APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1985, p. 1550–1552
0099-2240/85/121550-03$02.00/0

Vol. 50, No. 6

Production of N_2O and CO_2 During the Reduction of NO_2^- by *Lactobacillus lactis* TS4

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Received 18 March 1985/Accepted 30 July 1985

N_2O was produced during the reduction of NO_2^- by resting cells of *Lactobacillus lactis* TS4. At an initial NO_2^- concentration of 69 μg/ml, the rate of N_2O production was 1.97 nmol/min per mg of protein, and the recovery of reduced NO_2^--N as N_2O-N after 24 h was 77%. Higher initial NO_2^- concentrations decreased both the rate of production of N_2O and the recovery of reduced NO_2^--N. CO_2 production increased during NO_2^- reduction.

There are three major types of NO_2^- reduction by microorganisms. NO_2^- is reduced to NH_4^+ in an assimilatory process by some organisms, such as *Neurospora crassa* (4), and in a dissimilatory process by others, such as *Escherichia coli* (3). Many soil bacteria reduce NO_2^- to the gaseous end products NO, N_2O, and N_2 in the process of denitrification (14, 15, 20), coupling the reduction to oxidative phosphorylation.

Although denitrification was thought to be the primary source of N_2O, N_2O is also produced by other bacteria. Nitrifying bacteria, such as *Nitrosomonas* spp., produce significant amounts of N_2O when grown at reduced O_2 concentrations (10). Many nondenitrifying NO_3^- reducers, including *Bacillus*, *Enterobacter*, *Escherichia*, *Erwinia*, *Klebsiella*, and *Serratia* strains, can also produce N_2O (1, 19). Several *Propionibacterium* species (13) and a soil *Citrobacter* species (18) reduce NO_2^- to N_2O but are not normally considered denitrifiers (13, 18).

We have shown that some lactobacilli isolated from cured meat products are capable of enzymatically reducing NO_2^- (6). A previous study (9) indicated that lactobacilli produce various nitrogenous oxides but not NH_4^+ during NO_2^- reduction. In *Lactobacillus lactis* TS4, nitrite reductase appears to be inducible and requires an electron donor, such as glucose or NADH (8). The characteristics of nitrite reductase in *L. lactis* TS4 resemble those of the enzyme from denitrifying bacteria rather than those of the enzyme from NH_4^+-producing bacteria. The function of the enzyme in *L. lactis* TS4 has not been determined. Lactobacilli are microaerophilic or anaerobic, are cytochrome negative, and ferment carbohydrates; they do not normally reduce NO_3^- (16) and are therefore different from other organisms capable of reducing NO_2^-.

The production of N_2O is often associated with the dissimilatory reduction of NO_2^- (18). The purpose of this work was to determine the end products and possible intermediary products of NO_2^- reduction by *L. lactis* TS4 and to study some of the kinetics of the process.

The organism studied was originally isolated from Thuringer sausage and classified as *L. lactis* TS4 by standard biochemical tests (6). Cells were grown in a 2-liter fermentor in APT broth (Difco Laboratories) with 100 μg of NO_2^- per ml, under a headspace of 95% N_2–5% CO_2, to a cell density of approximately 10^9 cells per ml as described previously (6).

Cells were harvested by centrifugation in the late logarithmic phase, washed, and suspended in 0.2 M sodium phosphate buffer (pH 7.0), and 5-ml aliquots were transferred to sterile 30-ml serum vials. Glucose was added to a final concentration of 5,000 μg/ml. The vials were capped with serum stoppers and evacuated and backfilled with helium three times. Nitrite was added with a syringe to a final concentration of 8, 34, 69, 240, or 500 μg/ml. Each treatment was prepared as four replicates, two to be analyzed at a time, to avoid the disruption of gas partial pressures due to a decrease in the volume of the gas phase during sampling. Negative controls were sterilized at 121°C and 15 lb/in^2 for 15 min. Controls without glucose or without NO_2^- were also prepared. All vials were incubated in a 20°C water bath with occasional shaking. The gas phase was sampled every 20 min by withdrawing 0.5 ml with a gas-tight Hamilton syringe fitted with a Mininert valve. NO_2^- was determined by withdrawing 0.1 ml of the liquid phase with a tuberculin syringe. The vials were sampled for up to 24 h after the addition of NO_2^-. The experiment was repeated three times.

The gas phase in experimental and control vials was analyzed by injection into a Gowmac series 150 gas chromatograph with Porapak Q columns (1.2 m by 6.35 mm; 80/100 mesh) (Waters Associates, Inc.) and a thermal conductivity detector, both at 50°C. The current was 150 mA. The carrier gas was helium, and the flow rate was 42 ml/min. The following gases can be separated and detected with this system (15): N_2, O_2, NO, CO_2, and N_2O. Retention times were used to identify gases. Peak height units were measured and compared with standard curves. Small, reproducible peaks of N_2 and O_2 were always seen. These peaks did not increase in size, and it was determined that they were due to the dead space of the sampling syringe. Determinations of the amount of gas produced included gas in solution, which was obtained by calculation (20). Rates of gas production were calculated from the slope of the line determined by least-squares linear regression through at least eight duplicate measurements at 20-min intervals. For all curves, $r \geqq 0.96$.

NO_2^- was determined by a colorimetric method (method 2 in reference 12). The rate of reduction was calculated from the slope of the line determined by least-squares linear regression through at least three duplicate measurements during the first hour of incubation.

Protein was assayed by the method of Bradford (2) with

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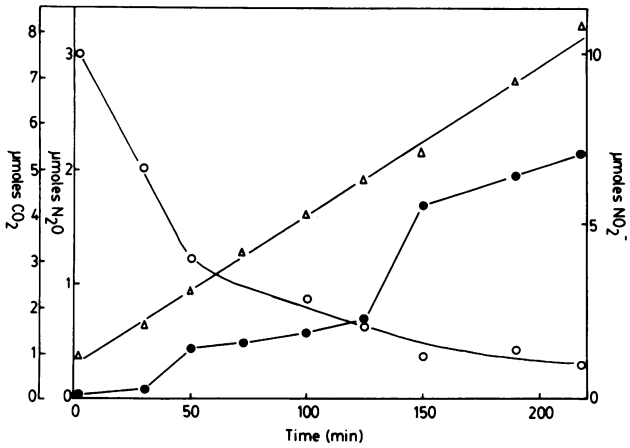

FIG. 1. Production of N_2O (●) and CO_2 (△) during reduction of NO_2^- (○) by suspensions of resting cells of *L. lactis* TS4 incubated anaerobically at 20°C. Reaction mixtures contained 5 ml of cells, 100 μg of NO_2^- per ml, and 5,000 μg of glucose per ml. All points represent the mean of two samples.

bovine serum albumin as the standard. To dissolve cells for protein assays, we mixed 1.0 ml of the cell suspension with 1.0 ml of 0.5 N NaOH and heated the mixture in a boiling water bath for 5 min. Standards were treated identically.

Both CO_2 and N_2O were produced by *L. lactis* TS4 during the reduction of NO_2^-. Neither NO nor N_2 was detected in this experimental system. No N_2O was produced by cells in the absence of NO_2^- or in the absence of glucose or by heat-killed cells.

The production of N_2O lagged behind the reduction of NO_2^- (Fig. 1.) This lag occurred in each experimental run. The rate of NO_2^- reduction was initially high, decreasing after the first 60 min. N_2O was not detected for approximately the first 30 min, and thereafter the rate of N_2O production was fairly constant, with some variation caused by changing the experimental vials sampled. The rate of N_2O production did not decrease as the rate of NO_2^- reduction decreased but only declined after 6 h of incubation. The initial delay in the detection of N_2O could reflect a delay in the production of N_2O or merely an inability to detect it. The limit of detection of the system used was approximately 3 nmol, and the headspace N_2O and solution-phase N_2O may not have reached an equilibrium.

After 3 h of incubation, at which point either NO_2^- reduction was essentially complete or the concentration remaining was constant, the recovery of lost NO_2^--N as N_2O-N was 20 to 69%, depending on the initial concentration of NO_2^- (Table 1). Recovery increased to between 77 and 98% after 24 h of incubation. Recovery increased as the initial concentration of NO_2^- decreased. The initial delay in N_2O production and the incomplete recovery at the time of NO_2^- depletion could implicate NO as an intermediate of NO_2^- reduction which remains enzyme bound. The production of NO as a free intermediate during denitrification is controversial (14, 20), but all data are supportive of an enzyme-bound NO intermediate (11, 20).

These recoveries are quite high. The recovery of reduced NO_3^--N as N_2O-N in many nondenitrifiers, such as *Bacillus*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia*, and *Erwinia* strains, has not been greater than 30%, and N_2 has not been detected (1, 19, 20). The recovery of at least 20% of reduced NO_3^- as N_2O has been considered to be confirmation of the presence of denitrifiers (20). Organisms which reduce NO_2^- to NH_4^+ may produce some N_2O but, typically, it accounts for less than 20% of the NO_2^- consumed (20). Propionibacteria also show high recoveries of reduced NO_2^--N as N_2O-N and are not normally considered to be denitrifiers (13). NO_2^- reduction to N_2O by propionibacteria has been considered to be a detoxication mechanism (13). However, of six strains of lactobacilli capable of reducing NO_2^-, the growth of three was not inhibited by NO_2^-, making the function of NO_2^- reduction as a detoxication mechanism questionable (7).

The rate of production of N_2O by *L. lactis* TS4 was highest, 1.97 nmol/min per mg of protein, when the initial NO_2^- concentration was 69 μg/ml (Table 2). This rate appeared to be higher than the rates of production of N_2O by other nondenitrifying bacteria, e.g., 2.5 nmol/h per mg by *Propionibacterium* sp. (13), 9.2×10^{-2} nmol/min per mg by *Citrobacter* sp. (18), or 4.4×10^{-6} nmol/day per cell by *Nitrosomonas* sp. (10) (for comparative purposes, all rates were converted to nanomoles of N_2O). Denitrifying bacteria produce N_2 at higher rates, e.g., 5.7×10^3 nmol of N_2 per h per mg by *Paracoccus denitrificans* (17).

The rate of reduction of NO_2^- and the rate of production of N_2O and CO_2 were highest when the initial NO_2^- concentration was 69 μg/ml (Table 2). Higher initial concentrations of NO_2^- decreased these rates, indicating the toxic effect that NO_2^- can have on the cell. The production of CO_2 was higher in the presence of any level of NO_2^- than in its absence.

TABLE 1. Reduction of NO_2^- and production of N_2O by suspensions of resting cells of *L. lactis* TS4 incubated anaerobically at 20°C

Initial NO_2^- concn (μg/ml)	Incubation time (h)	μmol of NO_2^- reduced	μmol of N_2O produced	% Recovery of NO_2^--N as N_2O-N
500	3	7.29	0.72	19.8
240	3	5.32	0.92	34.6
69	3	7.04	1.64	46.6
34	3	3.71	0.86	46.4
8	3	0.84	0.29	69.0
69	24	7.48	2.87	76.7
34	24	3.71	1.62	87.3
8	24	0.84	0.41	97.6

TABLE 2. Effect of different levels of NO_2^- on the initial rates of reduction of NO_2^- and production of N_2O and CO_2 by suspensions of resting cells of *L. lactis* TS4

Initial NO_2^- concn (μg/ml)	Rate (nmol/min per mg of protein) of reduction[a] of NO_2^-	Rate (nmol/min per mg of protein) of production[b] of:	
		N_2O	CO_2
0	0.0	0.00	1.67
8	5.6	0.22	2.13
34	11.1	0.87	3.53
69	24.0	1.97	6.12
240	5.8	1.06	4.56
500	5.3	0.90	4.22

[a] Determined by linear regression through at least three duplicate measurements during the first hour of incubation.

[b] Determined by linear regression through at least eight duplicate measurements at 20-min intervals. For all regressions, $r \geq 0.96$.

Homofermentative lactobacilli, such as *L. lactis*, do not usually produce gas from glucose (16). The increase in CO_2 production while NO_2^- was being reduced indicated that glucose was being dissimilated by a pathway other than the Embden-Meyerhof-Parnas pathway. Changes in normal metabolic processes in the presence of NO_3^- and NO_2^- have been reported, for example, in *Propionibacterium* species, in which a change from a fermentative to an anaerobic respiratory form of metabolism occurs (13, 15, 21). Changes in the metabolism of various lactic acid bacteria under different circumstances have been noted. In the presence of fumarate, significant increases in the production of acetate and CO_2 by *Streptococcus faecalis* occur (5). Fumarate can act as an alternate hydrogen acceptor, regenerating oxidized NAD^+ and averting the reduction of pyruvate to lactate. The subsequent catabolism of pyruvate via the dismutation and phosphoroclastic pathways would account for the alteration of products. These changes in pyruvate catabolism would yield additional energy, and small, but reproducible, increases in cell yields have been observed in the presence of fumarate (5).

The presence of NO_2^- seems to affect metabolism in *L. lactis* TS4 in a similar manner. The cell yield of *L. lactis* TS4 grown in the presence of NO_2^- was significantly higher ($P < 0.01$) than in the absence of NO_2^-, indicative of increased energy yields (7). The increase in cell yield and in CO_2 production suggest that NO_2^- reduction may serve a respiratory function in *L. lactis* TS4, a function often associated with the reduction of NO_2^- to NH_4^+ (3). However, the rate of production of N_2O and the high recovery of reduced NO_2^--N as N_2O-N observed in this study seem to indicate that *L. lactis* TS4 represents an atypical denitrifier, more like *Propionibacterium* sp. than *E. coli*.

This study was supported by the Natural Sciences and Engineering Research Council of Canada in the form of a grant to D.L.C.-T. and a postgraduate scholarship to K.L.D.

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