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# The effect of thiosulphate and other inhibitors of autotrophic nitrification on heterotrophic nitrifiers

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**Abstract.** It has been found that heterotrophic nitrification by *Thiosphaera pantotropha* can be inhibited by thiosulphate in batch and chemostat cultures. Allythiourea and nitrapyrin, both classically considered to be specific inhibitors of autotrophic nitrification, inhibited nitrification by *Tsa. pantotropha* in short-term experiments with resting cell suspensions. Hydroxylamine inhibited ammonia oxidation in chemostat cultures, but was itself fully oxidized. Thus the total nitrification rate for the culture remained the same.

Heterotrophic nitrification by another organism, a strain of "*Pseudomonas denitrificans*" has also been shown to be inhibited by thiosulphate in short term experiments and in the chemostat. During these experiments it became evident that this strain is able to grow mixotrophically (with acetate) and autotrophically in a chemostat with thiosulphate as the energy source.

## Introduction

Various inhibitors are commonly used in laboratory and field studies to distinguish between the activities of autotrophic and heterotrophic nitrifiers (Kuenen & Robertson 1987). However, these experiments are based on the assumption that some inhibitors are specifically active against the autotrophic ammonia oxidizers and do not affect heterotrophs. It has recently been found that the nitrification pathway used by *Thiosphaera pantotropha*, a heterotrophic nitrifier which simultaneously denitrifies (Robertson & Kuenen 1984; Robertson et al. 1988), is similar to that used by the autotrophs. Moreover, nitrification by *Tsa. pantotropha* is inhibited by thiosulphate (Robertson & Kuenen 1988; Robertson et al. 1988), a compound which is also effective against many autotrophic ammonia oxidizers (Sharma & Ahlert 1977). These observations prompted a survey of the effect of other nitrification inhibitors on *Tsa. pantotropha*, and of thiosulphate on other heterotrophic nitrifiers. The first results of this survey are reported here.

## Materials and methods

### *Organisms*

*Thiosphaera pantotropha* LMD 82.5 and *"Pseudomonas denitrificans"* LMD 84.60 were used. *Tsa. pantotropha* was described previously (Robertson & Kuenen 1983), *"Ps. denitrificans"* was obtained from the Delft Culture Collection.

### *Growth conditions*

Batch cultures were made in Kluyver flasks (Robertson & Kuenen 1984) which incorporated an oxygen electrode. Anaerobic cultures were made by sparging with argon instead of air. Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 30° C (for *"Ps. denitrificans"*) or 37° C (for *Tsa. pantotropha*).

### *Media*

The medium described for the growth of *Thiobacillus versutus* (formerly *Thiobacillus* A2) by Taylor & Hoare (1969) was used for batch culture. It contained (in $g\,l^{-1}$); $Na_2HPO_4.7H_2O$, 7.9; $KH_2PO_4$, 1.5; $NH_4Cl$, 0.3; $MgSO_4.7H_2O$, 0.1; 2 ml of trace element solution. The $MgSO_4$, trace element solution, substrates, $KNO_3$ and $KNO_2$ were all sterilized separately in a concentrated form and added as needed.

The medium supplied to the chemostats contained ($g\,l^{-1}$); $K_2HPO_4$, 0.8; $KH_2PO_4$, 0.3; $NH_4Cl$, 0.4; $MgSO_4.7H_2O$, 0.4; and 2 ml of trace element solution.

The trace element solution (Vishniac & Santer 1957) used with all batch and chemostat media contained (as $g\,l^{-1}$); EDTA, 50; $ZnSO_4$, 2.2; $CaCl_2$, 5.5; $MnCl_2.4H_2O$, 5.06; $FeSO_4.7H_2O$, 5.0; $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.1; $CuSO_4.5H_2O$, 1.57; $CoCl_2.6H_2O$, 1.61.

Heterotrophic batch and chemostat cultures were supplied with 10 and 20 mM acetate, respectively. Unless otherwise stated, the mixotrophic batch cultures were supplied with 5 mM acetate and 10 mM thiosulphate. Autotrophic cutures were supplied with 10 mM thiosulphate.

*Respiratory measurements*

Oxygen uptake was measured using a Clark-type electrode as described by Robertson & Kuenen (1988).

*Nitrification rates*

Nitrification rates were calculated from the nitrogen balances, dilution rate and protein concentrations after the amount of ammonia apparently lost from the cultures had been corrected for biomass formation and losses due to sparging.

*Biomass analysis*

Protein was measured by the Micro-Biuret method (Goa 1953).

Carbon dioxide fixation was measured using the uptake of $^{14}CO_2$ by extracts prepared by sonicating cells, as described by Robertson & Kuenen (1983).

*Analysis of medium*

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer). Thiosulphate and sulphate were measured by means of an HPLC fitted with a ionospher-TMA column (Chrompak) and a Waters RI detector.

Nitrite was measured colourimetrically, with the Griess-Romijn reagent (Griess Romijn van Eck 1966) or by means of the HPLC, as described above. Nitrate was also measured with the HPLC, as described above. Hydroxylamine was determined colourimetrically by means of the method described by Frear & Burrell (1955). Ammonia was determined by following the oxidation of NADH in the presence of α-ketoglutarate and L-glutamate dehydrogenase, using a test kit supplied by Sigma Chemical Co. As, at the pH values used in these experiments, ammonia and ammonium would both be present, the term "ammonia" will be used throughout to indicate both the protonated and unprotonated forms. Control experiments using sterile chemostats and a "worst case" situation with maximum levels of sparging and stirring and the lowest dilution rate used ($0.02\,h^{-1}$) showed that a maximum ammonia loss of 0.3 mM (or 6 $\mu$mol $min^{-1}$ $litre^{-1}$) could be expected from stripping (Robertson et al. 1989).

## Results and discussion

### *Thiosulphate as an inhibitor*

When *Tsa. pantotropha* was grown mixotrophically on 20 mM acetate and 5 mM thiosulphate, it was noticed that the nitrification rates were much lower than those obtained with similar cultures which did not receive thiosulphate (Robertson et al. 1988). Nitrification by *Tsa. pantotropha* was therefore checked in chemostat cultures receiving a range of acetate: thiosulphate ratios. It was found that the rate of nitrification decreased as the amount of thiosulphate in the influent increased. For example, when thiosulphate was not present, the nitrification rate was 11.9 nmol $min^{-1}$ mg $protein^{-1}$. With thiosulphate: acetate ratios of 0.25:1 and 2.0:1, the nitrification rates from cultures grown at similar dilution rates were 6.1 and 3.7 nmol $min^{-1}$ mg $protein^{-1}$, respectively.

Only short-term experiments to demonstrate the inhibitory effect of thiosulphate on nitrification by heterotrophically grown *Tsa. pantotropha* were possible because the organism induces its thiosulphate oxidizing pathway on prolonged exposure to thiosulphate (Robertson & Kuenen 1983; Robertson & Kuenen 1988; Robertson et al. 1988). During these short-term experiments, however, it was possible to show that thiosulphate prevented the expected stimulation of acetate-dependent oxygen uptake by ammonia (Robertson & Kuenen 1988). It thus seems likely that in this organism, the effect of thiosulphate is two-fold: firstly, a chemical inhibition analogous to that exerted on autotrophic nitrifiers (Kuenen & Robertson 1987), and secondly, a physiological change due to the induction of the enzymes of the pathway for thiosulphate oxidation. In the hope of inhibiting nitrification without the complication of mixotrophic growth, it was therefore decided to study the effect of thiosulphate on a strain from the heterogenous group previously known as "*Pseudomonas denitrificans*" (from here-on, simply termed *Pseudomonas* sp.; Doude-

*Table 1.* Thiosulphate and acetate dependent oxygen uptake by biomass from aerobic chemostat cultures of *Pseudomonas* sp. with $NH_3$ as the sole source of nitrogen and with acetate and/or thiosulphate limitation. nd = not determined.

| Acetate (mM) | $S_2O_3^{2-}$ (mM) | D ($h^{-1}$) | Maximum oxygen uptake rates ($nmol^{-1}$ $min^{-1}$ mg $protein^{-1}$) | |
|---|---|---|---|---|
| | | | Thiosulphate | Acetate |
| 20 | 0 | 0.06 | 0 | 252 |
| 20 | 5 | 0.06 | 615 | 209 |
| 0 | 5 | 0.03 | 2219 | nd |

roff et al. 1974; ICSB, 1982) which had also been found to possess the combined nitrification/denitrification pathways found in *Tsa. pantotropha* (Kuenen & Robertson 1987; Robertson et al. 1989). As expected, short-term experiments to measure the effect of ammonia on acetate-dependent oxygen uptake showed that thiosulphate, again, prevented any stimulation. Oxygen uptake rates in the presence of ammonia and thiosulphate were the same as those with neither additive, a rate 119% of this was obtained when only ammonia was added.

The addition of thiosulphate to the medium caused an increase in the yields obtained from acetate-limited, aerobic *Pseudomonas* sp. cultures (Table 1) and the nitrification rates fell by 50% (as shown by the reduced ammonia loss, Table 2). It has previously been shown that heterotrophic nitrification appears to be associated with low yields in *Tsa. pantotropha* (Robertson et al. 1988), and an increase in the yield was therefore to be expected if nitrification was inhibited. However, cells from these chemostat cultures were found to have induced a thiosulphate oxidizing capacity which was not present in biomass grown without thiosulphate (Table 1). Analysis of the effluent medium showed that all of the thiosulphate had been oxidized to sulphate (Table 2). It was clear that the increase in yield was due to mixotrophic growth. *Pseudomonas* sp. also grew autotrophically on thiosulphate in aerobic batch cultures. Anaerobic growth on thiosulphate with nitrate as the electron acceptor was not observed. Autotrophic, aerobic growth occurred in the chemostat (dilution rate = $0.03\,h^{-1}$) with a yield of 1.22 g protein/mol thiosulphate. This is lower than that found for chemostat cultures of *T. versutus* (2.75 g/mol; Gottschal & Kuenen 1980) but in the same range of magnitude as that found for *Tsa. pantotropha* (1.4 g/mol; HJ Nanninga, unpublished data). All of the

*Table 2.* Ammonia, thiosulphate and sulphate balances, together with the protein yields from mixotrophic cultures of *Pseudomonas* sp. (*Tsa. pantotropha* data from Robertson et al. 1988). All cultures were grown with ammonia as the nitrogen source, at a dissolved oxygen concentration of 80% air saturation, and with acetate or acetate/thiosulphate limitation. The $NH_3$ values have been corrected for nitrogen assimilation and losses due to sparging. n.a. = not applicable; − = utilization; + = production.

| Medium | Conc. (mM) | D ($h^{-1}$) | $NH_3$ (mM) | $S_2O_3^-$ (mM) | $SO_4^-$ (mM) | protein (mg/L) |
|---|---|---|---|---|---|---|
| *Pseudomonas* sp. | | | | | | |
| Acetate | 20 | 0.06 | − 2.9 | na | na | 120 |
| Acetate/thiosulphate | 20/5 | 0.06 | − 1.6 | − 4.4 | + 10.1 | 180 |
| Acetate/thiosulphate | 20/5 | 0.09 | − 0.4 | − 4.7 | + 8.1 | 197 |
| *Tsa. pantotropha* | | | | | | |
| Acetate | 20 | 0.05 | − 4.6 | na | na | 80 |

thiosulphate was oxidized to sulphate. $CO_2$ fixation rates around 16.5 nmol $min^{-1}$ mg $protein^{-1}$ were obtained using cell free extracts from the autotrophic cultures. This is slightly lower than, but in the same order of magnitude as the rates necessary to support the observed growth (30 nmol $min^{-1}$ mg $protein^{-1}$).

The nitrogen balances from the mixotrophic cultures of *Pseudomonas* sp. are also shown in Table 2. Only trace amounts of nitrite were detected in the cultures, and these have therefore not been shown. Results from heterotrophic *Pseudomonas* sp. cultures and *Tsa. pantotropha* cultures are also shown for comparison. It can be seen that the net ammonia loss (and thus the nitrification rate) in the mixotrophic *Pseudomonas* sp. cultures was considerably lower than in the heterotrophic cultures, and the yield was greatly increased. Nitrification did not take place in the autotrophic cultures.

### *Effect of other inhibitors on Tsa. pantotropha*

For the inhibitor studies, *Tsa. pantotropha* was selected as a representative heterotrophic nitrifier/aerobic denitrifier as its behaviour has been most extensively studied.

#### *Allylthiourea*

Allylthiourea (ATU) is one of the classical inhibitors of autotrophic nitrification which is commonly used in the field to test whether nitrification is due to heterotrophs or autotrophs. The rate of acetate-dependent oxygen uptake by resting *Tsa. pantotropha* cells in phosphate buffer with 10 $\mu$M ATU was the same as that of cells without ATU (115 $nmol^{-}$ $min^{-1}$ $ml^{-1}$). Oxygen uptake by a similar suspension which had been provided with 7.5 mM $NH_4Cl$ as well as

*Table 3.* Nitrification rates obtained with acetate-limited chemostat cultures of *Tsa. pantotropha* provided with hydroxylamine. Nitrification is expressed as nmol $NH_3$ oxidized $min^{-1}$ mg $protein^{-1}$ (ammonia) and nmol $NH_3$ and $NH_2OH$ oxidized $min^{-1}$ mg $protein^{-1}$ (total). $NH_3$ was present in all cultures. The acetate concentration in the influent was 20 mM.

| Hydroxylamine concentration (mM) | D $h^{-1}$ | Nitrification (ammonia) Rate | Nitrification (total) Rate | Protein (g/mole acetate) |
|---|---|---|---|---|
| 0 | 0.017 | 12.7 | 12.7 | 3.9 |
| 0.8 | 0.016 | 4.2 | 6.8 | 4.2 |
| 2.2 | 0.016 | 6.8 | 14.0 | 4.0 |
| 4.0 | 0.016 | 9.8 | 22.5 | 3.8 |
| 0 | 0.030 | 40.0 | 40.0 | 4.5 |
| 2.2 | 0.032 | 20.9 | 35.4 | 4.0 |

ATU, however, was only 90% of this (104 nmol$^{-}$ min$^{-1}$ ml$^{-1}$). In the absence of an inhibitor, $NH_3$ stimulates oxygen uptake by these cells, generating rates around 120–140% of those without $NH_3$ (Robertson & Kuenen 1988).

*Nitrapyrin*

Nitrapyrin (or N-serve) is also one of the classical inhibitors of autotrophic nitrification. Acetate-dependent oxygen uptake by *Tsa. pantotropha* in the presence of ammonia was inhibited both by nitrapyrin (50 $\mu$M) and by the acetone (0.0005% v/v end concentration) required to dissolve it (84% and 83%, respectively, of that when neither was present). This inhibition of nitrification by the solvent for nitrapyrin has also been observed for autotrophs (Hall 1984), and indicates that inhibition studies involving organic solvents should be evaluated with care. In this case (and that described by Hall), it is impossible to say how much effect the nitrapyrin actually has.

*Hydroxylamine*

Hydroxylamine appears to be intermediate between ammonia and nitrite in the nitrifying pathway of *Tsa. pantotropha*. However, ammonia oxidation by *Tsa. pantotropha* is inhibited by the presence of hydroxylamine (Robertson & Kuenen 1988; Robertson et al. 1988). Its effect on growth and nitrification in chemostat cultures was therefore tested. It proved possible to run a series of acetate-limited chemostat experiments if the concentration of hydroxylamine in the medium supply was gradually increased (in 1 mM "steps"), and the dilution rate was kept low (below 0.04 h$^{-1}$). All of the hydroxylamine was oxidized. However, it is possible that cells with a greater tolerance for hydroxylamine were being selected during these experiments because after the experiment with 4 mM hydroxylamine, it was found possible to start up a fresh culture directly with 4 mM hydroxylamine if cells from the old experiment were used, but not with a fresh culture which had not been in contact with hydroxylamine.

The inhibition of ammonia monooxygenase by hydroxylamine is reflected in the nitrification rates based on ammonia disappearance (Table 3). However, when the rates were recalculated on the basis of ammonia and hydroxylamine oxidation, together, they became (depending on the hydroxylamine concentration) comparable with, or greater than those obtained from the $NH_3/O_2$ cultures (Table 3). High nitrification rates appear to be associated with low yields in *Tsa. pantotropha* (Kuenen & Robertson 1987; Robertson et al. 1988), and all of the yields obtained with these hydroxylamine/ammonia cultures were also low (Table 3), compared with those obtained when nitrate was also present (5.9 g protein/mol acetate; Robertson et al. 1988).

## Conclusion

It is clear that thiosulphate, ATU and nitrapyrin are not specific inhibitors for autotrophic nitrification, but can also affect some heterotrophic nitrifiers. Possibly, the determining factor is whether the inorganic or organic pathways of nitrification are in use (Kuenen & Robertson 1987; Robertson & Kuenen 1988).

The ability of *Pseudomonas* sp. to metabolize thiosulphate does not seems to surprising when it is realised that all of the heterotrophic nitrifiers so far tested (6 strains) were able to oxidize thiosulphate to sulphate or tetrathionate in mixotrophic batch cultures (LA Robertson, R Zeng & JG Kuenen, unpublished results). However, not all facultatively autotrophic sulphur oxidizing bacteria are heterotrophic nitrifiers and it remains to be seen whether or not the combination of heterotrophic nitrification and thiosulphate oxidation is coincidental. As was shown for *Thiobacillus* Q (Gommers & Kuenen, 1988), chemostat experiments with thiosulphate limitation are frequently required before the significance of thiosulphate oxidation can be established for some apparent heterotrophs.

The strain of *"Ps. denitrificans"* studied here merits inclusion among the colourless sulphur bacteria. In view of the growing number of species which are being found to be able to oxidize reduced sulphur compounds (see also Kuenen 1989; Friedrich & Mitrenga 1981; Suylen & Kuenen 1986; Mason & Kelly 1988; Kelly & Harrison 1989), the use of this property as a primary taxonomic criterion will soon require revision, at least for the facultative chemolithotrophs and chemolithoheterotrophs.

The results obtained with *Tsa. pantotropha* cultures and hydroxylamine confirm that, as previously suggested, low protein yields are indeed associated with high nitrification rates (Kuenen & Robertson 1987; Robertson et al. 1988). However, whether these low yields are associated with loss of reducing equivalents in the ammonia mono-oxygenase reaction (Robertson & Kuenen 1988) or are related to hydroxylamine oxidation is not yet clear. The answer to this question must await the elucidation of the cytochrome chain in *Tsa. pantotropha*.

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