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Aerobic denitrification in various heterotrophic nitrifiers

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Abstract. Various heterotrophic nitrifiers have been tested and found to also be aerobic denitrifiers. The simultaneous use of two electron acceptors (oxygen and nitrate) permits these organisms to grow more rapidly than on either single electron acceptor, but generally results in a lower yield than is obtained on oxygen, alone. One strain, formerly known as "*Pseudomonas denitrificans*", was grown in the chemostat and shown to achieve nitrification rates of up to 44 nmol NH_3 min^{-1} mg $protein^{-1}$ and denitrification rates up to 69 nmol NO_3^{-1} min^{-1} mg $protein^{-1}$.

Unlike *Thiosphaera pantotropha*, this strain needed to induce its nitrate reductase. However, the remainder of the denitrifying pathway was constitutive and, like *T. pantotropha*, "*Ps. denitrificans*" probably possesses the copper nitrite reductase.

Introduction

The incidence and importance of heterotrophic nitrification, relative to that of the autotrophic nitrifiers, has been a subject of some dispute for a number of years (Verstraete 1975; Killham 1986; Kuenen & Robertson 1987). Among the hypotheses put forward to explain heterotrophic nitrification were the control of the citric acid cycle (Witzel & Overbeck 1979) and the synthesis of chelating agents (Verstraete 1975). Nitrifiers were evaluated according to the amount of oxidation products (usually nitrite or nitrate) accumulating in the culture medium, and it appeared that since most heterotrophic nitrifiers accumulated very little nitrite in comparison with the autotrophs, they were "poor" nitrifiers. Castignetti & Hollocher (1984) showed that many common soil denitrifiers were also heterotrophic nitrifiers which did not accumulate very high levels of nitrite when oxidizing pyruvic oxime or hydroxylamine, and thus seemed to be equally "poor" nitrifiers.

It has recently been shown that *Thiosphaera pantotropha* is capable of simultaneous heterotrophic nitrification and aerobic denitrification (Robert-

son et al. 1988). Our present insight into the process strongly indicates that under fully aerobic conditions, this organism oxidizes ammonia to nitrite and immediately reduces the nitrite to N_2, with the result that little or no nitrite accumulates in the cultures (Robertson & Kuenen 1984a, 1988; Robertson et al. 1988; Kuenen & Robertson 1987). As the end product of this combined pathway is gaseous (usually N_2), its nitrification rate can only be judged by the making of complete nitrogen balances. In this way, it has been shown that *T. pantotropha* was able to nitrify at rates up to at least 200 nmol min^{-1} mg $protein^{-1}$. This is sufficiently high to be compared with the autotrophic nitrifiers (Kuenen & Robertson 1987). If this combined nitrification/denitrification pathway is widespread among other bacteria, such as the denitrifying soil bacteria investigated by Castignetti & Hollocher (1984), its occurrence might require a reassessment of the relative importance of heterotrophic nitrifiers in the field, and in waste water treatment systems. A screening programme has therefore been underway to determine whether other heterotrophic nitrifiers are also aerobic denitrifiers (and vice versa), and how different environmental factors affect their performance. This paper presents the first results of the programme.

Materials and methods

Organisms

The strains used, together with their collection numbers, are shown in Table 1 or in the text. They were all obtained from the Delft Culture Collection (indicated by LMD).

Culture techniques

Batch cultures were made in Kluyver flasks (Robertson & Kuenen 1984A) which contained an oxygen electrode. Anaerobic batch cultures were carried out by sparging cultures in Kluyver flasks with oxygen-free argon or nitrogen. Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 30° C and the pH at 8.0, unless otherwise stated.

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, 5O; $ZnSO_4$, 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; $CuSO_4$, 1.57; $CoCl_2.6H_2O$, 1.61.

Heterotrophic batch and chemostat cultures were supplied with 10 and 20 mM acetate, respectively. When necessary, 38 mM nitrate was supplied in the medium. The mixotrophic batch cultures were supplied with 5 mM acetate and 10 mM thiosulphate. The concentrations of thiosulphate used for chemostat cultures are specified in the text.

Respiratory measurements

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

Biomass analysis

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

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 an ionosphor-TMA column (Chrompack) and a Waters RI detector.

Nitrite was measured colourimetrically, with the Griess-Romjn reagent (Griess-Romijn van Eck 1966) or by means of the HPLC, as described above. Nitrate was also measured colourimetrically, using diphenylamine sulphonic acid chromogene (Szechrome NAS reagent, Polysciences Inc.), or with the HPLC as described above. N_2O could be qualitatively determined in solution by means of a Clark-type oxygen electrode provided that the test mixture was kept anaerobic by means of a suspension of bakers yeast (Kučera et al. 1984).

Hydroxylamine was determined colourimetrically by means of the method described by Frear & Burrell (1955). Ammonia was determined with a test kit supplied by Sigma. 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. Analysis of the effluent gas using acid wash bottles confirmed that in all cases tested, loss of ammonia because of stripping was negligible.

Results and discussion

Heterotrophic nitrifiers as aerobic denitrifiers

It has been shown that the aerobic denitrifier, *T. pantotropha* grows more rapidly in the presence of oxygen and nitrate than when it is presented with a single electron acceptor. This is probably linked to a bottleneck in its respiratory chain to oxygen (Robertson & Kuenen 1984a,b; Robertson et al. 1988). *Paracoccus denitrificans* (LMD 22.21), which denitrifies only under anaerobic conditions, is unaffected by the presence of nitrate in aerobic cultures (Robertson & Kuenen 1984A; Robertson et al. 1988). In order to screen for the potential to denitrify aerobically, the growth rates of various known hetero-

Table 1. Comparison of the maximum specific growth rates (μ_{max}), protein concentrations and nitrate reduction obtained from aerobic or anaerobic batch cultures of bacteria known to be capable of heterotrophic nitrification. All of the media contained ammonia as the nitrogen source. The cultures were maintained at a dissolved oxygen concentration above 80% of air saturation. The growth rate and yield of a strain of *Pa. denitrificans* (which does not nitrify) were unaffected by the presence of nitrite. *T. pantotropha* data from Robertson & Kuenen 1984A.

Organism	μ_{max} (h^{-1})			Protein (mg/l)		$\Delta\ NO_3^-$
	O_2	O_2/NO_3^-	NO_3^-	O_2	O_2/NO_3^-	mM
Pseudomonas sp. LMD 84.60 (ex. *Ps. denitrificans*)	0.1	0.41	0.15	78	60	5.0
A. faecalis LMD 84.59	0.17	0.25	0.07	30	14	4.1
Ps. aureofaciens LMD 37.26	0.19	0.21	0.07	66	66	5.0
T. pantotropha LMD 82.5	0.28	0.34	0.25	81	60	5.5
Pa. denitrificans LMD 22.21	0.28	0.28	nd	92	88	< 1.0

nd = Not determined.

Table 2. Substrate-dependent oxygen uptake by various species in the presence of inorganic nitrogen compounds. For ease of comparison, the results are shown as the percentage of the uptake by a nitrogen-free sample.

Organism	Buffer	$+NH_3$	$+NO_3^-$	$+NO_2^-$	$+NH_2OH$
		7.5 mM	10 mM	5 mM	1 mM
Pseudomonas sp.	100	114	102	84	86
Pseudomonas aureofaciens	100	114	98	97	nt
Alcaligenes faecalis	100	109	89	48	nt
A. faecalis S6	100	123	58	87	104
Paracoccus denitrificans	100	105	100	92	48

trophs which were known to be nitrifiers were therefore measured in batch culture. It was found that *"Pseudomonas denitrificans"* and *Alcaligenes faecalis* both grew more rapidly when both electron acceptors were present, but *Ps. aureofaciens* did not (Table 1). Millimolar quantities of nitrate disappeared from all of the cultures which had been supplied with it. Moreover, in these experiments, lower protein yields were obtained from the nitrate/oxygen cultures than from those receiving oxygen alone (Table 1). These results show a strong similarity to the behaviour of batch cultures of *T. pantotropha* rather than that of *Pa. denitrificans* cultures which were run as a control (Table 1; Robertson & Kuenen 1984A).

A first confirmation that most of the strains were indeed heterotrophic nitrifiers was obtained from the fact that ammonia stimulated the rate of acetate-dependent oxygen uptake (Table 2), a phenomenon observed with *T. pantotropha*, but not to the same extent with *Pa. denitrificans*.

One of the strains was selected for detailed study in order to determine the degree of similarity between it and *T. pantotropha*. This strain is one of the heterogenous group previously known as *"Pseudomonas denitrificans"* whose taxonomic position is now considered as uncertain (Doudoroff et al. 1974; JCSB 1982). For convenience it will therefore be referred to as *Pseudomonas* sp.

Manometric experiments – nitrogen production

As already mentioned, *Pseudomonas* sp. resembled *T. pantotropha* in that it grew more rapidly with nitrate and oxygen together than with either electron acceptor individually (Table 1). However, it differed from *T. pantotropha*, which has a constitutive denitrifying system, in that its nitrate reductase appeared to be inducible. *Pseudomonas* sp. only produced gas from nitrate

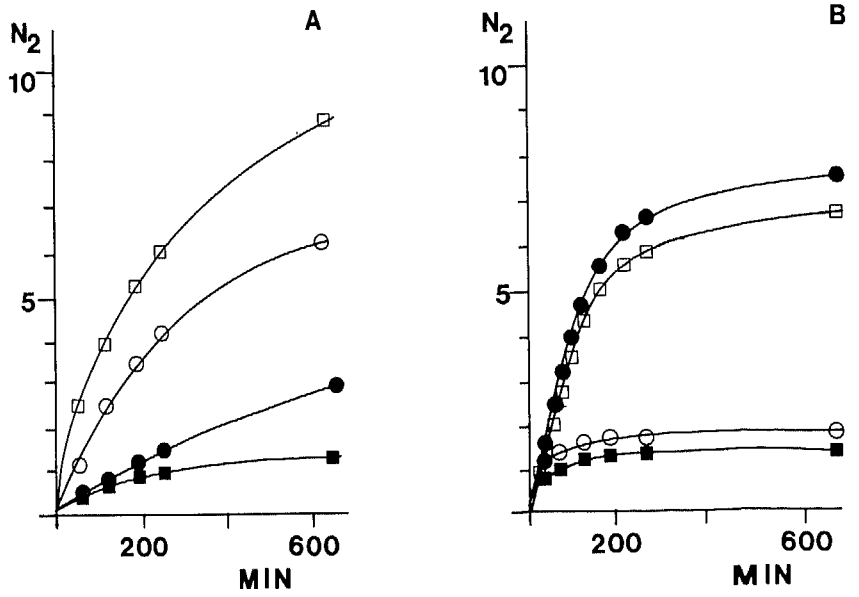

Fig. 1. Comparison of the effect of azide (closed circles) and DDC (open circles) on acetate-dependent nitrogen production by *Pa. denitrificans* (A) and *T. pantotropha* (B). Open squares = acetate present, no inhibitor; closed squares, endogenous, no inhibitor.

immediately if it had been grown (aerobically or anaerobically) in the presence of nitrate. Gas production rates varied with the growth history and the substrate concentrations provided. Cultures grown with ammonia as the sole source of nitrogen required an induction period of around 2.5 hours. Gas production from nitrite was immediate in all cases, indicating that the remainder of the denitrification pathway was constitutive.

With both *T. pantotropha* and *Pseudomonas* sp., the copper chelator, diethyldithiocarbamate (DDC), inhibited acetate-dependent gas production completely and azide had little or no effect. When cultures of *Pa. denitrificans* (a strain which only denitrifies under anaerobic conditions and does not nitrify to any significant extent) were used, the reverse was true. Figure 1 shows the results from *T. pantotropha* and *Pa. denitrificans*, the results from the *Pseudomonas* sp. experiments were essentially similar to those with *T. pantotropha* and are therefore not shown. Azide inhibits cytochrome cd and DDC inhibits the copper nitrite reductase (Shapleigh & Payne 1985). It is therefore likely that both *T. pantotropha* and *Pseudomonas* sp. contain the copper nitrite reductase rather than cytochrome cd.

Acetate-dependent oxygen uptake in the presence of ammonia and DDC was 95% of that in the absence of both compounds, whereas when the DDC was omitted the oxygen uptake was slightly stimulated by the addition of

ammonia (108%). Oxygen uptake was also considerably more sensitive to hydroxylamine in the presence of DDC than in its absence (62% and 84% of the control, respectively). This indicates that, like *T. pantotropha, Pseudomonas* sp. has a copper-based hydroxylamine oxidoreductase.

Because the cultures were simultaneously nitrifying and denitrifying, it was necessary to make complete nitrogen balances in order to appreciate the extent of nitrification taking place. This is most easily done in continuous cultures under controlled environmental conditions.

Chemostat experiments

In contrast to the batch results, acetate-limited cultures of *Pseudomonas* sp. gave higher yields in the presence of nitrate and oxygen than when oxygen was the sole electron acceptor (Table 3). This phenomenon has also been observed with *T. pantotropha*, where relatively low yields appear to be associated with high nitrification rates (Robertson 1988; Robertson et al. 1988). The *Pseudomonas* sp. yields (Table 3) were higher than those found with *T. pantotropha* (4.05 g/mol with and 5.15 g/mol without nitrate), and its nitrification rates were correspondingly lower than those obtained with *T. pantotropha*.

At a dissolved oxygen concentration of 80% of air saturation, *T. pantotropha* nitrifies harder in the absence of nitrate than in its presence (e.g. at a

Table 3. The yields, nitrogen balances and nitrification and denitrification rates obtained with acetate-limited chemostat cultures of *Pseudomonas* sp. at various dilution rates and dissolved oxygen concentrations in the presence or absence of added nitrate. All cultures contained ammonia as the nitrogen source. Yac = g protein/mol acetate; L = "lost" by nitrification and/or denitrification; nit.rate = nitrification rate and denit.rate = denitrification rate, both are given as nmol min^{-1} mg $protein^{-1}$.

Oxygen (% air)	D (h^{-1})	Yac	NH_3L mM	NO_3^-L mM	Nit. rate	Denit. rate
80	0.05	5.74	1.82		13.2	13.2
80	0.09	6.10	1.25		15.4	15.4
65	0.05	5.41	2.10		16.2	16.2
40	0.07	5.99	1.73		16.9	16.9
30	0.05	5.84	1.87		13.3	13.3
30	0.07	5.84	1.33		13.3	13.3
80	0.06	7.72	2.13	4.96	13.8	45.9
50	0.07	6.00	2.92	2.32	28.4	50.9
50	0.10	6.17	3.56	1.99	44.3	69.0
30	0.07	6.30	2.93	2.55	27.4	50.7
30	0.10	6.17	3.49	2.76	43.4	77.7

dilution rate of $0.05\,h^{-1}$, these were 43 and 12 nmol min^{-1} mg $protein^{-1}$, respectively; Robertson 1988; Robertson et al. 1988). This was not the case with *Pseudomonas* sp. where the rates were similar in both cases. Like *T. pantotropha*, however, both the nitrification and denitrification rates of the cultures which included both ammonia and nitrate increased as the growth rate increased. This effect was not apparent when ammonia was the sole nitrogen compound present.

Another difference between the two species was that the nitrification rates in the reduced-oxygen (30–50% of air saturation), nitrate-containing cultures were actually much higher than those in the other reduced-oxygen cultures.

Taxonomic status of Pseudomonas sp.

Pseudomonas sp. has been found to be able to utilize thiosulphate for mixotrophic and autotrophic growth (Robertson 1988; Robertson et al. 1989). In view of this, and its nitrifying/denitrifying capabilities it was desirable to establish that the strain was, indeed, different from *T. pantotropha*. Limited

Table 4. Comparison of the reactions shown by *Pseudomonas* sp. and *T. pantotropha* LMD 82.5, *Paracoccus denitrificans* LMD 22.21 and *Thiobacillus versutus* LMD 80.62 when screened with the API20B system. *T. pantotropha, Paracoccus denitrificans* and *Thiobacillus versutus* data from Robertson & Kuenen 1983.

Organism	GEL	NIT	ONPG	SAC	ARA	MAN	FRU	GLU	MAL	AMD	RHA	GAL
Pseudomonas sp.	−	+	−	+	+	+	+	+	+	−	−	+
T. pantotropha	−	+	−	+	−	+	+	+	+	−	−	−
Pa. denitrificans	−	+	−	−	−	−	−	−	−	−	−	−
T. versutus	−	+	−	+	+	+	+	+	+	−	−	−

	MNE	SOR	GLY	URE	IND	H2S	VP	CIT	OX	CAT	COC	GRAM
Pseudomonas sp.	−	+	+	−	−	−	−	−	+	+	+	+
T. pantotropha	−	+	−	−	−	−	+	−	+	+	+	−
Pa. denitrificans	−	−	−	−	−	−	−	+	+	−	+	−
T. versutus	−	+	−	−	−	−	−	+	+	+	−	−

Abbreviations: GEL – gelatine liquefaction; NIT – nitrate reduction; ONPG – β-galactosidase present. Acid produced from the following carbohydrates; SAC – saccharose; ARA – L(+)-arabinose; MAN – mannitol; FRU – fructose; GLU – glucose; MAL – maltose; AMD – starch; RHA – rhamnose; GAL – galactose; MNE – mannose; SOR – sorbitol; GLY – glycerol; URE – urease present; IND – indole produced by a tryptophanase; H2S – hydrogen sulphide produced; VP – acetoin produced; CIT – citrate utilization; OX – cytochrome oxidase present; CAT – catalase present; COC – coccoid form; GRAM – reaction to Gram's stain

taxonomic tests were therefore carried out in order to compare the strain with *T. pantotropha* and other denitrifying mixotrophs.

Pseudomonas sp. is gram negative, and is most often seen under substrate-limited growth in the chemostat as cocci 1.05 × 0.8 μm in size. During batch culture, single, very long polar flagellae were occasionally seen. However, these appeared to be easily lost and did not occur in the chemostat cultures.

Preliminary taxonomic tests with the API20B confirmed that the *Pseudomonas* sp. was, in fact, different from the three known facultatively chemolithotrophic sulphur bacteria which are able to denitrify (Table 4). It also differed from these species in various physiological reactions. *T. versutus* and most strains of *Pa. denitrificans* do not denitrify aerobically, and are not heterotrophic nitrifiers. *Pseudomonas* sp. differs from *T. pantotropha* in its need to induce its nitrate reductase, its lower nitrification rates and correspondingly higher yields, and in some of its responses to the dissolved oxygen concentration (Robertson et al. 1988).

Conclusions

Combined heterotrophic nitrification and aerobic denitrification has been found in unrelated bacteria from different genera and it is therefore obvious that *T. pantotropha* is not unique in this property. It is most likely that different denitrifying bacteria have different critical oxygen concentrations above which their denitrification systems do not function so efficiently. This is illustrated by the results obtained with a strain of *Alcaligenes* which has been shown to be capable of efficient aerobic denitrification only at dissolved oxygen concentrations below 50% of air saturation (Kuenen & Robertson 1987; E.W.J. van Niel & K. Braber, unpublished data). It is, as yet, too early to suggest that the coincidence of heterotrophic nitrification and aerobic denitrification is a universal rule. However, in the light of the results presented here, it is desirable that other organisms which have been found to be capable of aerobic denitrification (see, for example, Lloyd et al. 1987) be screened for heterotrophic nitrification.

The association of the copper nitrite reductase with aerobic denitrification may be more than coincidental. Nitrite reduction by the alternative nitrite reductase, cytochrome cd, is strongly inhibited by oxygen (Paitian et al. 1985) and denitrification at high concentrations of dissolved oxygen may not be possible for bacteria dependent on this enzyme. As cytochrome cd can also serve as a terminal oxidase (Km for oxygen 80 μm), it has been suggested that the inhibition of nitrite reduction by this cytochrome might be due to its reaction with oxygen (Kučera et al. 1983). Similar behaviour for the copper nitrite reductase has not been reported.

The strain of the former "species" "*Pseudomonas denitrificans*" studied during these experiments is clearly a facultatively chemolithotrophic member of the colourless sulphur bacteria. It differs from many members of the group in its ability to denitrify (Kelly & Harrison 1988). It seems likely that *Pseudomonas* sp. is most closely related to, but different from *T. pantotropha*. However, further, more detailed tests will be required before its precise taxonomic status can be determined.

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