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Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*

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Use of Clark-type electrodes has shown that, in cells of *Thiosphaera pantotropha*, the nitrous oxide reductase is active in the presence of O_2, and that the two gases involved (N_2O, O_2) are reduced simultaneously, but with mutual inhibition. Reduction of nitrate, or nitrite, to N_2O under aerobic conditions involves NO as an intermediate, as judged by trapping experiments with the ferric form of extracellular horse heart cytochrome *c* and the demonstration that the cells possess a nitric oxide reductase activity. The overall conversion of nitrate to N_2, the process of denitrification, under aerobic conditions, is thus not prevented by reaction of NO with O_2 and depends upon a nitrous oxide reductase system which differs from that in other organisms by being neither directly inhibited nor inactivated by O_2.

INTRODUCTION

Denitrification is the part of the nitrogen cycle in which nitrate and nitrite are reduced to N_2 by certain genera of bacteria. Enzymes for the reduction of nitrate, nitrite, nitric oxide (NO) and nitrous oxide (N_2O) have been identified (Ferguson, 1988). It is only recently that the formation of NO as a free intermediate between nitrite and N_2O has been strongly supported by trapping experiments and the characterization of a nitric oxide reductase (Goretski & Hollocher, 1988; Carr *et al.*, 1989; Heiss *et al.*, 1989; Carr & Ferguson, 1990*b*). The process of denitrification has generally been found to be inhibited in the presence of O_2 (Payne, 1981; Ferguson, 1987, 1988; Zumft *et al.*, 1988). In the case of the organism *Paracoccus denitrificans* there is evidence that the reduction of nitrate, nitrite and N_2O is inhibited in the presence of O_2 (John, 1977; Alefounder & Ferguson, 1982; Alefounder *et al.*, 1983). The mechanism of inhibition differs for each reductase enzyme, and it is only the nitrous oxide reductase that is seemingly directly inactivated by the presence of O_2 (Alefounder & Ferguson, 1982). Purification of nitrous oxide reductase from a number of sources has provided further evidence that it is an O_2 labile enzyme (Coyle *et al.*, 1985; Snyder & Hollocher, 1987). The possible functioning of nitric oxide reductase under aerobic conditions has not been extensively studied, although there is recent evidence that it may be active in *P. denitrificans* (Carr & Ferguson, 1990*a*).

In contrast with *P. denitrificans* and many other denitrifying organisms [but see Lloyd *et al.* (1987) and Davies *et al.* (1989) for a contrary view derived from an experimental approach differing from that used in other work], *Thiosphaera pantotropha* is reported to denitrify in the presence of O_2 (Robertson & Kuenen, 1984*a*,*b*). The experimental basis for this conclusion is that nitrate reduction and gas formation can be observed in the presence of O_2 (Robertson & Kuenen, 1984*a*,*b*, 1986), but direct evidence for the production of N_2 is not clearly available. As indicated above, reduction of N_2O to N_2 in the presence of O_2 would be at variance with the known behaviour of nitrous oxide reductases. Thus it is important to establish whether the nitrous oxide reductase of *T. pantotropha* can function under aerobic conditions; such activity would implicate either a novel type of nitrous oxide reductase or a mechanism for protecting the enzyme against O_2.

A second biochemical problem concerning conversion of nitrate or nitrite into gaseous products under aerobic conditions arises if NO is an intermediate between nitrite and N_2O, as has been strongly indicated under anaerobic conditions for *P. denitrificans* and other organisms (Goretski & Hollocher, 1988; Carr *et al.*, 1989; Zafiriou *et al.*, 1989). NO reacts rapidly with O_2 (Carr & Ferguson, 1988), and thus conversion of nitrite into N_2O via NO could be retarded in the presence of O_2. Thus, if *T. pantotropha* is capable of aerobic denitrification, it is essential to ascertain whether this organism reduces nitrite to N_2O via NO with the participation of a nitric oxide reductase, especially under aerobic conditions.

MATERIALS AND METHODS

T. pantotropha strain LMD 82.5 was obtained from Dr. L. A. Robertson, Department of Microbiology, Technical University of Delft, Delft, The Netherlands, and grown anaerobically at 37 °C with acetate as carbon source and nitrate as added electron acceptor in the basal medium described by Robertson & Kuenen (1983). Cells in the late exponential phase of growth were harvested by centrifugation at 4000 *g* and washed once with 200 mM-sucrose/10 mM-Hepes/NaOH, pH 7.3, before being resuspended in the same buffer. *P. denitrificans* N.C.I.B. 8944 was grown and harvested in the same way.

Procedures for measuring dissolved concentrations of O_2, NO and N_2O with Clark-type electrodes fitted to reaction chambers that were closed systems, except for a very narrow entry port that allowed additions to be made with a Hamilton syringe, were as described previously (Alefounder & Ferguson, 1982; Carr *et al.*, 1989).

Nitrite reductase activity was measured by periodically withdrawing samples from a reaction mixture containing 1 mM-nitrite plus cells suspended in 200 mM-sucrose/10 mM-Hepes/NaOH (pH 7.3)/10 mM-potassium acetate, and determining the nitrite concentration colorimetrically as previously described (Alefounder *et al.*, 1983). Nitrate reductase activity was measured by using the same assay, but with myxothiazol present to inhibit electron transport to nitrite reductase and 1 mM-nitrate instead of nitrite, to determine the formation of nitrite. Alternatively a nitrate-specific electrode was used.

Cell protein was determined by lysing the cells in 0.1 M NaOH

Abbreviation used: TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.
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at 100 °C before assaying by the Lowry method (with BSA as standard). Horse heart cytochrome *c* was used as supplied in the oxidized form.

Spectroscopic measurements were made at 30 °C with a Kontron Uvikon 810 spectrophotometer using a 1 cm light path and a split-beam mode. NO, N_2O and acetylene solutions were prepared by bubbling these gases through N_2-saturated buffer at room temperature. The concentrations were taken to be 3 mM, 24.8 mM and 45 mM respectively.

RESULTS

Simultaneous reduction of N_2O and O_2 by cells of *T. pantotropha*

A standard Clark-type oxygen electrode with platinum cathode shows only a very small response to N_2O at polarizing voltages in the region of −0.7 V. Consequently the effect of N_2O on the rate of O_2 reduction can be readily studied with this type of electrode. Fig. 1(*a*) shows that introduction of N_2O caused a 57% inhibition of the rate of O_2 reduction. That this inhibition was a consequence of diversion of electrons to nitrous oxide reductase was established by the finding that acetylene, an inhibitor of nitrous oxide reductase, reversed the effect of added N_2O (Fig. 1). Acetylene alone had no effect on the rate of reduction of O_2. A complementary experiment was done using the N_2O electrode. This electrode responds to both O_2 and N_2O, with the former being detected at higher sensitivity. Addition of H_2O_2, from which O_2 was generated by the action of catalase, caused the electrode to respond to O_2, but a partial inhibition of the rate of N_2O reduction could be clearly observed (Fig. 1*b*). The extent of the inhibition can be calculated in the following way. The record of N_2O concentration as a function of time can be extrapolated beyond the point where O_2 was introduced. It is evident (Fig. 1*b*) that, after reduction of the added O_2, the rate of N_2O reduction was indistinguishable from the original rate. However, the amount of N_2O remaining was 26 nmol higher (displacement of point B above point C in Fig. 1*b*) than would have been the case if anaerobic conditions had been maintained throughout the ~ 75 s period of aerobic N_2O reduction. The average rate of N_2O reduction during this period was 0.65 nmol of N_2O reduced·s^{-1}. This compares with the linear rate of 1 nmol of N_2O reduced·s^{-1} observed at the beginning and end of the experiment under anaerobic conditions. Thus comparison of the results shown in Fig. 1(*a*) with those in Fig. 1(*b*) indicates that the reduction of O_2 is more strongly inhibited (57%) by N_2O than is N_2O reduction by O_2 (35% inhibition in this experiment). The observations shown in Fig. 1 were made consistently and, although the exact extent of inhibition of oxidase activity by N_2O varied between 40 and 65% when ten different batches of cells were compared, established that nitrous oxide reductase is very significantly active in the presence of O_2. The specific rate of N_2O reduction for a given batch of cells was higher (in the range 90–130 nmol of $N_2O \cdot min^{-1} \cdot mg$ of $protein^{-1}$ for ten batches of cells) than the rate of O_2 reduction (in the range 60–100 nmol of $O \cdot min^{-1} \cdot mg$ of $protein^{-1}$), and so the typical results shown can probably be explained by a competition for electrons between the reductases for O_2 and N_2O, especially as the concentrations of the two gases throughout the experiments were essentially considerably above their respective low K_m values seen with intact cells, as judged by the linearity of respective electrode recordings (Robertson & Kuenen, 1986; see also Fig. 3 below).

The effect of N_2O at inhibiting the reduction of O_2 was also clear when isoascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) was used as substrate with cells of *T. pantotropha* (Fig. 2*a*). Again, the strong inhibitory effect of N_2O was reversed by acetylene. It has been shown previously that reduction of N_2O by cells of *P. denitrificans*, using physiological substrates, is blocked by O_2. Fig. 2(*b*) shows that reduction of O_2 by *P. denitrificans* was, in contrast with *T. pantotropha*, not inhibited by N_2O, even when electrons were donated to the electron-transport system at the level of cytochrome *c* from isoascorbate plus TMPD.

Reduction of NO by *T. pantotropha*

Recently the role of NO in anaerobic denitrification has been substantiated through trapping experiments carried out mainly with *P. denitrificans*, but also with several other genera (Goretski & Hollocher, 1988; Carr *et al.*, 1989). The reactivity of NO with O_2 might mean that, in an organism capable of aerobic denitrification, nitrite would be directly converted by its

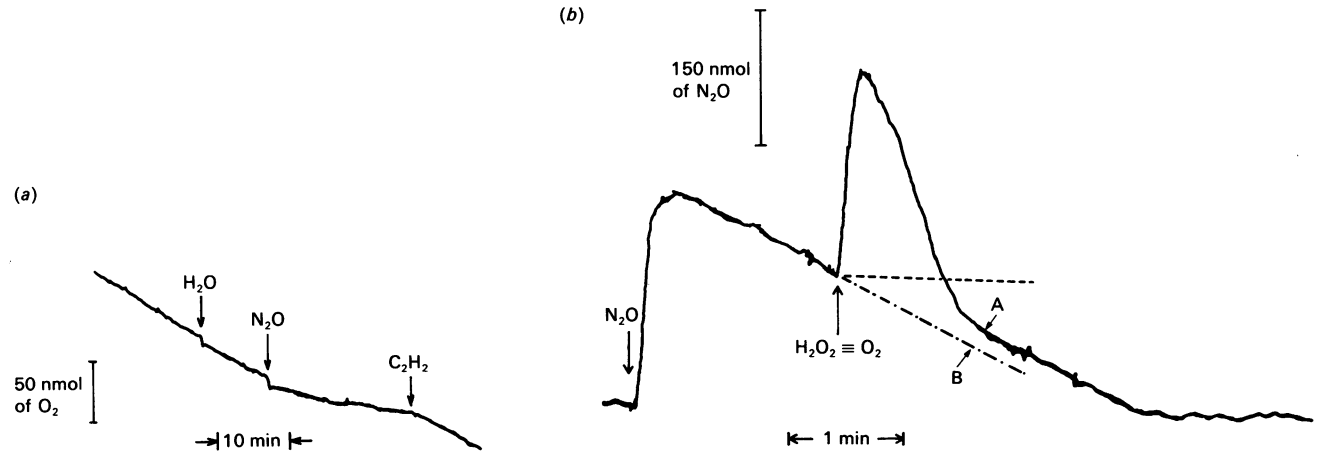

Fig. 1. Simultaneous reduction of N_2O and O_2 by cell suspensions of *T. pantotropha*

(*a*) Record of oxygen-electrode response: a 2 ml reaction mixture contained 200 mM-sucrose, 10 mM-Hepes/NaOH, pH 7.3, 10 mM-potassium acetate and 0.12 mg dry weight of cells. N_2O and acetylene (C_2H_2) were added where shown as saturated aqueous solutions to give final concentrations of 0.62 mM and 1 mM respectively. (*b*) Record of N_2O response: the 2 ml reaction mixture had the same composition as in (*a*), except that 50 units of catalase and 0.6 mg dry weight of cells were present. On exhaustion through cell respiration of the dissolved O_2, N_2O (final concn. 0.12 mM) was added, followed by H_2O_2, from which O_2 was generated by the activity of catalase. The amount of H_2O_2 added was calculated to release approx. 12 μM-O_2, close to what was observed by the electrode. ------, [N_2O] at the time of O_2 addition; –·–·, extrapolated rate of reduction of N_2O.

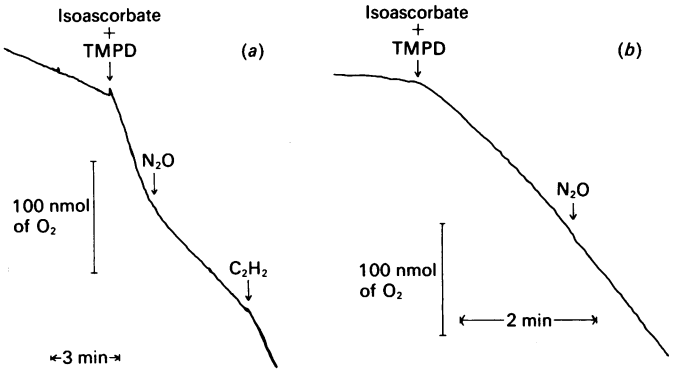

Fig. 2. (*a*) Inhibition by N_2O of the reduction of O_2 by cells of *T. pantotropha* with sodium isoascorbate plus TMPD as substrate and (*b*) lack of inhibition by N_2O of the rate of O_2 reduction by *P. denitrificans*

(*a*) Cells (0.53 mg dry wt.) were added to a reaction chamber containing, at 30 °C, 2 ml of 200 mM-sucrose, 10 mM-Hepes/NaOH, pH 7.3, 0.2 mM-TMPD, 1 mM-sodium isoascorbate; 0.6 mM-N_2O and 1 mM-acetylene (C_2H_2) were added as shown. (*b*) Apart from the use of cells of *P. denitrificans* (0.5 mg dry wt.) and the absence of an addition of acetylene, the conditions were as for (*a*).

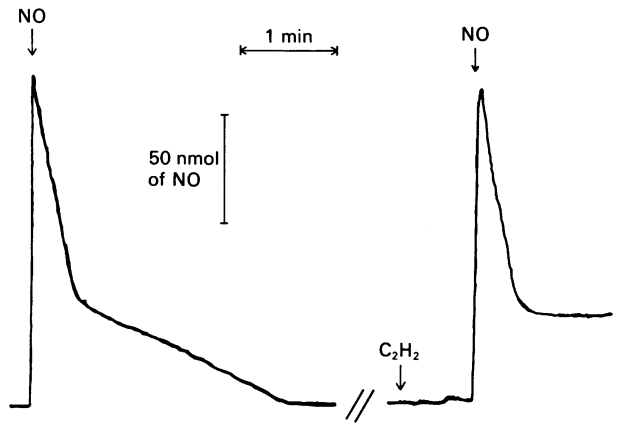

Fig. 3. Reduction of NO to N_2O by cells of *T. pantotropha*

Cells (15 μg dry wt.) were suspended in 2 ml of medium containing 200 mM-sucrose, 10 mM-Hepes/NaOH, 16 mM-D-glucose, 10 mM-potassium acetate, 50 units of catalase and 4 units of glucose oxidase at pH 7.3. When the silver-cathode electrode indicated that the reaction mixture had become anaerobic, NO to a final concentration of 75 μM was added. Subsequently acetylene (C_2H_2; final concn. 1 mM) was introduced, followed by a second addition of NO.

reductase into N_2O, as previously and frequently suggested for denitrification in general (Ferguson, 1987, 1988).

A silver-cathode Clark-type electrode detects both NO and N_2O. Fig. 3 shows that addition of NO to anaerobic cells was followed by a two-phase response of the electrode with time. When NO was added in the presence of acetylene, only a single-phase response was observed, but the final signal from the electrode was considerably displaced from the baseline. As acetylene inhibits reduction of N_2O, but not NO, the observations reported in Fig. 3 clearly show that, under anaerobic conditions, NO is reduced to N_2O by *T. pantotropha* and that reduction of N_2O is fully inhibited by the presence of NO. From the time courses for disappearance of NO and N_2O shown in Fig. 3, it is clear that the rate of NO reduction is, on a molar basis, approximately ten times that of N_2O. Other experiments (results not shown) established that the rates of nitrate and nitrite reduction were similar to that of N_2O reduction. Since reduction of NO is a one-electron reaction, but that of N_2O is a two-electron process, the rate of NO reduction can be calculated, on an electron basis, as approx. 5-fold greater than the rate of N_2O reduction. These comparisons assume that the products of the reduction of nitrate, nitrite, NO and N_2O are respectively nitrite, NO, N_2O and N_2.

Sensitivity to myxothiazol of denitrifying electron-transport reactions in *T. pantotropha*

Reduction of both NO and N_2O, as well as of nitrite, could be inhibited with myxothiazol by at least 95%, whereas nitrate reduction was unaffected by this compound. The amount of this inhibitor required for this extent of inhibition was 0.5 nmol of inhibitor/mg cell dry weight. This titre was also shown to be just sufficient for the maximum inhibition of oxidase activity (approx. 50%) of the cells, the residual insensitive rate presumably reflecting the presence of a second oxidase system, as observed in *P. denitrificans*, for which similar titres of myxothiazol are required to inhibit the same reduction reactions (Parsonage *et al.*, 1986; Carr *et al.*, 1989). In the latter organism myxothiazol acts on the cytochrome bc_1 complex, and it is reasonable to conclude, therefore, that this respiratory complex is also involved in the electron-transport reactions of denitrification in *T. pantotropha*. However, caution must be exercised before concluding that the cytochrome bc_1 complex is the sole pathway for electron transfer from respiratory substrates to nitrite, NO and N_2O. Fairly recent work (Richardson *et al.*, 1989) with *Rhodobacter capsulatus* has shown that two pathways can operate in that organism, a feature that can account for incomplete inhibition of N_2O reduction by myxothiazol in *R. capsulatus*. Nevertheless, the high percentage inhibition by myxothiazol observed for *T. pantotropha* suggests that the relative capacity of any alternative electron-transport pathway must be low.

The complete denitrification electron-transport pathway, including the formation and reduction of NO, operates under aerobic conditions

Fig. 4 shows that addition of nitrate to a suspension of

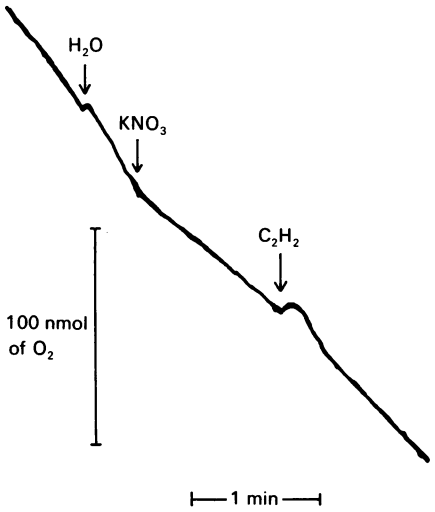

Fig. 4. Effect of nitrate upon reduction of O_2 by cells of *T. pantotropha*

Cells (3.6 mg dry wt.) were added to 2 ml of a solution containing 200 mM-sucrose, 10 mM-Hepes/NaOH, pH 7.3, and 10 mM-potassium acetate in the reaction chamber of an oxygen electrode. Potassium nitrate (KNO_3) and acetylene (C_2H_2) were added as shown to final concentrations of 0.1 mM and 1 mM respectively. The temperature was 30 °C.

anaerobically grown cells of *T. pantotropha* caused an immediate and sustained decrease in the rate at which O_2 was reduced. The rate of O_2 reduction was substantially, but not completely, restored by addition of acetylene (Fig. 4). As acetylene had been shown in separate experiments to inhibit only the nitrous oxide reductase among the enzymes of denitrification in *T. pantotropha* (results not shown), the interpretation of Fig. 4 is that reduction of nitrate through to N_2 diverts electrons from the oxidase system(s) and that introduction of acetylene partially alleviates this diversion by inhibiting nitrous oxide reductase. This result corroborates the conclusion from Fig. 1 that nitrous oxide reductase is active under aerobic conditions.

The clear evidence for both the formation and reduction of N_2O under aerobic conditions (Fig. 4) and the evidence for a highly active nitric oxide reductase activity (Fig. 3) raised the question of whether NO was formed as an intermediate of denitrification. Haemoglobin has been used as a trap for NO in recent work (Goretski & Hollocher, 1988; Carr *et al.*, 1989) with denitrifying bacteria, but this approach would be fraught with difficulties under aerobic conditions because the visible absorption spectra of oxy- and nitrosyl-haemoglobin are very similar. Consequently the ferric form of horse heart cytochrome *c* was adopted as a trap.

As previously exploited by Kucera *et al.* (1987), a ferric–nitrosyl complex of cytochrome *c* forms in the presence of NO. This can be identified from its strong absorption at 563 nm. Fig. 5 shows that the nitrosyl form of added cytochrome *c* was produced, consistent with the formation of NO, as suspensions of cells of *T. pantotropha* reduced nitrate under aerobic conditions. Parallel measurements made with an oxygen electrode showed that the reaction mixture remained aerobic throughout the period of the experiment. Addition of nitrate to cytochrome *c* in the absence of cells did not cause the formation of the species absorbing at 563 nm, whereas, as expected, addition of NO did so. The eventual loss of the absorption band at 563 nm is consistent with previous observations after addition of NO to ferricytochrome *c*, and it is thought to correspond to the dissociation of the nitrosyl cation with concomitant formation of ferrocytochrome *c* (Ehrenberg & Szczepkowski, 1960). The same experimental approach also showed that NO formation could be detected during reduction of nitrate under anaerobic conditions by both *T. pantotropha* and *P. denitrificans*. However, as predicted from reports of the inability of *P. denitrificans* to denitrify aerobically (see the Introduction), the formation of the nitrosyl adduct of cytochrome *c* was not observed when nitrate was added to an aerobic suspension of this organism. This observation confirmed the distinction between the ability of these two organisms to denitrify aerobically.

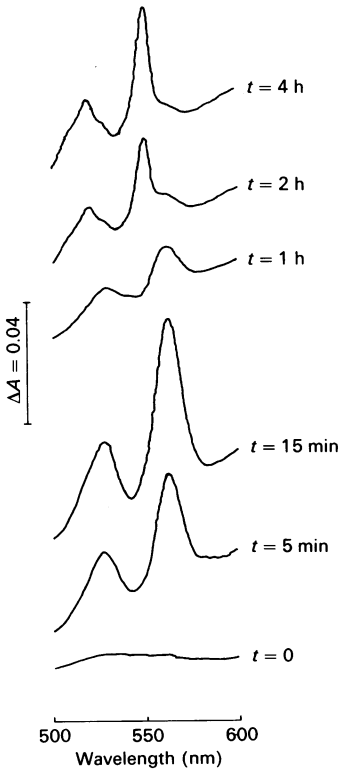

Fig. 5. Formation of nitrosyl–ferric horse heart cytochrome *c* during reduction of nitrate by cells of *T. pantotropha* under aerobic conditions

Cells (33 μg dry wt.) were suspended in cuvettes containing 1 ml of 200 mM-sucrose/10 mM-Hepes/NaOH (pH 7.3)/10 mM-potassium acetate/50 μM ferric horse heart cytochrome *c*. KNO_3 was added to the sample cuvette to a final concentration of 20 μM. The spectra were scanned at 1 nm/s at the times shown.

DISCUSSION

The present work provides definite evidence for *T. pantotropha* that N_2O is reduced to N_2 under aerobic conditions and that the whole sequence of denitrification reactions from nitrate to N_2 is operative under aerobic conditions. The ability to reduce nitrate under aerobic conditions correlates with the possession by *T. pantotropha* of a periplasmic nitrate reductase that is not found in anaerobic denitrifiers such as *P. denitrificans* (Bell *et al.*, 1990). Particularly striking is the formation of NO, as judged by trapping experiments, under aerobic conditions. This observation, together with the new evidence that cells of *T. pantotropha* have a high nitric oxide reductase activity, strongly indicates that NO is an intermediate of denitrification under both aerobic and anaerobic conditions. For this to be the case under aerobic conditions, the rate of reaction between NO and O_2 must be sufficiently lower than the rate of reduction of NO by the cells. It has been suggested that this condition can be met in aqueous conditions when the concentration of NO is low (Carr & Ferguson, 1988). The concomitant operation of both the denitrification pathway and the oxidase reactions indicates that either NO does not inhibit the oxidase pathways, in contrast with the situation with *P. denitrificans* (Carr & Ferguson, 1990*a*), or the steady-state concentration of NO is confined to a level that is ineffective as an inhibitor.

The nitrous oxide reductases that have been isolated from several genera of bacteria have proved to be inactivated by O_2 (Coyle *et al.*, 1985; Snyder & Hollocher, 1987). Such inactivation would be consistent with the generally held view that bacteria do not reduce N_2O in the presence of O_2. Therefore, the clear evidence that N_2O reduction occurs aerobically in *T. pantotropha* implies either (*a*) that this organism possesses a nitrous oxide reductase that is insensitive to the inactivating effect of O_2 or (*b*) that the nitrous oxide reductase is protected from the effects of O_2 by a mechanism akin to respiratory protection that has been suggested for nitrogenase in *Azotobacter* species. The finding that reduction of N_2O is inhibited in *T. pantotropha* by both acetylene and myxothiazol may suggest that the nitrous oxide reductase and the underlying electron-transport system are similar to those in anaerobic denitrifiers, which are also susceptible to these inhibitors; however, extensive further characterization will be necessary for an understanding of this intriguing point.

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