

Aerobic denitrification: a controversy revived*

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Abstract. During studies on the denitrifying mixotroph, *Thiosphaera pantotropha*, it has been found that this organism is capable of simultaneously utilizing nitrate and oxygen as terminal electron acceptors in respiration. This phenomenon, termed aerobic denitrification, has been found in cultures maintained at dissolved oxygen concentrations up to 90% of air saturation.

The evidence for aerobic denitrification was obtained from a number of independent experiments. Denitrifying enzymes were present even in organisms growing aerobically without nitrate. Aerobic yields on acetate were higher (8.1 g protein/mol) without than with (6.0 g protein/mol) nitrate, while the anaerobic yield with nitrate was even lower (4 g protein/mol). The maximum specific growth rate of *Tsa. pantotropha* was higher (0.34 h^{-1}) in the presence of both oxygen (>80% air saturation) and nitrate than in similar cultures not supplied with nitrate (0.27 h^{-1}), indicating that the rate of electron transport to oxygen was limiting. This was confirmed by oxygen uptake experiments which showed that although the rate of respiration on acetate was not affected by nitrate, the total oxygen uptake was reduced in its presence. The original oxygen uptake could be restored by the addition of denitrification inhibitors.

Key words: Aerobic denitrification – *Thiosphaera pantotropha* – Nitrate reduction – Bacterial selection – Ecology – Oxygen

It has commonly been accepted that denitrification requires completely anoxic conditions (Tiedje et al. 1982; Payne 1981) because some well studied bacteria completely shut down their denitrifying capacity upon exposure to oxygen. However, there have been periodic reports of aerobic denitrification (Marshall et al. 1953; von Meschner and Wuhrmann 1963; Krul 1976). These have often been dismissed since the dissolved oxygen concentration was often not monitored and may have been limiting (Watahiki et al. 1983). In other cases the inhomogeneity of the culture, for example because of clumping by the bacteria, has been held responsible for the creation of anaerobic microniches which would allow denitrification. However, in more recent experiments the dissolved oxygen was measured, and the cultivation con-

ditions were such that dissolved oxygen was present in homogeneously suspended bacterial cultures at concentrations ranging from 10% to twice air saturation. The results of these experiments clearly indicate that aerobic denitrification does indeed occur (Krul and Veeningen 1977; Meiberg et al. 1980).

During studies on an industrial waste water treatment pilot plant (patent number EP0051888A1) in which hydrogen sulphide is anaerobically oxidized by denitrifying bacteria, the predominant organisms were isolated. Preliminary studies with one of these, the facultatively chemolithotrophic *Thiosphaera pantotropha*, indicated that this organism might, in common with *Thiomicrospira denitrificans* (Timmer ten Hoor 1977) have a constitutive nitrate reductase (Robertson and Kuenen 1983). To discover whether this was indeed the case and whether the enzyme was active under fully aerobic conditions (allowing aerobic denitrification), further investigations were made.

Materials and methods

The isolation and identification of *Thiosphaera pantotropha* LMD 82.5 has been described (Robertson and Kuenen 1983). *Thiobacillus versutus* (formerly *Thiobacillus* A2, Taylor and Hoare 1969; Harrison 1983) was originally obtained from B. F. Taylor.

Cultures were grown in Kluuyver flasks which incorporated an oxygen electrode. Aerobic cultures were sparged with air, and the dissolved oxygen did not fall below 80% of air saturation. Anaerobic cultures were sparged with nitrogen. The mineral salts medium contained (g l^{-1}): $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 7.9; K_2HPO_4 , 1.5; NH_4Cl , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; and 2 ml of a trace element solution (Vishniac and Santer 1957). All cultures were provided with 10 mM sodium acetate. 20 mM KNO_3 was provided when required. *Tsa. pantotropha* was grown at 37°C and *T. versutus* at 30°C .

Protein was measured by means of the micro-biuret method (Goa 1953). Nitrate was determined colourimetrically, using diphenylamine sulphonic acid chromogene (Szechrome NAS reagent, Polysciences Inc.). Nitrite was measured using the Griess-Romijn reagent (Griess-Romijn van Eck 1966). Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer). Ammonia was monitored by following the oxidation of NADH in the presence of α -ketoglutarate and L-glutamate dehydrogenase using a test kit (Sigma).

The rate of gas production under anaerobic conditions by cells suspended in phosphate buffer, pH 8.0, was mea-

* Dedicated to Professor Dr. H.-G. Schlegel on the occasion of his 60th birthday

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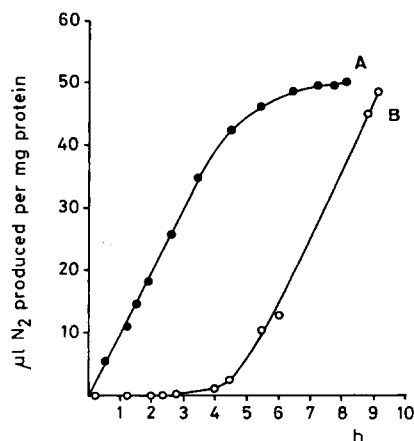


Fig. 1. Gas production by aerobically grown *Thiospaera pantotropha* and *Thiobacillus versutus* cultures in anaerobic manometric experiments. (Data from Robertson and Kuenen 1983) Curve A: *Tsa. pantotropha*, curve B: *T. versutus*

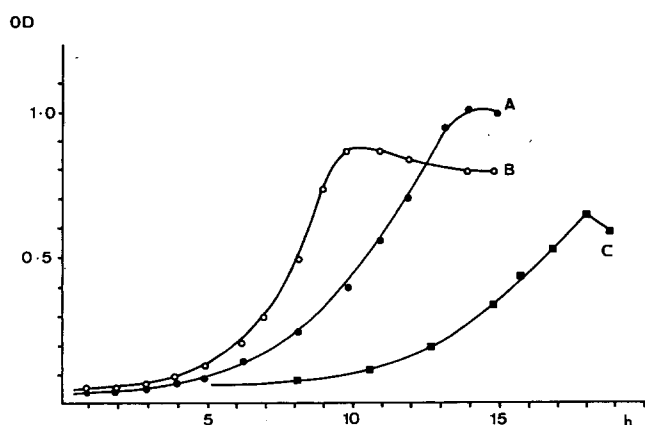


Fig. 2. Growth curves from *Thiospaera pantotropha* cultures grown: A aerobically with acetate; B aerobically with acetate and nitrate; C anaerobically with acetate and nitrate. OD, Optical density at 430 nm. Acetate and nitrate concentrations 10 and 20 mM, respectively

sured using standard manometric techniques. N_2O was detected using a Poropak Q column in a gas chromatograph equipped with a thermal conductivity detector. Oxygen uptake was measured in a Biological Oxygen Monitor. This consists of a Clark-type oxygen electrode mounted in a cell which is closed except for a channel through which additions can be made. Different amounts of acetate (10–20 μM), all small enough to allow the total oxygen uptake to be measured, were used.

Nitrate reductase activity in cell extracts was measured by determining nitrite production in the presence of NADH (Sawhney and Nicholas 1977).

Results and discussion

As has been reported previously (Robertson and Kuenen 1983), it was found that although aerobically-grown *T. versutus* required the expected 4–5 h induction period before gas production from nitrate began, *Tsa. pantotropha* began releasing gas immediately (Fig. 1). That the enzymes of denitrification were not only synthesized under aerobic

Table 1. Growth rates and protein yields obtained from the growth experiments with *Thiospaera pantotropha* shown in Fig. 2

Growth conditions	Specific growth rate (h^{-1})	Protein (mg/l)
Aerobic, no nitrate	0.28	81
Aerobic, with nitrate	0.34	60
Anaerobic, with nitrate	0.25	40

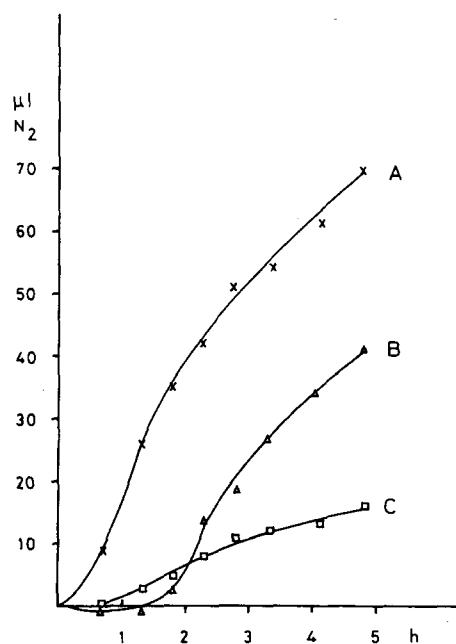
as well as anaerobic conditions (i.e. constitutive), but also were active was indicated by the results of the growth experiments. From Fig. 2 it can be seen that the acetate culture provided with both nitrate and oxygen grew faster than the cultures receiving either, and that the final optical density of this culture lay between those of the other two. The specific growth rates and the protein yields obtained with these cultures (Table 1) confirmed this. As expected, aerobic growth of *T. versutus* was unaffected by the presence of nitrate ($\mu = 0.12 h^{-1}$, results not shown). The upward flow of gas in the Kluver flasks maintained the culture as a homogenous suspension in which clumps did not occur. 5.5 mM nitrate disappeared from the aerobic, nitrate-containing, culture. As the assimilatory nitrite reductase was not present ($< 5 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$), and as the ammonium in the medium was depleted but not exhausted at the end of the experiment, it was concluded that this disappearance was not due to nitrate assimilation. Only trace amounts of nitrite were present, and it was therefore concluded that the nitrate had been reduced to nitrogen gas. From the biomass yield it can be calculated that 65% of the acetate was oxidized. Of this, about one half must have been oxidized through nitrate, the remainder presumably through oxygen. If only the oxygen respiration produced respiratory energy, a yield of half that obtained from the aerobic, nitrate-free culture could be expected. As the actual yield was considerably higher, it was concluded that respiration with nitrate as the terminal electron acceptor also contributed to the energy budget of the cells. The lower yield obtained under anaerobic conditions confirms that, as with other species, denitrification provides less energy than oxygen-linked respiration (Stouthamer 1980).

Low levels of nitrate reductase were found in the cells grown without nitrate (18 nmol nitrite produced $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$), confirming that the enzyme is constitutive. The increased level in the aerobic, nitrate containing culture (50 nmol nitrite produced $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$) provides additional evidence that nitrate functioned as a terminal electron acceptor for respiration.

The simultaneous use of oxygen and nitrate by *Tsa. pantotropha* was further tested by means of the Biological Oxygen Monitor. The results of the first experiment are shown in Table 2. It can be seen that the total amount of oxygen used by *Tsa. pantotropha* was lower in the presence of nitrate than in its absence. When antimycin A or low concentrations of cyanide, both inhibitors of denitrification in *Paracoccus denitrificans* (Stouthamer 1980), were added, the oxygen uptake was restored to nearly the level of the nitrate-free culture. That these inhibitors were also effective against the denitrifying enzymes of *Tsa. pantotropha* was confirmed by measuring the amount of gas produced by the cells in their presence (Fig. 3). It can be seen that over the first h of the experiment (the maximum duration of an

Table 2. The effect of additives on the oxygen uptake by *Thiosphaera pantotropha*

Test conditions	Oxygen uptake (as the percentage of uptake on acetate alone)
Acetate	100
Acetate, nitrate	83
Acetate, nitrate, 10 μ M cyanide	95
Acetate, nitrate, antimycin a	96

**Fig. 3.** Gas production by aerobically grown *Thiosphaera pantotropha* cells in anaerobic manometric experiments with denitrification inhibitors: A no inhibitor; B 10 μ M cyanide; C antimycin a

oxygen uptake experiment) little or no gas was produced in the presence of the inhibitors. The identity to the gas accumulating in the presence of cyanide was checked and found to be nitrous oxide. It has been reported (Stouthamer 1980) that low concentrations of cyanide specifically inhibit nitrous oxide reductase in *P. denitrificans*, and this is obviously also the case for *Tsa. pantotropha*. Although the total amount of oxygen taken up in the presence of nitrate was lower in these experiments, the initial rate of oxygen uptake was the same whether nitrate was present or not. This indicates that *Tsa. pantotropha* was able to increase its rate of respiration, and consequently its rate of energy production, in the presence of nitrate. This might explain the higher specific growth rate of the organism in the presence of both oxygen and nitrate. Detailed studies of the electron transport chain should confirm this and are currently in progress.

In a second experiment involving the Biological Oxygen Monitor, nitrite formation from nitrate in the presence of antimycin a, an inhibitor of nitrite rather than nitrate

(Stouthamer 1980), was followed while oxygen was also monitored. It was found that even cells which had been grown aerobically in the absence of nitrate produced significant amounts of nitrite (7.2 nmol min⁻¹mg⁻¹ protein) while oxygen uptake continued (249 nmol min⁻¹ mg⁻¹ protein). This level of nitrite production corresponds well with the amount of nitrate reductase found in the cells.

The findings presented here, when considered with those of other workers (Krul and Veeningen 1977; Meiberg et al. 1980; Ottow and Fabig 1983) confirm that the denitrification enzymes can be constitutive in some organisms, and can also be operative in the presence of oxygen. A possible advantage conferred by this might lie in a higher specific growth rate or a greater adaptability to fluctuating degrees of anaerobiosis. *Tsa. pantotropha* was isolated from a waste water treatment plant which, although normally anaerobic, receives a certain amount of oxygen in some of the influents. The selective pressure would favour bacteria possessing constitutive enzymes for both denitrification and oxygen respiration. Oxygen would always be a limiting factor and therefore species whose denitrifying enzymes required re-induction or re-activation after a period of aerobiosis would be at a disadvantage. Alternatively, these organisms might have evolved as specialists in denitrification and lost their ability to shut off their denitrification system in the presence of oxygen. Recent work has shown that other isolates from the same source can also denitrify aerobically.

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References

- Goa J (1953) A microbiuret method for protein determination; determination of total protein in cerebrospinal fluid. *J Clin Lab Invest* 5:218–222
- Griess-Romijn-van Eck (1966) Physiological and chemical tests for drinking water. NEN 1056, IV-2 Nederlands Normalisatie Instituut Rijswijk
- Harrison AP (1983) Genomic and physiological comparisons between heterotrophic *Thiobacilli* and *Acidiphillum cryptum*, *Thiobacillus versutus* sp. nov., and *Thiobacillus acidiphillum* nom. rev. *Int J Syst Bacteriol* 33:211–217
- Krul JM (1976) Dissimilatory nitrate and nitrite reduction under aerobic conditions by an aerobically and anaerobically grown *Alcaligenes* sp. and by active sludge. *J Appl Bacteriol* 40:245–260
- Krul JM, Veeningen R (1977) The synthesis of the dissimilatory nitrate reductase under aerobic conditions in a number of denitrifying bacteria isolated from active sludge and drinking water. *Water Res* 11:39–43
- Marshall RO, Dishburger HJ, MacVicar R, Hallmark GD (1953) Studies on the effect of aeration on nitrate reduction by *Pseudomonas* species using N¹⁵. *J Bacteriol* 66:254–258
- Meiberg JBM, Bruinenberg PM, Harder W (1980) Effect of dissolved oxygen tension on the metabolism of methylated amines in *Hyphomicrobium* X in the absence and presence of nitrate: evidence for "aerobic" denitrification. *J Gen Microbiol* 120:453–463
- Meschner K von, Wuhrmann K (1963) Beitrag zur Kenntnis der microbiellen Denitrification. *Pathol Microbiol* 26:579–591
- Ottow JCG, Fabig W (1983) Influence of oxygen aeration on denitrification and redox level in different bacterial batch cultures. Abstracts of the Proceedings of the 6th International Symposium on Environmental Biogeochemistry, p 61
- Payne WJ (1981) In: Denitrification. John Wiley & Sons, New York, pp 61–65

- Robertson LA, Kuenen JG (1983) *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. *J Gen Microbiol* 129:2847–2855
- Sawhney V, Nicholas DJD (1977) Sulphite and NADH dependant nitrate reductase from *Thiobacillus denitrificans*. *J Gen Microbiol* 100:49–58
- Stouthamer AH (1980) Bioenergetic studies on *Paracoccus denitrificans*. *Trends in Biochemical Sciences* 5:164–166
- Taylor BF, Hoare DS (1969) New facultative *Thiobacillus* and a reevaluation of the heterotrophic potential of *Thiobacillus novellus*. *J Bacteriol* 100:487–497
- Tiedje JM, Sextstone AJ, Myrold DD, Robinson JA (1982) Denitrification; ecological niches, competition and survival. *Antonie van Leeuwenhoek* 48:529–607
- Timmer ten Hoor A (1977) In: Denitrificerende kleurloze bacterien. PhD thesis, University of Groningen
- Vishniac W, Santer M (1957) The *Thiobacilli*. *Bacteriol Rev* 21:195–213
- Watabiki M, Hata S, Aida T (1983) N₂O accumulation and inhibition of N₂O reduction by denitrifying *Pseudomonas* sp. 220A in the presence of oxygen. *Agric Biol Chem* 47:1991–1996

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