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Inhibition by Sulfide of Nitric and Nitrous Oxide Reduction by Denitrifying *Pseudomonas fluorescens*†

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The influence of low redox potentials and H_2S on NO and N_2O reduction by resting cells of denitrifying *Pseudomonas fluorescens* was studied. Hydrogen sulfide and Ti(III) were added to achieve redox potentials near −200 mV. The control without reductant had a redox potential near +200 mV. Production of ^{13}NO, [^{13}N]N_2O, and [^{13}N]N_2 from $^{13}NO_3^-$ and $^{13}NO_2^-$ was followed. Total gas production was similar for all three treatments. The accumulation of ^{13}NO was most significant in the presence of sulfide. A parallel control with autoclaved cells indicated that the ^{13}NO production was largely biological. The sulfide inhibition was more dramatic at the level of N_2O reduction; [^{13}N]N_2O became the major product instead of [^{13}N]N_2, the dominant product when either no reductant or Ti(III) was present. The results indicate that the specific action of sulfide rather than the low redox potential caused a partial inhibition of NO reduction and a strong inhibition of N_2O reduction in denitrifying cells.

In a search of environmental factors that influence the overall activity and the differential release of gas products during denitrification, reference is most often given to parameters such as O_2, available carbon, pH, and temperature (2). Much less attention has been paid to the parameters characteristic of the reduced environment, e.g., iron and sulfur compounds. In some environments, in particular marine sediments, denitrification takes place in close proximity to zones of active transformation of iron and sulfur.

In an earlier study in coastal marine sediments, significant accumulation of the denitrification intermediates, NO and N_2O, was noted in the redox transition zone near the sulfide-rich deeper layers; it was suggested that these accumulations were caused by either the low redox potential or the presence of sulfide in this zone (5).

The present study was undertaken to establish whether a low redox potential or the presence of sulfide caused accumulation of NO, N_2O, or both. It was found that sulfide and not a low redox potential caused an increase in proportion of N_2O and NO at the expense of N_2 in denitrifying *Pseudomonas fluorescens*.

MATERIALS AND METHODS

The denitrifying bacterium used was *P. fluorescens* (strain 72), isolated from poorly drained Minnesota maize soil by Gamble et al. (3). The organism was grown anaerobically in tryptic soy or nutrient broth (Difco) with nitrate (3.5 mM KNO_3) or nitrite (5 mM KNO_2) as the electron acceptor. The cells were harvested at early stationary phase by centrifugation at 5°C. Cells were washed three times in 0.02 M phosphate buffer (pH 7.0) and suspended to an optical density of 0.2 to 0.5 at 660 nm.

It was anticipated that gas samples taken in syringes might be subject to O_2 contamination during the short wait before injection into the ^{13}N detection system. This risk was minimized by the admission of excess, unlabeled NO into the syringes, a step which also improved the elution of ^{13}NO from the chromatographic column. The possible lack of a quantitative recovery of ^{13}NO should not exclude a relative comparison of ^{13}NO production between the individual treatments, since all samples of a given incubation time were treated in a similar manner.

Any loss of free sulfide was negligible during the experimental time of 5 min, since a significant decrease of H_2S in similar incubations could not be detected by iodine titration until several hours had elapsed (Sørensen, unpublished data). Both reducing agents provided a measured redox potential (Eh value) near −200 mV. The reaction mixtures without reducing agent gave a positive but variable Eh value between +100 and +300 mV. It was likely that the short exposure to air during the redox assay gave values that overestimated the actual Eh during the incubation. The possible error was less important in the present context, however, where comparisons were made to the strongly reduced series with Ti(III) or H_2S and Eh values near −200 mV.

Five milliliters of the cell suspension was transferred to 25-ml serum vials with 1 ml of a 1% glucose solution. The vials were capped, made anaerobic by repeated evacuation, and purged with helium gas. This proce-

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dure most likely left traces of O_2 in the vials, but constitutive enzymes for aerobic respiration and the intermittent storage of the vials for a few hours before ^{13}N addition apparently exhausted the residual O_2. Autoclaved reaction mixtures served as sterile controls for experiments in which chemical production of N gases was examined.

Ti(III) citrate or -oxalate and H_2S were added in separate series of vials to achieve low and comparable redox potentials. Concentrations of Ti(III) + Ti(IV) and H_2S in the cell suspensions were 15 mM and 0.3 mM, respectively, as calculated from the addition of 0.5 ml of 0.2 M titanium solution (7) or 0.1 ml of pure H_2S gas (solubility, 13 M at 25°C) to the 6-ml cell suspensions. The reducing agents were added about 15 min before the ^{13}N substrate to allow the compounds to reach equilibrium. The approximate redox potential in the vials was measured with platinum and calomel reference electrodes. For this assay, a vial was uncapped and the sample was rapidly poured into a small beaker under the measuring electrodes. The Eh value was recorded immediately since a prolonged exposure to air gradually increased the redox potential in the samples.

Radioactive ^{13}N (half-life, 10 min) was produced at the Michigan State University Cyclotron using the ^{16}O (p, α) ^{13}N reaction as described elsewhere (6). The $^{13}NO_3^-$ substrate used contained 80 to 90% $^{13}NO_3^-$ and 10 to 20% $^{13}NO_2^-$. In other experiments pure $^{13}NO_2^-$ was used, which was produced by reduction of the $^{13}NO_3^-$ (+$^{13}NO_2^-$) in a column of copperized cadmium, as described by McElfresh et al., (3a), but modified to a smaller scale. The ^{13}N in the effluent was more than 99% NO_2^-, as determined by high-pressure liquid chromatography (6).

The vials were incubated at room temperature (about 25°C) on a rotary shaker to facilitate exchange of gases between the gaseous and liquid phases. Each reaction mixture representing a specific treatment received about 100 μl of carrier-free radioactive substrate, typically 0.1 to 1 mCi of ^{13}N (5 to 50 fg of N), by injection to start the experiment. Gas samples (0.5 ml) were taken after 1, 3, and 5 min into helium-purged 1-ml syringes equipped with Mininert closure valves (Precision Scientific Co., Baton Rouge, La.). About 0.2 ml of pure NO (Matheson) was then carefully drawn into each syringe before the gas samples were analyzed for mass and radioactivity in a combined gas chromatograph-proportional counter detection system. This instrument, described previously (6), was modified to improve the ^{13}NO detection for the present study. A liquid-N_2-cooled loop (50 by 0.32 cm, stainless-steel tubing) was inserted between the injection port and the chromatographic column (Porapak Q) to retain ^{13}NO and [^{13}N]N_2O while [^{13}N]N_2 was eluted from the column; then the loop was warmed, and ^{13}NO and [^{13}N]N_2O were separated by the column and detected. The reported results have been observed repeatedly, and data of only a few representative experiments are reported here. Corrections were made for solubility of the gases, and all reported activities were corrected for decay and background. All quantities reported are total disintegrations in the gas chromatographic peak.

RESULTS AND DISCUSSION

Only carrier-free $^{13}NO_3^-$ and $^{13}NO_2^-$ were used in this study since high concentrations of NO_3^- and NO_2^- have been shown to influence NO reduction (4) and the differential release of N_2O and N_2 from soil denitrification (1a).

$^{13}NO_3^-$ as substrate. A first experiment, with one treatment without a reducing agent and two treatments with Ti(III) and H_2S, respectively, was performed where the nitrogenous substrate for denitrification was about 80% $^{13}NO_3^-$ and 20% $^{13}NO_2^-$. No attempt was made to obtain a $^{13}NO_2^-$-free source at this stage, since the bacterium was shown to accumulate some $^{13}NO_2^-$ during $^{13}NO_3^-$ reduction (M. R. Betlach, personal communication).

The pattern of ^{13}N gas production is shown in Fig. 1. All three gases (^{13}NO, [^{13}N]N_2O, [^{13}N]N_2) were produced in all three treatments, but the production pattern was influenced by treatment. The upper part of Fig. 1 shows the accumulation of ^{13}NO in the three treatments (note smaller vertical scale than for [^{13}N]N_2O and [^{13}N]N_2 in lower part of figure). The most pronounced accumulation of ^{13}NO was observed in the sulfide-containing series, which showed a threefold increase in NO.

Sulfide, however, exerted a stronger inhibitory effect on the reduction of [^{13}N]N_2O since [^{13}N]N_2O accumulated at the expense of [^{13}N]N_2 in the H_2S-containing treatment. This was opposite to the result from the two other treatments, where the production of [^{13}N]N_2O was small compared to that of [^{13}N]N_2. The total gas production was similar in the three treatments, which indicates that there was no major effect by H_2S at the level of $^{13}NO_3^-$ and $^{13}NO_2^-$ reduction. A most important result was the similar gas composition in the presence of Ti(III) reductant and in the absence of any reductant. This suggested that the inhibitions were not caused by the low redox potential but rather induced by some specific action of the sulfide compound.

$^{13}NO_2^-$ as substrate. To confirm the findings above and to determine the significance of chemical processes, in particular the reduction of $^{13}NO_2^-$ to ^{13}NO, two parallel experiments were performed using pure $^{13}NO_2^-$ as the substrate for denitrification and with live and autoclaved cells, respectively. Decay during $^{13}NO_2^-$ preparation explains the lower activity of total ^{13}N gas production in the $^{13}NO_2^-$-amended (versus $^{13}NO_3^-$) vials.

The results from an experiment with live cells in which Ti(III) and H_2S were supplied as reducing agents are shown in Fig. 2. The gas production patterns were similar to those ob-

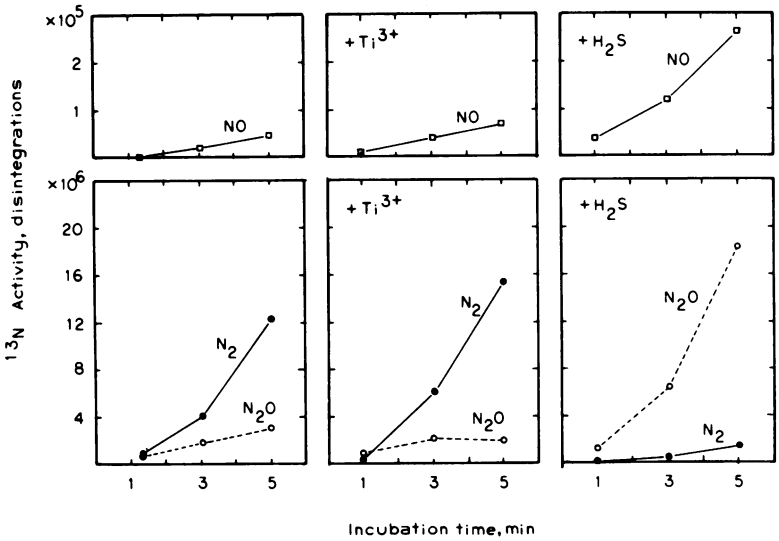

FIG. 1. *^{13}NO, [^{13}N]N_2O, and [^{13}N]N_2 production by P. fluorescens from $^{13}NO_3^-$ substrate without reducing agent (left column), with 15 mM Ti(III) (middle column), and with 0.3 mM H_2S (right column).*

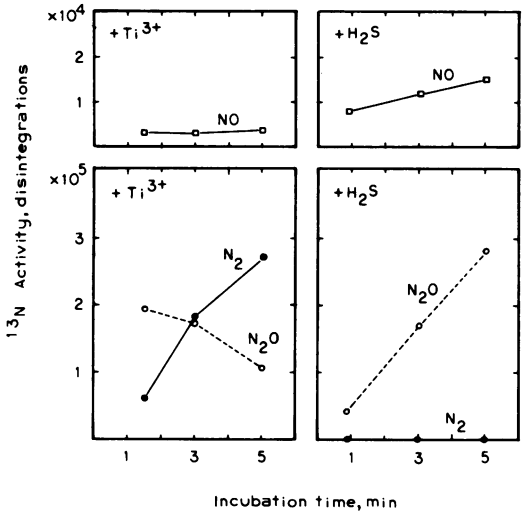

FIG. 2. *^{13}NO, [^{13}N]N_2O, and [^{13}N]N_2 production by P. fluorescens from pure $^{13}NO_2^-$ substrate with 15 mM Ti(III) (left column) and with 0.3 mM H_2S (right column).*

tained in the first experiment with $^{13}NO_3^-$. In short, the ^{13}NO accumulation was most significant in the presence of H_2S, and the accumulation of [^{13}N]N_2O was transient, though initially higher, in the Ti(III)-containing series. In this experiment, almost complete inhibition of the [^{13}N]N_2O reduction was found in the H_2S-containing series. Thus, no qualitative difference was observed between the two experiments with $^{13}NO_2^-$ and $^{13}NO_3^-$ substrates.

The use of strong reducing agents, such as Ti(III) and H_2S, involves a risk of chemical reactions and general toxic effects to the bacterium, but the inclusion of sterile controls and observations for significantly altered rates of the total gas production should provide a control for any chemical reactions caused by these compounds. Since nitrite (as opposed to nitrate) is more prone to chemical reactions, a source of pure $^{13}NO_2^-$ was used in the sterile control experiment. This experiment is directly comparable to the previous one in terms of treatments and $^{13}NO_2^-$ substrate. Table 1 shows the activities of ^{13}NO, [^{13}N]N_2O, and [^{13}N]N_2 in the treatment without reductant and in two treatments with Ti(III) and H_2S. Only traces of ^{13}NO and no [^{13}N]N_2O or [^{15}N]N_2 were detected in the treatments without reducing agent and with H_2S. The production of ^{13}NO in the sterile H_2S-containing control was much lower than in the viable experiments. Though ^{13}NO was detectable in the sterile controls, it was apparent that most ^{13}NO production in the viable experiments was biological. Further, in light of the low ^{13}NO levels in the sterile control, the increased accumulation of ^{13}NO in the viable H_2S-containing treatment would seem to result from an inhibition of the ^{13}NO reduction.

The Ti(III)-containing sterile treatment showed a significant accumulation of ^{13}NO prior to a further reduction to [^{13}N]N_2O and [^{13}N]N_2. The accumulations of the latter were insignificant as compared to the [^{13}N]N_2O and [^{13}N]N_2 production in the viable Ti(III)-containing treat-

TABLE 1. *^{13}N gas production from $^{13}NO_2^-$ under sterile conditions (autoclaved cells) compared to live cells as influenced by reductant*

Reductant	Cells	Time (min)	^{13}N gases detected (10^4 disintegrations)		
			^{13}NO	$[^{13}N]N_2O$	$[^{13}N]N_2$
None	Dead	1	0.01	ND[a]	ND
	Dead	3	ND	ND	ND
	Dead	5	ND	ND	ND
H_2S	Dead	1	0.04	ND	ND
	Dead	3	0.03	ND	ND
	Dead	5	0.01	ND	ND
	Live	5	1.53 (>100)[b]	28.0 (>1,000)	0.01
Ti(III)	Dead	1	0.90	0.05	0.01
	Dead	3	1.34	0.10	0.01
	Dead	5	0.96	0.12	0.03
	Live	5	0.36	10.9	27.2

[a] ND, None detected.
[b] Fold increase in ^{13}N gases in vial of live cells over that found in analogous sterile control.

ment, but the result illustrates the strength of Ti(III) as a reducing agent. The results also indicated that further biological reduction of any chemically produced ^{13}NO to $[^{13}N]N_2O$ and $[^{13}N]N_2$ kept the ^{13}NO levels low in the viable experiment. Any concurrent chemical reduction of $^{13}NO_2^-$ to ^{13}NO in the viable, Ti(III)-containing series was most likely inferior to the biological, since the total gas production (mostly as $[^{13}N]N_2$) was much higher than the rate of $^{13}NO_2^-$ reduction to ^{13}NO in the chemical controls.

In conclusion, the experiments showed that sulfide rather than the associated low redox potential was responsible for the increased accumulations of ^{13}NO and $[^{13}N]N_2O$ during denitrification by *P. fluorescens* resting cells. A partial inhibition of ^{13}NO reduction and a strong inhibition of $[^{13}N]N_2O$ reduction is suggested. This finding seems to be generally true for all denitrifiers, since we noted this same response to H_2S for three other denitrifiers, *Alcaligenes faecalis* 191, *Flavobacterium* strain 175, and *P. aeruginosa* 156, all isolated by Gamble et al. (3).

Our results suggest that sulfide may influence the production of N_2O and NO in natural environments. Further studies are needed to determine in which environments this effect is significant and to define what concentrations of sulfide could account for elevated concentrations of these gases in nature. It is suggested that this mechanism could account for the accumulations of NO and N_2O reported earlier (5) for a coastal marine sediment. Perhaps there are situations where sulfide rather than acetylene could be used more favorably in assays of denitrification which rely on quantifying N_2O.

ACKNOWLEDGMENTS

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