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# Influence of Volatile Fatty Acids on Nitrite Accumulation by a *Pseudomonas stutzeri* Strain Isolated from a Denitrifying Fluidized Bed Reactor

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**Intermediate nitrite accumulation during denitrification by *Pseudomonas stutzeri* isolated from a denitrifying fluidized bed reactor was examined in the presence of different volatile fatty acids. Nitrite accumulated when acetate or propionate served as the carbon and electron source but did not accumulate in the presence of butyrate, valerate, or caproate. Nitrite accumulation in the presence of acetate was caused by differences in the rates of nitrate and nitrite reduction and, in addition, by competition between nitrate and nitrite reduction pathways for electrons. Incubation of the cells with butyrate resulted in a slower nitrate reduction rate and a faster nitrite reduction rate than incubation with acetate. Whereas nitrate inhibited the nitrite reduction rate in the presence of acetate, no such inhibition was found in butyrate-supplemented cells. Cytochromes *b* and *c* were found to mediate electron transport during nitrate reduction by the cells. Cytochrome *c* was reduced via a different pathway when nitrite-reducing cells were incubated with acetate than when they were incubated with butyrate. Furthermore, addition of antimycin A to nitrite-reducing cells resulted in partial inhibition of electron transport to cytochrome *c* in acetate-supplemented cells but not in butyrate-supplemented cells. On the basis of these findings, we propose that differences in intermediate nitrite accumulation are caused by differences in electron flow to nitrate and nitrite reductases during oxidation of either acetate or butyrate.**

A problem often encountered in denitrifying reactors is the accumulation of nitrite due to incomplete reduction of nitrate to nitrogen gases. Maximum admissible nitrite concentrations in potable water are as low as 0.03 mg of $NO_2$ nitrogen per liter. This stringent requirement has triggered studies aimed at obtaining an understanding of the factors underlying nitrite accumulation in denitrifying organisms. These studies, which were carried out both in situ and with denitrifying isolates, revealed that a number of factors underlie nitrite accumulation. In denitrifying bacteria exposed to low levels of oxygen, nitrite accumulation resulted from differential repression of the synthesis and activities of nitrite and nitrate reductases (7, 11). Shifts in the pH values of the medium were also found to affect nitrite accumulation (2, 4, 27). Competition between nitrate and nitrite reductases for common electron donors is an additional factor which causes nitrite accumulation in some denitrifiers (12, 27). A kinetic explanation for nitrite accumulation in denitrifying isolates based on differences in maximum reduction rates of nitrate and nitrite reductase activities was provided by Betlach and Tiedje (5). A model for nitrite accumulation combining both kinetic parameters of and competition for electrons between these reductases was recently provided by Almeida et al. (2). Finally, the choice of carbon source was shown to affect the level of nitrite accumulation in denitrifying reactors (15). The carbon source may lead to specific enrichment of nitrite-accumulating bacteria, as was found in a study on denitrifying reactors, in which addition of fermentable substrates enhanced the growth of nitrate-respiring fermentative bacteria, causing nitrite accumulation (30). Alternatively, individual denitrifiers may accumulate nitrite when grown on different carbon sources. The latter reason for nitrite accumulation has been examined little (6, 16, 17).

The operation of most denitrifying reactors involves the addition of external carbon sources, such as methanol, glucose, and acetate. Volatile fatty acids (VFA) are major degradation products of the fermentation of organic materials in reduced environments (10) and constitute an excellent carbon source for denitrifying bacteria (8, 9). In previous studies we described the performance of fluidized bed reactors used for removal of nitrate from intensive fish culture systems (1, 3). VFA were found to be the main carbon and electron donors fueling denitrification in these reactors, and occasionally elevations in effluent nitrite levels were observed.

In this study, data are presented on a *Pseudomonas stutzeri* strain isolated from a fluidized bed reactor. It is shown that during reduction of nitrate by this strain, competition for common electron donors and differences in the nitrate and nitrite reduction rates caused the accumulation of nitrite. In addition, nitrite accumulation by this bacterium was found to depend on the type of VFA supplied. Distinct differences in cytochrome *c* redox kinetics were found between nitrite-reducing *P. stutzeri* incubated with acetate and that incubated with butyrate. It is concluded that differences in electron flow to cytochrome *c* underlie the differences in nitrite accumulation by cells grown on either one of these VFA.

## MATERIALS AND METHODS

**Organism.** *P. stutzeri* was isolated from granules derived from a denitrifying fluidized bed reactor (1) after standard enrichment procedures under anoxic conditions at 30°C in medium containing (per liter) 7.2 g of $Na_2HPO_4 \cdot 7H_2O$, 1.5 g of $K_2HPO_4$, 0.3 g of $NH_4Cl$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $KNO_3$, and 2 ml of a trace element solution (29). Acetate, propionate, and butyrate were used in combination as carbon sources, each at a concentration of 5 mM. Identification was conducted with the API NE system, consisting of 8 conventional tests and 12 assimilatory tests (18), as well as by gas chromatography of whole-cell fatty acids (22). Similar results were scored with both identification tests, each with a high probability.

**Culture conditions and nitrate and nitrite reduction studies.** *P. stutzeri* was

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cultured anaerobically at 30°C in the aforementioned synthetic medium supplemented with acetate (5 mM), propionate (5 mM), butyrate (5 mM), valerate (2 mM), or caproate (2 mM). The pH of the medium was 7.2. The initial nitrate concentration in the medium was 2.85 mM. Nitrate was the growth-limiting nutrient under these conditions, since addition of nitrate at concentrations stoichiometrically required for complete oxidation of the various VFA leads to the accumulation of nitrite to growth-inhibiting concentrations (27a).

Nitrate and nitrite reduction studies were conducted on cells harvested during the late log phase of growth (after 4 to 5 days). Cells were washed twice and resuspended in medium similar to that used for isolation, except that either nitrate or nitrite was added at various concentrations. Although nitrate and nitrite were added at concentrations less than that required for complete oxidation of the VFA, their initial concentrations were sufficient to allow determination of maximum nitrate and nitrite reduction rates. Experiments were conducted in a temperature-controlled (30°C) incubation vessel (300 ml) which was placed on a magnetic stirrer and fitted with nitrate, pH, and oxygen-temperature electrodes. Anaerobic conditions in the vessel were obtained by continuous flushing with prepurified nitrogen gas. Overpressure within the incubation vessel prevented oxygen penetration, as verified by continuous oxygen monitoring. The experiments were initiated by the addition of one of the carbon sources. The initial carbon concentration varied, depending on the carbon sources, but was always in excess of the concentration required for complete reduction of the nitrate or nitrite in the medium. Periodically, samples were withdrawn for VFA and nitrite determinations. Changes in nitrate levels and pH were monitored every 2 to 5 min, whereas protein and ammonia concentrations in the vessel were determined in aliquots withdrawn at the beginning and end of the experiment. A minimum of five runs were performed for each electron donor-acceptor combination; nitrate reduction rates, nitrite reduction rates, and VFA uptake rates (expressed per gram of protein) for the runs did not differ by not more than 10%. During the various experiments, the bacterial biomass (as measured by protein analysis) did not increase by more than 15%. Decreases in ammonia concentrations corresponded with the increases in bacterial biomass in the medium. An increase in pH (not exceeding 0.4 units) was measured in all experiments.

Maximum values for nitrate and nitrite reduction rates during each run were obtained by nonlinear regression analyses of at least 30 datum points based on Michaelis-Menten kinetics, using the Enzfitter software program (Elsevier-Biosoft, Amsterdam, The Netherlands).

**Cytochrome studies.** Cells used for cytochrome studies were grown with acetate as the electron and carbon donor and nitrate as the electron acceptor. Cells were harvested in the late log phase of growth, washed with 50 mM phosphate buffer (pH 7.1), and resuspended in the same buffer at various concentrations. Cytochrome studies were performed in closed 3-ml cuvettes with a Hitachi (model U-3000) double-beam spectrophotometer. Difference spectra (400 to 600 nm) of cells supplemented with various combinations of electron donor and acceptor were obtained by comparing these cells with dithionite-reduced cells. Cytochrome *c* redox kinetics were studied at 553 nm by comparison of electron donor- and acceptor-supplemented cells with cells which had been fully oxidized by the addition of solid ferricyanide. Antimycin A was solubilized in *N,N*-dimethylformamide and was added to a final concentration of 20 μg/ml.

**Analytical procedures.** Total ammonia ($NH_3$ and $NH_4^+$) was determined as described by Scheiner (23), nitrite was measured according to the method of Strickland and Parsons (26), and nitrate was measured with a specific nitrate electrode (Radiometer, Copenhagen, Denmark) amplified with a pH meter (Radiometer model PHM92). VFA were determined with a Hewlett-Packard (model 5890) gas chromatograph equipped with a stainless steel column (inside diameter, 1/8 in. [0.3175 cm]; length, 200 cm) packed with 60- to 80-mesh Chromosorb W. The injector, oven, and flame ionization detector temperatures were set at 170, 140, and 175°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. Analytical-grade VFA were used as standards. Protein was determined according to the method of Lowry et al. (14) with bovine serum albumin as the standard. Oxygen levels and temperature were measured with a YSI (model 57) temperature-oxygen probe (Yellow Springs Instruments Co., Yellow Springs, Ohio).

## RESULTS

**Denitrification characteristics of *P. stutzeri* with acetate as carbon and electron donor.** A total of 15 denitrifying strains were isolated from biofilm-coated sand derived from a denitrifying fluidized bed reactor. Incubation of these isolates under anaerobic conditions with nitrate as the electron acceptor and acetate as the carbon and electron donor resulted in intermediate nitrite accumulation in most, but not all, of the isolates. With acetate as the carbon and electron source, nitrate and nitrite reduction rates were determined in an isolate in which nitrate reduction coincided with intermediate nitrite accumulation in the medium (Fig. 1). In this strain, identified as *P. stutzeri*, the maximum nitrate reduction rate (± the standard deviation) was 375.7 ± 35.7 μmol of $NO_3^-$ reduced per g of protein per min while the maximum nitrite reduction rate was 162.9 ± 29.3 μmol of $NO_2^-$ reduced per g of protein per min. When this strain was incubated with nitrite as the sole electron acceptor, the maximum nitrite reduction rate rose to 326.4 ± 20.0 μmol of $NO_2^-$ reduced per g of protein per min, approximately twice as fast as that measured in the presence of nitrate. Various studies (2, 12, 27) have presented evidence that in some denitrifiers, intermediate accumulation of nitrogen oxides is caused by competition among the terminal reductases for common electron donors. To test whether competition between nitrate and nitrite reductases for electrons occurs during nitrate reduction by *P. stutzeri*, the following experiment was conducted. *P. stutzeri* was incubated in the presence of acetate in medium in which the nitrate concentration fluctuated between 71.4 and 280 μM because of periodic nitrate amendments (Fig. 2). Maximum nitrate and nitrite reduction rates were determined. As might be expected from Michaelis-Menten kinetics, at low ambient nitrate concentrations, rates of nitrate reduction in *P. stutzeri* were lower (257.9 ± 2.9 μmol of $NO_3^-$ reduced per g of protein per min) than at high ambient nitrate concentrations (375.7 ± 35.7 μmol of $NO_3^-$ reduced per g of protein per min) (Fig. 1). However, faster nitrite reduction rates were obtained with cells incubated at low ambient nitrate concentrations than with cells incubated at high ambient nitrate concentrations (250.0 ± 64.2 and 162.8 ± 29.3 μmol of $NO_2^-$ reduced per g of protein per min,

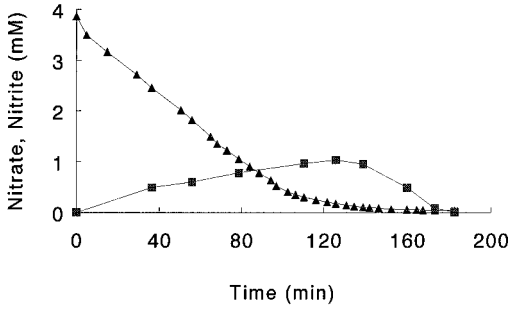


FIG. 1. Changes in nitrate (▲) and nitrite (■) concentrations upon incubation of *P. stutzeri* (protein content, 77.6 mg/liter) under anoxic conditions in culture medium (see Materials and Methods) containing acetate (5 mM) as the carbon and electron donor and nitrate (3.9 mM) as the electron acceptor.

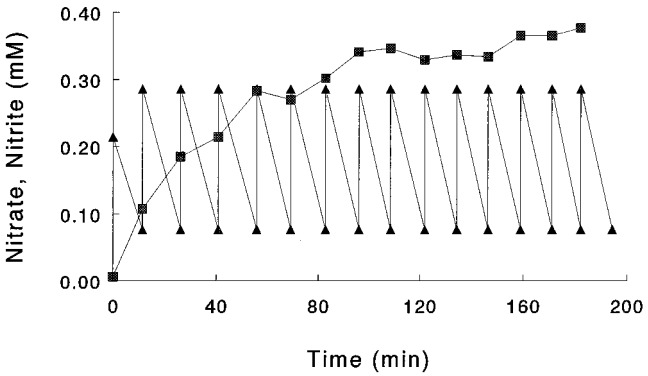


FIG. 2. Changes in nitrate (▲) and nitrite (■) concentrations upon incubation of *P. stutzeri* (protein content, 61.3 mg/liter) under anoxic conditions in culture medium (see Materials and Methods) with acetate (5 mM) as the carbon and electron donor and nitrate as the electron acceptor. Nitrate (as $KNO_3$) was added in 16 periodic amendments of 0.21 mmol of $NO_3^-$ per liter.

TABLE 1. Maximum rates (± standard deviations) of nitrate and nitrite reduction by *P. stutzeri* incubated with nitrate and different volatile fatty acids

| Carbon source | Maximum reduction rate for: | |
|---|---|---|
| | Nitrate (μmol of $NO_3^-$/g of protein/min) | Nitrite (μmol of $NO_2^-$/g of protein/min) |
| Acetate | 375.7 ± 35.7 | 162.9 ± 29.3 |
| Propionate | 267.9 ± 50.0 | 194.3 ± 29.3 |
| Butyrate | 252.9 ± 12.1 | 328.6 ± 47.9 |
| Valerate | 235.7 ± 27.9 | 265.0 ± 30.0 |
| Caproate | 109.3 ± 36.4 | 185.7 ± 11.4 |

respectively). This finding supports the notion that during acetate oxidation, respiratory electron flow is insufficient for both nitrate and nitrite reductase activities to be maximal. This suggests that intermediate nitrite accumulation by this *P. stutzeri* strain with acetate as the sole carbon and electron donor is caused by some metabolic regulatory process that results in inhibition of nitrite reduction under conditions in which the rate of respiratory electron flow is insufficient to allow both nitrate and nitrite reduction rates to be maximal. Because the rate of nitrate reduction depends on its concentration, in this specific case, the concentration of nitrate in the medium is an additional factor influencing the extent of intermediate nitrite accumulation.

**Denitrification characteristics of *P. stutzeri* with different VFA.** The effects of individual VFA on the denitrification characteristics of *P. stutzeri* were tested, and intermediate nitrite accumulation was found to depend on the type of VFA supplied. Incubation with propionate, like acetate, resulted in intermediate nitrite accumulation. In contrast, nitrite did not accumulate when cells were incubated with butyrate, valerate, or caproate if nitrate served as the electron acceptor. Maximum nitrite reduction rates for bacterial suspensions grown on butyrate, valerate, or caproate were higher than the maximum nitrate reduction rates with these VFA (Table 1). Therefore, in addition to the factors already mentioned, the type of carbon source oxidized by this bacterium was yet another factor which determined intermediate nitrite accumulation. The question of how the nature of the carbon source exerts this influence in this *P. stutzeri* strain was addressed by comparing its denitrification characteristics in the presence of acetate with those exhibited in the presence of butyrate (Table 2). The maximum nitrate reduction rate during incubation of the cells with butyrate was slower than that obtained when acetate was provided, whereas in the absence of nitrate, acetate- and butyrate-supplemented cells reduced nitrite at similar rates. Contrary to what was found in bacterial suspensions incubated with acetate, nitrate did not affect the nitrite reduction rate in cells incubated with butyrate. The maximum acetate consumption rate was higher than the maximum butyrate consumption rate. Butyrate oxidation did not lead to intermediate VFA accumulation (acetate or propionate); therefore, electron flow rates during oxidation of acetate and butyrate can be compared when considering that the electron donor potential of acetate is 8 mole-electrons per mole and that of butyrate 20 mole-electrons per mole. The calculated maximum electron flow rates were $1.76 \times 10^{-3}$ and $1.20 \times 10^{-3}$ mole-electrons/g of protein per min in the presence of acetate and butyrate, respectively. Finally, incubation of *P. stutzeri* with acetate and butyrate (in combination) as carbon and electron donors and nitrate as the electron acceptor resulted in no intermediate nitrite accumulation in the medium (Fig. 3). The maximum nitrate reduction rate was close to that obtained when cells were grown on acetate alone, while the maximum nitrite reduction rate was close to that of cells grown on butyrate alone (Table 2).

On the basis of these differences, we propose that in this *P. stutzeri* strain, differences in electron flow along the respiratory chain underlie the difference between nitrite accumulation during oxidation of acetate and that during oxidation of butyrate. Were the oxidations of acetate and butyrate to follow the same pathway, intermediate nitrite accumulation by this bacterium would be higher with butyrate than with acetate, since electron limitation during butyrate oxidation is more severe than it is during acetate oxidation. Furthermore, inhibition of nitrite reduction rates by nitrate in the presence of acetate, but not butyrate, is not consistent with the view that terminal reductases are reduced through a similar electron flow pathway with acetate and butyrate.

**Cytochrome *c* redox transitions during nitrite reduction by *P. stutzeri* in the presence of acetate or butyrate.** Cytochrome studies were performed during nitrate and nitrite reduction in the presence of either acetate or butyrate. Difference spectra (absorbance of dithionite-reduced cells minus absorbance of nitrate-oxidized cells [in the presence of either acetate or butyrate]) revealed that cytochromes *b* (529 nm) and *c* (553 nm) were involved in the reduction of nitrate (Fig. 4). The oxidation states of both cytochromes during nitrate reduction were higher in acetate-supplemented cells than in butyrate-supplemented cells (Fig. 4). The redox kinetics of cytochrome *c* were examined after addition of nitrite to either acetate- or butyrate-supplemented cells. Cytochrome *c* redox transitions

TABLE 2. Rates of nitrate reduction, nitrite reduction, and VFA consumption by *P. stutzeri* incubated with different combinations of electron acceptor(s) and donor(s)

| Carbon source(s) | Electron acceptor(s) | Reduction rate for: | | VFA consumption rate (μmol of VFA/g of protein/min) |
|---|---|---|---|---|
| | | Nitrate (μmol of $NO_3^-$/g of protein/min) | Nitrite (μmol of $NO_2^-$/g of protein/min) | |
| Acetate | Nitrite | NR[a] | 326.4 ± 20.0 | ND[b] |
| | Nitrate + nitrite | 375.7 ± 35.7 | 162.9 ± 29.3 | 222 ± 11.3 |
| Butyrate | Nitrate | 252.9 ± 12.1 | NR | ND |
| | Nitrite | NR | 342.9 ± 36.4 | ND |
| | Nitrate + nitrite | 253.6 ± 0.7 | 328.6 ± 47.9 | 60 ± 4.0 |
| Acetate + butyrate | Nitrate + nitrite | 335.0 ± 17.1 | 417.9 ± 50.0 | ND |

[a] NR, not relevant.
[b] ND, not determined.

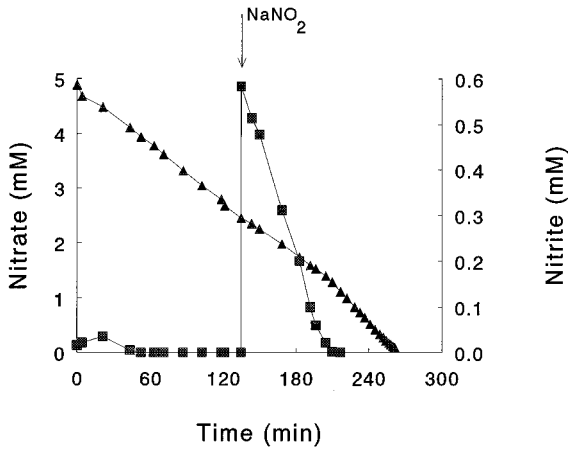


FIG. 3. Changes in nitrate (▲) and nitrite (■) concentrations upon incubation of *P. stutzeri* (protein content, 79.1 mg/liter) under anoxic conditions in culture medium (see Material and Methods) with acetate (5 mM) and butyrate (5 mM) in combination as carbon and electron donors and nitrate (5 mM) as the electron acceptor. Sodium nitrite (0.6 mM) was added to the medium at the indicated time.

were distinctively different, as it was found (Fig. 5) that nitrite reduction in acetate-supplemented cells resulted in a considerably higher degree of cytochrome *c* oxidation than it did in butyrate-supplemented cells. It was of interest to examine whether the observed differences in nitrite accumulation by acetate- and butyrate-oxidizing cells could be explained by these differences in redox transitions. A difference spectrum (absorbance of cells incubated with butyrate, acetate, and nitrite minus absorbance of cells incubated with acetate and nitrite) resulted in a positive difference in absorbance; i.e., during the redox transition, cytochrome *c* remained more reduced in butyrate-supplemented cells (Fig. 6). A similar difference spectrum (absorbance of cells incubated with butyrate, acetate, and nitrite minus absorbance of cells incubated with butyrate and nitrite) revealed no significant differences in absorption (data not shown). It is concluded, therefore, that cytochrome *c* accepts electrons more readily from butyrate than from acetate. A possible explanation for this finding would be that butyrate donates electrons in closer proximity to cytochrome *c* than does acetate. This possibility was examined by following cytochrome *c* redox transitions in nitrite-reducing cells incubated with either acetate or butyrate, with or without antimycin A, an inhibitor known to block electron flow between cytochromes *b* and *c*. A clear difference was noticed between the cytochrome *c* redox transitions in nitrite-reducing cells incubated with acetate in the presence of antimycin A and those in cells incubated with acetate in the absence of antimycin A. During reduction of nitrate by cells incubated with acetate and antimycin A, cytochrome *c* became more oxidized, and subsequent reduction back to its original reduction state took longer than in similar cells without antimycin A (Fig. 7A). In contrast to those of acetate-incubated cells, redox transitions during nitrite reduction in butyrate-incubated cells were similar in the absence or presence of antimycin A (Fig. 7B), a finding which suggests that during butyrate oxidation by this *P. stutzeri* strain, electrons enter the respiratory chain at a more downstream region of the electron transport chain than during acetate oxidation.

## DISCUSSION

Betlach and Tiedje (5) described intermediate nitrite accumulation during nitrate reduction of *Pseudomonas fluorescens* by Michaelis-Menten kinetics. According to this concept, the reduction rate of any nitrogen oxide depends only on its concentration. In the present study, a similar explanation for nitrite accumulation in *P. stutzeri* can be ruled out, since nitrate and nitrite reduction rates were controlled not only by the concentrations of their substrates. Two findings support the notion that additional factors influence nitrite accumulation in this organism. First, nitrate was shown to influence nitrite reduction rates in cells incubated in the presence of acetate (Table 2), and second, nitrite accumulation was influenced by the type of carbon source supplied. The finding that the presence of nitrate lowered nitrite reduction rates indicates competition between nitrate and nitrite reductases for electrons. Such a competition has been described in studies with *Paracoccus denitrificans* (12, 13, 27) and with *Pseudomonas fluorescens* (2). On the basis of these studies, it was suggested that

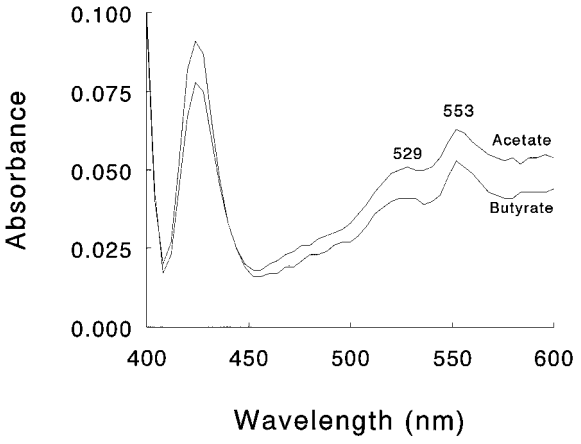


FIG. 4. Difference spectra (400 to 600 nm) (absorbance of dithionite-reduced cells minus absorbance of nitrate-oxidized cells) for *P. stutzeri* grown on acetate, harvested in the late exponential phase of growth, and washed and resuspended in phosphate buffer (pH 7.1). Spectra of cells incubated with acetate (5 mM) or butyrate (5 mM) in the presence of nitrate (3.6 mM) were examined. The protein content of each of the bacterial suspensions was 1,063 mg/liter.

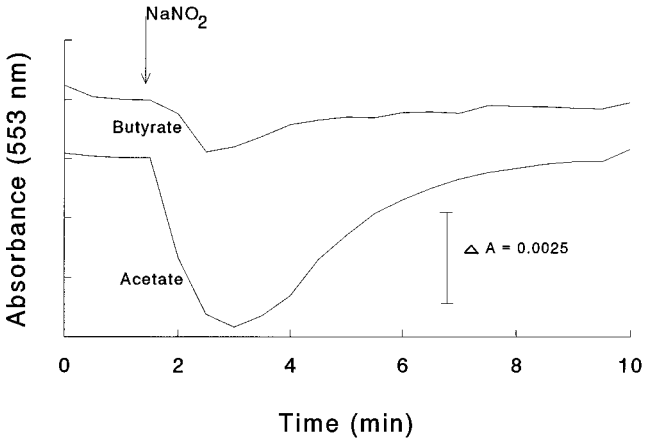


FIG. 5. $A_{553}$ (cytochrome *c*) of *P. stutzeri* cells grown on acetate, washed and resuspended in phosphate buffer (pH 7.1), and incubated in the presence of either acetate (5 mM) or butyrate (5 mM). At the indicated time, sodium nitrite (0.71 mM) was added. The absorbance was read against a reference cuvette containing ferricyanide-oxidized *P. stutzeri* at the same density (protein content, 1,561 mg/liter) as the sample cuvette.

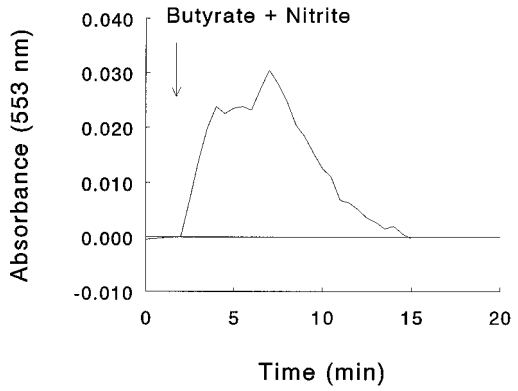


FIG. 6. Differences in $A_{553}$ values of *P. stutzeri* cells incubated in medium with acetate (5 mM) to which butyrate (5 mM) and sodium nitrite (0.71 mM) were added (as indicated) versus readings of a reference cuvette containing cells incubated with acetate (5 mM) and supplemented with sodium nitrite (0.71 mM) at the same time point. Cells were grown on acetate and then washed and resuspended in phosphate buffer (pH 7.1) at a protein concentration of 1,063 mg/liter.

respiratory nitrate reduction in these denitrifiers follows a branched electron transport chain in which alternative terminal reductases compete for a common reductant.

In this study, incubation of nitrate-reducing cells with butyrate, caproate, or valerate did not lead to nitrite accumulation, in contrast to incubation of the cells with acetate or propionate. A comparison of the denitrification characteristics of acetate- and butyrate-supplemented cells clearly shows differences in nitrate and nitrite reduction. In principle, during the metabolism of the various carbon sources, different primary hydrogenases and electron-transferring proteins are involved. Therefore, it seems reasonable to assume that metabolic differences among the carbon sources lead to differences in electron flow velocities which, in turn, lead to different nitrate and nitrite reduction rates. Butyrate oxidation results in a slower electron flow velocity than does oxidation of acetate (Table 2). Consequently, the slower nitrate reduction rate in butyrate-supplemented cells compared with that in acetate-supplemented cells can be explained on kinetic grounds. However, on the basis of differences in the rates of acetate and butyrate oxidation, one cannot explain the observed differences in nitrite reduction rates. In the presence of nitrate, the nitrite reduction rate in *P. stutzeri* was faster with butyrate than with acetate.

Relatively little information is available on the effect of the carbon source on nitrite accumulation in denitrifiers. In a study on *Paracoccus denitrificans* (6), nitrite accumulation changed when alterations in the nutritional composition of the medium were made, a finding which was explained by differences in the induction times required for synthesis of nitrate and nitrite reductases in cells incubated in various culture media. Nishimura et al. (16, 17) found that when *Pseudomonas denitrificans* was grown on formate instead of citrate, nitrite accumulation was markedly depressed. This was attributed to lower nitrate and higher nitrite reductase activities in the presence of formate. Finally, the extent of the reduction state of the carbon source was found to influence a periplasmic nitrate reductase in *Thiosphaera pantotropha* (21) and *Paracoccus denitrificans* (24). In these organisms, which are capable of aerobic denitrification, both a periplasmic and a membrane-bound nitrate reductase were present. The activity of periplasmic nitrate reductase (operating under aerobic conditions) was shown to be influenced by the nature of the carbon substrate, whereas membrane-bound nitrate reductase (operating under anoxic conditions) was not.

In most denitrifiers, nitrate reductase accepts electrons in the upstream region of the respiratory chain from either ubiquinone or cytochrome *b* whereas nitrite reduction occurs in a more downstream region, at the site of cytochrome *c* (25). In the present study, we found that antimycin A affects the cytochrome *c* redox transition in nitrite-reducing cells incubated with acetate whereas in the presence of butyrate no such effect was evident. Considering the different locations of the terminal reductases, these findings could be explained by acetate donating electrons closer to nitrate reductase (upstream) and butyrate donating electrons closer to nitrite reductase, downstream of the site of antimycin A blockage. In this context, the findings on methanol oxidation by *Paracoccus denitrificans* (28) illustrate the existence of different electron transfer routes. In the latter study, methanol oxidation was shown to be coupled to electron transport via cytochrome *c* and, compared with other electron donors, was not inhibited by antimycin A.

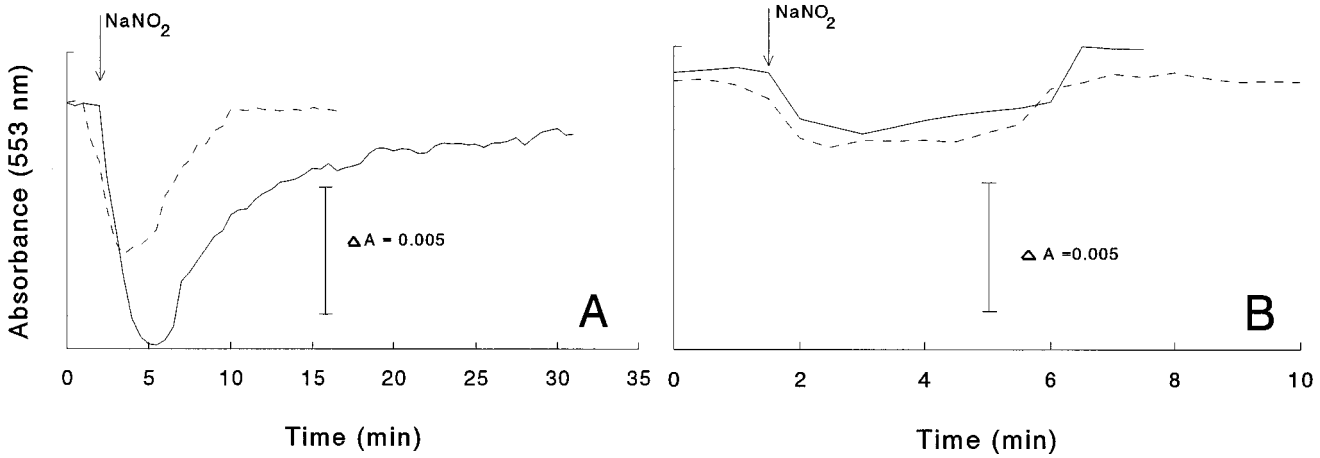


FIG. 7. $A_{553}$ (cytochrome *c*) of *P. stutzeri* cells grown on acetate and washed and resuspended in phosphate buffer (pH 7.1) at a protein concentration of 2,942 mg/liter. (A) Acetate (5 mM)-supplemented cells with (solid line) and without (broken line) antimycin A (20 μg/ml) to which sodium nitrite (0.71 mM) was added as indicated. (B) As in panel A except cells were supplemented with butyrate (5 mM) instead of acetate. Reference cuvettes contained ferricyanide-oxidized *P. stutzeri* cells.

Studies on butyrate oxidation by denitrifiers have shown that butyrate (and probably longer-chain VFA) is metabolized by either β-oxidation and subsequent incorporation into the tricarboxylic acid cycle or condensation of butyrate to form β-ethylmalate (19, 20). To the best of our knowledge, the question of the electron transfer route involved during either of these oxidation pathways has not been examined.

In conclusion, in this study we provide evidence for the fact that in addition to previously known factors, the type of carbon source may also influence nitrite accumulation in denitrifiers. The significance of the finding that different VFA cause differences in nitrite accumulation lies in the fact that in many treatment plants designed for nitrate removal, VFA are the most abundant carbon and electron sources available for denitrifiers. Although this study was conducted with a bacterial strain isolated from a denitrifying fluidized bed reactor, it is impossible to predict the effect of VFA on nitrite accumulation in such reactors on the basis of these results. Follow-up studies aimed at discerning the relative abundance of this *P. stutzeri* strain and bacterial strains with similar metabolic characteristics under in situ conditions are in progress.

## REFERENCES


1. **Aboutboul, Y., R. Arbiv, and J. van Rijn.** 1995. Anaerobic treatment of fish culture effluents: volatile fatty acid mediated denitrification. Aquaculture **133:**21–32.
2. **Almeida, J. S., M. A. M. Reis, and M. J. T. Carrondo.** 1995. Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens*. Biotechnol. Bioeng. **46:**476–484.
3. **Arbiv, R., and J. van Rijn.** 1995. Performance of a treatment system for inorganic nitrogen removal in intensive aquaculture systems. Aquacult. Eng. **14:**189–203.
4. **Beccari, M., R. Passino, R. Ramadori, and V. Tandoi.** 1983. Kinetics of dissimilatory nitrate and nitrite reductase in suspended growth culture. J. Water Pollut. Control Fed. **55:**58–63.
5. **Betlach, M. R., and J. M. Tiedje.** 1981. Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. Appl. Environ. Microbiol. **42:**1074–1084.
6. **Blaszczyk, M.** 1993. Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*. Appl. Environ. Microbiol. **59:**3951–3953.
7. **Coyne, M. S., and J. M. Tiedje.** 1990. Induction of denitrifying enzymes in oxygen limited *Achromobacter cycloclastes* continuous culture. FEMS Microbiol. Ecol. **73:**263–270.
8. **Eilersen, A. M., M. Henze, and L. Kloft.** 1995. Effect of volatile fatty acids and trimethylamine on denitrification in activated sludge. Water Res. **29:** 1259–1266.
9. **Fass, S., V. Ganaye, V. Urbain, J. Manem, and J. C. Block.** 1994. Volatile fatty acids as organic carbon sources in denitrification. Environ. Technol. **15:**459–467.
10. **Kaspar, H. F., and K. Wuhrmann.** 1978. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl. Environ. Microbiol. **36:**1–7.
11. **Korner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
12. **Kucera, I., V. Dadak, and R. Dobry.** 1983. The distribution of redox equivalents in the anaerobic respiratory chain of *Paracoccus denitrificans*. Eur. J. Biochem. **130:**359–364.
13. **Kucera, I., L. Lampordova, and V. Dadak.** 1987. Control of respiration rate in non-growing cells of *Paracoccus denitrificans*. Biochem. J. **246:**777–782.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **McCarthy, P. L., L. Beck, and S. P. Amant.** 1969. Biological denitrification of wastewaters by additions of organic materials, p. 1271–1285. *In* 24th Annual Purdue Industrial Waste Conference, Purdue University, Lafayette, Ind.
16. **Nishimura, Y., T. Kamihara, and S. Fukui.** 1979. Nitrite reduction with formate in *Pseudomonas denitrificans* ATCC 13867. Biochem. Biophys. Res. Commun. **87:**140–145.
17. **Nishimura, Y., T. Kamihara, and S. Fukui.** 1980. Diverse effect of formate on the dissimilatory metabolism of nitrate in *Pseudomonas denitrificans* ATCC 13867: growth, nitrite accumulation in culture, cellular activities of nitrite and nitrate reductases. Arch. Microbiol. **124:**191–195.
18. **Peladan, F., and H. Monteil.** 1988. Identification of *Pseudomonas*, *Flavobacterium* and *Alcaligenes* sp. with API 20 NE. Syst. Pathol. Biol. **36:**187–192.
19. **Rabin, R., and S. J. Ajl.** 1965. Enzymatic hydration of ethylfumaryl-CoA by *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **97:**388–391.
20. **Rabin, R., I. I. Salamon, A. S. Bleiweis, and S. J. Ajl.** 1968. Metabolism of β-ethylmalic acid by *Pseudomonas aeruginosa*. Biochemistry **7:**377–388.
21. **Richardson, D. J., and S. J. Ferguson.** 1992. The influence of carbon substrate on the activity of the periplasmic nitrate reductase in aerobically grown *Thiosphaera pantotropha*. Arch. Microbiol. **157:**535–537.
22. **Sasser, M.** 1990. Identification of bacteria through fatty acid analyses, p. 199–204. *In* Z. Klement, K. Rudolph, and D. C. Sands (ed.), Methods in phytobacteriology. Akademiai Kiado, Budapest.
23. **Scheiner, D.** 1976. Determinations of ammonia and Kjeldahl nitrogen by the indophenol method. Water Res. **10:**31–36.
24. **Sears, H. J., S. J. Ferguson, D. J. Richardson, and S. Spiro.** 1993. The identification of a periplasmic nitrate reductase in *Paracoccus denitrificans*. FEMS Microbiol. Lett. **113:**107–112.
25. **Stouthamer, A. H.** 1988. Dissimilatory reduction of oxidized nitrogen compounds, p. 245–303. *In* A. J. B. Zehnder (ed.), Environmental microbiology of anaerobes. John Wiley and Sons, New York.
26. **Strickland, J. D., and T. R. Parsons.** 1972. A practical handbook of seawater analysis. Bull. Fish. Res. Board Can. **167:**77–80.
27. **Thomsen, J. K., T. Geest, and R. P. Cox.** 1994. Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans*. Appl. Environ. Microbiol. **60:**536–541.

27a. **van Rijn, J., and Y. Tal.** Unpublished data.

28. **Van Verseveld, H. W., and A. H. Stouthamer.** 1978. Electron-transport chain and coupled oxidative phosphorylation in methanol-grown *Paracoccus denitrificans*. Arch. Microbiol. **118:**13–20.
29. **Visniac, W., and M. Santer.** 1957. The thiobacilli. Bacteriol. Rev. **21:**195–213.
30. **Wilderer, P. A., W. L. Jones, and U. Dau.** 1987. Competition in denitrification systems affecting reduction rate and accumulation of nitrite. Water Res. **21:**239–245.