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Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria

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Abstract

Reports of the simultaneous use of oxygen and denitrification by different species of bacteria have become more common over the past few years. Research with some strains (e.g. *Thiosphaera pantotropha*) has indicated that there might be a link between this 'aerobic denitrification' and a form of nitrification which requires rather than generates energy and is therefore known as heterotrophic nitrification. This paper reviews recent research into heterotrophic nitrification and aerobic denitrification, and presents a preliminary model which, if verified, will provide at least a partial explanation for the simultaneous occurrence of nitrification and denitrification in some bacteria.

Introduction

Recent laboratory studies have resulted in some progress being made in the understanding of the physiology behind heterotrophic nitrification and aerobic denitrification. Among the findings has been the discovery of a group of bacteria, typified by *Thiosphaera pantotropha*, which are both heterotrophic nitrifiers and aerobic denitrifiers. The combination of the two pathways means that little or none of the nitrite or nitrate produced by the nitrification accumulates in the cultures. This review will concentrate on the work with *Tsa. pantotropha* and on complementary laboratory studies on other bacteria, and only brief introductions to the two phenomena will be given. For information on fungal nitrification, environmental studies, ammonia oxidation by methanotrophs and details on the historical background to heterotrophic nitrification and the controversy which previously surrounded aerobic denitrification, the reader should consult the reviews by Verstraete (1975), Killham (1986), Robertson & Kuenen (1984a) and Kuenen & Robertson (1987).

Physiology of aerobic denitrification and heterotrophic nitrification

Aerobic denitrification

The traditional view that bacteria must show biphasic consumption of electron acceptors when given the choice between oxygen and nitrate dominated the literature for many years. At first glance, biphasic growth on two electron acceptors (e.g. oxygen and nitrate) seems so logical from a bioenergetic point of view that it has blocked the consideration of alternative metabolic strategies which, from an ecological rather than a biochemical point of view, might have survival value under particular environmental conditions. An analogous situation, in some respects, might be the consideration of growth on two electron donors which

causes diauxy in batch cultures where the substrates are in excess. Under the right conditions (e.g. in substrate-limited continuous cultures) these two electron donors are utilized simultaneously, a phenomenon known as mixotrophy. It is now widely accepted that during competition for limited resources in nature, diauxy may be the exception rather than the rule and mixotrophy may be an important metabolic parameter in determining the competitive success of a species (see, for example, Beudeker et al. 1982). In addition, metabolic flexibility and reactivity, which can be conferred by the possession of consitutive rather than inducible enzymes, may often play a role in the competitive success of a species.

In this context, it is apparent that the versatility conferred by the simultaneous presence of alternative metabolic properties is no more surprising in electron accepting systems (e.g. simultaneous oxygen and nitrate respiration) than it is in electron donation. Much of the controversy regarding aerobic denitrification was due to confusion created by inadequate experimental design and techniques. However, equipment is improving all the time, and new techniques are rapidly becoming available. The use of sensitive and accurate electrodes for the measurement of dissolved oxygen has allowed the precise definition of the 'aerobic' status of the immediate environment of bacteria. Furthermore, the use of steady state continuous cultures in well-mixed fermentors permits the reproducible measurement, under controlled conditions, of phenomena which were, in batch culture, often transient.

As with earlier reports of aerobic denitrification (see the review by Robertson & Kuenen 1984a), the original indications that *Tsa. pantotropha* might have a constitutive denitrification pathway came from anaerobic respirometric experiments designed to compare denitrification by *Tsa. pantotropha* with that by the physiologically similar *Thiobacillus versutus* (Robertson & Kuenen 1984b). As expected, aerobically-grown *T. versutus* required an induction period before gas production began. However, *Tsa. pantotropha* began denitrifying immediately it was supplied with acetate and nitrate (Fig. 1). That the enzymes were active under aerobic conditions was shown in two ways. Firstly, the behaviour of *Tsa. pantotropha* cultures in Kluyver flasks (Robertson & Kuenen 1984a) in the presence of oxygen and/or nitrate was checked (Robertson & Kuenen 1984b). As may be seen from Fig. 2, the cultures receiving both electron acceptors grew more rapidly, and gave a final optical density which was intermediate between those obtained from the two single acceptor cultures. Subsequent analysis confirmed that the difference between the optical densities reflected a difference in the biomass yield and, moreover, sufficient nitrate had disappeared from the culture to account for half of the acetate dissimilated. Secondly, electrodes were used to show the simultaneous use of oxygen and nitrate in a well-mixed suspension of aerobically-grown *Tsa. pantotropha* cells (Fig. 3).

In order to further quantify the process under accurately controlled and reproducible conditions, and to allow the investigation of other factors such as growth rate, dissolved oxygen and medium composition, continuous cultures were grown under acetate limitation in well-mixed fermentors fitted with dissolved oxygen and pH control. As with the batch cultures, experiments were done with single and double electron acceptors. As expected, significant amounts of nitrate were reduced in the oxygen/nitrate cultures, the actual quantity increasing as the growth rate increased. This did not happen with similar cultures of *Pa. denitrificans* used as controls. Unexpectedly, the yield pattern found with the batch cultures was not repeated. Although the oxygen/nitrate cultures were higher than the anaerobic cultures by the expected amount (extrapolating from the batch culture results), the culturres receiving oxygen as their sole electron acceptor only produced about 60% of their expected protein (Fig. 4). As will be discussed later, there appears to be an inverse correlation between nitrification (rather than denitrification) rates and biomass yields in these cultures. The provision of nitrate or nitrite in the medium, permitting denitrification, resulted in lower nitrification rates and, as mentioned above, biomass yields at roughly expected levels. In addition to increasing with growth rate, the amount of nitrate denitrified by the *Tsa. pantotropha* cultures also increased as the dissolved

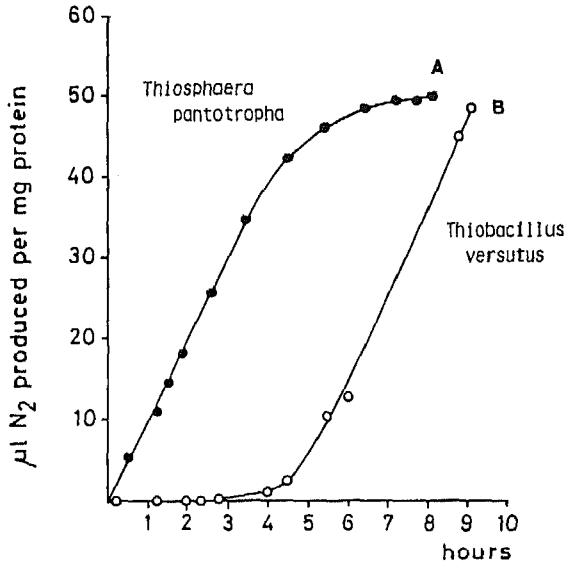

Fig. 1. Gas production by aerobically grown *Tsa. pantotropha* and *T. versutus* cultures during anaerobic respirometry experiments. A: *Tsa. pantotropha*, B: *T. versutus* (from Robertson & Kuenen 1984b).

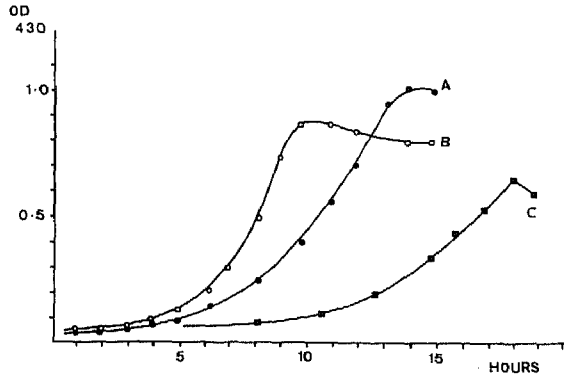

Fig. 2. Growth of *Tsa. pantotropha* in batch cultures on acetate and ammonia. A: oxygen supplied, B: oxygen and nitrate supplied, C: nitrate supplied (from Robertson & Kuenen 1984b).

oxygen fell, and reached its anaerobic value at a dissolved oxygen concentration around 25–30% of air saturation. Similar results were obtained with saturating amounts of nitrite, except that the denitrification rates were somewhat lower (Table 1). When 'limiting' concentrations of nitrite (i.e. below the maximum that *Tsa. pantotropha* could reduce so that nitrite was not detectable in the culture) were used, both the nitrification rates and the protein yields were similar to those obtained when ammonia was the sole nitrogen source. If the cultures were forced to assimilate nitrate rather than using ammonia as the source of nitrogen, the rate of aerobic denitrification fell to less than 10% of that found with similar cultures which had been provided with ammonia as well (Table 1).

Experiments to establish whether or not *Tsa. pantotropha* was unique in its ability to denitrify aerobically were carried out using a *Pseudomonas* sp. (formerly *Ps. denitrificans*). These experiments showed that, indeed, this strain was also an aerobic denitrifier, but that it differed from *Tsa. pantotropha* in that its nitrate reductase needed to be induced by the presence of nitrate, although anaerobiosis was not necessary. The remainder of its denitrification pathway was constitutive (Robertson et al. 1989a).

^{15}N-labelled compounds and mass spectrometry, have been used (Degn et al. 1985; Lloyd et al. 1986; 1987), to analyse the products of denitrification (aerobic or otherwise) in detail. Lloyd et al. (1987) used this technique to investigate the persistence of denitrification under aerobic conditions and came to the conclusion that the phenomenon is very widespread among bacteria. They found that strains of *Paracoccus denitrificans* (in contrast to the results described above), *Ps. aeruginosa, Ps. stutzeri, Propionibacterium thoenii* and newly isolated *Pseudomonas* species all continued to denitrify at air saturation. In most of the strains, the rate of N_2 evolution was low (0.13–0.40 nmol min^{-1} mg $protein^{-1}$), but *Ps. stutzeri and Pr. thoenii* produced N_2 at somewhat higher rates (3.42–5.61 nmol min^{-1} mg $protein^{-1}$). Hence, their rate of aerobic denitrification was much lower than that observed with *Tsa. pantotropha* (30–300 nmol min^{-1} mg $protein^{-1}$). In chemostat experiments with such cultures, deviations in the nitrogen balance of that order of magnitude would not have been detectable. All of the strains investigated by Lloyd and co-workers also produced small amounts of N_2O. In a subsequent study using aerobically and anaerobically grown cells (Davies et al. 1989). N_2O appeared in *Pa. denitrificans* suspensions (provided with nitrate and succinate) when oxygen was added, and increased as the dissolved oxygen rose (from 0 to 100

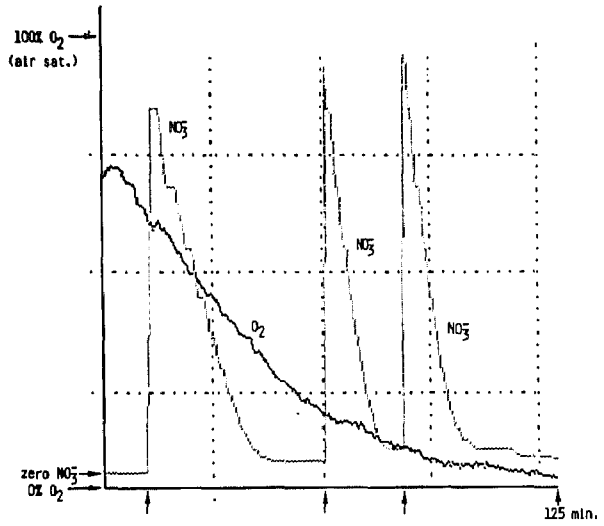

Fig. 3. Simultaneous use of oxygen and nitrate by well-mixed suspensions of aerobically-cultured *Tsa. pantotropha*, as monitored by electrodes (from Robertson et al. 1986).

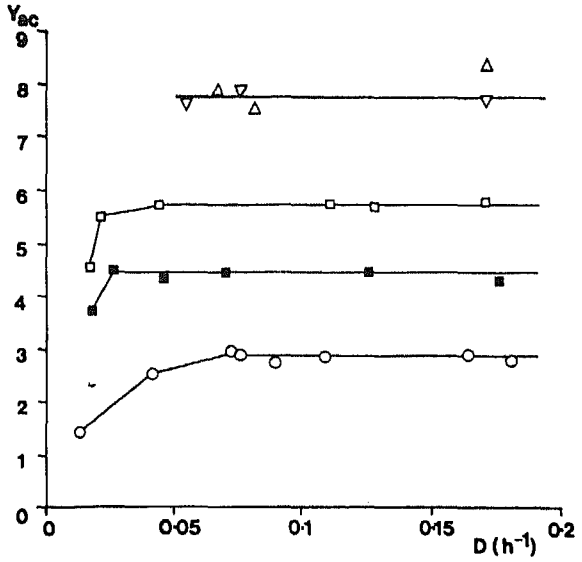

Fig. 4. Protein yields from acetate-limited chemostat cultures of *Tsa. pantotropha* and *Pa. denitrificans*, with oxygen and/or nitrate, as a function of the dilution rate (D). Yac: grams protein per mol acetate. *Tsa. pantotropha* cultures: open circles, nitrate only; closed squares, oxygen only; open squares, nitrate and oxygen. *Pa. denitrificans* cultures: triangles, oxygen only; inverted triangles, oxygen and nitrate (adapted from Robertson et al. 1988).

and then 150 μM). Similar results were obtained with a *Ps. aeruginosa* strain, indicating that the N_2O reductase in these organisms was more oxygen sensitive than the other denitrifying enzymes. The position of *Pa. denitrificans* as a typical specialist denitrifier is further complicated by results published by Kawakami et al. (1985). The authors showed that N_2O production in the presence of acetylene by anaerobically grown cells was only partially inhibited by oxygen, even at 50% of air saturation. N_2O production continued at reduced levels for periods of at least 4 hours. Moreover, it took approximately 1 hour for inhibition by oxygen to reach its maximum. These observed differences in the denitrifying abilities of *Pa. denitrificans* may be due to strain variation. Oxygen sensitivity of the denitrification pathway in its most extreme form ('all or nothing') may only actually occur in a few strains. During studies of N_2O and NO production (without acetylene) by common soil bacteria, Anderson & Levine (1986) found that *Rhizobium japonicum* and *Pseudomonas fluorescens* only continued to generate these gases up to a dissolved oxygen concentration of 5% air, but that their strain of *Alcaligenes faecalis* continued to do so at air saturation and, in other words, must be an aerobic denitrifier. Robertson et al. (1989a) have also shown that some *A. faecalis* strains are aerobic denitrifiers. Stimulation of denitrification by the presence of oxygen was shown by Abou Seada & Ottow (1985) for *Aeromonas 'denitrificans'*, *Azospirillum lipoferum* and *Bacillus licheniformis*. They attributed this phenomenon to oxygen stimulated mineralisation rates. Unfortunately, only headspace analysis of the oxygen concentration was used during these experiments, and it is therefore not possible to relate the results to those described above.

It should be remembered that the situation is not a clear-cut choice between denitrification anaerobically or at full air saturation. Different strains appear to have different oxygen maxima above which their denitrifying systems begin to be inhibited by the oxygen. Figure 5 summarizes the responses of various denitrifying bacteria to dissolved oxygen whereby the vertical arrows indicate the oxygen concentration at which that particular species begins to have difficulty in denitrifying under the growth conditions tested.

Heterotrophic nitrification

During the investigations into aerobic denitrification by *Tsa. pantotropha* in batch cultures described above, nitrate was replaced by nitrite in a series of experiments. Surprisingly, the nitrite concentration in the medium of the aerobic cultures went up during the early log phase, before eventually dropping to almost 0 at the onset of the stationary phase (Robertson & Kuenen 1984b). Enzyme tests showed that *Tsa. pantotropha* is able to oxidize ammonia to nitrite via hydroxylamine (Robertson & Kuenen 1988). Given the capacity of *Tsa. pantotropha* to denitrify aerobically, nitrite would not normally accumulate, but instead be immediately reduced to nitrogen. Unlike the autotrophic nitrifiers, *Tsa. pantotropha* only carried out this oxidation when a source of energy was supplied, and did not appear to gain any energy from the reaction. This is typical of heterotrophic nitrification. It was thus abundantly clear that during the chemostat experiments it was essential to make nitrogen balances which were as complete as possible. One of the first observations was that, like the denitrification rates, the nitrification rate increased with the growth rate, but this relationship was not linear. Moreover, there was a direct correlation between high nitrification rates and the unexpectedly low yields mentioned above (Table 1). When nitrification was inhibited by the presence of nitrate, nitrite or thiosulphate, the yields were restored to their expected values (taking into account that *Tsa. pantotropha* can gain energy from the oxidation of thiosulphate). Hence, it would appear that heterotrophic nitrification actually costs *Tsa. pantotropha* energy (Robertson et al. 1988). Similar results were obtained with chemostat cultures of *Pseudomonas* sp. (formerly '*Ps. denitrificans*'; Robertson et al. 1989a; 1989b).

Table 1. Correlation of chemostat yields (mg/L) with their respective nitrification and denitrification rates (nmol^{-1} min^{-1} mg protein^{-1}). All cultures were acetate limited, at a dissolved oxygen level of 80% air saturation and supplied with NH_4^+, except in the last case.

Additive	Nitrification	Denitrification	Yield
–	43	43	81*
NO_3^-	12	107	103
NO_2^- (limiting)	48	85	80*
NO_2^- (saturating)	25	98	115
NH_2OH	45	45	75*
$S_2O_3^{2-}$	21	21	145
$S_2O_3^{2-}/NO_3^-$	6	36	120
NO_3^-	na	7	118

D = 0.04 h^{-1}.
* Indicates yield lower than expected.
na = nor applicable.
(Most data from Robertson et al. 1988.)

As with denitrification, the nitrification rate in the *Tsa. pantotropha* cultures increased as the dissolved oxygen fell, until a value of 25–30% air was reached. At this point, several factors became apparent in the cultures which were reliant on oxygen as the sole electron acceptor:

- the cells appeared to become oxygen limited;
- the nitrification rate fell;
- the cells began to accumulate PHB;
- biofilm formed on all surfaces in the chemostat.

As mentioned above, cultures which were supplied with both electron acceptors at this dissolved oxygen concentration achieved denitrification rates equivalent to those found under anaerobic conditions, and it could be calculated that all of the oxygen uptake was being used for nitrification.

Castignetti (1988) examined proton translocation in pseudomonads, *Arthrobacter* sp. and *Alcaligenes faecalis* in order to confirm that nitrification by these strains did not generate energy. In three cases, hydroxylamine oxidation yielded only stoichiometric amounts of protons, and the fourth bacterium, *A. faecalis*, did not oxidize the hydroxylamine at a significant rate. Tests for proton translocation with *Arthrobacter* sp. when oxidizing ammonia yielded only stoichiometric amounts of protons. There was thus no evidence for energy generation during nitrification by any of these heterotrophs.

The combined pathways

It is clear that aerobic denitrification and heterotrophic nitrification are intimately linked in *Tsa. pantotropha* as well as many other bacteria (Kue-

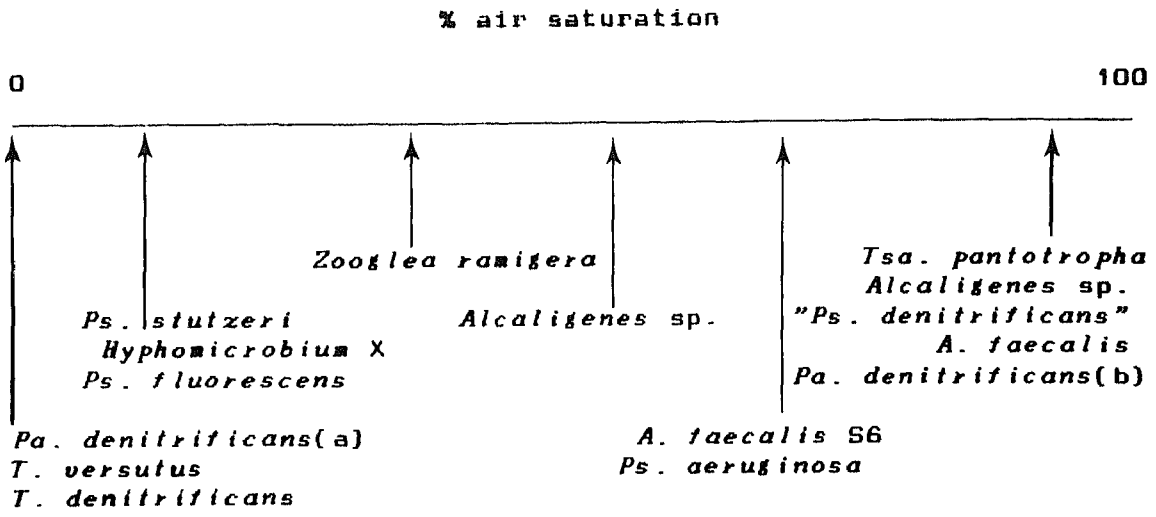

Fig. 5. 'Spectrum' showing the different oxygen sensitivities of the denitrifying systems of various bacteria.

nen & Robertson 1987; Robertson et al. 1989a). Certainly, in *Tsa. pantotropha* and *'Ps. denitrificans'* they appear to be inseparable and, as will be discussed below, may possibly be different ways of overcoming the same problem.

Once the combination of aerobic denitrification and heterotrophic nitrification in one organism has been accepted, it immediately becomes clear that nitrification rates obtained with other heterotrophic nitrifiers require fresh evaluation as many were obtained from product (i.e. nitrate or nitrite) accumulation data and may be underestimates. Several strains which were known to be able to denitrify and had been described in the literature as 'poor nitrifiers' (Castignetti & Hollocher 1984) were screened for aerobic denitrification in batch cultures, as described for *Tsa. pantotropha,* above. As can be seen from Table 2, all of them showed the stimulation of μ_{max} and nitrate disappearance which characterized the behaviour of *Tsa. pantotropha* in this type of experiment (Robertson & Kuenen 1984b; Robertson et al. 1989a), and it may well be that their 'poor nitrifying activity' is due to immediate denitrification of NO_x. Table 3a and b shows the result of recalculating published nitrification rates on the basis of ammonia disappearance rather than product accumulation. Of course, neither *Tsa. pantotropha* or the strain of *'Ps. denitrificans'* described above would have been identified even as 'poor' heterotrophic nitrifiers if nitrite accumulation had been the only criterion under investigation. That this may be a widespread problem is illustrated by a recent paper (Strand et al. 1988) in which aerobic denitrification by chemostat-grown cultures of *Zooglea ramigera* at a range of dissolved oxygen concentrations was examined. As with *Tsa. pantotropha* the rate of denitrification by *Z. ramigera* achieved its maximum at an oxygen concentration somewhat lower than 50% of air saturation. Furthermore, even though this species had not been previously recognised as a heterotrophic nitrifier, it appears from the data provided in the paper that ammonia was disappearing from their cultures at about half the rate found with similar cultures of *Tsa. pantotropha.* It would appear that this is yet another species which can combine heterotrophic nitrification and aerobic denitrification.

Enzymology

Denitrification enzymes

Nitrate reduction in denitrifying bacteria proceeds via nitrite and nitrous oxide to N_2. Nitric oxide is also generated in some cases. The enzymology of denitrification has been reviewed in detail (Payne

1981; Stouthamer 1988a; 1988b), and will only be briefly discussed here.

The nitrate reductases from the denitrifying and dissimilatory nitrate reducing bacteria appear to be surprisingly similar (Stouthamer 1988a; 1988b). Nitrate reductase is a molybdoprotein located on the cytoplasmic side of the cell membrane. In *E. coli*, one of the subunits has been found to be a cytochrome b which is necessary not only for electron transport, but also for the association of the enzyme with the membrane. The main discrimination between denitrification and dissimilatory nitrate reduction appears at the level of nitrite reduction. In denitrification, two types of nitrite reductase are known to occur. The best-known is cytochrome cd which occurs in, among others, *Pa. denitrificans* (Payne 1981; Stouthamer 1980; 1988a; 1988b). Purified cytochrome cd produces a mixture of NO and N_2O. The second nitrite reductase is a soluble, copper-containing enzyme which occurs in the periplasm. It has only been found in a limited number of bacteria including *Rhodopseudomonas sphaeroides* var *denitrificans*, *Corynebacterium nephridii*, *Achromobacter cycloclastes* and *Alcaligenes* sp. (Iwasaki & Matsubara 1972; Iwasaki et al. 1963; Iwasaki et al. 1975; Reuner & Becker 1970; Sawada et al. 1978). The product of the purified enzyme is NO. Both nitrite reductases display cytochrome oxidase activity, but have a considerably lower K_m for nitrite than for oxygen. The third enzyme, N_2O reductase, seems to not be very stable in the organisms investigated. Like one of the nitrite reductases, it is a copper-containing enzyme with a periplasmic location. Various molecular weights and subunit combinations have been reported (Zumft & Matsubara 1982; Stouthamer 1988a; 1988b), but it is not clear whether this is due to a range of different enzymes or the effect of different preparatory methods on a relatively labile enzyme.

Körner & Zumft (1989) used immunological techniques to follow the synthesis of the denitrifying enzymes by chemostat-grown *Ps. stutzeri* at different dissolved oxygen concentrations. They found that the expression of the nitrate, nitrite (cytochrome cd) and nitrous oxide reductases was controlled by both different oxygen concentrations and the identity of the nitrogen oxide provided for denitrification. In all cases, nitrite reductase was the most sensitive to oxygen. N_2O reductase was present in low concentrations in all of the cells, even when they had been grown at air saturation in the absence of a nitrogen oxide, but was found at its highest levels when nitrate (and not N_2O) was provided. Indeed, N_2O reductase appears to be constitutive. At 50% air saturation, nitrate reductase was also detectable.

Another report which serves as a reminder that there is considerable variation in the behaviour of denitrifying bacteria is that by Castignetti & Hollocher (1982) in which they showed that their strain of *Alcaligenes* contained nitrate reductase and nitric oxide reductase when grown as a heterotrophic

Table 2. 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 significantly) were unaffected by the presence of nitrate (data from Robertson et al. 1989a).

Organism	μ_{max} (h^{-1})			Protein (mg/l)		Delta NO_3^- mM
	O_2	O_2/NO_3^-	NO_3^-	O_2	O_2/NO_3^-	
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.

nitrifer, but neither nitrite nor N_2O reductase was present.

Using the method described by Shapleigh & Payne (1985) in which cytochrome cd and the Cu^{2+} nitrite reductases are selectively inhibited by azide and chelating agents, respectively, it was possible to show that both *Tsa. pantotropha* and *Pseudomonas* sp. possessed the Cu^{2+}-based enzyme (Fig. 6).

Nitrifying enzymes

Because of the wide variety of organisms which carry out heterotrophic nitrification, it is not unexpected that more than one pathway occurs. A pathway involving organic and inorganic intermediates was proposed by Verstraete & Alexander (1972) and there is some evidence that the fungi use an almost entirely organic route (Killham 1986). Although somewhat unexpected, *Tsa. pantotropha* appears to have a nitrification pathway which resembles that of the autotroph in many ways (Robertson & Kuenen 1988). Experiments with crude extracts revealed the presence of a light-sensitive, NADPH-dependent ammonia monooxygenase which appeared to require the presence of Mg^{2+} ions, all points of similarity with the autotrophs (Hooper 1981). Unlike the enzyme from *Nitrosomonas europaea*, the *Tsa. pantotropha* ammonia monooxygenase appears to be inhibited by hydroxylamine (Suzuki et al. 1976; Robertson & Kuenen 1988).

Using crude extracts, biological, hydroxylamine-dependent oxygen uptake could be shown in the presence of cytochrome c. The end product of hydroxylamine oxidation by the extracts and by whole cells was nitrite. Hydrazine, phosphate and nitrite all inhibited this activity (Robertson & Kuenen 1988). Hydrazine has been shown to inhibit hydroxylamine oxidoreductase activity in *Nitrosomonas europaea* (Dua et al. 1979). Similar enzyme activity was observed in *Arthrobacter globiformis* extracts (Kurokawa et al. 1985). After purification, the enzyme involved could be shown to reduce cytochrome c. It required iron and was inhibited by the chelator, EDTA.

Another similarity between the behaviour of the heterotrophic and autotrophic nitrifiers was found during the experiments referred to in the section on aerobic denitrification, when chemostat cultures were made with a *Tsa. pantotropha* mutant, known as TP43 (Chandra & Friedrich 1986). As it lacks nitrite reductase, it had been assumed that this organism would accumulate nitrite, thus allowing nitrification to be measured without the complication of simultaneous denitrification. This did not prove to be the case, and the organism continued to nitrify/denitrify, although at a reduced rate. The ability to use its hydroxylamine oxidoreductase both to make nitrite and to combine nitrite and hydroxylamine to make N_2O has been reported for the autotrophic ammonia-oxidizing bacteria (Hooper 1968), and a potential explanation for this

Table 3A. Nitrification rates calculated from the published results of batch culture experiments. For ease of comparison, where other nitrogen compounds were used the results have been recalculated as though for ammonia. Nitrifying activity is expressed as nmol NH_3/min/mg dry weight (most data from Robertson et al. 1988).

Organism	Activity	N-compound used
Ps. aeruginosa	12– 28	hydroxamate
Ps. aeruginosa	70– 90	hydroxylamine
Ps. denitrificans	2.6	pyruvic oxime
Ps. aureofaciens	2.8	pyruvic oxime
Al. faecalis	11.9	pyruvic oxime
Alcaligenes sp.	33	pyruvic oxime
Arthrobacter sp.	0.8	ammonia
T. pantotropha	35.4	ammonia
N. europaea	50– 100	ammonia
Nitrosomonas sp.	590–2300	ammonia

Table 3B. Nitrification rates calculated from published results from chemostat culture experiments. Nitrifying activity is expressed as nmol NH_3/min/mg dry weight. (data from Robertson et al. 1988.)

Organism	Activity	N-compound used
T. pantotropha	6– 47	ammonia
N. europaea	670– 835	ammonia
N. europaea	130–1550	ammonia
N. europaea	1385–5290	ammonia
N. europaea	400–1020	ammonia

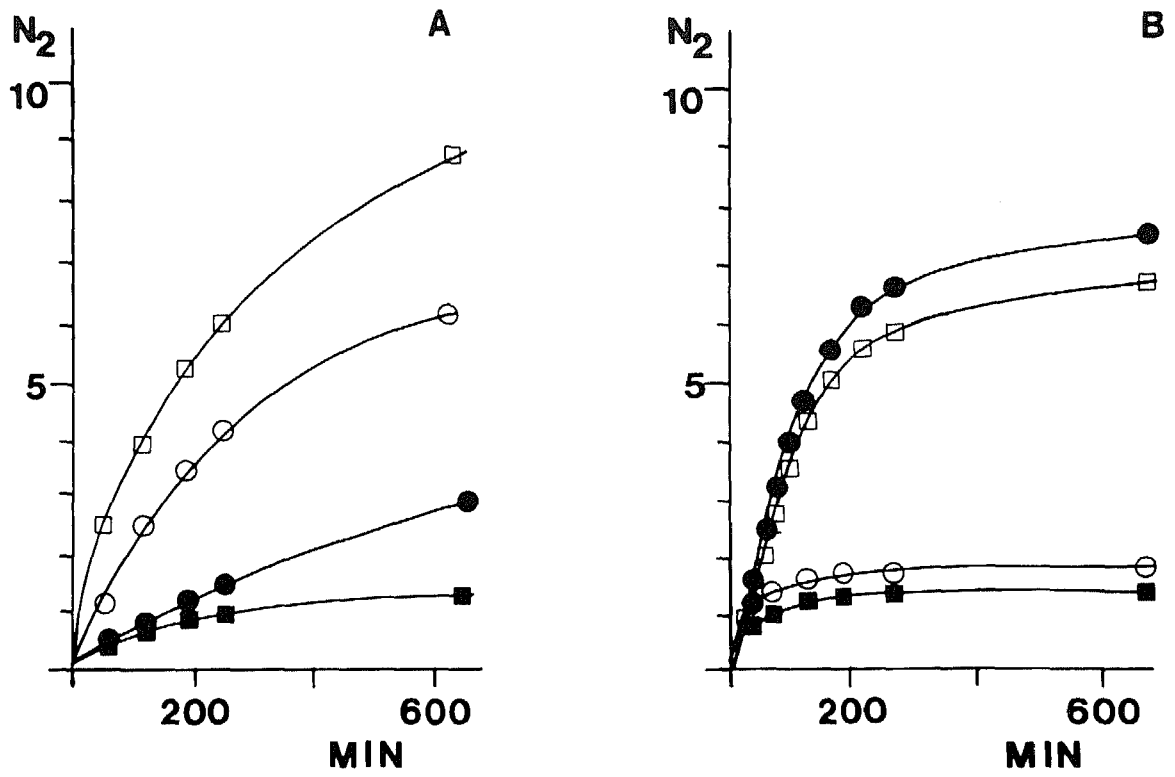

Fig. 6. Comparison of the effect of azide (closed circles) and DDC (open circles) on acetate-dependent denitrification by (A) *Pa. denitrificans* and (B) *Tsa. pantotropha*. The open squares show the response with acetate but no inhibitor, the closed squares show the endogenous behaviour in the absence of inhibitors (from Robertson et al. 1989a).

behaviour is that this also occurs in *Tsa. pantotropha*.

It is not yet clear whether the apparent similarities between the nitrifying enzymes of the autotrophs and the heterotrophs described here is real. Methane oxidizing bacteria including *Methylococcus* species have been reported to be capable of NADH-dependent ammonia oxidation and cytochrome c-dependent hydroxylamine oxidation (Dalton 1977; Drozd et al. 1978). In this case, part of the similarity is demonstrably fortuitous since the enzyme involved in ammonia oxidation is methane monooxygenase, and *Tsa. pantotropha* cannot oxidize methane (Robertson & Kuenen 1983). It remains to be seen whether the monooxygenase activity detected in this organism is a secondary role of another enzyme.

Control/cytochromes

Cytochromes and denitrification

As with oxygen respiration, denitrification proceeds via the cytochrome chain. Only a few cytochrome chains have been completely mapped (see, for example, Stouthamer 1988a; 1988b).

In *Pa. denitrificans* the appearance of the various cytochromes appears to be controlled by the redox levels within the cytochrome chain. For example, when the dissolved oxygen concentrations are high, cytochrome aa_3 is the dominant terminal oxidase. As the dissolved oxygen falls, cytochrome o becomes more important, and as the dissolved oxygen concentration approaches o, cytochrome cd is synthesized (Sapshead & Wimpenny 1972). As has already been mentioned, because of results obtained with a few species, it was considered for many years that bacteria used either oxygen or

nitrate (or nitrite), and that the presence of oxygen automatically excluded the possibility of denitrification occurring. As yet, the mechanism by which oxygen affects denitrification in many bacteria is not fully understood, and it is not yet clear if one or several mechanisms are involved. In some species, anoxia alone is sufficient to induce nitrate reductase, but in others the presence of nitrate is required. Again, in some species the denitrifying enzymes appear to be inactivated by oxygen, whereas in others synthesis is repressed but the existing enzymes only gradually disappear (Payne 1981; Knowles 1982).

Much of the work on oxygen inhibition has been done with *Pa. denitrificans* (Boogerd 1984; Alefounder & Ferguson 1981; Alefounder et al. 1983; Alefounder et al. 1984; Stouthamer 1988a; 1988b) and it appears that two factors may be interacting in the inhibition of denitrification by oxygen in this species. The redox level in the cytochrome chain has been shown to control the flow of electrons to the different cytochromes, and thus to determine whether or not electrons are available for denitrification (Kučera & Dadák 1983). However, it has also been shown that the cell membrane alters its permeability to nitrate in response to dissolved oxygen (Alefounder et al. 1984). Since the dissimilatory nitrate reductase is located on the inside of the cell membrane (Stouthamer 1980), a permeability barrier between the enzyme and its substrate would be a very effective controlling factor. However, the dissimilatory nitrite reductase is located on the outside of the membrane (Stouthamer 1980; Boogerd 1984; Stouthamer 1988a; 1988b) and therefore lack of access cannot provide the full explanation as denitrification from nitrite would not be affected by changes in the conformation of the membrane. Nitrite has been reported to inhibit oxygen uptake, possibly by interacting with cytochrome aa_3, thus causing the cytochrome chain to become more reduced (Kučera & Dadák 1983; Kučera et al. 1984). Thus in cells containing significant amounts of nitrite, rather than nitrite reduction being inhibited by oxygen, the reverse effect (with oxygen uptake being inhibited sufficiently to permit electron flow to the denitrification enzymes) might be predicted.

Cytochromes and nitrification

In *N. europaea*, it appears that ammonia monooxygenase accepts electrons from cytochrome b, and hydroxylamine oxidoreductase gives electrons to cytochrome c (Wood 1986). As was discussed in the section on enzymology, cytochrome c-linked hydroxylamine oxidation had been shown for at least two heterotrophic nitrifiers in vitro, but whether this actually occurs in vivo has not yet been established.

Control

Two questions concerning the biochemistry and physiology which have arisen during this work are:

- Why do *Tsa. pantotropha* and other heterotrophically nitrifying bacteria not gain energy from a reaction which can support the growth of autotrophs such as *N. europaea*? The resemblance of the nitrification pathway of *Tsa. pantotropha* to that of the autotrophic nitrifiers, as discussed above, has only added to this puzzle.
- Why are the denitrifying enzymes of some bacteria sensitive to oxygen, and others not?

A preliminary model to describe electron transport in *Tsa. pantotropha* has been developed in order to provide at least partial answers to both of these questions. The model (Fig. 7) is based on the assumption that when growing heterotrophically, *Tsa. pantotropha* has a redox problem which leads to the accumulation of NAD(P)H, possibly because of a 'bottleneck' in the flow of electrons to oxygen via cytochrome aa_3. Cells grown mixotrophically (on acetate and thiosulphate) or autotrophically (on thiosulphate) do not appear to have the same difficulties (Robertson et al. 1988; H.J. Nanninga unpublished data).

Figure 8 outlines the various options *Tsa. pantotropha* has for producing and reoxidizing NAD(P)H. They will be discussed in a clock-wise sequence:

- Under normal growth conditions, the organism

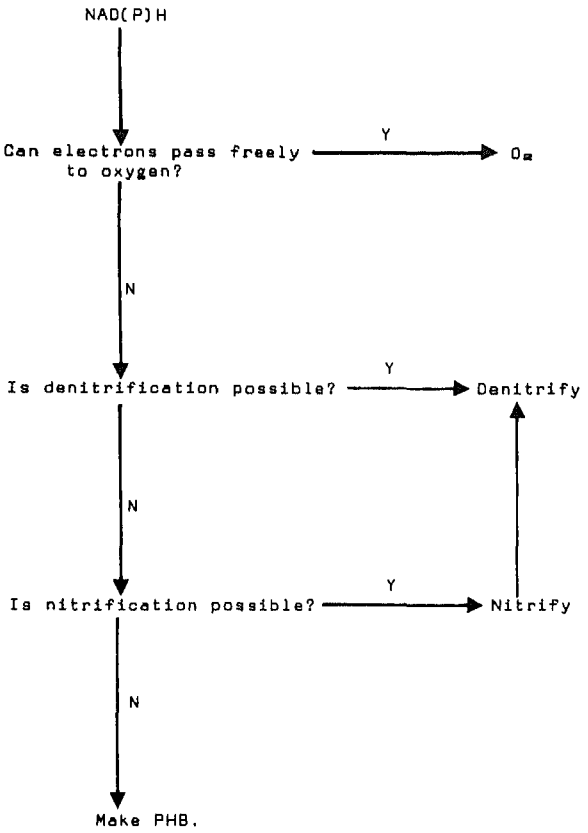

Fig. 7. Flow chart to present, as a verbal model, the working hypothesis developed to explain the control of aerobic denitrification and heterotrophic nitrification in *Tsa. pantotropha*, assuming that there is a 'bottleneck' in the flow of electrons to oxygen (from Robertson et al. 1988).

must strike a balance between biomass production and NAD(P)H generation. In heterotrophic chemostat cultures, approximately 25% of the acetate is assimilated (Robertson et al. 1988), and the remainder is used to generate (primarily) NAD(P)H. During mixotrophic growth, greater amounts of organic substrate are assimilated. The amount depends on the organic: inorganic ratio in the medium supply. Once the maximum possible assimilation is achieved, CO_2 fixation begins (Gommers et al. 1988). Thus, during mixotrophic growth, less NAD(P)H will be generated from acetate and any potential overload would be, at least partially, relieved.

- Similarly, cultures assimilating nitrate or nitrite would use NAD(P)H for the assimilatory nitrite reductase (Payne 1981), and some of the overload would be 'tapped off'.
- NADPH is required by the ammonia monooxygenase of the nitrification pathway (Robertson & Kuenen 1988). Nitrification, therefore, is also a means of reoxidizing NAD(P)H.
- Once electrons from NAD(P)H enter the cytochrome chain, a 'bottleneck' in their flow through cytochrome aa_3 to oxygen would result in an increase in the degree of reduction of the chain to such an extent that electrons can flow to the denitrification pathway. All of the aerobic denitrifiers so far tested have had the copper nitrite reductase rather than cytochrome cd (Kuenen & Robertson 1987; Robertson et al. 1989a; Robertson 1988), and these properties might be linked if the cytochrome cd-linked nitrite reducing system was more sensitive to oxygen than the copper nitrite reductase. However, the apparent induction of the nitrate reductase-linked cytochrome b_{590} in aerobic as well as anaerobic cultures indicates that at least one other factor plays an important part (Robertson 1988). This induction may be possible even under aerobic conditions if, in contrast to other denitrifiers, the cells remain permeable to nitrate (Alefounder et al. 1983).
- The final option for the reoxidation of NAD(P)H by *Tsa. pantotropha* could be defined as 'If all else fails, make PHB'.
- Another possible means of overcoming redox

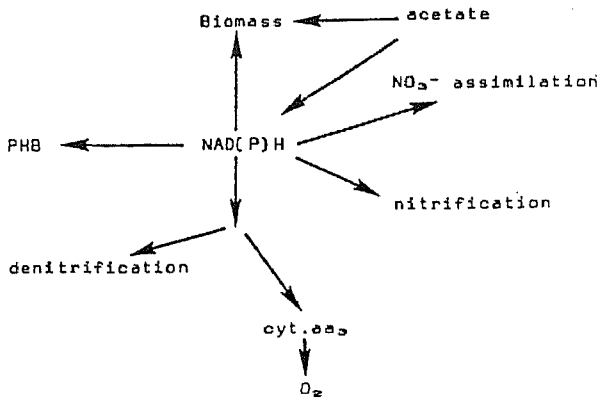

Fig. 8. Simplified scheme to outline the possible means of NAD(P)H utilization available to *Tsa. pantotropha* (Robertson 1988).

problems in the respiratory chain, not shown in Fig. 8, is the synthesis of additional cytochromes, most obviously those associated with the metabolism of reduced sulphur compounds. Preliminary measurements have shown that the composition of the cytochrome chains of heterotrophically and mixotrophically grown cells are very different, with at least one additional cytochrome c being synthesized during thiosulphate metabolism (L.A. Robertson, J.E. van Wielink, J. Frank, L.F. Oltman, A.H. Stouthamer & J.G. Kuenen, unpublished data).

These points have been dealt with in an arbitrary sequence. The experimental evidence indicates that redox problems are preferentially avoided either by mixotrophic growth or by co-respiration of O_2 and NO_x. Nitrification seems to be a 'second choice' mechanism when redox problems arise, and its inhibition leads to the formation of PHB.

Concluding remarks

As yet, our understanding of heterotrophic nitrification and arobic denitrification on a physiological basis is in its infancy. Possibly, as with many other microbiological phenomena, apparently identical reactions are carried out for different reasons, and there is no single explanation for their occurrence. Almost all of the work done thus far has been in the laboratory, with axenic cultures of a few species, and it is dangerous to generalize on the basis of so small a sample. It is, perhaps, time to increase the number of strains being examined, to build ecological models and to test them with work on deliberately mixed and enrichment cultures, as well as samples from different environments in order to determine the ecological significance of bacteria of this type in the natural world as well as in wastewater treatment systems. For example, it seems likely that bacteria with constitutive denitrifying enzymes would be favoured where the available dissolved oxygen was either low, or fluctuating in a cycle where the aerobic periods were sufficiently long to inactivate the denitrifying enzymes of the 'classical' denitrifying bacteria. This supposition has been supported by recent work using cultures grown to steady state in acetate-limited chemostats at a dissolved oxygen concentration 80% that of air, and then abruptly switched to anaerobiosis. *Tsa. pantotropha* was able to smoothly adjust to the new growth conditions, and was equally unaffected by a sudden return to aerobic conditions. *Pa. denitrificans*, however, washed out with the dilution rate during the anaerobic period and was, moreover, unable to respond to the return of the oxygen supply. This latter failure was presumably due to the accumulation of up to 14 mM nitrite during the anaerobic period. 10 mM nitrite was sufficient to give 50% inhibition of oxygen uptake by this strain. Cultures grown to steady state anaerobically did not contain these high levels of nitrite, and adjusted smoothly to the onset of aerobiosis (Robertson & Kuenen, in prep.).

Experiments currently underway at Delft involve the use of ^{15}N labelled compounds and mass-spectrometry in order to establish exactly which intermediates are formed, and under what conditions. In addition, we have now begun a new programme of research into the enzymes and cytochromes of *Tsa. pantotropha* which aims to establish the existence of the postulated bottleneck in the cytochrome chain, and to discover its identity (Robertson, van Wielink, Frank, Oltmann, Stouthamer & Kuenen, in prep.).

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