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Production of Nitric Oxide and Nitrous Oxide During Denitrification by *Corynebacterium nephridii*

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Resting cells of *Corynebacterium nephridii* reduce nitrate, nitrite, and nitric oxide to nitrous oxide under anaerobic conditions. Nitrous oxide production from nitrite was optimal from *p*H 7.0 to 7.4. The stoichiometry of nitrous oxide production from nitrite was 99% of the theoretical—two moles of nitrite was used for each mole of nitrous oxide detected. Hydroxylamine increases gas evolution from nitrite but inhibits the reduction of nitric oxide to nitrous oxide. Hydroxylamine is converted to nitrogenous gas(es) by resting cells only in the presence of nitrite. Under certain conditions nitric oxide, as well as nitrous oxide, was detected.

Although several investigators have observed nitrous oxide production by denitrifying bacteria, the main end product of dissimilatory nitrate reduction is nitrogen gas (11). Nitrous oxide has been discounted as a normal intermediate in denitrification by several workers (1, 10). However, Kluyver and Verhoeven (6) concluded that nitrous oxide is an intermediate in denitrification by at least some bacterial species.

This study was undertaken to characterize the general features of denitrification by *Corynebacterium nephridii*. This organism was chosen for investigation because it possesses a unique denitrifying system, inasmuch as growing cells reduce nitrate to nitrous oxide (4). It was hoped that knowledge gained with this organism would aid in evaluating the pathway of dissimilatory nitrate reduction in other bacterial species.

MATERIALS AND METHODS

C. nephridii (ATCC 11425) was grown in trypticase soy broth (TSB) supplemented with 1% potassium nitrate and 0.5% glucose. For studies with growing cells, 15 ml of medium was dispensed into 50-ml Warburg flasks and sterilized. A 1-ml amount of a 20% KOH solution was added to the center well and the flasks were inoculated with 1 ml of a 24-hr broth culture of *C. nephridii*. A rubber serum stopper was used to seal one side arm of the flasks; the flasks were connected to manometers, flushed with helium for 30 min, and incubated at 30 C. Gas evolution was measured manometrically. To determine the composition of the gas produced, the atmosphere above the medium was sampled periodically by means of a gas syringe inserted into the flask through the serum stopper. The gas withdrawn was then analyzed by gas chromatography. Nitrate and nitrite in the medium were detected by detaching flasks from the manometers and analyzing samples of the culture fluid. Growth was measured by centrifuging a sample of the culture and determining the nitrogen content of the washed cells.

For studies with resting cells, 300 ml of TSB medium with nitrate and glucose contained in 500-ml Erlenmeyer flasks was inoculated with 20 ml of a 24-hr *C. nephridii* culture grown in a screw-cap test tube. After anaerobic incubation at room temperature for 38 hr, the cells were harvested by centrifugation, washed twice with saline, and suspended in saline so that a 1:100 dilution gave a Klett reading of approximately 90 Klett units, using the blue filter.

Denitrification by resting cells was measured manometrically in 15-ml double-side arm Warburg flasks. Each flask usually contained 100 μmoles of phosphate buffer (*p*H 7.2) and 0.5 ml of standardized cell suspension in the main compartment. Sodium lactate (30 μmoles) and the desired concentration of nitrite or nitrate (13.5 μmoles unless otherwise specified) were contained in the side arms until the reaction was begun. The center well contained 0.2 ml of 20% KOH. When the effect of hydroxylamine on denitrification was studied, nitrite, freshly neutralized hydroxylamine, and lactate were placed in the main compartment, and the cell suspension was tipped in from the side arm. The total volume in the flasks was 3.0 ml, and contents of the flasks were incubated in an atmosphere of helium.

Samples for gas analysis were withdrawn through the serum stoppers attached to the Warburg flasks by means of a gas-tight syringe and injected into a model 29 Gas Partitioner (Fisher Scientific Co., Pittsburgh, Pa.). The columns consisted of Poropak S (0.64 by 39.6 cm; 150 to 200 mesh) in the first position and a 3.65-m column with a 1.53-m section of inert packing

and 2.13 m of activated molecular sieve 13X in the second position. The detector was a thermal conductivity cell.

Nitrite was measured with sulfanilic acid and *N*-1-naphthylethylene diamine dihydrochloride (2). Nitrate was estimated by reduction to ammonia with reduced iron, steam distillation of a sample into 5% boric acid solution, and subsequent nesslerization. Hydroxylamine was assayed with 8-hydroxyquinoline as outlined by Frear and Burrell (3). Cellular nitrogen was estimated by the Kjeldahl method (8).

RESULTS

Nitrate disappearance and gas evolution were most rapid during the first 36 hr of growth of *C. nephridii* (Table 1). At all times, nitrate was stoichiometrically converted to nitrous oxide. For example, after 72 hr of incubation 78.5 μmoles of nitrate nitrogen per ml had disappeared from the medium and 75.8 μmoles of nitrous oxide nitrogen per ml was detected. During this experiment, the nitrite nitrogen concentration never exceeded 3 μg/ml and the *p*H rose from 7.2 to 9.5. The only gas produced from the reduction of nitrate was nitrous oxide, as determined by gas chromatography. Nitrogen, nitrous oxide, and carbon dioxide were detected in gas samples obtained from growing cultures of *C. nephridii* (Fig. 1B). The presence of nitrogen was attributed to incomplete evacuation of the culture after inoculation since nitrogen was detected immediately after evacuation (Fig. 1B, insert) and the peak area did not increase upon further incubation of the culture.

The rate of nitrous oxide production and nitrite disappearance by resting cells of *C. nephridii* is shown in Fig. 2. The rate of each was rapid and linear during the first 20 min of reaction. No nitrite was detectable after 30 min of incubation and nitrous oxide was the only gas detected. After 20 min of incubation, 6.8 μmoles of nitrous oxide was evolved and 13.5 μmoles of nitrite was consumed. Thus, the observed stoichiometry is close to the theoretical, that is, 1 mole of nitrous oxide is produced by the reduction of 2 moles of nitrite.

TABLE 1. *Denitrification during growth of C. nephridii*

Hours of incubation	Cell-N[a]	NO_3^--N[b]	N_2O-N[b]
0	0	85.7	0
12	80	80.0	5.7
24	150	67.1	18.5
36	220	31.4	52.8
48	250	20.0	63.5
72	270	7.2	75.8

[a] Expressed in micrograms per milliliter.
[b] Expressed in micromoles per milliliter.

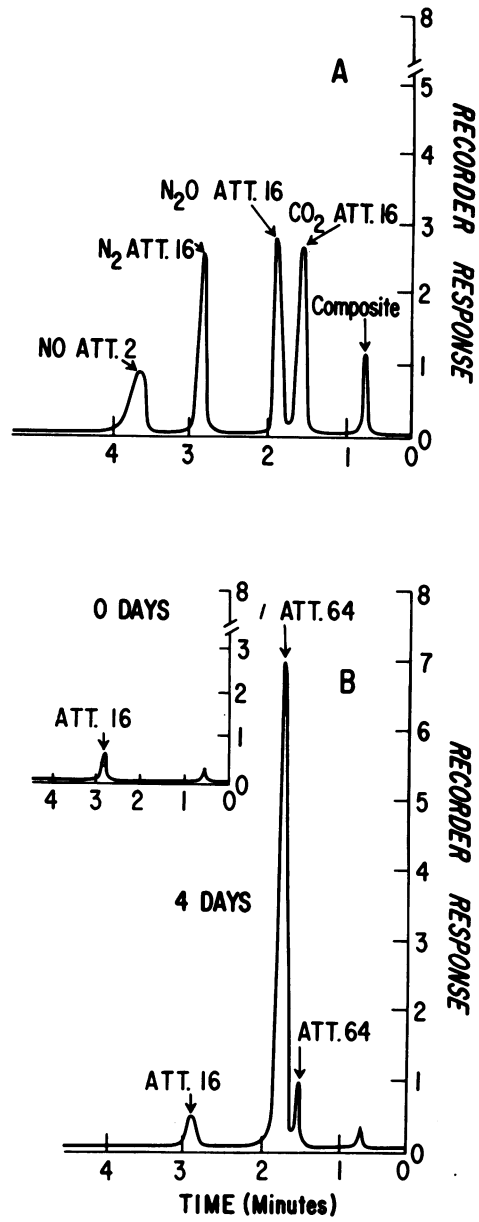

FIG. 1. *Gas liquid chromatograms of gas samples: (A) mixture of known gases; (B) sample from a culture of C. nephridii grown for 4 days; (Insert) sample from uninoculated control. The symbol ATT refers to attenuation of detector.*

The effect of *p*H on gas formation was investigated and the results are shown in Fig. 3. Denitrification occurred from *p*H 6 to 9, with a peak of activity between *p*H 7.0 and 7.4. Nitrous oxide

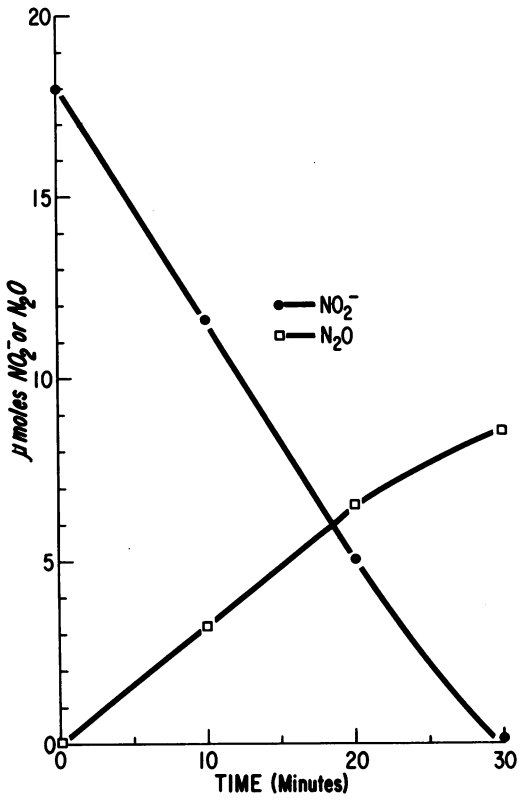

FIG. 2. *Rate of nitrous oxide formation and nitrite disappearance by resting cells of C. nephridii.*

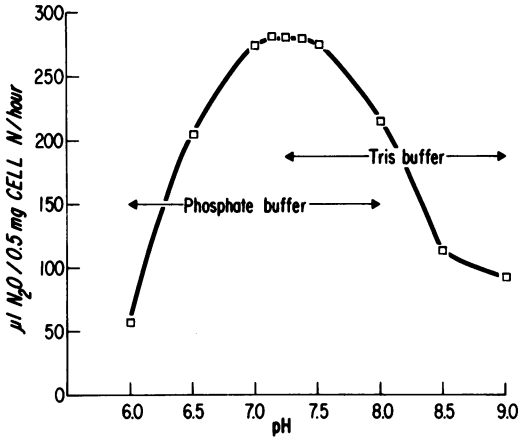

FIG. 3. *Effect of pH on nitrous oxide production by resting cells of C. nephridii. The concentration of buffer was 35 mM.*

was the only gas detected regardless of the *p*H of the reaction or the buffer used.

The effect of nitrite concentration on denitrification is shown in Table 2. Concentrations of nitrite higher than 50 μmoles per flask inhibited the rate of denitrification and, as shown by gas analysis, also caused the formation of different gaseous end products than had been previously observed. When 50 μmoles of nitrite was present in each Warburg flask, 240 μliters (10.7 μmoles) of nitrous oxide was produced in 1 hr. Doubling the nitrite concentration not only decreased the rate of gas evolution but also resulted in the production of a gas mixture comprised predominantly of nitric oxide. Higher concentrations of nitrite decreased gas evolution still further and increased the percentage of nitric oxide present in the end products until as much as 98% was nitric oxide. No nitric oxide was detected in control flasks containing 50 μmoles of nitrite and 1,000 μmoles of sodium chloride. When nitrate was used as an electron acceptor, high concentrations of nitrate also decreased the rate of denitrification but nitrous oxide was the only gas detected (Table 3).

Since it was reported that hydroxylamine had a pronounced effect on denitrification by *Pseudomonas denitrificans* (5), the effect of hydroxylamine on denitrification by *C. nephridii* was investigated. The addition of hydroxylamine markedly increased the rate of gas production as well as the total amount of gas evolved from nitrite (Table 4). Since the actual amount of gas produced exceeded the amount theoretically possible from the amount of added nitrite, it is

TABLE 2. *Effect of nitrite concentration on gas production by resting cells of C. nephridii*

NO_2^- added	Rate of gas evolution[a]	N_2O	NO
μmoles		%	%
50	240	100	0
100	225	13	87
500	128	3	97
1,000	70	3	97

[a] Expressed as microliters of gas per hour per milligram of cell nitrogen.

TABLE 3. *Effect of nitrate concentration on gas production by resting cells of C. nephridii*

NO_3^- added	Rate of gas evolution[a]	N_2O	NO
μmoles		%	%
50	204	100	0
100	206	100	0
500	134	100	0
1,000	63	100	0

[a] Expressed as microliters of gas per hour per milligram of cell nitrogen.

TABLE 4. *Effect of hydroxylamine concentration on gas production from nitrite by resting cells of C. nephridii*

NH_2OH added	Rate of gas evolution[a]	Total gas evolved	N_2O	NO	NH_2OH disappearance
μmoles		*μliters*	%	%	*μmoles*
0	245	155	100	0	
10	245	200	54	46	1.5
30	320	260	59	41	3.8
100	640	290	77	23	5.0
500	1,430	320	88	12	10.0
5,000	2,100	355	100	0	19.0

[a] Expressed as microliters of gas per hour per milligram of cell nitrogen.

clear that the additional gas observed in those flasks containing hydroxylamine must be derived from hydroxylamine. This was further supported by the results of hydroxylamine determinations, which showed that hydroxylamine actually disappeared when nitrite was present. No gas evolution or hydroxylamine disappearance was observed in flasks containing hydroxylamine and lactate but no nitrite.

Gas chromatographic analysis demonstrated that hydroxylamine had yet another effect on gas evolution from nitrite. When hydroxylamine and nitrite were both present in the Warburg flasks, nitric oxide, as well as nitrous oxide, was formed (Table 4). At a relatively low level of hydroxylamine (10 μmoles per flask), the gas evolved was composed of approximately 50% nitrous oxide and 50% nitric oxide. The percentage of nitrous oxide in the gas mixture increased as the hydroxylamine concentration was increased until, at the highest hydroxylamine concentration employed (5,000 μmoles/flask), no nitric oxide was detected. Hydroxylamine was also observed to have similar effects on gas production with nitrate as terminal electron acceptor.

Increased gas evolution in the presence of hydroxylamine may have resulted from increased nitric or nitrous oxide formation, or from an increase in both. To distinguish these possibilities, as well as to confirm the gas chromatographic identification of nitric oxide, 0.3 ml of a 10% solution of alkaline sulfite, which absorbs nitric oxide (13), was added to one side arm of a Warburg flask. The addition of alkaline sulfite to flasks containing hydroxylamine, nitrite, and lactate decreases gas production to almost the same level found in flasks containing nitrite and lactate and in which only nitrous oxide was formed (Fig. 4). It appears, therefore, that gas production is increased by relatively low levels of hydroxylamine by an increase in nitric oxide formation. This interpretation, however, does not explain increased gas production at higher hydroxylamine levels.

Resting cells of *C. nephridii* also produce nitrous oxide from nitric oxide, but this reaction is inhibited by 30 μmoles of hydroxylamine (Fig. 5), unlike gas production from nitrite. Nitrous oxide formation from nitric oxide is rapid and linear with no apparent lag. Gas analysis of a sample from this reaction showed that nitrous oxide was the sole detectable product.

DISCUSSION

Since nitrate is necessary for anaerobic growth and is reduced stoichiometrically to nitrous oxide by growing cultures of *C. nephridii*, this bacterium possesses a dissimilatory nitrate reductase. The use of *C. nephridii* for studying dissimilatory nitrate reduction possesses several unique advantages. Although nitrous oxide has been detected in experiments with other denitrifying organisms, its role as an intermediate in denitrification has not been definitely established. Since *C. nephridii* cannot reduce nitrous oxide to nitrogen gas (Renner, *unpublished data*), the end product of nitrate or nitrite reduction is nitrous oxide; thus, investigations with this organism may help elucidate the importance of nitrous oxide in respiratory nitrate reduction. Furthermore, nitrous oxide is the only gas produced from nitrate by growing or resting cells of *C.*

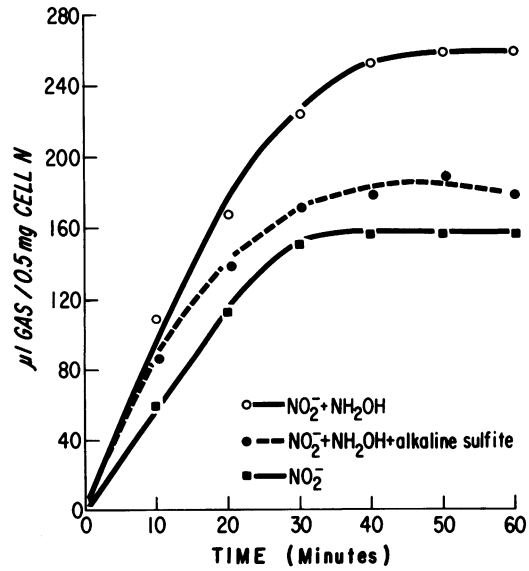

FIG. 4. *Effect of alkaline sulfite on gas production from nitrite and hydroxylamine by resting cells of C. nephridii.*

nephridii, so that studies of the pathway of denitrification are not complicated by the production of two gases as often occurs with other denitrifying organisms (6, 7).

A tentative scheme, consistent with the data for dissimilatory nitrate reduction in *C. nephridii*, is depicted in Fig. 6. The hypothetical nitroxyl intermediate of the reduction of nitric oxide to nitrous oxide is designated by "[X]."

Nitric oxide has been implicated as an intermediate in denitrification with other organisms (9, 12) and it appears also to be an intermediate of nitrite reduction in *C. nephridii*. Resting cells of *C. nephridii* reduce nitric oxide to nitrous oxide (Fig. 5); furthermore, nitric oxide can sometimes be detected during the reduction of nitrite to nitrous oxide. High concentrations of nitrite added to resting cells result in a decrease in the rate of gas production accompanied by the accumulation of nitric oxide (Table 2). A possible explanation for these results is that a high nitrite concentration inhibits the enzyme(s) which reduces nitric oxide to nitrous oxide. High concentrations of nitrate as electron acceptor similarly decrease the rate of gas production, but in this instance nitrous oxide but no nitric oxide can be detected. High nitrate concentrations, therefore, appear to inhibit gas production by a different mechanism than high nitrite concentrations, perhaps by inhibiting nitrate reductase.

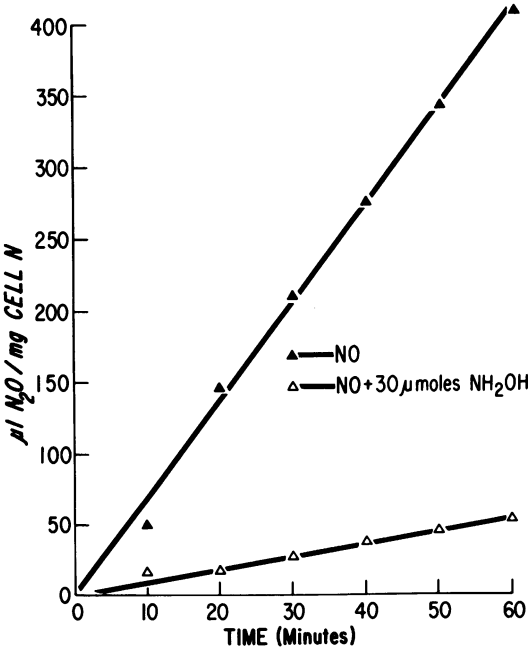

FIG. 5. *Effect of hydroxylamine on the reduction of nitric oxide to nitrous oxide by resting cells of C. nephridii.*

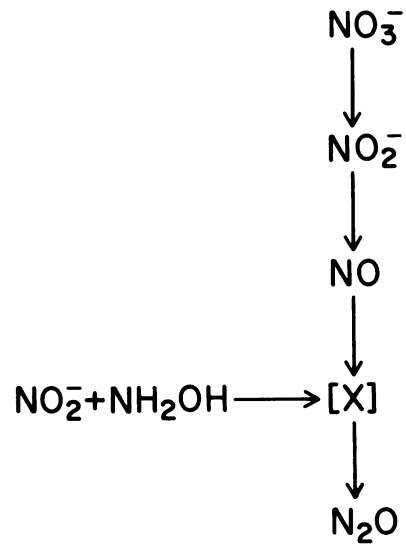

FIG. 6. *Proposed pathway of nitrate reduction in C. nephridii.*

Iwasaki and Mori (5) reported that gas production from nitrite by *P. denitrificans* was increased by hydroxylamine. They proposed that an enzymatic reaction between nitrite and hydroxylamine resulted in the production of nitrogen gas, if an electron donor (e.g., lactate) was present, or nitrous oxide if no electron donor was present. The results of the present investigation suggest that *C. nephridii* can also convert hydroxylamine and nitrite into nitrous oxide, but unlike *P. denitrificans* this occurs in the presence of lactate. The reaction between nitrite and hydroxylamine may involve the formation of an intermediate compound which is then converted to nitrous oxide. This intermediate may be identical to the hypothetical intermediate of nitric oxide reduction and is depicted as such in Fig. 6, although there is no evidence to support this assumption. This interpretation would explain why the rate of gas evolution and also the total gas production were greater than those obtained from nitrite alone when both hydroxylamine and nitrite were added to cells (Table 4). As indicated in Fig. 6, nitrous oxide can be produced from nitrite by *C. nephridii* in two ways. Accordingly, in the presence of low concentrations of hydroxylamine, all of the nitric oxide and some of the nitrous oxide are formed via the pathway through nitric oxide and some of the nitrous oxide by the reaction between nitrite and hydroxylamine. As the concentration of hydroxylamine is in-

creased, most (and finally all) of the nitrous oxide is produced via the latter pathway.

It would appear that hydroxylamine is also an inhibitor of nitrite reduction in *C. nephridii*. A likely site for the inhibitory effect of hydroxylamine is the conversion of nitric oxide to the hypothetical intermediate "[X]" in Fig. 6. Evidence to support this interpretation includes results which demonstrated that nitric oxide is not reduced to nitrous oxide in the presence of hydroxylamine (Fig. 5) and also the observation that nitric oxide can be detected when hydroxylamine is added to a reaction mixture containing resting cells, lactate, and either nitrite or nitrate (Table 4).

Finally, the role of nitrous oxide as a "true" intermediate of denitrification is not yet certain. Under normal conditions, nitrous oxide is produced in stoichiometric amounts from nitrate by either growing or resting cells of *C. nephridii*. Furthermore, nitrous oxide can be produced from nitric oxide and, therefore, may be formed via a nitric oxide reductase. However (Fig. 6), the possibility that nitrous oxide may be formed nonenzymatically from the hypothetical intermediate "[X]" has not been excluded at the present time.

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