APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1982, p. 854–860
0099-2240/82/040854-07$02.00/0
Vol. 43, No. 4

Dissimilatory Reduction of NO_2^- to NH_4^+ and N_2O by a Soil *Citrobacter* sp.†

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Received 17 September 1981/Accepted 1 December 1981

Dissimilatory reduction of NO_2^- to N_2O and NH_4^+ by a soil *Citrobacter* sp. was studied in an attempt to elucidate the physiological and ecological significance of N_2O production by this mechanism. In batch cultures with defined media, NO_2^- reduction to NH_4^+ was favored by high glucose and low NO_3^- concentrations. Nitrous oxide production was greatest at high glucose and intermediate NO_3^- concentrations. With succinate as the energy source, little or no NO_2^- was reduced to NH_4^+ but N_2O was produced. Resting cell suspensions reduced NO_2^- simultaneously to N_2O and free extracellular NH_4^+. Chloramphenicol prevented the induction of N_2O-producing activity. The K_m for NO_2^- reduction to N_2O was estimated to be 0.9 mM NO_2^-, yet the apparent K_m for overall NO_2^- reduction was considerably lower, no greater than 0.04 mM NO_2^-. Activities for N_2O and NH_4^+ production increased markedly after depletion of NO_3^- from the media. Amendment with NO_3^- inhibited N_2O and NH_4^+ production by molybdate-grown cells but not by tungstate-grown cells. Sulfite inhibited production of NH_4^+ but not of N_2O. In a related experiment, three *Escherichia coli* mutants lacking NADH-dependent nitrite reductase produced N_2O at rates equal to the wild type. These observations suggest that N_2O is produced enzymatically but not by the same enzyme system responsible for dissimilatory reduction of NO_2^- to NH_4^+.

In anaerobic ecosystems the predominant fate of NO_3^- is generally believed to be reduction to N_2O and N_2 by bacterial respiration, that is, denitrification. However, it is known that numerous bacterial genera can catalyze an alternative reduction pathway, dissimilatory reduction of NO_3^- to NH_4^+ (9, 14). A few studies suggest that this latter reaction may be of some significance (2–4, 12, 13), yet little is known about the physiology and ecology of the process. Recently, we reported that the majority of soil isolates capable of dissimilatory NO_3^- reduction could also reduce NO_2^- under the appropriate conditions and that organisms which can produce NH_4^+ from NO_2^- are apparently more numerous than denitrifiers in the soils we studied (11).

In this previous study it was observed also that most non-denitrifying NO_3^- reducers can produce N_2O in addition to NH_4^+. Evidence was presented suggesting that this type of N_2O production is enzymatic but not necessarily growth associated. Kaspar and Tiedje have recently suggested that N_2O production in the rumen is also associated with NO_2^- reduction to NH_4^+ and not denitrification (6). Production of N_2O in the biosphere is of current interest because of its potential for degradation of atmospheric ozone (5). Terrestrial production of N_2O has been attributed primarily to denitrification and more recently to nitrification (1). Since N_2O production associated with dissimilatory reduction of NO_2^- seems to be a widespread characteristic of bacteria, and so may be an important alternative source of N_2O, we further investigated this process with a *Citrobacter* sp. isolated from soil. In this paper we examine the relationship between NH_4^+ and N_2O production and the effect of energy substrate, NO_3^-, and NO_2^- concentrations on N_2O production.

MATERIALS AND METHODS

Organism and growth conditions. The organism used in these investigations was isolated from a Kentucky agricultural soil and classified as a *Citrobacter* sp. (isolate C48) in an earlier study (11). The growth media for most experiments consisted of: 50 mM phosphate buffer (K salt) at pH 7.0, 5 mM $(NH_4)_2SO_4$, 0.1 mM $Na_2MoO_4 \cdot 2H_2O$, 0.35 mM $CaSO_4$, 0.02 mM $FeSO_4$ with 0.025 mM citrate, and 0.005 mM $MnSO_4$, and the desired concentrations of glucose or sodium succinate and KNO_3 or $NaNO_2$. In some experiments molybdate was replaced with 10 mM $Na_2WO_4 \cdot 2H_2O$. Replacement of molybdate with tungstate results in an inactive NO_3^- reductase (10); this was confirmed in preliminary experiments with this *Citrobacter* isolate. Media for tungstate-grown cells contained 5 mM NO_2^-. All

† Journal article no. 81-3-166 of the Kentucky Agricultural Experiment Station.

ments with this *Citrobacter* isolate. Media for tungstate-grown cells contained 5 mM NO_2^-. All defined media were sterilized by filtration. Media were inoculated with a late-log-phase anaerobic culture grown in nitrate broth (Difco). Cultures were incubated at 24°C. Anaerobiosis was achieved either by growing cultures (10 ml) in Hungate tubes (Bellco Glass, Inc.) which were aseptically evacuated and flushed with N_2 or by placing culture flasks in an anaerobic chamber (BBL Microbiology Systems).

To determine the effect of chloramphenicol on expression of N_2O-producing activity, cells were grown aerobically in 0.3% tryptic soy broth. They were then centrifuged and resuspended in 0.15% tryptic soy broth with 1 mM NO_2^-, with and without 200 μg of chloramphenicol per ml, and then made anaerobic.

Escherichia coli mutants were supplied by J. A. Cole. These mutants were reported to be defective in the *nirB* gene, which appears to be the structural gene for NADH-dependent nitrite reductase, probably the particular NO_2^--reducing enzyme associated with dissimilatory or fermentative NH_4^+ production (4). These organisms were grown anaerobically in 3% tryptic soy broth with 5 mM KNO_3 added.

Resting cell assays. Unless otherwise noted, cells were harvested in early stationary phase, within 12 h after NO_3^- and NO_2^- disappeared from the media. Harvesting was by repeated centrifugation and washing in 50 mM phosphate buffer, pH 7.0, with 200 μg of chloramphenicol per ml. Tungstate-grown cells were washed and assayed in a solution also containing 1 mM tungstate since inactivation of NO_3^- reductase by tungstate was observed to be at least partially reversible. Chloramphenicol at 200 μg/ml was previously observed to completely inhibit the growth of this organism. Washed cells were resuspended in buffer and chloramphenicol with 50 mM glucose, or succinate in some assays. Cell density was determined by comparing optical density to a previously prepared standard curve relating optical density at 420 nm of cell suspensions to dry weight, the latter having been measured by washing cells in distilled water and drying at 80°C.

Cell density usually ranged from 0.1 to 0.4 mg (dry weight)/ml, but it was necessary to vary this in some of the experiments to obtain convenient reaction rates. Suspensions (5 or 10 ml) were placed in Hungate tubes and made anaerobic by evacuating and flushing with N_2. After a 30-min preincubation, assays were initiated by adding the appropriate quantity of anaerobic NO_2^- solution (final concentration, 1 mM in most experiments) or NO_3^- and NO_2^-. To observe the effect of sulfite, sufficient Na_2SO_3 was added before preincubation to give a final concentration of 1 mM. Hydroxylamine was added as $NH_2OH \cdot HCl$ in distilled water, with or without cells or NO_2^-, after tubes were made anaerobic. Assays were conducted at room temperature (20 to 22°C), and tubes were rapidly shaken lengthwise. Gas samples were obtained with 1-ml tuberculin syringes. Clear liquid samples were obtained by filtration and were refrigerated for up to 1 week before analysis.

Nitrous oxide was normally sampled three times at 10-min intervals from three replicate tubes. Liquid samples were taken from four replicate tubes at 30-min intervals to measure rates of NH_4^+ production. Rates of NO_3^- reduction were estimated by sampling as for NO_2^- reduction to NH_4^+, but only NO_2^- was measured and the rates were not corrected for the small amount of NO_2^- that would be further reduced during the assay. Assays were designed so that no more than 20% of the substrate was consumed during the incubation.

TABLE 1. Products of NO_3^- reduction by *Citrobacter* sp. grown with different concentrations of NO_3^- and glucose or succinate[a]

Energy source (mM)	Initial NO_3^- conc (mM)	% of added NO_3^-–N recovered as:			NH_4^+ accumulation[b]
		NO_3^-	NO_2^-	N_2O	
Glucose					
1	25	50.6	52.2	0.2	0
3	25	40.9	47.1	0.7	0
5	25	39.1	60.3	1.4	0
10	25	0.9	92.4	4.5	0
40	25	0	96.7	3.3	0
40	10	0	0.8	23.5	+
40	5	0	0	6.5	+
40	1	0	0	1.4	+
Succinate					
1	25	61.4	30.3	0.2	0
3	25	50.6	54.2	1.4	0
10	25	26.6	79.7	1.9	0
40	25	12.6	92.4	1.5	0
40	10	0	100.0	2.6	0
40	3	0	88.7	18.8	0
40	1	0	75.4	14.1	0

[a] All numbers are means of three replicates. All cultures were grown for 14 days, anaerobically, at 24°C, in defined media.

[b] Because media initially contained 5 mM $(NH_4)_2SO_4$, it was not possible to quantitatively determine NH_4^+ production from NO_3^-. +, Significant increase in NH_4^+ during growth.

Chemical analysis. Nitrous oxide was measured by gas chromatography as described previously (11). Spot tests for the presence of NO_3^- or NO_2^- were done with diphenylamine in H_2SO_4. The presence of NO_3^- was detected by first removing NO_2^- with 5% sulfamic acid. Quantitative NO_2^- measurements were made either by autoanalyzer or manually, using the sulfanilamide–*N*-1-naphthylethylenediamine dihydrochloride procedure (8). Nitrate was measured by the same procedure after biological reduction to NO_2^- (7). A gas-sensing electrode (Orion) was used for NH_4^+ determinations, using procedures described by the manufacturers. Some anaerobic bacteria have been observed to produce sufficient volatile amines to interfere with this measurement (unpublished data), but this was not the case for this isolate.

RESULTS

In batch cultures of *Citrobacter* C48, as much as 23% of the NO_3^- reduced was released as N_2O (Table 1). Some N_2O was produced at all combinations of substrate concentrations, but the percentage of N_2O evolved was small except

TABLE 2. Nitrate- and nitrite-reducing activity in resting cell suspensions of *Citrobacter* harvested at different stages of growth[a]

Stage of growth			Resting cell activity (ng of N mg of cells^{-1} min^{-1})		
h	NO_3^- remaining	NO_2^- remaining	$NO_3^- \rightarrow NO_2^-$	$NO_2^- \rightarrow N_2O$	$NO_2^- \rightarrow NH_4^+$
13	+	+	125	0.02	7
24	−	+	101	0.05	8
47	−	+	89	0.82	84
64	−	+	161	2.58	141
108	−	−	220	0.71	18

[a] Cells were grown in 50 mM dextrose and 5 mM KNO_3, anaerobically. Resting cells were assayed with 1 mM NO_3^- or NO_2^- and 50 mM glucose. Presence of NO_3^- and NO_2^- in growth media was determined by spot test with diphenylamine before cell harvest.

when the molar ratio of glucose/NO_3^- was near 4. At lower ratios NO_2^- was the predominant end product of NO_3^- reduction, and only at higher ratios was NO_2^- reduced to NH_4^+. Because 5 mM $(NH_4)_2SO_4$ was initially added to the media to prevent assimilatory utilization of the NO_3^- and because some NH_4^+ was assimilated, it was not possible in these experiments to accurately measure NH_4^+ production from NO_3^-. It was previously observed that added NH_4^+ had no effect on the dissimilatory reduction of NO_2^- or N_2O production by this isolate (11).

Cells grown with the nonfermentable substrate succinate and NO_3^- also produced N_2O (Table 1). As the succinate/NO_3^- ratio increased, the percentage of NO_3^- reduced to N_2O tended to increase, a somewhat different pattern than observed with glucose. There was no indication of significant production of NH_4^+ with succinate even when the succinate/NO_3^- ratio was 40. This isolate did not grow anaerobically with succinate and NO_2^- or with succinate alone. (Glucose did support growth under these conditions.)

A resting cell experiment with succinate (50 mM)- and NO_3^- (10 mM)-grown cells assayed with succinate (50 mM) provided further evidence that this nonfermentable substrate allowed NO_2^- reduction to N_2O but not to NH_4^+. Cell suspensions evolved N_2O at a rate of 0.62 ng of N min^{-1} mg of cells^{-1}, which fell within the range of values observed with dextrose.

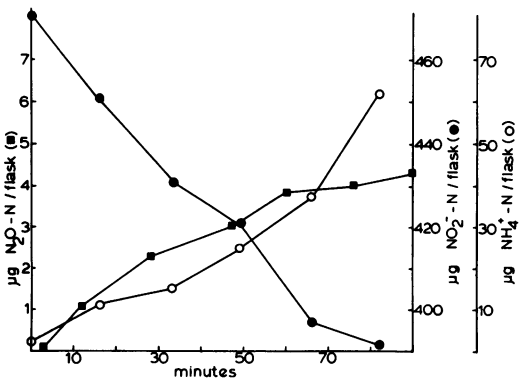

FIG. 1. Reduction of NO_2^- (●) and production of N_2O (■) and NH_4^+ (○) by resting cell suspensions of *Citrobacter* sp. C48. Flasks initially contained 1 mM NO_2^-, glucose, chloramphenicol, and pH 7.0 phosphate buffer. Points are means of three replicate flasks. Cells were grown in defined media with 50 mM glucose and 5 mM KNO_3.

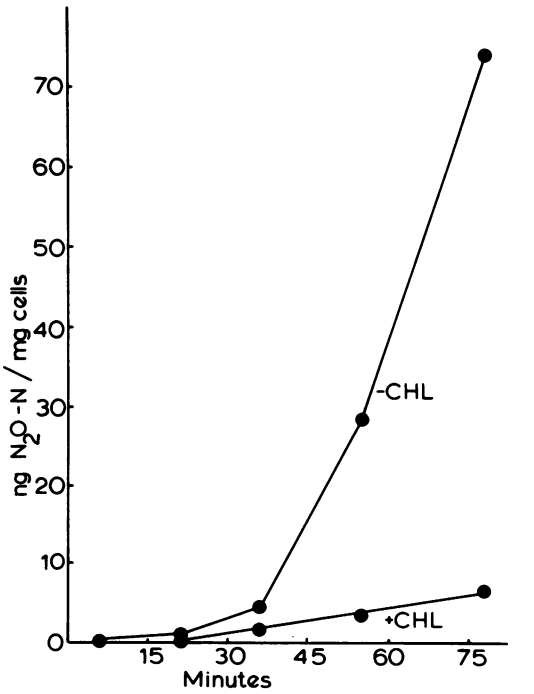

FIG. 2. Production of N_2O by aerobically grown cells transferred to anaerobic conditions with NO_2^-, with and without 200 μg of chloramphenicol (CHL) per ml. Points are means of three replications.

TABLE 3. Effect of NO_3^- on reduction of NO_2^- to N_2O and NH_4^+ by *Citrobacter* cells grown with tungstate or molybdate [a]

Cells grown in:	Resting cells with:	ng of N mg of cells^{-1} min^{-1}	
		N_2O production	NH_4^+ production
Tungstate	NO_2^-	0.012[b]	11.6[b]
Tungstate	NO_3^- + NO_2^-	0.011[b]	13.8[b]
Molybdate	NO_2^-	2.63[c]	10.1[b]
Molybdate	NO_3^- + NO_2^-	0.21[d]	2.3[c]

[a] Cells were grown with 50 mM glucose: tungstate-grown cells with 5 mM NO_2^-; molybdate-grown cells with 5 mM NO_3^-. Values in same columns with same letters are not significantly different at the 95% level of confidence.

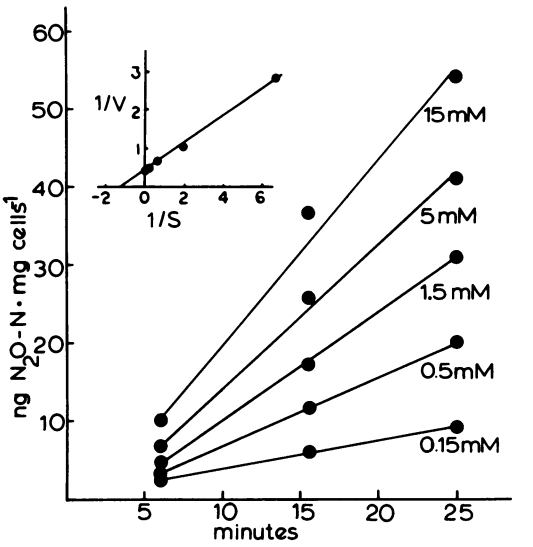

FIG. 3. Effect of NO_2^- concentration on rate of N_2O production by resting cell suspensions of *Citrobacter* sp. C48. Initial NO_2^- concentrations are shown next to curves. Inset is double-reciprocal plot of data with units of millimoles versus milligrams of cells per minute per nanogram of N_2O-N. Cells were assayed with 50 mM glucose, chloramphenicol, and pH 7.0 buffer.

Production of NH_4^+ was 0.9 ng of N min^{-1} mg of cells^{-1}, 10- to 100-fold less than rates with dextrose (see, for example, Table 2).

Anaerobic resting cell suspensions of *Citrobacter* C48 with glucose reduced NO_2^- simultaneously to N_2O and extracellular NH_4^+ (Fig. 1). Cells without added NO_2^- produced no detectable N_2O and did not release significant NH_4^+ (data not shown). After 80 min of incubation in the experiment shown, 76% of the NO_2^--N reduced was recovered as NH_4^+-N and 6% was recovered as N_2O-N. Some of the missing N might be accounted for as intracellular NH_4^+ but no attempt was made to measure this. Previous work indicated that this organism does not produce detectable N_2 (11).

Chloramphenicol inhibited the production of N_2O by aerobically grown cultures which were made anaerobic and amended with NO_2^- (Fig. 2). These results indicate that N_2O production is an enzymatic process and that it is inducible.

Previous studies have shown little reduction of NO_2^- and production of N_2O until most of the NO_3^- was depleted in batch cultures (11). This could be due to either differential rates of synthesis of the various reducing enzymes or NO_3^- inhibition of NO_2^--reducing activity. Table 2 shows that the reducing activities do appear to be expressed at different times in the growth cycle. Relative NO_3^--reducing activity of resting cells harvested at different stages of growth remained high throughout. In contrast, maximum N_2O-producing activity was 100-fold greater than the initial activity. A 20-fold increase in NH_4^+-producing activity was observed. The rate at which NO_2^- was reduced increased dramatically after the depletion of NO_3^-, which occurred shortly before 24 h in this experiment. After the depletion of NO_2^- by growing cells, activities for NO_2^- reduction decreased.

It appears that NO_3^- also affects NO_2^- reduction by cells which have already synthesized the NO_2^--reducing enzymes (Table 3). Molybdate-grown resting cells, which are capable of NO_3^- reduction, reduced NO_2^- to NH_4^+ and N_2O at significantly lower rates when 5 mM NO_3^- was added to the cell suspension. This effect could be a result of competition for reducing power between NO_3^-- and NO_2^--reducing systems, direct inhibition of NO_2^--reducing enzymes by NO_3^-, or, since the addition of NO_3^- resulted in an increase in NO_2^- from 1 to about 2.5 mM during the assay, a nonspecific decrease in cell viability due to NO_2^- toxicity. It was possible to test for direct inhibition of NO_2^--reducing enzymes by using tungstate-grown cells which cannot reduce NO_3^-. Production of NH_4^+ and N_2O was not slower with NO_3^- than without it. This suggests no direct inhibition of NO_2^--reducing enzymes by NO_3^-. Tungstate-grown cells produced N_2O at much lower rates than did molybdate-grown cells. The reason for this is not known. Tungstate (10 mM) added to resting cell suspensions of molybdate-grown cells did not inhibit N_2O production (data not shown).

Sulfite (1 mM) did have a marked differential effect on the two products of NO_2^- reduction. Nitrous oxide production was slightly increased when sulfite was added, from 2.7 to 4.2 ng of N

mg of cells^{-1} min^{-1}. Ammonium production was completely inhibited: from 59.0 to <0.5 ng of N mg of cells^{-1} min^{-1}, the limit of detection, with sulfite. Small quantities of N_2O were evolved when sulfite was added to autoclaved, anaerobic cells with NO_2^-, only about 2% of that produced with live cells. No attempt was made to determine whether sulfite was reduced to sulfide by these cell suspensions.

An examination of the dependence of N_2O and NH_4^+ production rates on NO_2^- concentration demonstrated another difference between these two reactions. Rates of N_2O production by resting cells increased with increasing NO_2^- concentrations in the range of 0.15 to 15 mM (Fig. 3). The approximately linear relationship observed in the double-reciprocal plot indicates that the data mathematically fit a Michaelis-Menten kinetic model. The K_m derived for NO_2^- reduction to N_2O was 0.9 mM NO_2^-. The V_{max} in this experiment was 2.4 ng of N_2O-N mg of cells^{-1} min^{-1}. Physical limitations to the distribution and diffusion of N_2O had no effect on these results; when this experiment was repeated with greatly increased rates of shaking the assay tubes, identical results were obtained.

The kinetics for reduction of NO_2^- to NH_4^+ apparently are markedly different than for N_2O production. The rate of NO_2^- reduction was independent of NO_2^- concentration between 0.17 and 0.96 mM, and the rate decreased by <20% at 0.04 mM (Table 4). Since NH_4^+ is the predominant product of NO_2^- reduction under these conditions, these data lead to the conclusion that the K_m for NO_2^- reduction to NH_4^+ is no greater than 0.04 mM and is more than an order of magnitude less than the K_m for NO_2^- reduction to N_2O. (This conclusion assumes Michaelis-Menten kinetics for NH_4^+ production, but this assumption is not necessary for the more general conclusion that the kinetics for N_2O and NH_4^+ production are different.) Attempts to more precisely define the kinetics of NH_4^+ production were limited by the sensitivity of our analytical techniques.

We tested the possibility that N_2O production by *Citrobacter* was due to chemical decomposition of enzymatically produced NH_2OH. When NH_2OH was added to cell-free assay media, N_2O was in fact produced. However, the rate of production was dependent on NH_2OH concentration: 0.1 mM NH_2OH was required to generate N_2O at a rate comparable to that observed with 0.3 mg of cells ml^{-1} plus 1 mM NO_2^- (10 and 8 ng of N min^{-1}, respectively). The rate was fourfold less (2 ng of N min^{-1}) with 0.01 mM NH_2OH. Under the conditions of our assays the presence of cells or of NO_2^- had no significant effect on the rate of NH_2OH decomposition to N_2O (data not shown).

TABLE 4. Rate of NO_2^- reduction by *Citrobacter* resting cell suspensions with various NO_2^- concentration[a]

NO_2^- concn (mM)	Rate of NO_2^- reduction (ng of N mg of cells^{-1} min^{-1})
0.04	118.8
0.17	147.1
0.39	131.1
0.57	154.8
0.80	148.1
0.96	140.9

[a] Cells were grown with 50 mM glucose and 5 mM KNO_3; assayed with 50 mM glucose.

Mutant strains of *E. coli* K-12 lacking NADH-dependent NO_2^- reductase activity were used to further examine the relationship between N_2O production and NH_4^+ production from NO_2^-. All strains of *E. coli* produced N_2O from NO_2^- at comparable rates (Table 5). Mutant strains produced NH_4^+ at much lower rates than did the wild type.

DISCUSSION

These results support our earlier hypothesis (11) that production of N_2O from NO_2^- by *Citrobacter* C48 and similar non-denitrifying soil bacteria is enzymatic. Chloramphenicol prevented the induction of N_2O-producing activity when aerobically grown cells were transferred to anaerobic conditions with NO_2^-. Previously it was observed that little or no N_2O was produced from NO_2^- by autoclaved cells, Hg-killed cells, cells grown aerobically, or cells grown with no NO_3^- or NO_2^- (11). Earlier we suggested that N_2O production might be attributed to chemical decomposition of enzymatically produced NH_2OH, a possible intermediate in the reduction of NO_2^- to NH_4^+ (14). Nitrous oxide was produced chemically from NH_2OH in our assay system, so this possibility cannot be definitely ruled out. However, it is improbable that NH_2OH accumulates in the concentrations seemingly required to generate N_2O at rates comparable to those observed with cells. This does not exclude the possibility of nonenzymatic generation of N_2O from some other enzymatic product of NO_2^- reduction.

A possible explanation for N_2O production is that it is a by-product of the same enzyme system which reduces NO_2^- to NH_4^+. More specifically, it could be a product of a partially reduced enzyme-substrate complex released when complete reduction to NH_4^+ is not favored (J. A. Cole, personal communication). However, several lines of indirect evidence suggest that N_2O and NH_4^+ are produced by separate enzyme systems. The K_m for NO_2^- reduction to

TABLE 5. Production of NH_4^+ and N_2O from NO_2^- by resting cell suspensions of *E. coli* mutants lacking NADH-dependent NO_2^- reductase[a]

E. coli strain	ng of N mg of cells^{-1} min^{-1}	
	N_2O production	NH_4^+ production
K-12, wild type	1.9[b]	250[b]
V563 *nirB*	2.1[b]	6[c]
CGSC4315 *nirB*	1.4[b]	14[c]
CB203 *nirB*	1.5[b]	17[c]

[a] Cells were grown anaerobically with tryptic soy broth plus 5 mM KNO_3; assayed in 50 mM dextrose and 1 mM NO_2^-. Numbers in the same column followed by the same letter are not significantly different at the 95% level of confidence. Strain designations were provided by J. A. Cole.

N_2O was 0.9 mM NO_2^-, but the overall reduction of NO_2^- had a K_m (assuming a Michaelis-Menten model) of no greater than 0.04 mM. Sulfite, which is known to inhibit some NO_2^- reductases (9), eliminated NH_4^+ production but did not inhibit N_2O production. With a nonfermentable energy source, succinate, little or no NO_2^- was reduced to NH_4^+ but N_2O was produced. (The results presented here are consistent with the conclusion made by others [4, 14] that NO_3^- reduction to NO_2^- is a respiratory process coupled to electron transport phosphorylation but that dissimilatory reduction of NO_2^- to NH_4^+ is fermentative.) The lack of a direct association between NH_4^+ and N_2O production is also suggested by results with *E. coli* mutants lacking NADH-dependent NO_2^- reductase activity. *nirB* strains produced N_2O at rates equal to the wild type but released NH_4^+ at greatly diminished rates. Even if the nitrite reductase of these strains retained the ability to bind NO_2^-, though lacking the ability to reduce it all the way to NH_4^+, it is unlikely that the rate of N_2O release would be unaltered.

The physiological function of N_2O production from NO_2^- is not apparent; perhaps there is none. It was observed previously (11) that N_2O production continued in batch cultures long after growth stopped. Increased growth by fermenting cells was associated with NO_2^- reduction to NH_4^+ but not necessarily with NO_2^- reduction to N_2O. In experiments described here anaerobic cells with succinate produced N_2O from NO_2^- even though succinate did not support detectable anaerobic growth with NO_2^-. The high K_m for NO_2^-reduction to N_2O, 0.9 mM NO_2^-, might also be taken as an indication that N_2O production does not have a significant physiological function. Nitrite concentrations this high would not be common in soils. Also, this is near the concentration at which NO_2^- toxicity becomes apparent (unpublished data). If N_2O production is, in fact, an effective mechanism of detoxifying NO_2^-, then more rapid and complete conversion of NO_2^- to N_2O might be anticipated.

A reliable assessment of the environmental significance of N_2O production by non-denitrifying dissimilatory NO_2^- reducers awaits the development of practical means of distinguishing this N_2O source from other sources in natural ecosystems. Previously it was suggested that non-denitrifying NO_2^- reducers are a potentially significant source of atmospheric N_2O since organisms of this type are numerous in soils and since production rates are high under some soil conditions (11). The observations by Kaspar and Tiedje (6) of N_2O production in the rumen suggest that at least in that anaerobic system this is the predominant mechanism of N_2O production. The experiments reported here lead to some suggestions about the environmental conditions which could favor N_2O production by this mechanism. Low NO_3^- supply and a nonlimiting supply of fermentable substrate favors rapid reduction to NH_4^+, with minimal N_2O production. High NO_3^- supply and limited energy source appear to permit reduction only to NO_2^-, again with limited N_2O evolved. In intermediate cases, particularly when high concentrations of NO_2^- accumulate and the NO_3^- supply is depleted before the energy source, N_2O production should be favored relatively. The much higher K_m for N_2O production than for overall NO_2^- reduction also suggests that the ratio of N_2O to NH_4^+ will increase as NO_2^- concentration increases.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Department of Agriculture-Science and Education Administration cooperative agreement 58-7B30-1-293.

I am grateful for the expert technical assistance of Jean Vincent.

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