Antonie van Leeuwenhoek **62**: 231–237, 1992.

Heterotrophic nitrification and aerobic denitrification in *Alcaligenes faecalis* strain TUD

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Received 11 March 1992; accepted in revised form 21 May 1991

Key words: Alcaligenes faecalis, aerobe, chemostat, denitrification, heterotroph, nitrification

Abstract

Heterotrophic nitrification and aerobic and anaerobic denitrification by *Alcaligenes faecalis* strain TUD were studied in continuous cultures under various environmental conditions. Both nitrification and denitrification activities increased with the dilution rate. At dissolved oxygen concentrations above 46% air saturation, hydroxylamine, nitrite and nitrate accumulated, indicating that both the nitrification and denitrification were less efficient. The overall nitrification activity was, however, essentially unaffected by the oxygen concentration. The nitrification rate increased with increasing ammonia concentration, but was lower in the presence of nitrate or nitrite. When present, hydroxylamine, was nitrified preferentially. Relatively low concentrations of acetate caused substrate inhibition ($K_I = 109\ \mu M$ acetate). Denitrifying or assimilatory nitrate reductases were not detected, and the copper nitrite reductase, rather than cytochrome cd, was present. Thiosulphate (a potential inhibitor of heterotrophic nitrification) was oxidized by *A. faecalis* strain TUD, with a maximum oxygen uptake rate of 140–170 nmol $O_2 \cdot min^{-1} \cdot mg\ prot^{-1}$. Comparison of the behaviour of *A. faecalis* TUD with that of other bacteria capable of heterotrophic nitrification and aerobic denitrification established that the response of these organisms to environmental parameters is not uniform. Similarities were found in their responses to dissolved oxygen concentrations, growth rate and ammonia concentration. However, they differed in their responses to externally supplied nitrite and nitrate.

Introduction

A variety of heterotrophic microorganisms are capable of nitrification, provided that an organic energy source is present. Studies have been made on the oxidation of various reduced nitrogen compounds, including ammonia, by fungi (e.g. Killham 1986) and bacteria (Verstraete & Alexander 1972; Rho 1986). Some heterotrophically nitrifying bacteria are also denitrifiers, and have been isolated from soil (Castignetti & Hollocher 1984) and waste water treatment systems (Robertson et al. 1988, 1989a). The subject has been reviewed by Verstraete (1975) and Kuenen & Robertson (1987). As with the autotrophs, heterotrophic nitrifying activity has commonly been estimated from the amount of product (usually nitrite) accumulation. However, because some heterotrophic nitrifiers also denitrify aerobically (Robertson et al. 1988, 1989a), some or all of the nitrite may be simultaneously reduced to nitrogen gas or one of the volatile nitrogen oxides. Thus, the amount of ammonia oxidized will not always be reflected in the amount of oxidation products accumulating in the medium,

and the activity of heterotrophic nitrifiers may be underestimated.

Recent work in this laboratory has shown that all six of the heterotrophic nitrifiers studied were also aerobic denitrifiers, implying a common link between the two properties. However, a recent report by Papen et al. (1989) has indicated that this link may not be universal. They observed that with some heterotrophic nitrifiers, aerobic nitrate and nitrite reduction did not occur, indicating that the denitrifying nitrate and nitrite reductase was either inactive or absent in the presence of oxygen.

Heterotrophic nitrification has generally been studied in batch cultures. Because of the relatively uncontrolled nature of this type of experiment, there can be difficulties in the interpretation of experimental results. Dissolved oxygen concentration, growth rate and pH are all important factors in these metabolic processes, and it is essential that they are controlled. In addition, the physiological status of batch cultures changes, with some pathways ceasing to operate earlier in the entry to stationary phase than others. If, for example, the denitrification enzymes ceased to function before those of heterotrophic nitrification, the processes would no longer be in balance, resulting in an accumulation of nitrite and an apparent association of nitrification (as indicated by nitrite accumulation) with the onset of the stationary phase (see, for example, Witzel & Overbeck 1979).

Since only a few heterotrophic nitrifiers have been studied under controlled conditions in continuous culture (Robertson et al. 1988, 1989a), and as it was desirable to further study a possible link between nitrification and aerobic denitrification, we decided to broaden our studies to include species from other genera. Therefore, the behaviour of a strain of *Alcaligenes faecalis* (a heterotrophic nitrifier commonly found in soil and activated sludge) was examined in continuous cultures at different oxygen concentrations, growth rates, and in the presence of various inorganic nitrogen compounds. This paper reports the results and compares them with those obtained with continuous cultures of another heterotrophic nitrifier and aerobic denitrifier, *Thiosphaera pantotropha,* (Robertson et al. 1988).

Materials and methods

Alcaligenes faecalis strain TUD, LMD 89.147, was kindly provided by Prof. D. Castignetti, Loyola University, Chicago, USA.

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.

The medium supplied to the chemostats contained ($g \cdot l^{-1}$): K_2HPO_4, 0.4; KH_2PO_4, 0.15; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4; and 2 ml of the trace element solution described by Vishniac & Santer (1957), but with 2.2 g instead of the originally reported 22 g $ZnSO_4 \cdot 7\ H_2O$. Substrate was 20 mM acetate. Where indicated, cultures were provided with 10 or 40 mM nitrate, 5 mM nitrite or 2 mM hydroxylamine.

Cultures tested for their thiosulphate oxidizing potential were grown as batch cultures in Kluyver flasks. The mineral salts medium is described elsewhere (Robertson & Kuenen 1984). The cultures were provided with 10 mM acetate and 5 mM thiosulphate.

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer) or as total organic carbon with a TOCA master 915-B. Nitrite was determined using the Griess-Romijn reagent (Griess-Romijn-van Eck 1966). Nitrate was measured colourimetrically, using diphenylamine sulphonic acid chromogene (Szechrome NAS reagent, Polysciences Inc.) or by means of a HPLC fitted with an ionospher-TMA column (Chrompack) and a Waters RI detector. Hydroxylamine was determined colourimetrically by means of the method described by Frear & Burrell (1955). Ammonia was determined colourimetrically by the method of Fawcett & Scott (1960). As ammonia and ammonium would both be present, at the pH values used in these experiments, the term 'ammonia' will be used throughout to indicate both the protonated and unprotonated forms. Ammonia loss from stripping due to air being sparged through the culture was found to be approximately 4.0 μmol $\cdot$ $l^{-1} \cdot h^{-1}$. N_2O could be qualitatively determined in solution by means of a Clark-type oxygen electrode provided that the test mixture was kept anaerobic

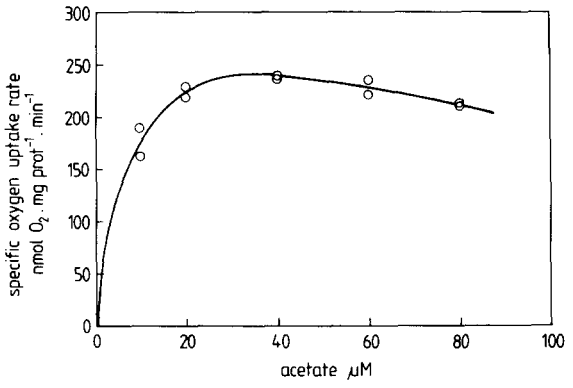

Fig. 1. Effect of acetate concentration on the specific oxidation rate of acetate by chemostat grown suspensions of *A. faecalis* strain TUD, as measured from oxygen uptake rates. Results of two experiments at each acetate concentration.

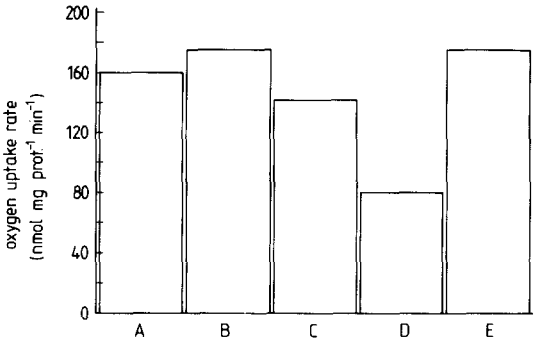

Fig. 2. Effect of the nitrogen compounds on the rate of acetate-dependent oxygen uptake by washed cells grown on acetate and ammonia. A, control; B, 10 mM ammonia; C, 100 μM nitrite; D, 600 μM hydroxylamine; E, 10 mM nitrate.

by means of a suspension of bakers yeast (Kučera et al. 1984).

Protein was measured spectrophotometrically, by means of the micro-biuret method (Goa 1953). The total organic carbon of washed cells was determined with a Beckmann TOCA master 915-B. The carbon, hydrogen, oxygen and nitrogen content of the biomass was measured with a Perkin Elmer 240 C.

Oxygen uptake by whole cells was measured polarographically with a Clark type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instruments, Ohio, USA) mounted in a thermostatically-controlled cell which is closed except for a small hole through which additions may be made. The acetate-dependent oxygen uptake rate was recorded by adding 10–80 μM acetate to a suspension of washed cells in phosphate buffer (pH 8.0). The rates were corrected for endogenous respiration.

The rate of gas production under anaerobic conditions by washed cells suspended in phosphate buffer, pH 8.0, was measured using standard manometric techniques. The suspensions were provided with 10 μM acetate and 40 μM nitrite. Azide (1 mM) and diethyldithiocarbamate (DDC) (10 mM) were used to examine the respiratory type of nitrite reductase present, as described by Shapleigh & Payne (1985).

Results and discussion

Oxygen uptake

As acetate was the electron donor for heterotrophic nitrification and denitrification in all experiments, the Monod constants for acetate oxidation by the *A. faecalis* strain were measured indirectly via oxygen uptake experiments. This method was adequate because the organism has a relatively low affinity constant ($K_s \approx 1$ μM) for oxygen, thus the oxidation rate is zero order for the oxygen concentration. The results with washed cells from a culture grown on acetate and ammonia clearly showed substrate inhibition by acetate at concentrations as low as 80 μM (Fig. 1). The affinity constant, maximum consumption rate and substrate inhibition constant, calculated using the Haldane equation (Han & Levenspiel 1988), were 12 μM, 200 nmol acetate. min^{-1}·mg protein^{-1} and 109 μM, respectively.

To determine whether ammonia or its oxidation products influenced acetate-dependent oxygen uptake, measurements were done in the presence of these nitrogen compounds (Fig. 2). Nitrate did not significantly affect (< 10%) the oxygen uptake rate. Hydroxylamine inhibited acetate-dependent oxygen uptake by 50%. The lack of a significant effect by ammonia (i.e. no increase in oxygen uptake by heterotrophic nitrification) was not unexpected as *T. pantotropha* cells grown under simi-

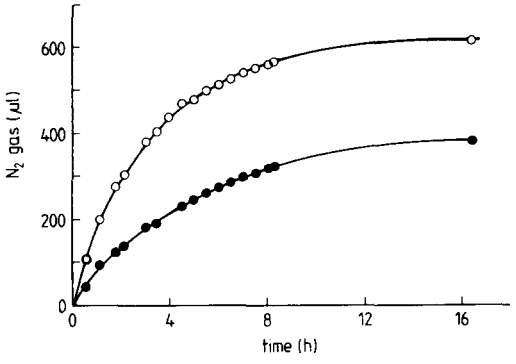

Fig. 3. Comparison of the effects of azide (1 mM) (open circles) and DDC (10 mM) (closed circles) on acetate-dependent nitrogen production by *A. faecalis* strain TUD. Inhibitor-free controls with acetate gave curves identical to that shown for azide.

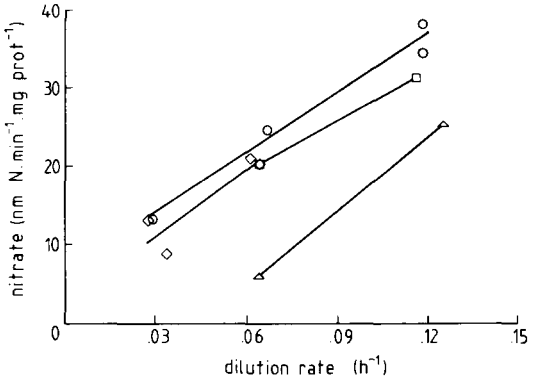

Fig. 4. The nitrification and denitrification rates of chemostat cultures in the presence of different nitrogen compounds as a function of the dilution rate. 7.5 mM ammonia (circles); 5 mM ammonia (triangles); 7.5 mM ammonia + 10 mM nitrate (squares); 7.5 mM ammonia + 40 mM nitrate (diamonds).

lar conditions also gave a very small reaction (Robertson & Kuenen 1988). This result might be explained by the observation that *T. pantotropha* cells under these conditions tend to make poly-β-hydroxybutyrate rather than reoxidize NADH by means of nitrification and/or denitrification (van Niel 1991).

A number of heterotrophic nitrifying species are able to oxidize thiosulphate (Robertson et al. 1989b). To test thiosulphate oxidation, *A. faecalis* TUD was grown in batch cultures provided with acetate and thiosulphate. Cells in exponential growth phase were harvested, washed, and assayed. The maximum thiosulphate-dependent oxygen uptake rate measured was 140–170 nmol $O_2 \cdot min^{-1} \cdot mg\ prot^{-1}$. This value, although significant, was lower than that observed with similar cultures of *T. pantotropha* (Robertson et al. 1989b).

Denitrification

Anaerobic growth experiments showed that this strain of *A. faecalis* could denitrify nitrite, but not nitrate, to nitrogen gas. Nitrous oxide could not be detected in the anaerobically growing cultures. The lack of a dissimilatory nitrate reductase contrasts with other *A. faecalis* strains (Knowles 1982; Anderson & Levine 1986), but is similar to that of *A. odorans* (Tilton 1981). Nitrite reduction by *A. faecalis* was sensitive to DDC rather than azide (Fig. 3), indicating that the nitrite reductase in *A. faecalis* strain TUD is likely to be of the copper-containing type (Shapleigh & Payne 1985).

Effect of the growth rate

In *T. pantotropha*, the nitrification and denitrification rates increased with increasing growth rate (Robertson et al. 1988). To determine whether this also occurred in *A. faecalis*, cultures supplied with 5 and 7.5 mM ammonia were grown at various dilution rates. Higher dilution rates, indeed, resulted in increased rates of nitrification and denitrification, indicating that, as with *T. pantotropha*, this process is linked to the metabolic activity of the cell. The dilution rate similarly affected the cultur-

Table 1. Nitrogen balances of acetate limited *A. faecalis* strain TUD chemostat cultures with ammonia as the only N-source, at different air saturations ($D = 0.07\ h^{-1}$). The ammonia disappearance has been corrected for assimilated nitrogen.

Air sat. (%)	NH_2OH (µM)	NO_2^- (µM)	NO_3^- (µM)	N-biomass (mM)	NH_4^+ (mM)
20	10	0	50	0.85	−3.25
46	221	20	60	0.83	−3.05
76	195	43	80	0.64	−4.43

es at different dissolved oxygen concentrations (not shown) and in the presence of nitrate (Fig. 4).

Effect of the dissolved oxygen

Cultures of *A. faecalis* strain TUD were grown in the chemostat at a dilution rate of 0.07 h^{-1} with ammonia as the sole nitrogen source. At low dissolved oxygen concentrations (< 20% air saturation), there was little accumulation of nitrification products such as nitrite or hydroxylamine (Table 1). As the dissolved oxygen was increased, hydroxylamine and nitrite appeared in the culture medium, indicating that the nitrification and denitrification pathways had become less efficient. Nitrate was produced in low amounts at every oxygen concentration (Table 1). Nitrate cannot be used by *A. faecalis* strain TUD as it lacks nitrate reductase. The amount of ammonia disappearing was similar at all oxygen tensions (Table 1). Less than 60 μM of this loss was due to stripping by the gas stream (see Materials and methods); it must therefore be concluded that the combined nitrification and denitrification pathways were still active, even at a dissolved oxygen concentration of 76% air saturation. These results contrast with those obtained with *T. pantotropha* which did not accumulate nitrification or denitrification intermediates at any oxygen concentration tested (up to 90% air saturation), indicating a more uniform efficiency of the enzymes involved (Robertson et al. 1988).

The nitrification and denitrification rates of *A. faecalis* chemostat cultures, grown on minerals medium with amonium in the presence of hydroxylamine or nitrate, were calculated from the nitrogen balances for the cultures (Table 2). These values have been corrected for the amounts of ammonia assimilated into biomass, and for the amount of stripping. In the presence of either hydroxylamine or nitrate, the dissolved oxygen concentration did not substantially affect the nitrification rate over most of the range tested. Below 5% air saturation, the oxygen concentration became limiting (not shown). Above 80% air saturation, oxygen appeared to have an inhibitory effect on the nitrification process, and the rates were lower (results not shown). These results are in contrast to previous observations with *T. pantotropha,* in which both the nitrification and denitrification rates increased as the dissolved oxygen concentration in the cultures decreased (Robertson et al. 1988), reaching a maximum at about 25% air saturation before dropping until oxygen became limiting.

Effect of inorganic nitrogen compounds

The effect of ammonia concentration on the nitrification activity of the cultures was determined by using different concentrations in the medium (Table 3). At a dilution rate of 0.06 h^{-1}, nitrification rates of 9.3 and 24.6 nmol $N \cdot min^{-1} \cdot mg\ prot^{-1}$ and yields of 10.5 and 7.4 g protein per mol acetate were

Table 2. Steady state nitrification and denitrification rates of acetate limited chemostat cultures of *A. faecalis* TUD (nmol $N \cdot min^{-1} \cdot mg\ protein^{-1}$) at different dilution rates (D) and in the presence of different nitrogen compounds and dissolved oxygen concentrations (% air saturation) ($[NH_4^+] = 7.5$ mM).

N-compounds	O_2 (%)	D (h^{-1})	Nitrif. rate	Denitrif. rate
+ NH_2OH 2 mM	77	0.05	21	17
	29	0.05	20	16
	5	0.05	17	16
+ NO_3^- 40 mM	72	0.03	9	9
	48	0.03	13	13
	28	0.03	8	7
	16	0.03	12	12

Table 3. Effect of different inorganic N-compounds on the nitrification rate (nmol $N \cdot min^{-1} \cdot mg\ prot^{-1}$) and biomass yield (g $prot \cdot mol\ acetate^{-1}$) ($D = 0.06\ h^{-1}$).

N-compound	Nitrif. rate	Yield
NH_4^+ 5 mM	9.3	10.5
NH_4^+ 7.5 mM	24.6	7.4
NH_4^+ 7.5 mM + NO_3^- 10 mM	15.5	9.4
NH_4^+ 7.5 mM + NO_3^- 40 mM	20.3	7.5
NH_4^+ 7.5 mM + NO_2^- 5 mM	9.7	7.6
NH_4^+ 7.5 mM + NH_2OH 2 mM	19.7	n.d.

n.d. = not determined.

obtained with 5 and 7.5 mM ammonia, respectively. A similar effect has been observed for a *Pseudomonas* species (L.A. Robertson & J.G. Kuenen, unpublished data). As with *T. pantotropha,* higher nitrification rates appear to correlate with lower yields of biomass, suggesting that nitrification is an energy-consuming rather than an energy-producing process (Robertson et al. 1988, 1989a). Hydroxylamine did not affect the total (i.e. ammonia and hydroxylamine) nitrification rate, but repressed ammonia oxidation.

Unlike *T. pantotropha,* in which nitrate reduces the nitrification rate, nitrate had little effect on nitrification by *A. faecalis* TUD (Fig. 4 and Tables 2 and 3). As with *T. pantotropha,* nitrification by cultures supplied with nitrite, however, was less than half of that found in cultures where ammonia was the sole nitrogen source. The differential effect of nitrate versus nitrite may be explained by the inability of *A. faecalis* to reduce nitrate (above). However, nitrite supplied in the medium was not aerobically denitrified by the culture, even at dissolved oxygen concentrations as low as 5% air saturation. This suggests that this organism is only able to denitrify internally-produced nitrification products under aerobic conditions, in contrast to *T. pantotropha.* It is possible that with *A. faecalis* TUD, only sufficient nitrite reductase is available to cope with nitrite produced internally by nitrification and, as it is saturated, externally supplied nitrite is not reduced. Alternatively, nitrite may not be taken up, although, as the known nitrite reductases are periplasmic, this is an unlikely explanation. The low growth yields obtained with cultures in the presence of nitrite (Table 3) do not correlate with high rates of nitrification, and thus must be due to other factors.

Conclusions

From the results presented here and elsewhere (Robertson et al. 1988, 1989a), it can be concluded that while there are many similarities between bacteria able to nitrify and denitrify simultaneously, the relationship is not completely straightforward as there are also many differences. It is, as yet, not possible to generalize because the number of species tested under the controlled conditions of the chemostat is too small, but the outlines of a pattern are emerging. For example, nitrification and denitrification by *T. pantotropha, Pseudomonas* sp. and *A. faecalis* strain TUD increase with increasing dilution rate and ammonia concentration, and with decreasing dissolved oxygen concentration. Moreover, in view of their sensitivity to DDC, all three strains are likely to posses the copper nitrite reductase. The pattern is less clear with regard to other factors such as nitrate and hydroxylamine. *T. pantotropha* has a constitutive nitrate reductase, that of *Pseudomonas* sp. is inducible by nitrate, and *A. faecalis* strain TUD does not have one. *T. pantotropha* could only grow at very low dilution rates in the presence of hydroxylamine, *A. faecalis* strain TUD did not have this problem.

Because only a small number of heterotrophic nitrifiers has thus far been tested (Robertson et al. 1989b), it is not yet clear whether the ability of these organisms to oxidize reduced sulphur compounds is significant. The occurrence of the two properties may be coincidental, especially as there are many species (e.g. *Paracoccus denitrificans*) which can grow on reduced sulphur compounds but do not significantly nitrify. However, despite the differences, it is a fact that many, if not most, heterotrophic nitrifiers simultaneously reduce at least part of the end product of their nitrification; thus their nitrifying potential cannot be estimated from product accumulation measurements. Before the ecological significance of bacteria of this type can be reassessed, however, further experiments on a broader range of species under controlled conditions is necessary.

Acknowledgement

The authors are grateful to Rong Zheng for experimental assistance.

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