Production of N₂O and CO₂ During the Reduction of NO₂⁻ by Lactobacillus lactis TS4

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N₂O was produced during the reduction of NO₂⁻ by resting cells of Lactobacillus lactis TS4. At an initial NO₂⁻ concentration of 69 μg/ml, the rate of N₂O production was 1.97 nmol/min per mg of protein, and the recovery of reduced NO₂⁻-N as N₂O-N after 24 h was 77%. Higher initial NO₂⁻ concentrations decreased both the rate of production of N₂O and the recovery of reduced NO₂⁻-N. CO₂ production increased during NO₂⁻ reduction.

There are three major types of NO₂⁻ reduction by microorganisms. NO₂⁻ is reduced to NH₄⁺ 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 NO₂⁻ to the gaseous end products NO, N₂O, and N₂ in the process of denitrification (14, 15, 20), coupling the reduction to oxidative phosphorylation.

Although denitrification was thought to be the primary source of N₂O, N₂O is also produced by other bacteria. Nitrifying bacteria, such as Nitrosomonas spp., produce significant amounts of N₂O when grown at reduced O₂ concentrations (10). Many nondenitrifying NO₃⁻ reducers, including Bacillus, Enterobacter, Escherichia, Erwinia, Klebsiella, and Serratia strains, can also produce N₂O (1, 19). Several Propionibacterium species (13) and a soil Citrobacter species (18) reduce NO₂⁻ to N₂O but are not normally considered denitrifiers (13, 18).

We have shown that some lactobacilli isolated from cured meat products are capable of enzymatically reducing NO₂⁻ (6). A previous study (9) indicated that lactobacilli produce various nitrogenous oxides but not NH₄⁺ during NO₂⁻ 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 NH₄⁺-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 NO₂⁻ (16) and are therefore different from other organisms capable of reducing NO₂⁻.

The production of N₂O is often associated with the dissimilatory reduction of NO₂⁻ (18). The purpose of this work was to determine the end products and possible intermediary products of NO₂⁻ 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 APF broth (Difco Laboratories) with 100 μg of NO₂⁻ per ml, under a headspace of 95% N₂-5% CO₂, to a cell density of approximately 10⁹ 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/in² for 15 min. Controls without glucose or without NO₂⁻ 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. NO₂⁻ 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 NO₂⁻. 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): N₂, O₂, NO, CO₂, and N₂O. Retention times were used to identify gases. Peak height units were measured and compared with standard curves. Small, reproducible peaks of N₂ and O₂ 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, \( r \approx 0.96 \).

NO₂⁻ 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
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 CO₂ and N₂O were produced by L. lactis TS4 during the reduction of NO₂⁻. Neither NO nor N₂ was detected in this experimental system. No N₂O was produced by cells in the absence of NO₂⁻ or in the absence of glucose or by heat-killed cells.

The production of N₂O laggered behind the reduction of NO₂⁻ (Fig. 1). This lag occurred in each experimental run. The rate of NO₂⁻ reduction was initially high, decreasing after the first 60 min. N₂O was not detected for approximately the first 30 min, and thereafter the rate of N₂O production was fairly constant, with some variation caused by changing the experimental vials sampled. The rate of N₂O production did not decrease as the rate of NO₂⁻ reduction decreased but only declined after 6 h of incubation. The initial delay in the detection of N₂O could reflect a delay in the production of N₂O or merely an inability to detect it. The limit of detection of the system used was approximately 3 nmol, and the headspace N₂O and solution-phase N₂O may not have reached an equilibrium.

After 3 h of incubation, at which point either NO₂⁻ reduction was essentially complete or the concentration remaining was constant, the recovery of lost NO₂⁻-N as N₂O-N was 20 to 69%, depending on the initial concentration of NO₂⁻ (Table 1). Recovery increased to between 77 and 98% after 24 h of incubation. Recovery increased as the initial concentration of NO₂⁻ decreased. The initial delay in N₂O production and the incomplete recovery at the time of NO₂⁻ depletion could implicate NO as an intermediate of NO₂⁻ 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 NO₂⁻-N as N₂O-N in many non-denitrifiers, such as Bacillus, Enterobacter, Klebsiella, Citrobacter, Serratia, and Erwinia strains, has not been greater than 30%, and N₂ has not been detected (1, 19, 20). The recovery of at least 20% of reduced NO₂⁻ as N₂O has been considered to be confirmation of the presence of denitrifiers (20). Organisms which reduce NO₂⁻ to NH₄⁺ may produce some N₂O but, typically, it accounts for less than 20% of the NO₂⁻ consumed (20). Propionibacteria also show high recoveries of reduced NO₂⁻-N as N₂O and are not normally considered to be denitrifiers (13). NO₂⁻ reduction to N₂O by propionibacteria has been considered to be a detoxification mechanism (13). However, of six strains of lactobacilli capable of reducing NO₂⁻, the growth of three was not inhibited by NO₂⁻, making the function of NO₂⁻ reduction as a detoxification mechanism questionable (7).

The rate of production of N₂O by L. lactis TS4 was highest, 1.97 nmol/min per mg of protein, when the initial NO₂⁻ concentration was 69 μg/ml (Table 2). This rate appeared to be higher than the rates of production of N₂O by other non-denitrifying 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 × 10⁻⁴ nmol/day per cell by Nitrosomonas sp. (10) (for comparative purposes, all rates were converted to nanomoles of N₂O). Denitrifying bacteria produce N₂ at higher rates, e.g., 5.7 × 10⁻⁵ nmol of N₂ per h per mg by Paracoccus denitrificans (17).

The rate of reduction of NO₂⁻ and the rate of production of N₂O and CO₂ were highest when the initial NO₂⁻ concentration was 69 μg/ml (Table 2). Higher initial concentrations of NO₂⁻ decreased these rates, indicating the toxic effect that NO₂⁻ can have on the cell. The production of CO₂ was higher in the presence of any level of NO₂⁻ than in its absence.

**TABLE 2. Effect of different levels of NO₂⁻ on the initial rates of reduction of NO₂⁻ and production of N₂O and CO₂ by suspensions of resting cells of L. lactis TS4**

<table>
<thead>
<tr>
<th>Initial NO₂⁻ concn (μg/ml)</th>
<th>Rate (nmol/min per mg of protein) of reduction of NO₂⁻</th>
<th>Rate (nmol/min per mg of protein) of production of</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>of NO₂⁻</td>
<td>N₂O</td>
</tr>
<tr>
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<td>0.00</td>
<td>0.00</td>
</tr>
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</tr>
<tr>
<td>500</td>
<td>5.3</td>
<td>0.90</td>
</tr>
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* Determined by linear regression through at least three duplicate measurements during the first hour of incubation.

* Determined by linear regression through at least eight duplicate measurements at 20-min intervals. For all regressions, r ≥ 0.96.
Homofermentative lactobacilli, such as *L. lactis*, do not usually produce gas from glucose (16). The increase in CO₂ production while NO₂⁻ 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 NO₃⁻ and NO₂⁻ 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 CO₂ 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 NO₂⁻ seems to affect metabolism in *L. lactis* TS4 in a similar manner. The cell yield of *L. lactis* TS4 grown in the presence of NO₂⁻ was significantly higher (P < 0.01) than in the absence of NO₂⁻, indicative of increased energy yields (7). The increase in cell yield and in CO₂ production suggest that NO₂⁻ reduction may serve a respiratory function in *L. lactis* TS4, a function often associated with the reduction of NO₂⁻ to NH₄⁺ (3). However, the rate of production of N₂O and the high recovery of reduced NO₂⁻-N as N₂O-N observed in this study seem to indicate that *L. lactis* TS4 represents an atypical denitrifier, more like *Propionibacterium* sp. than *E. coli*.

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LITERATURE CITED