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INHIBITION OF DENITRIFICATION AND OXYGEN UTILIZATION BY *THIOSPHAERA PANTOTROPHA*

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Mass spectrometry confirmed that the entire denitrification system of *Thiosphaera pantotropha* is present under aerobic conditions. Acetate-dependent oxygen uptake is totally inhibited by 0.3 mM cyanide. Nitrate reduction by cells grown with and without nitrate was inhibited by 10 and 15 mM azide, respectively. The nitrous oxide reductase was totally inhibited by 1% acetylene or 0.125 mM cyanide. This reductase was only temporarily inhibited by 10 mM azide. Frozen/thawed cell suspensions of *T. pantotropha* produced, under anaerobic conditions, only nitric oxide. The effects of various inhibitors on *T. pantotropha* are summarized and considered in relation to their effect on other denitrifiers.

The denitrifying enzymes of *Thiosphaera pantotropha* have been found to be constitutive (*14, 16*), and the stoichiometry of the reactions indicates that nitrogen gas is the end product of denitrification in this organism (*14*). Nitrous oxide has not been detected under normal growth conditions (*17*). Because of experimental difficulties in the direct identification of dinitrogen gas, ^{15}N-compounds offer an attractive way of confirming that N_2 is the end product of denitrification, and under what conditions other gases are produced. Gaseous ^{15}N-compounds and ^{14}N-compounds can be measured separately by means of mass spectrometry (*4, 10*). Inhibitors are frequently used to investigate the sequence of intermediates formed during denitrification. However, there is evidence that many of these inhibitors are not specific (for a review see (*9*)), and that different responses may be obtained from different species.

This short note describes the effect of various inhibitors on denitrification and oxygen uptake by *T. pantotropha* and the identification of the products of denitrification under different conditions using on-line mass spectrometry.

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MATERIALS AND METHODS

Organism. *Thiosphaera pantotropha* LMD 82.5 was originally isolated from a denitrifying, sulfide-oxidizing waste water treatment system (*13*).

Cultures. Cells were grown aerobically in acetate-limited continuous or batch cultures without pH- or dissolved oxygen control. The medium used for the cultures contained ($g\,l^{-1}$): NH_4Cl, 0.4; $K_2HPO_4 \cdot 3H_2O$, 1.05; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.4; tris(hydroxymethyl)aminomethane, 2.4; NaOH, 0.08; acetic acid, 10 mM; 2 ml trace element solution (*21*). KNO_3, when used, was supplied to the medium at a concentration of $3.2\,g\,l^{-1}$. The medium was adjusted to pH 8.0 with NaOH before autoclaving. The temperature was maintained at 37°C.

Analytical techniques. Protein was measured by means of the micro-biuret method (*6*). The total organic carbon of washed cells was determined with a TOCA master 915-B. Nitrite was determined using the Griess-Romijn reagent (*7*).

Respiration measurements. Oxygen and nitrous oxide respiration rates of washed suspensions of whole cells were measured polarographically with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instruments, Ohio, USA) at 37°C. The uptake rates were corrected for endogenous respiration. The oxygen electrode was adapted to obtain a better determination of N_2O. For this, a solution of 1 M KOH plus 100 mM KCl was used as electrolyte, and the polarizing voltage was set at a value of +1 V (*3*). Nitrous oxide was measured in anaerobic cell suspensions. Doubly concentrated cell suspensions were sparged for 3 min with N_2 gas. An equal volume of a phosphate buffer (50 mM, pH 8.0) saturated with 3% N_2O was then added.

Mass spectrometry. Use was made of an on-line, quadrupole mass spectrometer from Spectramass (Congleton, UK). This equipment was described in detail in (*4*) and (*5*). The reaction vessel, containing a cell suspension in 50 mM *N*-tris (hydroxymethyl) methylaminoethanesulfonic acid (TES) buffer (adjusted with NaOH to pH 8), was closed by an adjustable collar so that air bubbles were excluded. This collar contained the inlet probe and a capillary for additions to be made to the suspension. The temperature (37°C) was controlled by means of a water jacket. Cells from the cultures were washed twice with a 50 mM TES buffer (pH 8.0) before use.

The levels of dissolved O_2, $^{15,15}N_2$, $^{15,15}N_2O$ and in some cases, also $^{14,15}N_2$, $^{14,14}N_2$ and $^{14,15}N_2O$ in the suspension were followed. O_2, $^{15,15}N_2$, $^{15,15}N_2O$, $^{14,15}N_2$, $^{14,15}N_2O$ and $^{14,14}N_2$ were monitored at m/z (mass: charge ratio) values 32, 30, 46, 29, 45 and 28, respectively.

Inhibitor solutions. Distilled water (200 ml) was saturated with acetylene produced from carbide in water. A 1 M solution of sodium azide was made in distilled water. The concentrations of the inhibitors used are stated in the text.

RESULTS AND DISCUSSION

Constitutive nature of the denitrifying enzymes

A batch culture grown aerobically without nitrate or nitrite was added to the measuring vessel without washing of the cells. Acetate was not added as there was still substrate left in the batch culture. Then 1 mM $^{15}NO_3^-$ was added, and $^{15,15}N_2$ was produced as soon as the oxygen became depleted. $^{15,15}N_2O$ was not produced. With this direct proof, previous observations with indirect methods (*14,16*) were confirmed in that a complete denitrifying sysem is present under aerobic conditions.

Effect of inhibitors on denitrification

Acetylene. In the presence of 1% acetylene, $^{15,15}N_2O$, rather than $^{15,15}N_2$, accumulated at a rate of 80 nmol N mg-biomass^{-1} min^{-1} in anaerobic cell suspensions provided with $^{15}NO_3^-$. Under the same conditions without acetylene, $^{15,15}N_2$ was produced with a rate of 90 nmol N mg-biomass^{-1} min^{-1}. This showed that, like many other organisms including *Paracoccus denitrificans* (*2*), the N_2O reductase of *T. pantotropha* can be inhibited by acetylene.

Azide. Nitrogen gas production from nitrate by *T. pantotropha* cells (pregrown in a medium without nitrate) stopped immediately after the addition of 10 mM azide to the suspension. There was no accumulation of nitrite or nitrous oxide. Cells pregrown in a medium with nitrate were slightly less sensitive to azide. In this case, denitrification still took place in the presence of 10 mM azide, with nitrogen gas as the end product, but stopped when 15 mM was used. Small amounts of nitrous oxide accumulated temporarily, reflecting the relative insensitivity of the nitrous oxide reductase of *T. pantotropha* (not inhibited by 5.25 mM azide) in comparison to *P. denitrificans* (90% inhibited by 65 μM azide). Nitrite did not accumulate in the *T. pantotropha* cultures because this organism has the copper nitrite reductase (*15*) which is insensitive to azide, rather than cytochrome cd_1-nitrite reductase, which is sensitive (*18*).

Bell et al. (*1*) have reported that *T. pantotropha* uses a periplasmic nitrate reductase while denitrifying aerobically, and employs one similar to that found in other denitrifiers, including *P. denitrificans* for anaerobic denitrification. The latter nitrate reductase is inhibited by 20 μM azide (*11*). The nitrate reductase active under aerobic conditions is less sensitive to azide (*11*). The high resistance of *T. pantotropha* to azide as was found here reflects the active periplasmic nitrate reductase. The presence of nitrate during growth seemed to have reduced the sensitivity of the periplasmic nitrate reductase to azide. An explanation for this cannot yet be given.

Cyanide. Concentrations of 0.25 and 0.3 mM CN^- inhibited acetate-dependent oxygen uptake by 90 and 100%, respectively, in cell suspensions of *T. pantotropha* pregrown in the presence of ammonia as the only nitrogen source. These values lie close to that found (0.38 mM CN^-) for the 90% inhibition of

cytochrome aa_3 in *P. denitrificans* (*19*). Ninety percent inhibition of oxygen respiration via cytochrome *o* was found in cell suspensions of *P. denitrificans* at cyanide concentrations of 1.42 mM (*19*). Although cytochrome spectra of *T. pantotropha* cells grown under conditions similar to those used here revealed the possible presence of some cytochrome *o* in addition to cytochrome aa_3 (*12*), there is insufficient evidence to indicate whether the cytochrome *o* of *T. pantotropha* is more cyanide-sensitive than that of *P. denitrificans*, or whether there was simply not enough cytochrome *o* present to take over the total respiration load from cytochrome aa_3.

Ninety-five and hundred percent of nitrous oxide uptake was inhibited by 0.1 and 0.125 mM cyanide, respectively, in anaerobic cell suspensions of *T. pantotropha*. This reductase is rather insensitive to cyanide, compared to the nitrous oxide reductase of *P. denitrificans*. Only 10 μM CN^- was necessary to give 90% inhibition of the nitrous oxide reductase of *P. denitrificans* (*2*).

Nitric oxide production

An anaerobic experiment using $^{14}NO_2^-$ and cells which had been damaged by freezing and thawing, but which were still able to respire oxygen, was notable because it provided the sole instance in which NO production was detected as soon as the suspension became anaerobic. These results indicate that under normal conditions, NO may remain enzyme-bound, and may only become a free intermediate in the denitrification pathway of this organism when the enzyme system is disturbed, probably because NO-reductase became disassociated from the cell membrane. Cell membrane-associated NO-reductases in several denitrifiers have been reported (see e.g. (*8*)).

CONCLUSION

This study showed that oxygen respiration by *T. pantotropha* was as sensitive to cyanide as respiration via cytochrome aa_3 by *P. denitrificans*. However, the nitrate and nitrous oxide reductases of aerobically grown cells of *T. pantotropha* appeared to be relatively insensitive compared to *P. denitrificans* to the inhibitors tested.

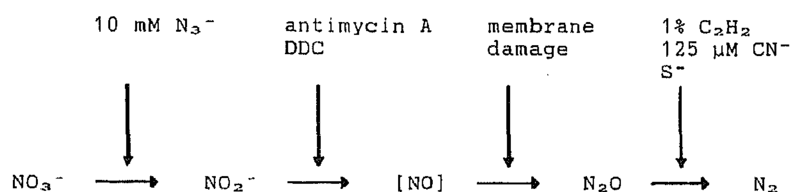

Fig. 1. The proposed sequence of intermediates in denitrification by *T. pantotropha*.
The inhibitors of these steps are also given, based on accumulated data.

In conclusion, Fig. 1 summarizes the data accumulated from these and other experiments (*14*). It can be seen that, despite their presence under aerobic conditions and their relatively lower sensitivity to the tested inhibitors, the denitrifying enzymes of *T. pantotropha* respond similarly to those of the classically studied, oxygen-sensitive denitrifiers (*18, 20*).

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