NaR of *E. coli* LE 392, NaR activity in MR-1 and MR-4 was not inhibited by 1 mM azide (data not shown).

NiR activity was detected in the crude extract and cytoplasmic and membrane fractions of MR-1 (Fig. 3A). When the protein band revealing NiR activity was partially purified, a 60-kDa band appeared in SDS gels (Fig. 3B). The NiR in MR-1 is a heme-containing enzyme, as shown by Laue et al. (22), who hybridized MR-1 chromosomal DNA with different NiR gene probes to distinguish between copper- and heme-containing NiRs. The latter probe revealed a strong hybridization signal showing that MR-1 possesses a NiR containing a heme *c* and a heme d_1 (data not shown).

DISCUSSION

In this study, the fate of nitrate under aerobic and anaerobic conditions was examined in *S. putrefaciens*. Anaerobic growth with nitrate led to rapid and complete reduction of nitrate to nitrite and the formation of N_2O in all of the *S. putrefaciens* strains tested (Fig. 1). This reduction was coupled to an increase in growth (Fig. 1), suggesting that *S. putrefaciens* MR-1, MR-4, and MR-7 can be confirmed as denitrifiers (27).

Nitrate reduction to ammonium was minor in *S. putrefaciens* MR-1, MR-4, and MR-7 under the low redox conditions and the low carbon-to-electron acceptor (C/NO_3^-) ratio used in our experiments. Samuelsson (36) has previously reported that *S. putrefaciens* dissimilates nitrate to ammonium in media with glucose and a high redox potential. Considering the effect of growth conditions on the fate of nitrate (34, 36), it seems reasonable that ammonium may be produced in MR-1, MR-4, and MR-7 cultures grown under high redox potential condi-

TABLE 1. Production of N_2O with and without acetylene by *S. putrefaciens*[a]

S. putrefaciens strain	Mean N_2O production (mmol liter^{-1}) ± SE	
	Without acetylene	With acetylene
MR-1	2.01 ± 0.22	2.34 ± 0.30
MR-4	4.01 ± 1.35	7.14 ± 2.43
MR-7	2.62 ± 1.32	6.34 ± 0.86

[a] Bacteria were grown in nutrient broth with 40 mM nitrate. N_2O was quantified after 48 h of incubation.

TABLE 2. Rates of nitrate reduction in *S. putrefaciens* and *E. coli* LE 392 cultures grown under aerobic and anerobic conditions

Bacterial strain	Incubation time (h)	Mean nitrate reduction[a] (μmol of NO_2^- mg of protein^{-1} h^{-1}) ± SE	
		Aerobic	Anaerobic
S. putrefaciens MR-1	4	1.42 ± 0.01	205.42 ± 6.15
	18	4.37 ± 0.50	37.61 ± 3.58
S. putrefaciens MR-4	4	13.96 ± 1.41	88.78 ± 18.07
	18	8.14 ± 0.93	29.17 ± 1.29
S. putrefaciens MR-7	4	14.72 ± 2.01	95.02 ± 21.19
	18	8.90 ± 0.09	25.84 ± 6.69
E. coli LE 392[b]	4	0.10 ± 0.01	6.99 ± 0.72
	18	1.16 ± 0.14	9.23 ± 1.70

[a] Nitrite production was determined in cultures grown in nutrient broth with 40 mM nitrate.
[b] Obtained from Promega, Madison, Wis.

tions and a high C/NO_3^- ratio. This indicates that *S. putrefaciens* may have an advantage over most other denitrifying bacteria because it may be able to grow in environments with fluctuating C/NO_3^- ratios.

S. putrefaciens has the unusual ability to respire nitrate under aerobic conditions (Table 2). Oxygen certainly inhibits nitrate respiration (31), but it does not completely suppress the nitrate respiratory system. Our data suggest that oxygen partially inhibits nitrate respiration, since rates of nitrite production were at least threefold higher in anaerobically grown cells. Anaerobic denitrification rates found in other denitrifying bacteria, such as *P. denitrificans* and *Pseudomonas aeruginosa*, were also three- to fourfold higher than rates found under aerobic conditions (10). It is conceivable that the corespiration of nitrate and oxygen carried out by *S. putrefaciens* and other denitrifiers may be advantageous in environments with fluctuating oxygen levels (5). Very little is known about the ecological role of aerobic nitrate reduction in the nitrogen cycle, but it has been suggested that this process may be essential in soils and sediments (5).

In addition to physiological studies, which showed that *S. putrefaciens* is a respiratory denitrifier, the enzymes associated with this process were examined. The NaR activities measured in intact cells of *S. putrefaciens* were equal to or higher than those in *E. coli* LE 392 (Table 3) or other denitrifiers (40, 46),

TABLE 3. Specific NaR activities and K_m values for nitrate reduction in intact cells of *S. putrefaciens* and *E. coli* LE 392[a]

Bacterial strain	Mean NaR sp act (μmol of NO_2^- min^{-1} mg of protein^{-1}) ± SE	Mean K_m (mM) ± SE
S. putrefaciens MR-1	30.79 ± 1.99	11.54 ± 0.96
S. putrefaciens MR-4	56.58 ± 1.95	1.32 ± 1.14
S. putrefaciens MR-7	59.70 ± 1.02	1.48 ± 0.06
S. putrefaciens MR-1[b]	BD[c]	ND[d]
E. coli LE 392	18.41 ± 1.93	5.43 ± 0.28

[a] Data were obtained by using reduced benzyl viologen as an artificial electron donor. Cells were grown anaerobically for 12 h in nutrient broth with or without 40 mM nitrate.
[b] Cells were grown anaerobically in nutrient broth with no additional electron acceptor.
[c] BD, below detection limit of nitrite assay (<1 μmol $liter^{-1}$).
[d] ND, not determined.

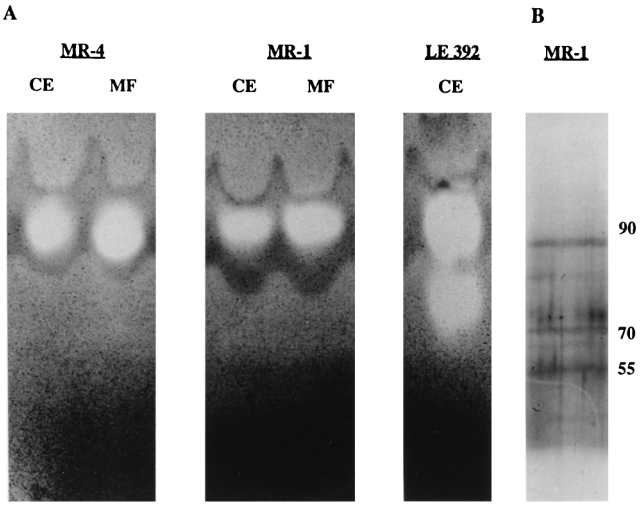

FIG. 2. (A) NaR activities in crude extracts (CE) and membrane fractions (MF) of *S. putrefaciens* MR-1 and MR-4 subjected to electrophoresis in 5% polyacrylamide gels. Enzymatic activity appeared as a band of clearing in native gels stained with reduced benzyl viologen. A crude extract of *E. coli* LE 392 was used as a positive control. (B) SDS–10% PAGE of the partially purified NaR of MR-1 revealed 55-, 70-, and 90-kDa protein bands.

indicating that *S. putrefaciens* is an active nitrate reducer. It seems that the rates of nitrite production measured in these assays (Table 3) were higher than those shown in Table 2. These results may be explained by the different methods used to determine the rates of nitrite production in *S. putrefaciens*. In the batch cultures, high nitrite concentrations may have accumulated (Table 2), possibly having a toxic effect on the metabolic activity of *S. putrefaciens*. This phenomenon has previously been described for other nitrate-reducing bacteria (see reference 2 and references therein). In contrast to this

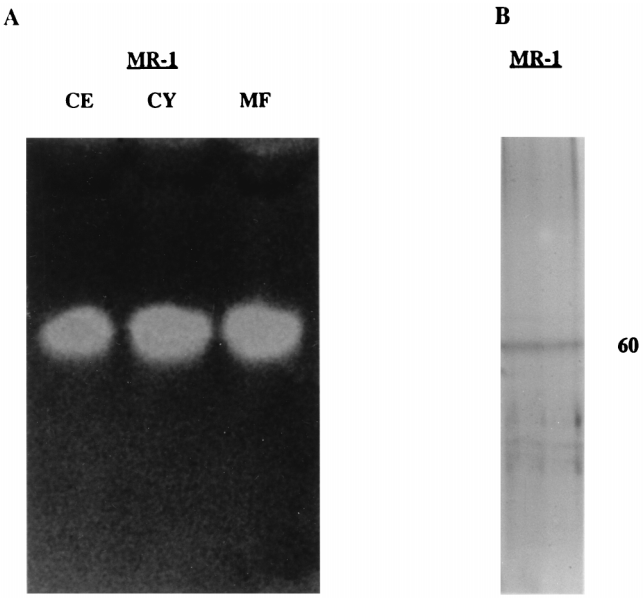

FIG. 3. (A) NiR activities in preparations of *S. putrefaciens* MR-1 subjected to PAGE (5% polyacrylamide). Enzymatic activity was determined in native gels by using a crude extract (CE) and cytoplasmic (CY) and membrane (MF) fractions. (B) The partially purified NiR appeared as a 60-kDa band on SDS–10% PAGE.

attempt, the specific NaR activities (Table 3) were obtained by using washed cells in a buffered reaction mixture containing lower nitrate and nitrite concentrations, respectively.

The K_m values for nitrate reduction found in intact cells of *S. putrefaciens* were higher than those found in other denitrifiers (2, 8, 13, 14) (Table 3). High K_m values may be a function of the benzyl viologen used as an artifical electron donor for NaR. Previous studies have shown that the use of viologen substrates for NaR resulted in significantly higher K_m values compared to assays utilizing reduced quinones as electron donors (8, 14, 29). For example, Morpeth and Boxer (29) reported K_m values of 420 and 2 μM with methyl viologen and duroquinol as the respective reductants for *E. coli* NaR. High K_m values with benzyl viologen as the reductant were also found in *E. coli* LE 392 (Table 3), suggesting that the artificial electron donor may have caused the high K_m values. The different quinones involved in anaerobic respiration in *S. putrefaciens* have recently been identified and quantified, but the particular quinone that reduces NaR has not been elucidated (23). Further investigations with reduced quinone as the reductant are required to provide more precise K_m values for nitrate respiration in *S. putrefaciens*.

In cell-free preparations, the NaRs of MR-1 and MR-4 appeared as membrane-bound enzymes. In general, both NaRs fit into the general concept of membrane-associated NaRs for the following reasons (14, 16, 46). First, they showed high sensitivity to oxygen, a feature described for other membrane-bound NaRs (5, 14). Second, the NaR of *S. putrefaciens* was positively regulated by the presence of its substrate (Table 3), a prerequisite for the activity of several NaRs (14, 42). Third, the NaR of MR-1 consists of three subunits (90, 70, and 55 kDa), resembling other NaRs purified from *E. coli* (26), *P. denitrificans* (8), and *Bacillus halodenitrificans* (17). In contrast to the NaR in *E. coli* LE 392 and most other NaRs, the NaRs of MR-1 and MR-4 were not inhibited by azide (8, 14). The NaR of *S. putrefaciens* revealed that this organism is a nitrate reducer, but further investigations were required to determine whether the bacterium is a denitrifier.

For this reason, the NiR of *S. putrefaciens* MR-1 was investigated by using biochemical and genetic tools. The dissimilatory reduction of nitrite in MR-1 was carried out by a membrane-bound protein. This enzyme activity was revealed in the crude extract and the membrane and cytoplasmic fractions (Fig. 3B). It was previously reported that NiR may be loosely associated with the membrane and may therefore be easily removed during preparation (38). Partial purification of NiR revealed a 60-kDa band in SDS-polyacrylamide gels, indicating that the enzyme may be composed of two 60-kDa subunits. This assumption is based on the observations that all known heme-containing NiRs (i) are composed of two identical subunits and (ii) reveal highly similar protein structures (14, 42). Further purification of the NiR and NaR of MR-1 is necessary to verify their composition, since both enzymes have been partially but not completely purified. However, it has been suggested that the presence of this type of NiR and its gene is evidence that an organism is a true denitrifier (27).

In this study, advances have been made concerning physiological and biochemical aspects of the dissimilatory nitrate reduction and denitrification of *S. putrefaciens*. The results showed that *S. putrefaciens* is indeed a respiratory denitrifier and possesses some of the unique enzymes associated with this process. Additionally, all isolates were capable of reducing nitrate aerobically, a phenomenon not previously reported for this species.

ACKNOWLEDGMENTS

This work was funded by grant 144DG40 from NASA awarded to K.H. Nealson and D. Saffarini.

We are very grateful to B. E. Laue for help with the molecular work, to P. D. Anderson for his skilled technical assistance with the gas chromatographic analysis, and to F. Vazquez from the EAWAG/Kastanienbaum for help with the calculations. We also thank C. C. Remsen and the reviewers for helpful discussions.

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