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Production of Nitrous Oxide from Nitrite in *Klebsiella pneumoniae*: Mutants Altered in Nitrogen Metabolism

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Under anaerobic conditions, *Klebsiella pneumoniae* reduced nitrite (NO_2^-), yielding nitrous oxide (N_2O) and ammonium ions (NH_4^+) as products. Nitrous oxide formation accounted for about 5% of the total NO_2^- reduced, and NH_4^+ production accounted for the remainder. Glucose and pyruvate were the electron donors for NO_2^- reduction to N_2O by whole cells, whereas glucose, NADH, and NADPH were found to be the electron donors when cell extracts were used. On the one hand, formate failed to serve as an electron donor for NO_2^- reduction to N_2O and NH_4^+, whereas on the other hand, formate was the best electron donor for nitrate reduction in either whole cells or cell extracts. Mutants that are defective in the reduction of NO_2^- to NH_4^+ were isolated, and these strains were found to produce N_2O at rates comparable to that of the parent strain. These results suggest that the nitrite reductase producing N_2O is distinct from that producing NH_4^+. Nitrous oxide production from nitric oxide (NO) occurred in all mutants tested, at rates comparable to that of the parent strain. This result suggests that NO reduction to N_2O, which also uses NADH as the electron donor, is independent of the protein(s) catalyzing the reduction of NO_2^- to N_2O.

Cultures of *Klebsiella pneumoniae* rapidly reduce nitrite (NO_2^-), which serves as the sole nitrogen source for growth. Anaerobic cultures of *Escherichia coli* and *Salmonella typhimurium* also reduce nitrite to ammonia, but at significantly lower rates compared with *K. pneumoniae*. In *E. coli*, two biochemically independent pathways for nitrite reduction are reported (15), an NADH-dependent nitrite reductase (EC 1.6.6.4) which has been purified (6, 8, 11) and a formate-dependent pathway that is reportedly involved in energy production by a chemiosmotic mechanism (15). An NADPH-sulfite reductase (EC 1.8.1.2) possessing gratuitous nitrite reductase activity was also detected in *E. coli* (7, 12). Cole (5) and Cole and Brown (6) reported that the NO_2^- reduction to NH_4^+ proceeds rapidly even when substantial quantities of NH_4^+ have accumulated in the medium. As a result, NO_2^- can serve as the sole nitrogen source for growth in *E. coli*. However, *K. pneumoniae*, *E. coli*, and other enteric bacteria were not known to produce products other than NH_4^+ from NO_2^-, although preliminary reports showed that these organisms are capable of producing nitrous oxide (N_2O) (J. M. Tiedje, N. V. Caskey, M. S. Smith, B. H. Bleakley, and F. B. Firestone, Agronomy Abstr., p. 165, 1979).

Caskey and Tiedje (3) recently reviewed in detail the basic concepts regarding denitrification versus dissimilatory NO_2^- reduction. These will not be discussed here. Suffice it to say that our previous experiments with enteric bacteria show that *K. pneumoniae*, *E. coli*, and *S. typhimurium* reduce NO_2^- and yield both N_2O and NH_4^+ under anaerobic conditions. Moreover, in these organisms, N_2O formation is not controlled by NH_4^+ (16).

In *K. pneumoniae*, NO_3^- metabolism and N_2 fixation pathways are interrelated (9, 17). Nitrogenase biosynthesis is completely repressed by nitrate, nitrite, and nitric oxide, even in mutants that are derepressed for nitrogenase biosynthesis, in the presence of NH_4^+. The influence of nitrogen oxides on nitrogenase biosynthesis was attributed to the dissimilatory reduction of NO_3^- and NO_2^- or NO to N_2O.

To gain further insight into the interaction between these two components of the nitrogen cycle, we investigated N_2O formation from NO_2^- in *K. pneumoniae* by isolating mutants affected in the NO_2^- reduction to NH_4^+ or to N_2O. In this communication, evidence is presented that NADH is the electron donor for the

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NO_2^- reduction to N_2O and that the enzyme system responsible for N_2O formation is distinct from the dissimilatory NO_2^- reductase.

MATERIALS AND METHODS

K. pneumoniae wild-type M5A1 and its chlorate-resistant derivative, strain SK-572, were used in this study. Other mutants were isolated as described below. The media used for growth (Luria broth and sucrose minimal medium) were described previously (20). Nitrogen sources were present at a concentration of 500 $\mu g \cdot ml^{-1}$, unless otherwise indicated. To increase the cell yield and to repress assimilatory NO_3^- reduction processes in some of the cultures, 1 mg of yeast extract (Difco Laboratories) $\cdot ml^{-1}$ was added to the sucrose minimal medium containing $NaNO_2$ or KNO_3.

Mutant isolation. After mutagenesis of *K. pneumoniae* wild-type M5A1 (18), strains SK-1500 and SK-1501 were isolated as mutants incapable of using NO_3^- as the sole nitrogen source for growth. Strain SK-1502 was isolated as a mutant incapable of using NO_2^- as the sole nitrogen source for anaerobic growth. Strain SK-512 was isolated as a chlorate-resistant mutant. Strain SK-1500 failed to grow in a medium containing NH_4^+ and a high concentration of $NaNO_2$ (500 $\mu g \cdot ml^{-1}$), due perhaps to NO_2^- toxicity. This property was exploited for the isolation of mutants which were affected in N_2O formation from NO_2^-. Very small colonies on sucrose minimal medium containing 20 μg of $NaNO_2 \cdot ml^{-1}$ under anaerobic conditions were isolated after mutagenesis of strain SK-1500 and tested further for their ability to reduce NO_2^- in sucrose-Luria broth medium containing 50 μg of $NaNO_2 \cdot ml^{-1}$ under anaerobic conditions. Mutants that are NO_2^- reduction defective were further tested for their ability to produce N_2O. Thus, strains SK-1503, SK-1504, and SK-1505 were chosen for further study.

Preparation of cell extracts. Cells were harvested during the exponential phase of growth in sucrose minimal medium containing 500 μg of $NaNO_2$ or $KNO_3 \cdot ml^{-1}$ and 1 mg of yeast extract $\cdot ml^{-1}$. After being washed with 0.1 M potassium phosphate buffer (pH 7.0), the cells were suspended in 2 to 3 volumes of the cell paste in identical buffer. Cells were broken by passage at 4°C through a French pressure cell at 10,000 lb/in^2. After centrifugation of the extract at 12,000 × *g* for 30 min, the supernatant was collected and used for enzyme assays.

Enzyme assays. Nitrate reductase was assayed by determining NO_2^- produced at 30°C under argon in a mixture containing 50 mM potassium phosphate buffer (pH 7.0), an appropriate quantity of the cell extract or the cell suspension, a 40 mM carbon source or 1 mM NADH or NADPH, and water to 0.99 ml. The reaction was initiated by the addition of 10 μl of 2 M KNO_3. When the cell suspension was used, the reaction was stopped at 3 min by the addition of the reagents for the NO_2^- determination (21). When the cell extract was used, the reaction was stopped at 3 min by the addition of 0.1 ml of saturated zinc acetate. After 4 ml of ethanol was added to precipitate NADH or NADPH, the treated reaction mixtures were centrifuged, and 1 ml of the supernatant of each was used for the NO_2^- determination.

NADH-nitrite reductase activity was assayed in open cuvettes at 30°C as described by Coleman and co-workers (8), except that potassium phosphate buffer was used. NADH-hydroxylamine reductase activity was determined in identical assay systems with $NH_2OH \cdot HCl$ replacing NO_2^-.

Benzyl viologen (BV)-linked nitrite reductase was assayed at 30°C in a 10-ml serum bottle containing 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM BV, 1 mM $NaNO_2$, an appropriate quantity of the cell extract, and water to 1.9 ml. The gas phase was argon. The reaction was initiated by the addition of 0.1 ml of sodium dithionite (10 mg $\cdot ml^{-1}$ in 0.2 M $NaHCO_3$). At 1-min intervals, 0.2-ml samples were removed with a syringe, aerated quickly until the blue color of reduced BV disappeared, and assayed for the nitrite content. When NO was used as the substrate, the reaction was initiated by the addition of NO gas to 0.1% (vol/vol) of the gas phase.

N_2O formation activity at 30°C was determined under argon in a 10-ml serum bottle containing 50 mM potassium phosphate buffer (pH 7.0), an appropriate quantity of the cell extract or the cell suspension, a 40 mM carbon source or 1 mM NADH or NADPH as the electron donor, and water to 1.9 ml. The reaction was initiated by the addition of 0.1 ml of 20 mM $NaNO_2$. At 5-min intervals, gas samples (50 μl) were taken with a gastight syringe, and the N_2O in the gas phase was determined by gas chromatography (16).

TABLE 1. Effect of electron donors on N_2O formation and NO_3^- reduction in strains M5A1 and SK-1500

Electron donor	N_2O formation[a]				NO_3^- reduction by strain SK-1500[b]	
	Whole cells		Cell extracts		Whole cells	Cell extracts
	SK-1500	M5A1	SK-1500	M5A1		
None	0.22	0.21	0.019	0.037	20	0.7
Glucose	0.78	0.75	0.095	0.063	146	5.3
Pyruvate	0.77	0.74	0.019	0.039	138	UD[c]
Acetate	0.23	0.29	0.018	0.031	49	3.0
Formate	0.25	0.13	0.018	0.033	257	59.2
Succinate	0.29	0.30	0.019	0.033	186	15.3
NADH	ND[d]	ND	0.133	0.101	ND	25.9
NADPH	ND	ND	0.117	0.118	ND	10.3

[a] Nanomoles of N_2O formed $\cdot$ minute^{-1} $\cdot$ milligram of protein^{-1}.
[b] Nanomoles of NO_2^- formed $\cdot$ minute^{-1} $\cdot$ milligram of protein^{-1}.
[c] UD, Undetectable.
[d] ND, Not determined.

RESULTS AND DISCUSSION

Effect of electron donors on N_2O formation and NO_3^- reduction. Rates of N_2O formation and NO_3^- reduction were determined using various carbon sources, NADH, and NADPH as the electron donors in wild-type strain M5A1 and strain SK-1500 (Table 1). Strain SK-1500 was defective in NO_2^- reduction to NH_4^+ when assayed in the presence of either NADH or reduced BV as the electron donor, but N_2O

TABLE 2. Reduction of NO_3^- and NO_2^- and formation of N_2O by whole cells of *K. pneumoniae* and *E. coli* mutants[a]

Strain	nmol · min^{-1} · mg of cell protein^{-1}		
	NO_3^- reduction[b]	NO_2^- reduction[c]	N_2O formation[c]
K. pneumoniae			
M5A1	103	77.0	0.31
SK-1500	172	4.5	0.55
SK-1502	165	7.5	0.80
SK-572	0	42.0	0
SK-1501	0	61.0	0
SK-1503	0	2.5	0.03
SK-1504	8	3.2	0.04
SK-1505	0	0.5	0.04
E. coli			
KL-16	ND[d]	ND	0.88
CB244 (*nirA*)	ND	ND	0.05
CB203 (*nirB*)	ND	ND	1.65

[a] The NO_3^- reduction activity of strain M5A1 is lower than that of strain SK-1500 because NO_2^- reduction was not blocked in the assay system for NO_3^- reduction. Glucose served as the electron donor in the experiments. *E. coli nirA* and *nirB* mutants were kindly provided by J. A. Cole.

[b] Cells were grown anaerobically in the presence of NO_3^-.

[c] Cells were grown anaerobically in the presence of NO_2^-.

[d] ND, Not done.

formation and NO_3^- reduction appeared to be unaffected (see Table 2). In contrast, strain SK-1502 reduced NO_2^- at the expense of reduced BV but not NADH (see Table 3). Strain SK-1500 may carry a mutation in the structural or regulatory gene for nitrite reductase, whereas strain SK-1502 appeared to be defective in an electron transport protein related to NO_2^- reductase. Since NO_2^- was not further metabolized in strain SK-1500, NO_3^- reduction activity could be determined by measuring the amount of NO_2^- produced. Glucose and pyruvate served as the electron donors for the N_2O formation in whole cells (Table 1). With cell extracts, glucose, NADH, and NADPH served as the electron donors. Formate failed to serve as an electron donor for the reduction of NO_2^- to N_2O. Nitrous oxide formation by wild-type strain M5A1 and strain SK-1500 was comparable. On the other hand, formate was the best electron donor for NO_3^- reduction in either whole cells or cell extracts. This result is consistent with the generally accepted view that formate dehydrogenation is closely coupled to NO_3^- reduction in enteric bacteria (1). These results suggest that formate metabolism is not involved in the electron donor system for N_2O formation. Although both NADH and NADPH served as the electron donors for N_2O production in cell extracts, NADH may be the physiological electron donor for N_2O formation. NADPH generally functions in cellular biosynthetic reactions but can transfer electrons to NAD^+.

Characterization of the mutants. Whole cells of strains defective in the NO_2^- metabolism were tested for their ability to reduce NO_3^- and NO_2^- and to produce N_2O with glucose as the electron donor (Table 2). Strains SK-1500, SK-1502, SK-1503, SK-1504, and SK-1505 were affected in NO_2^- reduction, probably to NH_4^+, and strains SK-1501 and SK-572 were not. Among these mutants, strains SK-1500 and SK-1502 were unchanged in N_2O formation, but strains SK-1501 and SK-572 were unable to produce N_2O. Strains SK-1503, SK-1504, and SK-1505, which were isolated as presumptive N_2O formation-minus mutants from strain SK-1500, were verified to be poor producers of N_2O. Nitrate reduction was not detected in strains SK-572, SK-1501, SK-1503, and SK-1505, although very low levels of nitrate reductase activity were detected in strain SK-1504. Strains SK-572 and SK-1501 were also found to be defective in NO_3^- reductase activity, even when assayed with reduced BV as the potential electron donor.

E. coli nirB mutants are defective in NADH-dependent NO_2^- reductase activity, and an *E. coli nirB* mutant (strain CB203 [6]) produced N_2O from NO_2^-. The high rate of N_2O production by the NO_2^- reductase mutant, compared with wild-type *E. coli* KL-16, may be due to the lack of NO_2^- reductase activity; this property is similar for *K. pneumoniae* SK-1500 and SK-1502. *E. coli nirA* mutants are defective in a wide range of anaerobic redox processes, including NO_3^- reduction, formic hydrogenlyase, fumarate reduction, and cytochrome c_{552} production (6). The product of the *nirA*$^+$ gene, which has also been referred to as the *fnr*$^+$ or *frdB*$^+$ gene (13), is postulated to be a protein essential for the proper function of the above redox reactions (14). An *E. coli nirA* mutant, strain CB244, was also found to be defective in N_2O formation from NO_2^- (Table 2).

Nitrous oxide formation activities (from NO_2^- and NO) and nitrite and hydroxylamine reductase activities of cell extracts of the various strains are summarized in Table 3. No N_2O formation from NO_2^- was observed in strains SK-572, SK-1501, SK-1503, SK-1504, and SK-1505 when NADH was used as the electron donor. Strain SK-1500 and its parent strain M5A1 produced N_2O from NO_2^- at comparable rates, whereas the activity of strain SK-1502 was higher than that of strain M5A1, as expected from the lack of NO_2^- reductase activity in

TABLE 3. N_2O formation activity from NO_2^- and NO and nitrite and hydroxylamine reductase activities of cell extracts of *K. pneumoniae* mutants

Strain	Parent	N_2O formation (NADH as electron donor)[a] from:		NO_2^- reduction with following electron donor:		NH_2OH reduction (NADH as electron donor)[b]
		NO_2^-	NO	NADH[b]	Reduced BV[c]	
M5A1	Wild type	0.081	0.070	390	420	85
SK-1500	M5A1	0.082	0.067	<40	<30	<7
SK-1502	M5A1	0.130	0.047	<40	650	<7
SK-1501	M5A1	<0.01	0.037	370	550	68
SK-572	M5A1	<0.01	0.032	280	390	56
SK-1503	SK-1500	<0.01	0.027	<40	<30	<7
SK-1504	SK-1500	<0.01	0.039	<40	<30	<7
SK-1505	SK-1500	<0.01	0.054	<40	<30	<7

[a] Nanomoles of N_2O formed · $minute^{-1}$ · milligram of $protein^{-1}$.
[b] Nanomoles of NADH oxidized · $minute^{-1}$ · milligram of $protein^{-1}$.
[c] Nanomoles of NO_2^- formed · $minute^{-1}$ · milligram of $protein^{-1}$.

whole cells (Table 2). NADH-dependent nitrite reductase activity was not detected in the crude extracts from strains SK-1500 and SK-1502. Strain SK-1500 also displayed no reduced BV-linked NO_2^- reduction activity. However, high levels of nitrite reductase activity were observed in the crude extracts from strain SK-1502 when reduced BV was used as the electron donor. The NADH or reduced BV-dependent nitrite reductase activity in strains SK-572 and SK-1501 and the parent was comparable. Rates of NADH-dependent production of N_2O from NO were comparable in all mutant and parent strains. Although NO is chemically reactive and can be easily transformed to other N-compounds (2), N_2O production in these experiments (Table 3) was dependent on the crude extract or cells. This result suggests that the reduction of NO to N_2O may be independent of the enzyme systems catalyzing the reduction of NO_2^- to N_2O or NH_4^+.

The NADH-nitrite reductase of *E. coli* is reported to have hydroxylamine reductase activity also (8), and a nitrite reductase from a denitrifying bacterium is reported to produce N_2O from NO_2^- and NH_2OH (10). Therefore, it is conceivable that there is a binding site for NH_2OH on the NADH-nitrite reductase and that if NH_2OH is an intermediate in the reduction of NO_2^- to NH_4^+, N_2O could be produced from NO_2^- as a side reaction of the NADH-nitrite reductase. To test this possibility, the NADH-hydroxylamine reductase activities of these mutants were determined. The rate of NH_2OH reduction in strain M5A1 was lower than the rate of NO_2^- reduction (Table 3); this property is similar for *E. coli* (4). NADH-hydroxylamine reductase activities of these mutants were similar to the NADH-NO_2^- reductase activities. If N_2O is produced from NO_2^- and NH_2OH by the NADH-nitrite reductase, then strains SK-572 and SK-1501 should also produce N_2O, but these strains did not produce detectable N_2O from NO_2^-. The possibility that N_2O is an intermediate in the reduction of NO_2^- to NH_4^+ can also be excluded, since strains opposite in the formation of N_2O and NH_4^+ were isolated (strains SK-1500 and SK-1502 versus SK-572 and SK-1501). Moreover, the NADH-nitrite reductase is firmly established as being capable of transferring six electrons to NO_2^- (8). These observations suggest that there are two separate NO_2^--reducing activities under anaerobic conditions, a dissimilatory nitrate reductase whose catalytic product is NH_4^+ and another protein generating N_2O.

In summary, all of the mutants tested which are defective in N_2O formation from NO_2^- were also blocked in NO_3^- reduction to NO_2^- (strains SK-572 and SK-1501; Table 2). Mutants isolated as N_2O production-defective mutants are also defective in NO_3^- reduction to NO_2^- (strains SK-1503, SK-1504, and SK-1505; Table 2). Although formate is the generally accepted electron donor for NO_3^- reductase, NADH is also found to be an electron donor in *K. pneumoniae*, an electron donor used by the organism for NO_2^- reduction to N_2O (Table 1). These results could suggest that the NO_3^- reductase is the enzyme catalyzing the reduction of NO_2^- to N_2O in *K. pneumoniae*. Smith (19) observed that a *Citrobacter* sp. grown in the presence of tungsten produced very little N_2O from NO_2^-, whereas cells grown in the presence of molybdate produced N_2O. These results suggest that N_2O production in *Citrobacter* sp. is catalyzed by a molybdoprotein, presumably NO_3^- reductase. If this type of NO_3^- reductase is involved in N_2O production also, it would be very interesting to study the mechanism by which NO_3^- reductase, an enzyme system catalyzing a two-electron transfer in the reduction of NO_3^- to NO_2^-, further reduces this product, although at a lower rate, by a four-electron step to N_2O,

using NADH as an electron donor. Additional experiments with purified nitrate reductase are essential to test this possibility.

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