AEROBIC DENITRIFICATION AND HETEROTROPHIC NITRIFICATION IN *THIOSPHAERA PANTOTROPHA* AND OTHER BACTERIA

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AEROBIC DENITRIFICATION AND HETEROTROPHIC NITRIFICATION IN *THIOSPHAERA PANTOTROPHA* AND OTHER BACTERIA.

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Technische Universiteit Delft op gezag van de
Rector Magnificus, prof. drs. P.A. Schenck, in het
openbaar te verdedigen ten overstaan van een
commissie aangewezen door het College van
Dekanen op donderdag 10 november 1988 te 16.00 uur

door

Lesley Anna Robertson

geboren te Nairobi

STELLINGEN

1: The suggestion of Sugio et al. that Fe^{3+} serves as an electron acceptor for sulphur oxidation in *Thiobacillus ferrooxidans* is not supported by the observation of Hazeu et al. that cells grown on reduced sulphur compounds (rather than pyrite) cannot oxidize Fe^{3+}, but are able to oxidize sulphur.

Sugio et al. (1985) Appl. Env. Microbiol. 49:1401-1406.
Hazeu et al. (1987) Proc. 4th Eur. Congress Biotech. 3:497-499.

2: As it is highly unlikely that three different biomass loadings in gel beads will give identical substrate gradients in the stagnant layer surrounding the beads, as shown by Brodelius & Vandamme, the model underlying their conclusions must be oversimplified.

Brodelius & Vandamme (1987) IN *Biotechnology* 7A:405-485.

3: Although the regulation of PQQ synthesis by leaky PQQ^- mutants of *Acinetobacter calcoaceticus* appears to vary with different substrates, the linkage of PQQ with the specific apoenzyme in the periplasm is not controlled.

van Kleef & Duine (1988) Arch. Microbiol. 150:32-36.

4: In view of the different numbers of cells per unit area, models developed for biofilms in wastewater treatment systems should only cautiously be used for the interpretation of bacterial behaviour in soil systems.

5: Changes in glyoxylate cycle enzymes and increased nitrite accumulation in *Arthrobacter* sp. batch cultures entering the lag phase are probably coincidental, rather than cause and effect.

Witzel & Overbeck (1979), Arch.Microbiol. 122:137-143.

6: The use of algae in photobioreactors for wastewater treatment is probably economically unfeasible.

Kosaric & Ngcakani (1988) 8th Int. Biotech. Symp. Abs. p.232.

7: The apparently different pH optima exhibited by free and attached cells cannot be explained by the theory of Hattori & Hattori since this does not take the differing dimensions of the ionic layer and bacterial cells into account.

Hattori & Hattori (1963), Ecol.Rev. 16:63-70.

8: The increasing number of electron donors which have been identified as potentially suitable for sulphate reducing bacteria considerably increases our recognition of the number of situations in which these organisms may contribute to corrosion.

Widdel & Pfennig (1984) Bergey's Manual of Determinative Bacteriology 1:663-697.
Hansen (1988) Microbiol. Sci. 5:81-84.

9: When the contents of their paper "Oxygen inhibition of nitrate uptake is a general regulatory mechanism in nitrate respiration" are considered, it is hard to see how the authors justify the title.

Hernandez & Rowe, J.Biol.Chem. 263:7937-7939.

10: The serendipity which gives research much of its excitement is clearly illustrated by the detection of neutrinos from Supernova 1987A in tanks of ultra-pure water which had been intended (so far unsuccessfully) for the detection of decaying protons.

Talcott (1988) Astronomy 16:6-23.

11: Government financial advisors who are only interested in supporting mission-oriented research should remember that Queen Victoria and her government considered the discoveries of Michael Faraday (electricity and magnetism) as somewhat arcane laboratory curiosities.

12: Once a country has decided that abortion is permissible, it is illogical to ban, on moral grounds, the use of foetal brain tissue from abortions for transplants to relieve Parkinson's disease if it is considered moral to use kidneys, hearts, retinas etc from other cadavers.

BBC Radio News, 18.4.88.

13: His slandering of Kings Macbeth of Scotland and Richard III of England reveal William Shakespeare as a political propagandist for the Tudors.

14: Since the editors of Nature have seen fit to publish the recent claims for biological activity in "solute-free solutions", one wonders about the standard of the manuscripts they reject.

Davenas et al. (1988) Nature 333:816-818.

15: The theoretical contention that "biotechnology is only a day job" is a clear example of practical results not fitting a proposed model.

Moser (1988) Tibtech 6:207-208.

Stellingen behorend bij het proefschrift van L.A. Robertson

Ecology is physiology carried into the actual habitat;
ecology is physiology under the worst possible conditions.

T.D.Brock (1966)

for my parents

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INTRODUCTION.

Methanogenisis from waste water has become popular over the last few years as a means of reducing the environmental impact of effluent with a high organic content. However, although this substantially reduces the problems associated with carbon compounds, it does not deal with those in which reduced sulphur or nitrogen compounds are involved. A combined series of reactors which aimed to tackle all three types of problem at the same time was patented (E.P.A. 0051 888) by Gist brocades in Delft. In this combination, the effluent passed through conventional, acidogenic and methanogenic fluidized bed columns, followed by denitrifying and nitrifying reactors. The nitrate generated by nitrification was recirculated to provide electron acceptors for denitrification. A study of the performance of a laboratory-scale model of the denitrifying reactor was the subject of a recent PhD thesis (Gommers, 1987).

It is on the third, denitrifying reactor that our research has focussed. The influent normally contained equivalent amounts of reduced sulphur compounds (sulphide) and organic acids (acetate), in addition to cell lysis products from the two preceding columns. The aim of the work was to analyse the behaviour of the system, as a black box, and to see if this could be related to the constitution of the microbial community it contained. The community, as a whole, was essentially behaving like a mixotrophic population in that organic and inorganic substrates were being simultaneously utilized. Microbiological analysis, indeed, confirmed that the dominant sulphur oxidizing bacteria in the community were facultative chemolithotrophs (i.e. mixotrophs). One of the dominant mixotrophs was selected for detailed study. This isolate proved to be the first member of a new genus of colourless sulphur bacteria, and was given the name *Thiosphaera pantotropha*. It was capable of aerobic and denitrifying growth on reduced sulphur compounds, hydrogen and a wide range of organic substrates. Given the environmental conditions, the selection of mixotrophic organisms could easily be explained (see section 4). However, it was quite unexpected that *T. pantotropha* would be able to denitrify aerobically (even at dissolved oxygen concentrations >80% air saturation), and nitrify heterotrophically at a relatively high rate, compared with published rates for heterotrophic nitrifiers.

The work described in this thesis has mainly concentrated on these last two aspects of the metabolism of *Tsa. pantotropha* (i.e. aerobic denitrification and heterotrophic nitrification). The remainder of this introduction will therefore give some of the necessary background information with regard to the microbial processes involved in the nitrogen cycle (especially nitrification and denitrification). In order to facilitate an appreciation of the physiology of *Tsa. pantotropha* in relation both to the other colourless sulphur bacteria, and to wastewater treatment, they will both be briefly discussed.

1. THE NITROGEN CYCLE.

In its most inert form, nitrogen makes up most of the atmosphere. It is an essential component of many of the compounds which are necessary for life (e.g. amino acids, proteins, nucleic acids). However, despite the enormous amount of N_2 fixation which takes place, only a few highly specialized microorganisms (e.g. *Rhizobium*, *Azotobacter* and *Azospirillum* species, and many of the

phototrophic cyanobacteria) are able to extract nitrogen from the air and turn it into biologically available compounds (nitrogen fixation). Conversely, nitrogen is lost to the atmosphere by the activities of bacteria which reduce oxidized nitrogen compounds to nitrogen gas and various other volatile compounds (denitrification). Between these two extremes, a host of reactions take place including the oxidation of ammonia to nitrite and nitrate (nitrification), the reduction of these oxidized nitrogen compounds to ammonia (dissimilatory nitrate reduction) and the incorporation of the various compounds in organic molecules (assimilation). The nitrogen cycle is shown in Figure 1.

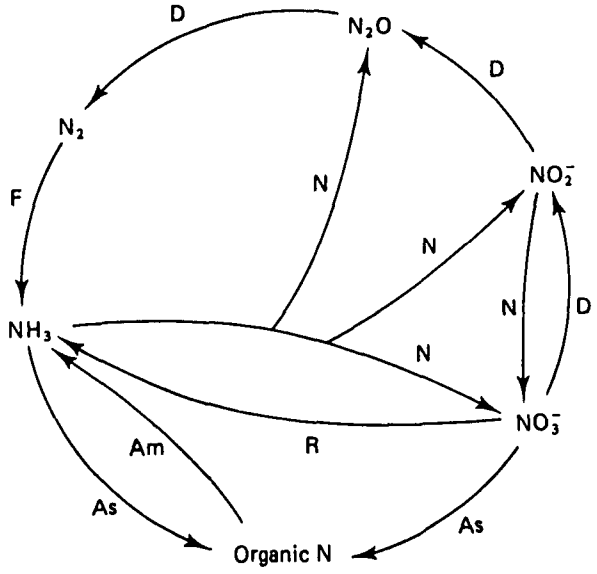


FIGURE 1. The nitrogen cycle. For simplicity, many intermediates have been omitted. As = assimilation; Am = ammonification; N = nitrification; F = Nitrogen fixation; R = dissimilatory nitrate reduction.

Like the other element cycles, the nitrogen cycle is normally in balance. However, the cycle can be deliberately or inadvertently boosted by agricultural or industrial activities, or by natural causes. This results in the undesirable accumulation of intermediates of the cycle, many of which are toxic. When the nitrogen cycle becomes unbalanced, it almost always results in ecological problems. For example, excess ammonia and the more oxidized nitrogen compounds all promote the

formation of algal blooms which, among other problems, cause oxygen depletion. Nitrite, and to some extent nitrate, can be toxic and have been implicated in various diseases including "blue baby syndrome" and cancer. Also, it has been recently shown that blooms of blue-green algae associated with nitrogen pollution produce certain toxins which can poison livestock and even people (Codd, 1984). Finally, of course, nitrogen compounds are implicated in the acid rain problem. For these reasons, the factors which affect the accumulation of nitrogen compounds in the environment are of great interest.

One of the basic requirements for understanding any element cycle is, of course, suitable methods for measuring the rates of turnover and the fluxes of the various compounds involved, together with the quantitative estimation of the contributions to the different processes of various populations as they occur in diverse ecosystems. Indeed, without quantification it is often difficult, if not impossible to interpret and extrapolate qualitative results. Habitats and communities tend to be complex and it is frequently difficult to separate phenomena being caused by different species or consortia. Additionally, environmental parameters such as pH and redox can change drastically over a very small area (e.g. in soil aggregates and sediments) and mini-cycles can exist within the more obvious macro-effects being measured. In heterogenous situations, representative sampling can be very difficult. In order to interpret results from complex situations, models based on simpler communities under well defined conditions are necessary. Laboratory studies can, of course, be well defined, rigidly controlled and highly quantitative. If care is taken with the experimental design, they may provide a considerable amount of evidence which can be of use in interpreting data from more complex situations. A certain amount of success has been achieved by various research teams in the extrapolation of results obtained with pure and simple mixed cultures of bacteria to allow the prediction of the outcome of enrichment cultures (see, for example, Gottschal & Kuenen, 1980; Kuenen et al., 1985; Mosser et al., 1973).

2: NITRATE REDUCTION.

The three main routes of nitrate reduction available to bacteria are shown in Figure 2. Although the reactions of the three pathways of nitrate reduction are superficially similar, the enzymes involved are all different, and a denitrifying species which is also using nitrate as its sole source of nitrogen will contain two different sets of nitrate and nitrite reductases (Payne, 1981).

Assimilatory nitrate reduction (1) takes place when nitrate rather than ammonia is the source of biosynthetic nitrogen. This pathway is found in eukaryotes as well as prokaryotes and, together with ammonia assimilation, is the beginning of organic nitrogen metabolism. In principle, any nitrate nitrogen which is processed via this pathway remains in the biomass.

Dissimilatory nitrate reduction (2) involves the conversion of nitrate to ammonia, and can also therefore be considered as nitrogen-conserving. It can be used by fermentative bacteria during fermentation, when it allows all of the substrate (e.g. glucose) to be converted to acetyl CoA (giving extra ATP) and serves as a means of re-oxidizing NADH. When fermentation is not possible, some bacteria can use nitrate as an alternative electron acceptor to oxygen, and the term "respiratory nitrate

reduction" has occasionally been used in connection with this pathway (Cole & Brown, 1980; Yordy & Ruoff, 1981; Payne, 1981; Stouthamer, 1988).

Both assimilatory and dissimilatory nitrate reduction have recently been reviewed (Cole, 1987).

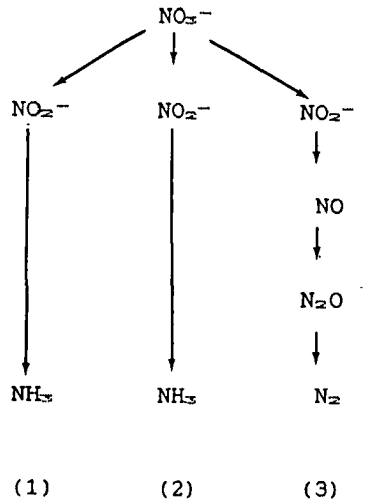


FIGURE 2. The pathways of nitrate reduction available to bacteria. From left to right, assimilatory nitrate reduction (1), dissimilatory nitrate reduction (2), denitrification (3).

Denitrification (3) may be defined as the reduction of nitrate via nitrite and nitrous oxide to nitrogen gas by bacteria using the pathway as a respiratory alternative to oxygen. Generally, oxygen respiration yields more energy than denitrification, and the yields of denitrifying bacteria are lower, rather than those obtained when the same species respires oxygen (Koike & Hattori 1975).

TABLE 1. Reactions of denitrification and dissimilatory nitrate reduction, and examples of the bacteria which use them.

Maximum possible reaction.	Species which use the reaction.
$NO_3^- \rightarrow NO_2^-$	*Thiobacillus thioparus, Lysobacter antibioticum*
$NO_3^- \rightarrow N_2O$	*Achromobacter (Corynebacterium) nephrii, Aquaspirillum itersonii*, various pseudomonads
$NO_3^- \rightarrow N_2$	*Paracoccus denitrificans, T. denitrificans Rhodopseudomonas spaeroides, Alcaligines eutropha, Hyphomicrobium, Pseudomonas* and *Halobacterium* species
$NO_2^- \rightarrow N_2$	*Neisseria* and *Flavobacterium* species
$N_2O \rightarrow N_2$	*Vibrio succinogenes*
$NO_3^- \rightarrow NH_3$	*Escherichia coli, Klebsiella pneumoniae, K. aerogenes.*

Examples of bacteria which use the denitrification and dissimilatory nitrate reduction pathways are shown in Table 1. Speculation that the denitrifying bacteria (such as *Paracoccus denitrificans*) and the eukaryotic mitochondrion are descended from a common ancestor has been fueled by the finding that members of at least one fresh-water protozoan genus, *Loxodes*, are capable of denitrification, and that the denitrifying enzymes seem to be associated with their mitochondria (Finlay et al., 1983; Finlay, 1985).

2.1. Denitrification.

It is not proposed to deal with the full history of denitrification. This was reviewed by Payne in 1981. However, various points of relevance will be considered here.

Gayon and Dupetit (1886) first introduced the term "denitrification" for a phenomenon which had, by then, been known for some years. There has since been a large number of papers published on the subject, to the extent that as long ago as 1953 Kluyver commented "It seems a somewhat risky enterprise to make bacterial nitrate reduction the subject of a contribution to a modern symposium on bacterial metabolism. Most bacteriologists will consider the subject distinctly demoded, and they are fully satisfied with their knowledge of the process." However, even 35 years later there are still many points of interest and also of controversy which have not yet been resolved. For example, the participation of nitric oxide as a free or bound intermediate in nitrate reduction to nitrogen is outside the scope of this thesis and those interested should consult other papers (e.g. Knowles, 1982; Payne, 1981; Hollocher, 1982).

Occurrence

The occurrence of denitrifying and other nitrate reducing bacteria is limited by the amount of nitrate or nitrite available, and thus, to some extent, by the activity of the nitrifiers. According to the model developed by Tiedje et al. (1982), dissimilatory nitrate reduction is the preferred route for nitrate reduction when the ratio of electron donor to electron acceptor is high. When the reverse is true, denitrification is favoured. This model appears to hold particularly well for sediments (Kuenen & Robertson, 1987).

The ubiquity of denitrifying bacteria is illustrated by the results of a study of bacteria found in agricultural soils made by Gamble et al. (1977). Denitrifiers were found to occur in all of the soil types studied, at a wide range of temperatures, pH values and moisture levels (Tables 2A and 2B). There was no clear correlation between the size of the denitrifying population and either the amount of organic material present or the number of different isolates which were found. Taxonomically, the majority of isolates fitted the description of the genus *Pseudomonas*.

Physiology and Electron Transport.

As already mentioned (Figure 2), 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, 1988), and will only be briefly discussed here.

TABLE 2A. Summary of soil types used for the survey of denitrifiers. (adapted from Gamble et al., 1977).
Abbreviations: c = clay, l = loam, m = muck, s = sand, si = silt, swp = somewhat poor, ann. = annual

	Area	Crop	Texture.	mean ann. rain (cm)	Drainage	Moisture
A	Minnesota	Corn	cl	50-100	poor	15.9
B	Conneticut	Tobacco	sl	100	well	8.7
C	Argentina	Corn	sil	50-100	swp	22.3
D	Argentina	Wheat/potato	sil	50-100	well	23.0
E	Michigan	Vegetables	m	50-100		84.5
F	Texas	Prairie	c	100-200	poor	6.55
G	Argentina	wheat/ sweet clover	c	50-100	swp	28.6
H	Brazil	Wheat	sicl	100-200	poor	24.7
I	Nigeria	Corn	sl	100-200	swp	12.75
J	Kansas	Corn (heavily manured)	sil	50-100		10.9
K	Nigeria	Rice/ corn	ls	100-200	poor	17.2
L	Columbia	Rice paddy	sicl	100-200	swp-poor	32.0
M	Philippines	Rice paddy	sicl	100-200	swp	17.5
N	Taiwan	Rice paddy	sil	100-200	swp-poor	19.6

TABLE 2B. Growth parameters and numbers of denitrifiers found in the soils shown in Table A. (adapted from Gamble et al., 1977).

	pH	mean annual temp.°C	organics (%)	Denitrifying population x 10^4	Number of isolates
A	7.2	5-10	2.34	290	34
B	5.2	10	1.45	14	5
C	5.92	15-20	3.34	140	12
D	5.69	15-20	4.08		
E	6.53	5-10	81.51	700	2
F	7.39	20-25	3.61	1.2	3
G	7.84	15-20	2.81	160	9
H	4.42	20-25	4.94	62	4
I	5.51	25-30	3.21	31	7
J	7.12	10-15	4.81	23	1
K	6.34	25-30	1.34	590	5
L	7.74	25-30	3.21		
M	6.49	25-30	3.34	220	7
N	5.09	20-25	1.20	57	4

The nitrate reductases from the denitrifying and dissimilatory nitrate reducing bacteria appear to be surprisingly similar (Stouthamer, 1988). 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

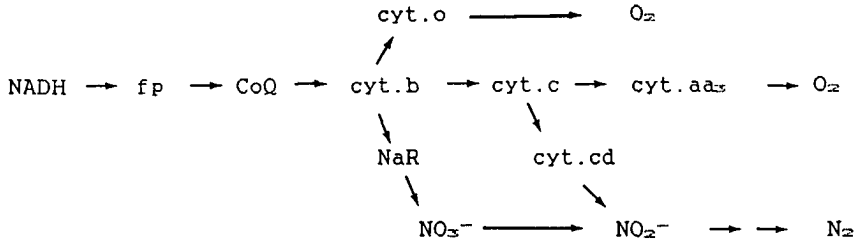
occur. The best-known is cytochrome cd which occurs in, among others, *Pa. denitrificans* (Payne, 1981; Stouthamer, 1980; 1988). Purified cytochrome cd produces a mixture of NO and N_2O. The second nitrite reductase is a soluble, copper-containing enzyme which occurs in the periplasm. It has only been 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). NO is the product of the purified enzyme. Both nitrite reductases display cytochrome oxidase activity, but have a considerably lower K_m for nitrite than for oxygen. N_2O reductase is not very stable. 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 (Stouthamer, 1988), 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.

As with oxygen respiration, denitrification proceeds via the cytochrome chain. Only a few cytochrome chains have been completely mapped (see, for example, Stouthamer 1988). Those of *Pa. denitrificans*, a mixotroph, and *T. denitrificans*, an autotroph, are shown in Figure 3 . *Tsa. pantotropha*, the species at the centre of this thesis, is mixotrophic and the cytochrome chain of *Pa. denitrificans* is thus of greatest relevance. However, because *Tsa. pantotropha* can denitrify on reduced sulphur compounds and *Pa. denitrificans* cannot, the cytochrome chain of *T. denitrificans* may also be relevant. It is believed that the electrons from thiosulphate metabolism in *Pa. denitrificans* and another mixotroph, *T. versutus*, enter the cytochrome chain at cytochrome c and would therefore not generate any energy during their passage to the denitrification system. *Tsa. pantotropha* must differ at this point. It should be remembered that the linear type of chains shown in the figures are a great simplification. In reality, the cytochromes are distributed over the cell membrane and periplasm, and their location is crucial for their function. Moreover, bacteria such as *Pa. denitrificans* are known to contain several cytochromes b and c, each presumably with a different function. In addition to the linear flow of electrons (from b to c, etc), these cytochromes may also be able to pass electrons among themselves.

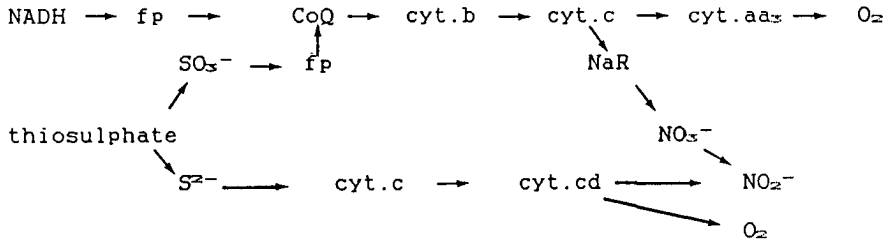
In *Pa. denitrificans* the appearance of the various cytochromes appears to be controlled by the redox levels within the cytochrome chain. For example, when the dissolved oxygen concentrations are high, cytochrome aa_3 is the dominant terminal oxidase. As the dissolved oxygen falls (or during growth on very rich media), cytochrome o becomes more important, and as the dissolved oxygen concentration approaches 0, cytochrome cd is synthesized (Sapshead & Wimpenny, 1972). As will be discussed below, because of results obtained with a few species, it was considered for many years that bacteria either used 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).



Paracoccus denitrificans



Thiobacillus denitrificans

FIGURE 3. Cytochrome chains from different bacteria (adapted from Stouthamer, 1980).

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, 1988) 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, 1988) and therefore lack of access

cannot provide the full explanation. Nitrite has been reported to inhibit oxygen uptake, thus altering the redox level of the cytochrome chain (Kučera & Dadák, 1983; Kučera et al., 1984). Should nitrite enter the cell, the reverse effect, with oxygen uptake being inhibited sufficiently to permit electron flow to the denitrification enzymes, might be predicted.

Aerobic denitrification

Bréal (1892) began another controversy when, in a paper on the denitrifying ability of straw-based cultures, he reported that nitrate reduction continued even when the culture vessels were provided with oxygen. This claim was received with disbelief, and the ensuing prejudice against aerobic denitrification has lasted for almost 100 years. It is to the arguments surrounding the occurrence of aerobic denitrification that the focus of the next part of this introduction will be directed.

Despite fairly frequent reports of bacteria able to denitrify in the presence of measurable amounts of oxygen, it has often been stated that *denitrification occurs only in anaerobic* conditions (e.g. Christensen & Tiedje, 1988), and that the presence of oxygen inhibits the activity of the denitrifying enzymes and suppresses their synthesis (see for example Bryan, 1981). This generalization is based on studies with pure cultures of a few well known organisms.

In the early days of the work on denitrification, one line of research was based on the view that the sole function of nitrate reduction was to provide "oxygen" for use in respiration (see for example, Cranston & Lloyd, 1930, Lloyd, 1936). If this was correct, it would be logical that in the presence of more readily accessible oxygen, there would be no requirement for nitrate reduction. It is now known that nitrate, nitrite and the other oxides of nitrogen serve as electron acceptors in their own right, and that the respiratory pathways to oxygen or to the nitrogen oxides are to some extent separate (see reviews such as Stouthamer, 1980; Payne, 1981; Knowles, 1982; Stouthamer, 1988) Some species of bacteria are known to have denitrification systems which are very sensitive to the presence of oxygen, and the reasons for this inhibition are not yet completely clear. It is not practicable to deal with all of the papers demonstrating oxygen inhibition, but to provide a balanced view a few will be mentioned.

Jannasch (1960) used a very simple experiment to demonstrate that the denitrifying enzymes of *Ps. stutzeri* are inactivated on exposure to oxygen. In a mixed culture of *Ps. stutzeri* and a *Chlorella* species, nitrate disappeared as long as the culture was incubated in the dark. However, if the culture vessel was illuminated, denitrification stopped immediately. As the denitrification in a similar vessel containing a pure culture of *Ps. stutzeri* was unaffected by light, it was concluded that the oxygen generated during photosynthesis was inhibiting nitrate reduction by the pseudomonad.

Using electrodes to measure oxygen, nitrate and nitrite, John (1977) showed that *Pa. denitrificans* and *E. coli* both ceased reducing nitrate immediately they were supplied with oxygen, and began again as soon as the oxygen was depleted.

It has been shown that the different enzymes of the denitrification pathway in *Pa. halodenitrificans* respond to different concentrations of oxygen. Hochstein et al. (1984) grew this species to steady state in anaerobic chemostats, and then

established steady states at different dissolved oxygen concentrations. They found that although with 5% oxygen in the influent gas there was no detectable dissolved oxygen in the culture, production of dinitrogen had been almost completely replaced by the production of N_2O. By the time the dissolved oxygen level became measurable (1 nmol/litre with an influent gas concentration of 7.5% oxygen), the products of nitrate reduction were nitrite and N_2O in approximately equal parts. When the culture was supplied with air, only a small amount of nitrite was produced, and the remainder of the nitrate was untouched.

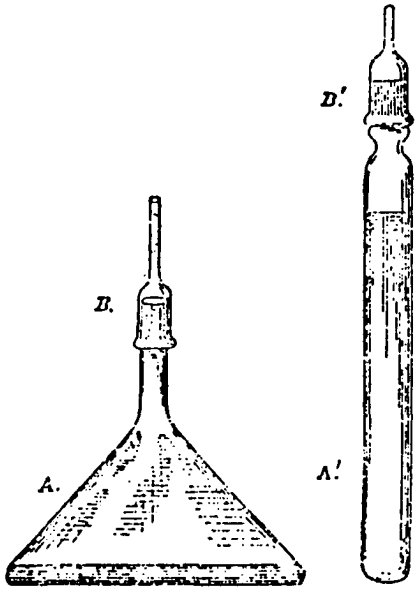


FIGURE 4. Examples of culture vessels used to produce "aerobic" and "anaerobic" conditions in early denitrification experiments (from Gayon & Dupetit, 1886).

Schulp & Stouthamer (1970) showed the gradual inactivation of nitrate reductase in anaerobically grown cells of *Bacillus licheniformis* (a dissimilatory nitrate reducer) when these were incubated in a buffer, on a shaker, in the presence of air. A similar culture which was not shaken did not loose its activity. Growing cultures began nitrate reduction after the dissolved oxygen in the medium had fallen to below 20% of air saturation, but cells grown at higher concentrations of oxygen did not reduce nitrate, and did not contain nitrate reductase. Similarly, cultures of *Thiobacillus denitrificans* growing on thiosulphate in a chemostat did not reduce nitrate while oxygen was present in the cultures, and cultures maintained at various levels of dissolved oxygen required an induction period, the length of which was related to the amount of oxygen present in the growth medium (5 hours at 88% of air saturation and 1 hour at 30%)

before they were able to reduce nitrite (Justin & Kelly, 1978). Finally, Alefounder et al. (1981) used a nitrate electrode and H_2O_2 to show that nitrate reduction by *Pa. denitrificans* stopped immediately oxygen was added and resumed only when the available oxygen was exhausted.

Many of the early reports of "aerobic denitrification" were dismissed, probably with reason, as examples of poor aeration or poor experimental technique. Most anaerobic experiments were done in completely filled, glass-stoppered bottles or by the use of alkaline pyrogallol (which can give off CO). "Aerobic" samples, including those of Bréal in the paper previously referred to, were frequently just exposed to the air and not shaken or sparged with air. Typical culture vessels are shown in Figure 4. Such procedures clearly resulted in an oxygen gradient in the culture, and it is thus likely that most of the observed denitrification occurred in the anaerobic areas of the medium. The dangers of relying on estimates of the degree of aerobiosis can be seen in Table 3. The cultures used in experiment A were described as having had "moderate aeration". These cultures involved 100 ml amounts of medium in 300 ml conical flasks which were incubated with a current of air passing over the surface of the liquid, but without shaking. The results in experiment B were obtained with cultures described as having had "excessive aeration". In this case, 50 ml. amounts of medium were used in 300 ml conical flasks which were incubated on a shaker with air flowing over the surface of the liquid at twice the rate of flow used in the previous cultures.

TABLE 3. The results presented by Verhoeven (1956) to show the effect of different degrees of aeration on denitrification by different strains of bacteria.

	N as % initial NO_3^- nitrogen					
	NO_3^- start	NO_3^- end	NO_2^-	protein	NH_4^+	gas products
A						
B. megaterium	100	95.7	traces	4.3	0	0
M. denitrificans	100	0	traces	14.8	0	85.2
Ps. aeruginosa	100	0	traces	10.6	0	89.2
B. licheniformis	100	0	traces	15.7	89.4	0
B. licheniformis	100	33.8	4.5	10.9	54.8	0
B						
B. megaterium	100	93.6	0	5.9	0	0
M. denitrificans	100	72.3	0.2	16.6	4.2	6.9
Ps. aeruginosa	100	72.6	0.2	14.5	9.6	3.1
B. licheniformis	100	72.8	0.4	19.7	0.4	6.6
B. licheniformis	100	87.0	0.4	8.5	0.7	3.4

The degree of denitrification or dissimilatory nitrate reduction varied both with the experimental method and with the organism used. It should be noted that even in the cultures which received "excessive aeration", *Micrococcus* (now *Paracoccus*) *denitrificans* appeared to retain at least some of its denitrifying ability. This organism has now been established as having a denitrification system which is extremely sensitive to oxygen (John, 1977; Alefounder et al., 1981). The degree of aerobiosis within a culture is dependent on the rate of oxygen transfer from the gas to the liquid phase (determined by the

oxygen transfer coefficient) as counteracted by the rate of oxygen uptake by the organisms. Thus, growth conditions sufficient to provide aerobiosis in one shaken culture may be inadequate for another, faster growing species. The results shown in Table 3 emphasize that dissolved oxygen measurements are essential in experiments of this type.

TABLE 4. Summary of the experimental conditions described by Collins (1955).

vessel number	shape	vol. (ml)	liquid vol. (ml)	%filled
1	straight-sided bottle	1000	200	20
2	conical	1000	200	20
3	conical	500	200	40
4	round-bottomed	500	200	40
5	round-bottomed	490	200	41
6	round, anaerobic	500	200	40

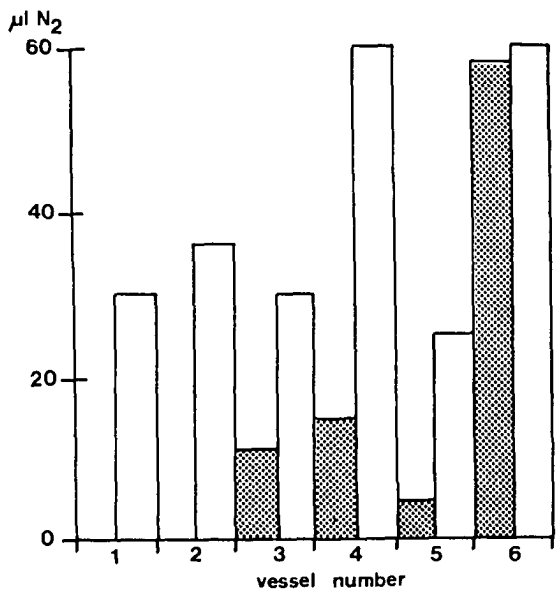


FIGURE 5. The amount of gas produced by cells using yeast autolysate and nitrate in Warburg manometers after having been grown under the conditions described in Table 4, washed and resuspended in phosphate buffer. Open bars, *Ps. aeruginosa* NCTC 6750; shaded bars, *Ps. aeruginosa* T8. N_2 production as µl N_2 h^{-1} mg^{-1} wet weight of cells (data from Collins, 1955).

A paper (Collins, 1955) which is frequently quoted as showing that the shape of the culture vessel can determine the efficiency of aeration in a culture is another case where the results cannot be fully interpreted because the dissolved oxygen concentration is not known. Various strains and species of

denitrifying bacteria were grown on a shaker in culture vessels of different shapes and sizes (summarized in Table 4). The denitrifying ability of the resulting biomass was then compared with that of a similar culture grown anaerobically. The results obtained with two strains of *Ps. aeruginosa* are shown in Figure 5.

Since *Ps. aeruginosa* NCTC 6750, and some of the other organisms tested had no denitrifying ability when cultured in the bottle and 1000ml conical flask, it was assumed that the shape of the vessel was the determining factor. However, for efficient oxygen transfer, the surface to volume ratio of the liquid is at least as important as the mixing efficiency. As all of the vessels received the same volume of liquid (200 ml) it appears that the ratio between the volume of the vessel and the volume of the culture liquid was of importance in these experiments. Unfortunately, a 1000 ml round-bottomed flask was not included in the study. That *Ps. aeruginosa* strain T8 retained about half of its denitrifying ability under all conditions further demonstrates the variability of the response of different species to exposure to air.

Despite their lack of dissolved oxygen measurements, a few early reports include sufficiently clear experimental descriptions to allow the results to be viewed with a certain degree of confidence. One such is the report by Meiklejohn (1940) in which the effect of various environmental effects on denitrification by two *Pseudomonas* species was measured. Table 5 summarizes one of the experiments.

TABLE 5. The fate of nitrate supplied to a *Pseudomonas* species under different degrees of aeration as found by Mieklejohn (1940).

Culture conditions	Time (days)	Total N mg/L	Protein N mg/L
2 cm deep culture in cotton plugged conical flask.	0	237	–
	7	53	45
2 cm deep culture in cotton plugged conical flask with continuous air sparging.	0	235	–
	7	66	66
anaerobic culture incubated in the presence of alkaline pyrogallol.	0	222	–
	7	33	24

It can be seen that, under all three growth conditions, virtually all of the nitrate not assimilated by the cells had disappeared from the medium. Moreover, as denitrification provides less energy than oxygen respiration (Koike & Hattori, 1975), the increase in yield with the increasing supply of air to the culture could be expected if the cells were altering the balance between oxygen and nitrate respiration according to oxygen availability. In contrast to Meiklejohn, Marshall et al. (1953) supplied both ammonium and nitrate to their cultures. By using the N^{15} form of each nitrogen compound in turn, they attempted to trace the fate of the ammonia and nitrate nitrogen

atoms in sparged and unsparged cultures grown in shallow (25 mm) layers in conical vessels with cotton plugs. They found that when the N^{15} was supplied in the ammonium, its disappearance from the medium was matched by its appearance in the biomass. However, if the N^{15} was incorporated in the nitrate, it did not appear in the biomass while ammonium was present, but 97% of the nitrate disappeared from the medium. In common with the results of Meiklejohn, the biomass yield was higher in the sparged culture. Although, because of the lack of dissolved oxygen measurements, neither of these papers can be taken as evidence for fully aerobic denitrification, they certainly indicate that denitrification can proceed in cultures which are well-mixed, and which are being continuously supplied with air.

Even now, 30 years after these studies were done, and despite the considerable improvements in the equipment available, investigations into the effect of oxygen on denitrification in which only the oxygen level in the gaseous phase and not in solution are still being published (see, for example Nakajima et al., 1984). This is especially undesirable when a rich medium, in which bacterial oxygen uptake can outstrip oxygen transfer from the gas to the liquid phases, is used without shaking or sparging (see, for example, Watahiki et al., 1983).

Some researchers tried to investigate oxygen inhibition of denitrification by measuring the redox potential in their cultures. Korochinka (1936) found that *Ps. denitrofluorescens* (now *Ps. fluorescens*) continued to denitrify at rH values of 24-25, but that the rate was reduced at a rH value of 35. She concluded that the presence of air was not sufficient to stop denitrification. Kefauver & Allison (1956) used a complex apparatus in an attempt to ensure good aeration. Although, they did not measure dissolved oxygen, they did measure redox, and found, as did Elema (1932), a drop in the redox of the medium with the onset of nitrite reduction. However, during experiments in which they controlled the Eh in suspensions of resting cells at values between +100 and + 350 mv, they still obtained nitrite reduction. They concluded that the redox of the medium was not a controlling factor, and that their test species could use oxygen and nitrate simultaneously when less than 6% oxygen was present in the influent gas. More recently, Ottow & Fabig (1983) established that the rH level in cultures of *Moraxella* sp. and *Acinetobacter* sp. was related to the metabolic activity of the culture rather than the degree of anaerobiosis. Experiments relying on redox measurements in the growth medium will therefore not yield information regarding denitrification in the presence of measureable amounts of oxygen.

Skerman et al. (1951) appear to have been the first to include the measurement of dissolved oxygen in their investigation of the effect of oxygen on denitrification. Some of their results are shown in Figure 6. They varied the amount of oxygen supplied to their batch culture of a *Pseudomonas* sp. by varying the stirring rate, and measured growth, oxygen, nitrate and nitrite. Nitrite appeared in the culture before the dissolved oxygen concentration reached 0, and in at least one case (see Figure 6b), significant amounts of nitrate had been reduced before the dissolved oxygen became depleted. The increase in the level of dissolved oxygen present (Figure 6b) as growth stopped serves as a reminder that oxygen was being supplied to these cultures continuously, and that oxygen and nitrate reduction must have been taking place simultaneously. Another indication of this is the increasing yield of cells as the ratio of oxygen to

nitrate used increased, with the highest yield being obtained in the culture with the highest stirring rate, when little or no nitrate was reduced.

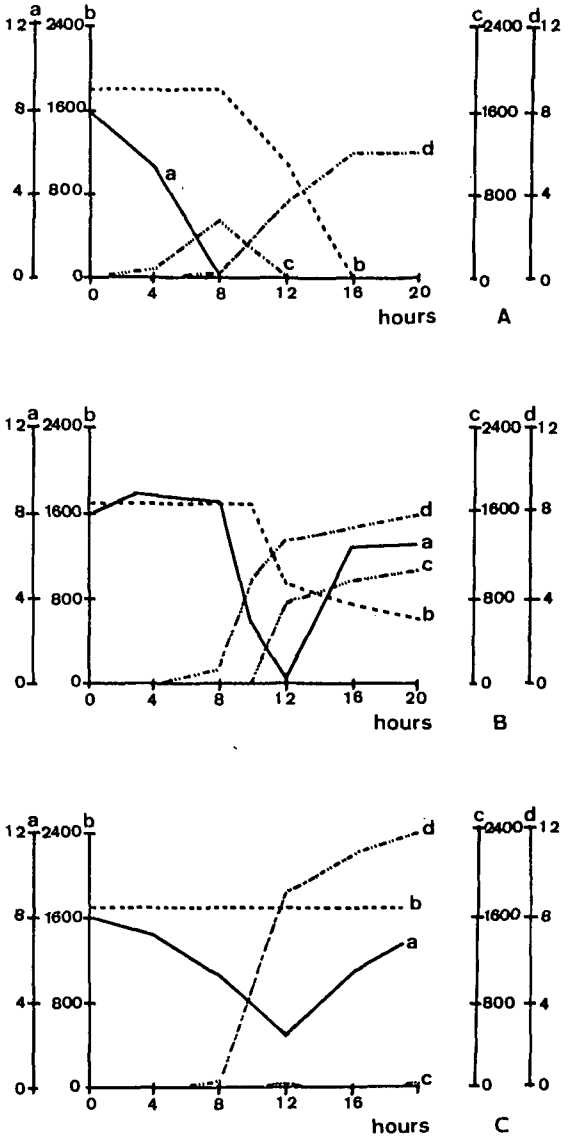


FIGURE 6. Results of experiments using an oxygen electrode to measure the respiratory activity of a *Pseudomonas* species at different stirring rates in peptone yeast broth at 25 C in the presence of nitrate. Curve a = dissolved oxygen (ppm); curve b = nitrate present (ppm); curve c = nitrite present (ppm); curve d = cells numbers ($1X10^9$). A = Stirring rate 100 rpm; B = Stirring rate = 300 rpm; C = 500 rpm. (data from Skerman et al., 1951).

A second example of denitrification in the presence of low, but significant concentrations of dissolved oxygen was reported by Meiberg et al. (1980). They found that with *Hyphomicrobium* X in chemostat cultures growing on dimethylamine, the induction of the denitrifying enzymes required the presence of nitrate, and also appeared to be linked to the growth rate. At a dilution rate of 0.05 h^{-1}, the "threshold concentration" of dissolved oxygen below which nitrate reductase was induced was about 28% of air saturation. At a dilution rate of 0.1 h^{-1}, this "threshold concentration" had fallen to about 9% and at μ_{max} (0.18 h^{-1}) the enzymes were only induced if the culture became virtually anaerobic. This derepression of the denitrifying enzymes at low growth rates may be ecologically significant with respect to soil denitrification.

Aquaspirillum magnetotacticum (Bazylinski & Blakemore, 1983a) presents an interesting example of denitrification in the presence of low concentrations of oxygen. This is a microaerophillic species with an absolute requirement for O_2. It does not grow anaerobically. However, cells grown in the presence of nitrate with between 0.2 to 1% oxygen (initial headspace concentration) reduced the nitrate to nitrogen with a transient appearance of N_2O, while at the same time consuming oxygen. Furthermore, if acetylene was included, nitrogen production was replaced by the generation of N_2O, a common phenomenon among denitrifying species (Payne, 1981). Cells cultured with ammonia, only, did not produce any of the nitrogen oxides. It is clear that *A. magnetotacticum* is capable of true denitrification, despite its requirement for oxygen. It is, perhaps of interest to note that growing cells of *A. magnetotacticum* are also capable of fixing atmospheric nitrogen (Bazylinski & Blakemore, 1983b).

The examples given so far have involved denitrification in the presence of reduced amounts of dissolved oxygen. However, at least two species have been shown to denitrify in the presence of much higher concentrations of oxygen.

TABLE 6. Nitrate reduction and oxygen uptake by an *Alcaligenes* sp. In all cases the initial dissolved oxygen concentration was 5 mg/litre (data from Krul, 1976). redn. = reduction.

Growth conditions	O_2 uptake nmol/min/mg protein	NO_3^- redn. O_2 present nmol/min/mg protein	NO_3^- redn. O_2 exhausted nmol/min/mg protein
1: aerobic ammonium as nitrogen source	146	6	43
2: anaerobic nitrate present	151	15	90
3: cells from 2 aerated for 24 hours in N-free medium	81	6	39

Krul (1976) found that both aerobically and anaerobically grown cells of an unidentified species of *Alcaligenes* were able to reduce nitrate and oxygen at the same time. The reduction rates he obtained, by using oxygen and nitrate electrodes together to measure the respiratory activity of cells supplied with glycerol in a closed, thermostatically controlled cell, are summarized in Table 6. The rate of nitrate reduction occurring at the same time as oxygen uptake was much lower than when all of the the oxygen had been utilized. Nitrate reductase is presumably constitutive in these cells since even the cells grown aerobically in the absence of nitrate were able to reduce nitrate immediately.

In a subsequent experiment, Krul & Veeningen (1977) found that nitrate had no effect on the growth rate or final optical density of aerobic cultures grown in the presence of ammonium as nitrogen source. Additionally, cells grown aerobically (dissolved oxygen concentrations 53% and 200% of air saturation) in the presence of nitrate did not contain more nitrate reductase than similar cultures grown without nitrate. Figure 7 shows the rates of oxygen uptake and anaerobic nitrate reduction obtained with cultures of this organism grown at different dissolved oxygen concentrations. It can be seen that even the cells grow at dissolved oxygen concentrations twice air saturation still contained 20% of their anaerobic nitrate reducing capacity.

It is clear, from these examples, that aerobic denitrification merits further study before its existence is dismissed.

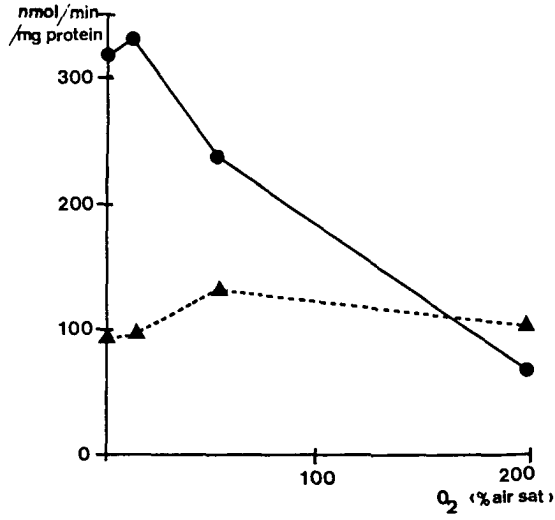


FIGURE 7. The effect of the dissolved oxygen concentration on the rate of oxygen uptake and nitrate reduction by *Alcaligines* strain 15 as measured using oxygen and nitrate electrodes. Circles = oxygen uptake, triangles = nitrate reduction (data from Krul & Veeningen, 1977).

3: NITRIFICATION.

Nitrification may be defined as the oxidation of reduced nitrogen compounds. Although Pasteur (1862) first suggested that nitrification in soil was probably biological, it was not experimentally identified as a biological process until the work of Schloesing and Muntz (1877a; 1877b). The earliest claim for the isolation of nitrifying bacteria was made by Heraeus (1886) but other workers were unable to repeat his work. There were other claims, but it is the work of Winogradsky (1890a, 1890b, 1890c) which is generally regarded as providing the foundation for the modern study of nitrification. This very early work was reviewed by Nelson (1929), Kingma-Boltjes (1934) and MacDonald (1986).

TABLE 7. Examples of autotrophic nitrifying bacteria.

Ammonia to nitrite.	Nitrite to nitrate.
Nitrosomonas europaea	*Nitrobacter winogradskyi*
Nitrosocystis javanensis	*Nitrobacter agilis*
Nitrospira briensis	*Nitrococcus mobilis*
Nitrosolobus multiformis	*Nitrospina gracilis*

The autotrophic nitrifying bacteria can be divided into two groups. Those in one group obtain energy for growth from the oxidation of ammonia to nitrite, and the others oxidize nitrite to nitrate. In both cases, CO_2 serves as the carbon source. These groups are summarized in Table 7. The physiology of the autotrophic nitrifying bacteria has been relatively well studied (see, for example, reviews by Hooper, 1984; Wood, 1986; Bock et al., 1986), but one subject which remains a matter of controversy is their sensitivity or insensitivity to organic compounds. It has been claimed that even small amounts of organic chemicals are sufficient to inhibit ammonia oxidation, and indeed it has been shown that organic solvents such as acetone or ethanol which are used to dissolve the nitrification inhibitor, N-serve, have almost as much effect without the inhibitor (Hall, 1984). However, extensive studies have shown that low concentrations of organic compounds do not inhibit nitrification, although higher concentrations of compounds such as organic acids may indeed be inhibitory. The effect of inhibitors on nitrification has been extensively reviewed by Sharma & Ahlert (1977) and Painter (1986).

The bacteria and fungi known as the heterotrophic nitrifiers are only able to oxidize reduced nitrogen compounds when an external energy supply (e.g. acetate) is available. Examples of their potential substrates and products are shown in Table 8. The existence and ecological significance of this phenomenon has been a subject of controversy since the time of Winogradsky. Doubts expressed about the purity of cultures led Kingma-Boltjes (1934) to dismiss the description of a heterotrophic nitrifier by Stutzer and Hartleb (1894) as valueless. In some ways the controversy over heterotrophic nitrification resembles that over "aerobic denitrification" in the poor documentation of experimental methods and occasional spurious claims (perhaps because of a mixed culture) which over-shadows the early literature and makes it very difficult to assess the validity of results. Kingma-Boltjes (1934) extensively reviewed the published

work on heterotrophic nitrification and concluded that the only authentic nitrifiers which had been isolated up until the time of his writing were the obligate autotrophs isolated by Winogradsky.

The more modern reviews such as those by Verstraete (1975) and Killham (1986) recognise not only the existence of heterotrophic nitrification, but also the surprisingly heterogenous group of prokaryotes and eukaryotes which are involved. Representatives of this group are shown in Table 8, but for an exhaustive list, the reader is referred to Verstraete (1975). As heterotrophic nitrification does not appear to yield energy for growth, these organisms must have other reasons for carrying out the reactions.

For a long time, it was considered that as the apparent rates of nitrification by heterotrophs appeared to be very low compared with those of the autotrophs, the phenomenon was of little significance outside the laboratory. However, some researchers have claimed that in certain types of soil (e.g. very acid soils) where autotrophic nitrifiers were unable to grow, the heterotrophs were responsible for the bulk of nitrification taking place (for example, Strayer et al., 1981). Additionally, many common soil denitrifiers have been shown to be heterotrophic nitrifiers (Castignetti & Hollocher, 1984). Table 9 shows the nitrite accumulation reported for batch cultures of some of these organisms when grown on pyruvic oxime and hydroxylamine, both of which are likely to occur in soil during the breakdown of plant material.

TABLE 8. Examples of prokaryotic and eukaryotic heterotrophs which nitrify (data mainly from Verstraete, 1975).
comps. = compounds. a. = acids

Species	Substrate	Product
Arthrobacter globiformis	ammonium	hydroxylamine
Aspergillus flavus	ammonium	monohydroxamic a.
Streptomyces sp.	ammonium	monohydroxamic a.
Mycobacterium phlei	ammonium	dihydroxamic a.
Aerobacter aerogenes	ammonium	dihydroxamic a.
Rhodotorula sp.	ammonium	dihydroxamic a.
Ustilago sphaerogena	ammonium	trihydroxamic a.
Neurospora crassa	ammonium	trihydroxamic a.
Streptomyces griseus	ammonium	trihydroxamic a.
Thiosphaera pantotropha	ammonium	nitrite
Proteus sp.	hydroxylamine	nitrite
Alcaligenes sp.	oximes	nitrite
Pseudomonas aeruginosa	aliphatic nitro comps.	nitrite
Flavobacterium sp.	aromatic nitro comps.	nitrite
Nocardia sp.	aromatic nitro comps.	nitrite
Chlorella sp.	ammonium	nitrate
Aspergillus parasiticus	ammonium	nitrate
Aspergillus wentii	nitrite	nitrate
Aspergillus flavus	aliphatic nitro comps.	nitrate
Pseudomonas sp.	aromatic nitro comps.	nitrate

Physiology and electron transport.

The enzymology of autotrophic ammonia and nitrite oxidation has recently been reviewed (Bock et al., 1986; Wood, 1986), and only a brief overview will be given here.

The autotrophic nitrifiers oxidize ammonia via hydroxylamine to nitrite. Ammonia oxidation is carried out by means of an

ammonia monooxygenase which probably reacts with NH_3 rather than NH_4^+ (Suzuki et al., 1974). Experiments with stable isotopes have shown that the oxygen comes from molecular oxygen rather than water (Dua et al., 1979). The reaction is energetically unfavourable, and it is the oxidation of hydroxylamine to nitrite by means of hydroxylamine oxidoreductase which actually generates energy for growth. In addition to the usual production of nitrite, hydroxylamine oxidoreductase can also form N_2O for hydroxylamine and nitrite (Hooper, 1984). Nitrite oxidation proceeds to nitrate by means of nitrite oxidoreductase without any detectable intermediates (Bock et al., 1986). The oxygen involved has been shown to come from water.

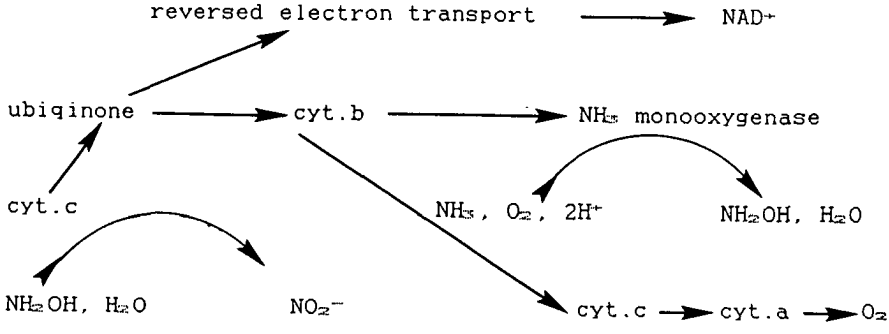


FIGURE 8. The cytochrome chain of *Nitrosomonas* (adapted from Wood, 1986).

The biochemistry of heterotrophic nitrification has been studied by several research teams (e.g. Doxtader, 1965; Aleem, 1975) and there is evidence that at least two different (organic and inorganic) pathways exist (Killham, 1986). It seems likely that most of the fungi use an organic route as follows:

$$RNH_2 \longrightarrow RNHOH \longrightarrow R\text{-}NO \longrightarrow RNO_2 \longrightarrow NO_3^-$$

However, evidence for an inorganic pathway has also been found (e.g. Aleem, 1975):

$$NH_4^+ \longrightarrow NH_2OH \longrightarrow NOH \longrightarrow NO_2^- \longrightarrow NO_3^-$$

It is, of course, also possible that some organisms use combinations of these pathways, as proposed by Verstraete (1975).

The linkage of the nitrification reactions to the cytochrome chain in *Nitrosomonas* is shown in Figure 8. The terminal oxidases

appear to be cytochrome a_1 in *N. europaea* (Erikson & Hooper, 1972; Yamazaki et al., 1985) and cytochrome aa_3 in *Nitrobacter agilis* (Yamanaka et al., 1981). Cytochromes o and a_1 have also been found in *Nitrobacter* (Wood, 1986). NADH is generated by reversed electron flow in which the proton motive force drives electrons "backwards" along the cytochrome chain, generating NAD^+.

Effect of oxygen concentration on nitrification

It is generally accepted that nitrification does not proceed efficiently at low dissolved oxygen concentrations, but the reports of the actual concentrations are variable. Values for K_m (the dissolved oxygen concentration at which the specific growth rate is half that obtained when oxygen is not limiting) vary from 0.5 µM to 6.25 µM (Winkler, 1981). This variability can be attributed to many factors including oxygen diffusion through biolayers, competition for oxygen by other organisms and various physical parameters. Strain or even species differences among the nitrifiers present may also be a factor. Gundersen et al. (1966) showed that *Nitrosomonas oceanus* lost 25% of its nitrifying capacity when the oxygen concentration of cultivation was reduced from 100% to about 10% of air saturation. However, under the same conditions *Nitrosomonas europaea* greatly increased its nitrification rate. The amount of nitrite oxidized by *Nitrobacter agilis* dropped by 50% when cultivated at 10% rather than 100% air saturation. As has already been pointed out, some heterotrophic nitrifiers nitrify most efficiently at low dissolved oxygen concentrations, and this might indicate a possible niche for them.

TABLE 9. Nitrite production from hydroxylamine and pyruvic oxime by resting cells of common denitrifying bacteria which had been aerobically grown on pyruvic oxime and yeast extract (from Castignetti & Hollocher, 1984).

Organism	Rate of nitrite accumulation (as nmol NO_2^- $min^{-1}mg$ $protein^{-1}$)	
	from pyruvic oxime	from hydroxylamine
Alcaligenes faecalis	8	0
Chromobacterium violaceum	0	1
Flavobacterium sp.	0	7
Paracoccus denitrificans	0	0
Pseudomonas aeruginosa	1	6
Pseudomonas aureofaciens	2	108
"*Pseudomonas denitrificans*"	1	31
Pseudomonas fluorescens 400	1	41
Pseudomonas fluorescens 401	0	0
Pseudomonas fluorescens 402	2	6
Pseudomonas stutzeri	1	1

4: OXIDATION OF REDUCED SULPHUR COMPOUNDS.

As already mentioned, both *Tsa. pantotropha* and the microbial community from which it was isolated were oxidizing reduced sulphur compounds. A brief consideration of the various

physiological types of sulphur bacteria, and the factors which influence their selection is therefore appropriate at this stage. Sulphide, thiosulphate and sulphur can all be oxidized by bacteria. In most cases the oxidation serves as a source of energy for growth, and the end product is usually sulphate, although sulphur or tetrathionate accumulates in some cultures.

The sulphur oxidizing bacteria are readily separated into two groups by the fact that many species contain bacteriochlorophyll and utilize light for photosynthesis. These phototrophic species are not relevant to this work, and will not be discussed further. Their physiology and behaviour have been reviewed (Trüper & Fisher, 1982; van Gemerden, 1983; Kuenen et al, 1985; Kelly & Kuenen, 1984). Other species, known as the colourless sulphur bacteria, are not phototrophic and are dependent on chemical oxidations for their energetic and biosynthetic requirements. Table 10 defines these physiological types, but does not include the sulphur-oxidizing heterotrophs which do not derive energy from the oxidation. The different physiological groups will be discussed in detail below.

TABLE 10. Definition of metabolic types of colourless sulphur bacteria (from Kuenen et al, 1985).

	Energy Source		Carbon source	
	Reduced sulphur compounds	Organic compounds	CO_2	Organic compounds
obligate autotroph	+	−	+	−
facultative autotroph	+	+	+	+
chemolithoheterotroph	+	+	−	+

Table 11 summarizes the subdivisions among the colourless sulphur bacteria, together with examples of the physiological types which occur within those groups.

The obligate chemolithotrophs.

The physiological types represented within the colourless sulphur bacteria (Table 11) include obligately chemolithoautotrophic bacteria. These highly specialized bacteria are dependent on an inorganic source of energy and obtain their cell carbon from the fixation of carbon dioxide by means of the Calvin cycle (Schlegel, 1981). The citric acid cycle in these bacteria is incomplete, and its enzymes serve a purely biosynthetic function (Beudeker, 1981). It has been shown that many of these species can use small amounts of exogenous carbon compounds as a supplementary carbon source (Matin, 1978), or can ferment endogenous storage compounds such as glycogen (Beudeker et al, 1981 ; Kuenen & Beudeker, 1982), but these are both secondary metabolic activities and these organisms are primarily dependent on autotrophic growth. Many of the *Thiobacilli*, and all of the known species of *Thiomicrospira*, fall into this group.

The facultative chemolithotrophs.

A second group of colourless sulphur bacteria is made up by the facultative chemolithotrophs. These bacteria can grow chemolithoautotrophically with an inorganic energy source and carbon dioxide, heterotrophically with complex organic compounds

providing both carbon and energy, and mixotrophically. Mixotrophy is the simultaneous use of two or more different metabolic pathways for energy and carbon (Gottschal & Kuenen, 1980). It occurs during continuous culture on limiting mixtures of substrates. The usual view of mixotrophy involves autotrophic and heterotrophic growth (e.g. on thiosulphate and acetate), but the simultaneous use of any mixture of substrates which in batch culture might produce diauxy (e.g. glucose and lactose, succinate and glucose), or which require separate metabolic pathways (e.g. iron and sulphur, hydrogen and sulphide, acetate and lactate) could be considered as mixotrophy.

Some of the *Thiobacilli*, *Paracoccus denitrificans* (Friedrich & Mitrenga, 1981) and certain *Beggiatoa* species (Nelson & Jannasch, 1983) are typical examples of organisms able to grow on mixtures of reduced sulphur compounds and organic substrates.

TABLE 11. The sub-divisions among the colourless sulphur oxidizing bacteria (from Kuenen et al, 1985). a = acidophilic; b = microaerophilic; c = thermophilic; d = only denitrifies heterotrophically.

Metabolic Definition	Genera Involved	Examples of species in the group	Respiratory Type
Obligate chemolithotroph	*Thiobacillus*	*T. neapolitanus*	O_2
	Thiomicrospira	*T. ferrooxidans*[a]	O_2
		T. denitrificans	O_2/NO_3^-
		Tms. denitrificans[b]	O_2/NO_3^-
Facultative chemolithotroph	*Thiobacillus*	*T. intermedius*	O_2
	Sulfolobus	*Beggiatoa* sp.[b]	O_2
	Thermothrix	*T. acidophilus*[a]	O_2
	Paracoccus	*S. acidocaldarius*[a,b]	O_2
	Thiosphaera	*T. versutus*[d]	O_2/NO_3^-
		Tsa. pantotropha	O_2/NO_3^-
Chemolithotroph	*Thiobacillus*	*T. perometabolis*	O_2
	Pseudomonas	*Thiobacillus* Q	O_2
Heterotroph	*Beggiatoa*	*Beggiatoa* sp.	O_2/S^0

The chemolithoheterotrophs.

This is a little known group of bacteria which is characterized by an ability to generate energy from the oxidation of reduced sulphur compounds, but which cannot fix carbon dioxide. Until recently, *Thiobacillus perometabolis* was considered to be a member of this group, but recent reports have shown that under certain conditions it can grow autotrophically (Katayama-Fujimura et al, 1984). However, unnamed species which seem to lack a Calvin cycle but do obtain energy from the oxidation of reduced sulphur compounds do exist, and at least one strain of *Thiobacillus* has been well characterized (Gommers & Kuenen, 1988; Tuttle et al, 1974). Some *Beggiatoa* strains may also belong in this group (Larkin & Strohl, 1983).

The sulphur-oxidizing heterotrophs

Some heterotrophic bacteria can oxidize reduced sulphur compounds, but do not derive energy from them. However, one possible benefit that some of these organisms may obtain from the

oxidation of reduced sulphur compounds is the detoxification of metabolically produced hydrogen peroxide (e.g. some *Beggiatoa* and *Thiothrix* species) (Larkin & Strohl, 1983). The oxidation of thiosulphate to tetrathionate by a number of heterotrophic bacteria has recently been described (Mason & Kelly, 1988).

The denitrifying sulphur bacteria.

Most of the species described above are obligate aerobes, that is, they can only use oxygen for respiration. However, there are two known obligately chemolithotrophic sulphur bacteria which denitrify. *Thiobacillus denitrificans* is relatively versatile in being able to grow under fully aerobic conditions with oxygen, and fully anaerobic conditions with nitrate or nitrite (Aminuddin & Nicholas, 1973; Ishaque & Aleem, 1973). *Thiomicrospira denitrificans* is more fastidious. It grows well anaerobically with nitrate or nitrite, but can only use oxygen for growth if its concentration is kept extremely low (i.e.below the detection level of normal oxygen electrodes) (Timmer ten Hoor, 1975). Another obligate autotroph, *T. thioparus*, has the denitrifying nitrate reductase, but cannot denitrify any further as it lacks the denitrifying nitrite reductase. Among the facultative chemolithotrophs, *Thiobacillus versutus* and *Paracoccus denitrificans* can both denitrify heterotrophically (and *Paracoccus denitrificans* can denitrify autotrophically on hydrogen) but neither species can use nitrogen oxides as electron acceptors for anaerobic growth on reduced sulphur compounds (Taylor & Hoare, 1969; Friedrich & Mitrenga, 1981).

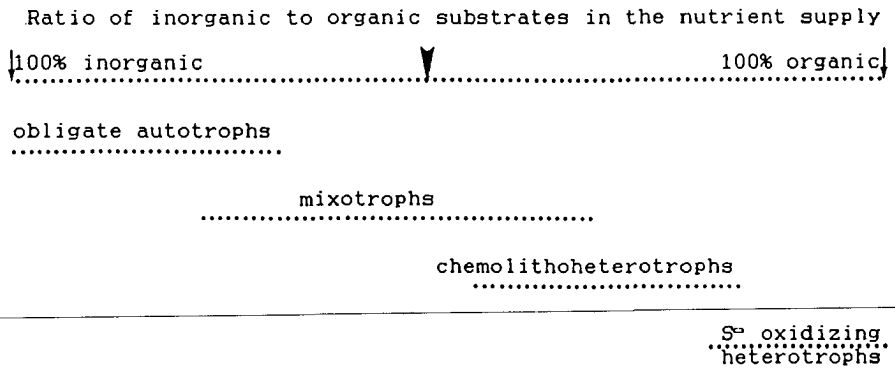


FIGURE 9. Selection of physiological types according by means of the substrate content.

As with other sorts of bacteria, the appearance of the different types of sulphur-oxidizing bacteria in different

situations will depend on the environmental conditions, and also on the possible interactions with other species present. A brief discussion of bacterial interactions is therefore given in the next section.

Selection of physiological types.

It has been shown (Gottschal & Kuenen, 1980; Kuenen & Robertson, 1984a, 1984b; Robertson & Kuenen, 1983) that in freshwater environments, the occurrence of the different physiological types of colourless sulphur bacteria can, to some extent, be predicted from the turnover ratio of the organic and inorganic substrates available at growth limiting concentrations. Where the nutrient supply is exclusively inorganic or organic, the dominant population could be expected to be obligately autotrophic or heterotrophic, respectively. However, when the turnover rates of organic and heterotrophic compounds are roughly equal, facultative chemolithotrophs should dominate. Chemolithoheterotrophs have been obtained from cultures where the amount of inorganic substrate is sufficient to provide an additional source of energy, but insufficient to make the energetically expensive operation of the Calvin cycle advantageous. Figure 9 summarizes the predicted dominant population for organic/reduced sulphur substrate mixtures or nutrient limitations in fresh water-type media. Marine enrichments are, for unknown reasons, generally less predictable. An example of this is the non-appearance of mixotrophs in thiosulphate/acetate limited cultures (Kuenen et al, 1985). That marine mixotrophs do exist has been shown by the isolation of a facultatively chemolithotrophic marine strain of *T. intermedius* from a thiosulphate limited culture (Smith & Finazzo, 1981).

5: WASTEWATER TREATMENT.

This is not the place for an exhaustive account of wastewater treatment. There are many relatively recent reviews and books on the subject available (e.g. Wanielista & Eckenfelder, 1978; Winkler, 1981). However, the species central to this thesis was isolated from a wastewater treatment plant, and the results obtained during this study may have implications for nitrogen removal from waste. The subject will therefore briefly be discussed.

The different systems in use for maintaining aerobic nitrification reactors include trickling filters where the water percolates through a bed with growing biomass (Focht & Chang, 1975), rotating discs to which the biomass is attached (Antonie, 1978), and fluidized beds in which the bacteria form pellets around a carrier material (often sand) and are kept in suspension by the high flow rates used (Andrews, 1982; Heijnen, 1984). The activated sludge process is not always ideal for nitrification because the abundant heterotrophic growth present in the reactor tends to out-grow, and thus out-compete for oxygen, the autotrophic nitrifiers. Perhaps, more importantly, the rate of growth of the sludge defines the rate at which it must be harvested. As the growth rate of the autotrophic nitrifiers tends to be much slower than those of most heterotrophs, they will gradually be "diluted out" by the harvesting process.

Denitrification reactors should contain low amounts of oxygen or be anaerobic. Even "aerobic denitrifiers" perform more efficiently at low dissolved oxygen concentrations. Denitrification systems currently in use include fluidized beds,

anaerobic activated sludge and packed bed reactors (Francis & Callahan, 1975; Heijnen, 1984; Gommers, 1987).

Denitrifying bacteria are fairly catholic in their range of usable substrates, but many of these are too expensive to be economic choices for wastewater treatment. In addition to methanol, acetate and sulphur have been used, the latter relying on *Thiobacillus denitrificans* and related bacteria (Batchelor & Lawrence, 1978). Another, cheaper source of energy is the wastewater itself. Organic carbon is frequently removed early in a treatment sequence so that the effluent can undergo nitrification before denitrification. Sequential carbon removal and nitrification reactors allow the operation of the different reactors under the optimal conditions for each individual process involved. As already mentioned, a multiple stage system which also includes a denitrification stage has been patented (E.P.A. 0051 888) by Gist brocades, in Delft. This system recovers energy from the effluent in the form of methane, and then uses the sulphide generated by sulphate reducing bacteria during methanogenisis, together with any organic material passing through the methanogenic reactors, as the energy sources for denitrification. The reactor configuration avoids the problems associated with nitrifying bacteria in a high BOD effluent by placing the nitrification reactor last in the sequence, and then recirculating the nitrate generated therein to the denitrification reactor. Figure 10 shows an outline of the system. The behaviour of a model of the denitrifying reactor with different influent compositions has been extensively studied with regard to black box behaviour, mixing and modelling (Gommers, 1987).

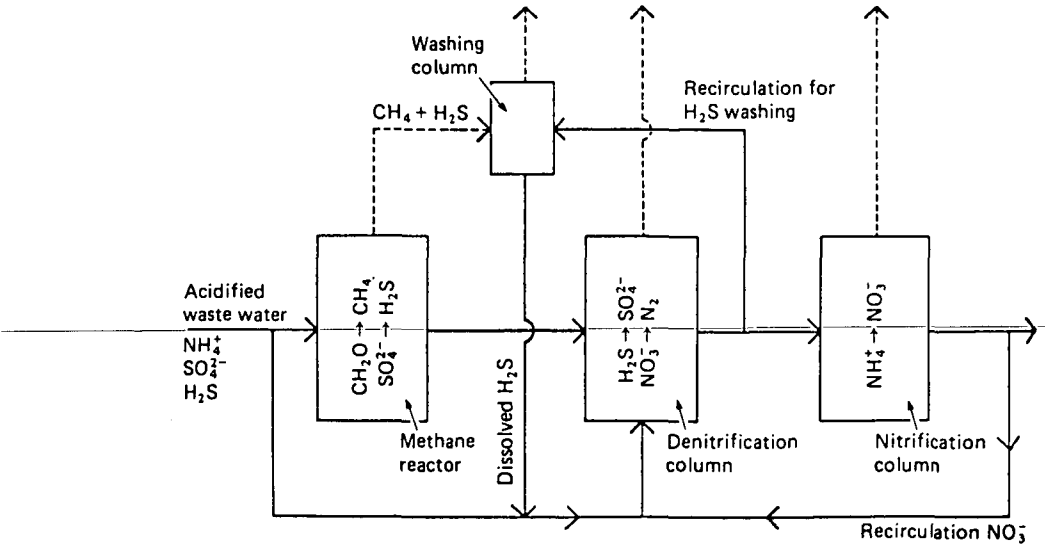


FIGURE 10. Outline of the sequential wastewater treatment system which includes the denitrification reactor under study.

Studies on the pilot-plant scale reactor showed that a model developed for the selection of different types of aerobic sulphur bacteria, based on the ratio between organic and inorganic constituents in the influent (Figure 9), could also be successfully applied to predict the type of population in the denitrifying reactor (Robertson & Kuenen, 1983; Kuenen & Robertson, 1986). The influent to the reactor contained roughly equivalent amounts of simple organic compounds and reduced sulphur compounds, together with a variable, but significant amount of complex organic material including dissolved protein, presumably from cell lysis within the acidogenic and methanogenic reactors. As both the inorganic and the organic substrates were consumed by the microbial community, the denitrifying reactor as a whole could be considered to be behaving like a culture of mixotrophic bacteria. Table 12 shows the proportions of the different physiological types within the microbial community in the denitrifying reactor. Obligately chemolithotrophic sulphur bacteria were not found. The dominant heterotroph was a member of the genus *Klebsiella*. This fermentative species was capable of growth on the complex cell lysis products in the influent. One of the dominant mixotrophs was *Thiosphaera pantotropha*.

TABLE 12. The relative proportions of the physiological types present in the pilot plant denitrifying, desulphurizing column.

Metabolic type	% of the total population.
obligate chemolithotroph	0
facultative chemolithotroph	29
chemolithoheterotroph	1
heterotroph	65
miscellaneous isolates (each less than 1% of the community)	5

6: AIM OF THE THESIS

This work aims to establish the occurrence, mechanism of control and consequences of aerobic denitrification and heterotrophic nitrification (both individually and in combination) in *Tsa. pantotropha*. The effect of the metabolism of reduced sulphur compounds on these phenomena will also be considered. In addition, the extent of the simultaneous nitrification and denitrification pathways in other species will be considered and the ecological implication discussed.

Chapter 2 describes the isolation and characterization of *Tsa. pantotropha*.

Chapter 3 provides the evidence for aerobic denitrification and heterotrophic nitrification gained from batch cultures of *Tsa. pantotropha*.

Chapter 4 describes a computer-linked electrode monitoring system which was used to demonstrate simultaneous oxygen uptake and nitrate reduction, thus confirming the existence of aerobic denitrification in homogenous cell suspensions from batch and chemostat cultures.

Chapter 5 and its appendix report the results obtained with chemostat cultures of *Tsa. pantotropha* and a control species, *Pa. denitrificans* under different environmental parameters, with

special attention being paid to nitrification and denitrification rates, and protein yields.

In Chapter 6 the enzymology of heterotrophic nitrification by *Tsa. pantotropha* is described.

Chapter 7 provides an account of the changes in the cytochromes of *Tsa. pantotropha* under various environmental changes.

The preceding chapters have concentrated on the physiological and biochemical aspects of aerobic denitrification and heterotrophic nitrification. In order to gain insight into the ecological advantages (or disadvantages) which are gained from aerobic denitrification, *Tsa. pantotropha* must be compared with a strain whose denitrification system is sensitive to oxygen. Competition experiments between *Tsa. pantotropha* and *Pa. denitrificans* under various environmental regimes in the chemostat are therefore described in Chapter 8, together with the kinetic data which can be used to predict the outcome of such experiments.

In Chapter 9, heterotrophic nitrification and aerobic denitrification in other species, especially a species formerly known as "*Pseudomonas denitrificans*", is reported.

Thiosulphate and sulphide are commonly used to inhibit autotrophic nitrification. Thiosulphate, at least, is also effective against heterotrophic nitrification by *Tsa. pantotropha*. A survey of its effect on other heterotrophic nitrifiers is therefore presented in Chapter 10.

Chapter 11 is a general discussion which aims to review the results described here and correlate them with those found by other workers.

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Journal of General Microbiology (1983), **129**, 2847–2855. *Printed in Great Britain*

Thiosphaera pantotropha gen. nov. sp. nov., a Facultatively Anaerobic, Facultatively Autotrophic Sulphur Bacterium

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(*Received 21 March 1983; revised 20 April 1983*)

During studies on a desulphurizing, denitrifying effluent-treatment system, an organism which is able to grow aerobically and anaerobically on reduced sulphur compounds and hydrogen, while fixing carbon dioxide, was isolated. The new isolate is also capable of mixotrophic and heterotrophic growth on a wide range of substrates, and is therefore a facultatively aerobic, facultative autotroph. Comparisons with two similar species, *Thiobacillus* A2 and *Paracoccus denitrificans*, showed that the new isolate is significantly different from the other two, and merits separate classification. In view of its ability to oxidize reduced sulphur compounds, and because it is a chain-forming coccus rather than a rod, the new isolate has been given the generic name of *Thiosphaera*, and the species name *pantotropha* in recognition of its wide range of possible substrates.

INTRODUCTION

In various types of industrial effluent, both nitrate and reduced sulphur compounds can be an environmental problem. Until now, methanol has been a popular electron donor in denitrification plants (Rhee & Fuhs, 1978), but in view of the rising cost of methanol, attention is turning to cheaper additives such as sulphur (Batchelor & Lawrence, 1978). This type of system would clearly involve bacteria similar to the thiobacilli and, where the sulphur compounds are already present in the effluent, the advantages are obvious. We have studied a pilot-plant-scale reactor of this type from a practical management viewpoint and as an ecophysiological problem.

The population in such a sulphide-oxidizing effluent treatment system is likely to be complex, and in addition to the 'black box' type of approach, it is therefore necessary to start with pure culture studies of suitable model organisms.

Almost all of the work on denitrification by colourless sulphur bacteria has concentrated on the obligately chemolithotrophic species such as *Thiobacillus denitrificans* (Aminuddin & Nicholas, 1973; Ishaque & Aleem, 1973) and, with only a few exceptions (Timmer ten Hoor, 1977; Justin & Kelly, 1978), was done in batch culture. Very little is known about other physiological types and, with the exception of *Thiobacillus* A2 (Taylor & Hoare, 1969), the only recognized denitrifying sulphur bacteria are obligate autotrophs (Timmer ten Hoor, 1975; Vishniac, 1974; Taylor *et al.*, 1971). A thermophilic organism, *Thermothrix thiopara*, was reported to be able to oxidize but not to grow anaerobically on reduced sulphur compounds (Brannan & Caldwell, 1980). Taylor & Hoare (1969), when describing *Thiobacillus* A2, stated that although this organism behaved as a facultative autotroph under aerobic conditions, it was only able to denitrify when growing heterotrophically.

Recent studies on enrichment cultures and simple mixtures of physiologically different thiobacilli indicated that the facultative chemolithotrophs may have a competitive advantage over their obligately autotrophic counterparts if mixotrophic growth on inorganic sulphur

Abbreviation: RuBPcase, ribulosebisphosphate carboxylase

0022-1287/83/0001-1144 $02.00

compounds and organic substrates is possible, or if autotrophic and heterotrophic growth conditions frequently alternate (Gottschal *et al.*, 1979; Beudeker *et al.*, 1982). If a similar principle can be applied to anaerobic cultures of denitrifying bacteria, the effluent treatment system under study would appear to provide conditions which would promote the selection of denitrifying, facultatively chemolithotrophic, sulphur bacteria. In order to test this, the first phase of study was to attempt the isolation of such an organism from the effluent-treatment system, and the second stage to compare the properties and competitive success with those of an obligately autotrophic denitrifyer such as *T. denitrificans*.

This paper describes the isolation and characterization of an organism which is capable of both facultatively autotrophic and facultatively anaerobic growth. This isolate is compared with two species which appear to be closely related, *Thiobacillus* A2 and *Paracoccus denitrificans*.

METHODS

Organisms. Thiobacillus A2 (Delft culture collection LMD 80.62) was originally obtained from B. F. Taylor. *Paracoccus denitrificans* was obtained from the Delft culture collection (LMD 22.21) and is the strain originally isolated by Beijerinck (1910). Bacterium A1 was a kind gift from P. W. Trudgill and is one of the strains used by Taylor *et al.* (1980).

Media. Thiobacillus A2 and the new isolate were grown in the medium described by Taylor & Hoare (1969), but with phenol red omitted. It contained (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; and 2 ml trace element solution (Vishniac & Santer, 1957). *Paracoccus denitrificans* and bacterium A1 were grown in the medium described by Taylor *et al.* (1971) for *T. denitrificans*. This contained (g l^{-1}): KNO_3, 2·0; KH_2PO_4, 2·0; $NaHCO_3$, 1·0; NH_4Cl, 1·0; $MgSO_4.7H_2O$, 0·8; and 2 ml trace element solution (Vishniac & Santer, 1957). In both cases, the trace element solution used was as described by Vishniac & Santer (1957), except that it contained 2·2 g rather than the original 22 g of $ZnSO_4.7H_2O$. Solid media were prepared by the addition of 2% Difco Bacto agar to the appropriate liquid medium. Unless otherwise stated, all chemicals were of analytical grade.

The utilization of different substrates by strain GB17 (substrate screening) was tested in liquid medium as it was found that the presence of agar gave false positive results. Substrates for testing were used at a concentration of 0·1%, unless otherwise stated. Thiosulphate and sulphide were used at concentrations of 10 mM. Growth on sulphite was tested at concentrations between 2·5 and 20 mM. Growth on H_2 was tested in a mineral medium containing (g l^{-1}): KH_2PO_4, 2·3; $NaHPO_4.2H_2O$, 2·9; NH_4Cl, 1·0; $MgSO_4.7H_2O$, 0·5; $NaHCO_3$, 0·5; $CaCl_2.2H_2O$, 0·01; and ferric ammonium citrate, 0·05. Unless otherwise stated, 20 mM-KNO_3 was added to all batch cultures for anaerobic growth. KNO_2 utilization was tested at concentrations between 1 and 20 mM. Nitrogen sources were tested using the normal media, but with the NH_4Cl omitted.

The medium supplied to the chemostat 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 trace element solution. Acetate (10 mM) and thiosulphate (10 mM) were used as the electron donors, and KNO_3 (32 mM) as the electron acceptor.

Thiobacillus A2 and *P. denitrificans* were incubated at 30 °C. Except during the determination of its growth range, strain GB17 was incubated at 37 °C. All aerobic cultures were incubated on a rotary shaker.

For the continuous flow enrichment, a Biolafitte glass chemostat with a working volume of 1·5 l was used. Dissolved oxygen, pH and OD of the culture were continuously monitored. The pH was maintained at 8·0 by automatic titration with 1 M-NaOH. Anaerobic conditions were maintained by continuous flushing with nitrogen from which all traces of oxygen had been removed by a catalyst (BASF-Katalysator-R3-11). The medium reservoirs and NaOH supply were also kept under nitrogen. To prevent oxygen contamination, all tubing on the fermenter except the pump tubing was made of Teflon with butyl rubber joints. The pump tubing was of Tygon. The dilution rate was 0·03 h^{-1}. The enrichment culture was inoculated with 10 ml of material from a denitrifying, desulphurizing effluent-treatment column.

Miscellaneous methods. Sulphide and sulphite were determined as described by Trüper & Schlegel (1964) and thiosulphate by the method described by Sörbo (1957). Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer). Nitrate was measured with a nitrate electrode (Radiometer Selectrode S) and nitrite with the Griess-Romijn reagent (Griess-Romijn-van Eck, 1966). Standard taxonomic parameters were checked with the API20B and API50CH arrays of routine metabolic tests (API Benelux). The percentage GC was determined from the melting point (T_m) of the DNA. Anaerobic conditions for batch culture were established using the Becton-Dickinson GasPak or the Oxoid G.A.S. system. In all cases, inoculated medium which lacked a substrate was used as a control. The gas mixture used for aerobic growth on hydrogen contained (%, v/v): H_2, 65; N_2, 24; CO_2, 5; and O_2, 6. For anaerobic growth, 95% H_2 and 5% CO_2 (v/v) was used.

The rate of gas production under anaerobic conditions was measured using standard manometric techniques.

Nitrate was added at the same time as the substrate to prevent endogenous gas production before the start of the experiment.

Ribulosebisphosphate carboxylase (RuBPcase) activity in cell extracts was determined by the method described by Beudeker *et al.* (1980).

RESULTS

The new isolate was obtained from a denitrifying, sulphide-oxidizing, effluent-treatment pilot plant at Gist Brocades, Delft, by enrichment in an anaerobic chemostat with thiosulphate and acetate as growth-limiting substrates, followed by selective streaking on thiosulphate and acetate plates, which were then incubated anaerobically. It was chosen from a group of isolates for further study since it lacked any requirement for vitamins or other supplements, and because it was capable of both autotrophic and heterotrophic growth under aerobic and anaerobic conditions. Its distinctive morphology allows it to be easily distinguished in phase-contrast microscope preparations of material from the effluent-treatment column where it seems to form a significant part of the population. It has since been re-isolated from many samples taken from the enrichment culture, and also from samples direct from the column. For convenience, it is referred to as strain GB17.

Strain GB17 is a non-motile, Gram-negative coccus (0·7 × 0·9 μm) which is frequently seen in pairs and long chains (Figs 1 and 2). It does not contain a complex membrane system, and carboxysomes have not been observed (Fig 3). The isolate has a % GC ratio of 65·8–66·0, which is in the same range as other facultative chemolithotrophs such as *Thiobacillus novellus* (62–68), *Thiobacillus* A2 (65–68) and *Paracoccus denitrificans* (66·5).

On acetate or thiosulphate agar, strain GB17 grows as off-white, translucent, round, entire, wet-looking colonies. It can grow over a pH range between 6·5 and 10·5 with an optimum at 8·0, and the temperature range permitting growth lies between 15 and 42 °C with an optimum at 37 °C. It is both oxidase- and catalase-positive. The results of a comparison between strain GB17, *Thiobacillus* A2 and *P. denitrificans* using the API20B system are shown in Table 1.

All three organisms can use nitrate and nitrite as electron acceptors for anaerobic growth on heterotrophic substrates, but only strain GB17 could do so on sulphide or thiosulphate. Strain GB17 and *Thiobacillus* A2 can also use fumarate instead of nitrate, whereas *P. denitrificans* cannot. Fermentation did not occur.

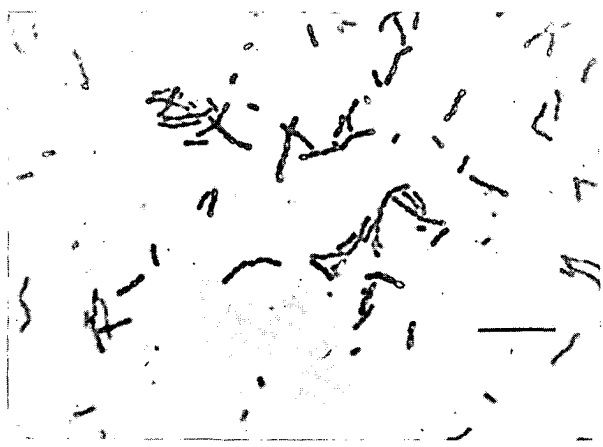

Fig. 1. *Thiosphaera pantotropha* (strain GB17). Positive phase-contrast micrograph showing the chain-forming characteristic of growth on rich media. The bar marker represents 10 μm.

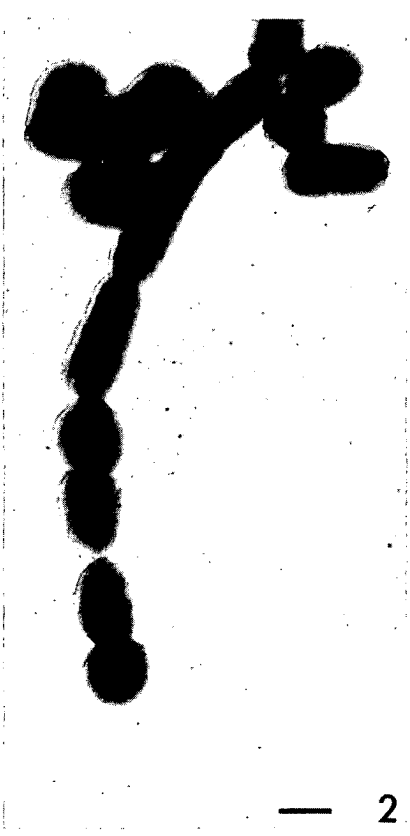

Fig. 2

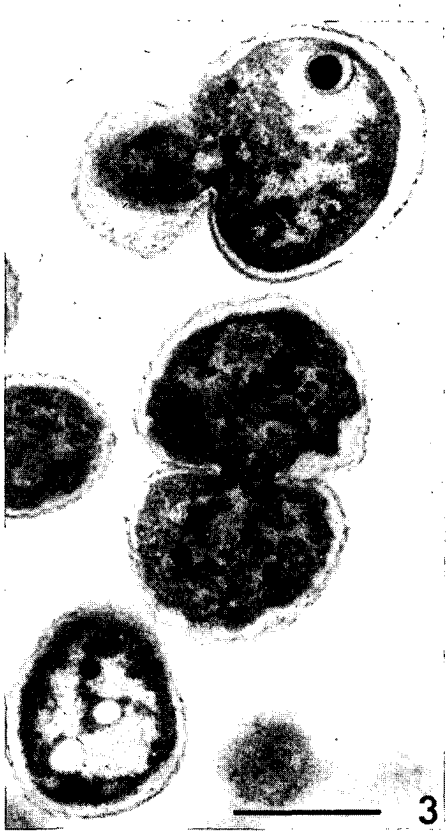

Fig. 3

Fig. 2. *Thiosphaera pantotropha* (strain GB17). Platinum-shadowed to show atypical forms produced during rapid growth. The bar marker represents 0·5 μm.

Fig. 3. *Thiosphaera pantotropha* (strain GB17). Electron micrograph of a thin section (1% OsO_4, uranyl acetate, epon and lead citrate) showing lack of carboxysomes. The bar marker represents 0·5 μm.

All three organisms could use NH_4^+, NO_3^-, urea, Casamino-acids and glutamate as a sole source of nitrogen. None of them grew without an added nitrogen supply. *Thiobacillus* A2 could utilize methylamine, but strain GB17 could not. Neither organism could use dimethylamine or trimethylamine as a source of nitrogen.

The major points of difference between the growth of the three organisms on organic substrates are shown in Table 2.

In addition to the substrates shown in the Tables, strain GB17 grew both aerobically and anaerobically on hydrogen, fructose, mannose, glucose, acetate, lactate, pyruvate, succinate, aspartate, fumarate, gluconate, glutamate, alanine, histidine, isoleucine, leucine, proline, yeast extract, Casamino acids, acetone, propane-1,2-diol, propan-2-ol, acetol and propionate. It grew aerobically, but not anaerobically on benzoate, and only anaerobically on propionaldehyde. Strain GB17 did not grow on arabinose, lactose, methanol, methane, pimelate, oxalate, methyl acetate, methyl ethyl ketone or propylene oxide.

All three strains could grow aerobically with thiosulphate or sulphide as an electron donor, but only strain GB17 grew anaerobically with either substrate as the sole source of energy. Sulphur

T. pantotropha, a sulphide-using denitrifier

Table 1. *Different reactions shown by the three species when screened with the API20B system*

Organism	Property*											
	GEL	NIT	ONPG	SAC	ARA	MAN	FRU	GLU	MAL	AMD	RHA	GAL
Strain GB17	−	+	−	+	−	+	+	+	+	−	−	−
P. denitrificans	−	+	−	−	−	−	−	−	−	−	−	−
Thiobacillus A2	−	+	−	+	+	+	+	+	+	−	−	−
	MNE	SOR	GLY	URE	IND	H_2S	VP	CIT	OX	CAT	COC	GRAM
Strain GB17	−	+	−	−	−	−	+	−	+	+	+	−
P. denitrificans	−	−	−	−	−	−	−	+	+	−	+	−
Thiobacillus A2	−	+	−	−	−	−	−	+	+	+	−	−

* 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. H_2S, hydrogen sulphide production. VP, acetoin production. CIT, citrate utilization. OX, cytochrome oxidase present. CAT, catalase present. COC, coccoid form. GRAM, reaction to Gram's stain.

Table 2. *Major differences in the growth of the three species on organic substrates*

Substrate	Strain GB17		*Thiobacillus* A2		*P. denitrificans*	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Adipate	+	−	−	−	+	+
Arabinose	−	−	−	−	+	+
Citrate	−	−	+	+	+	−
Galactose	−	−	+	+	+	+
Serine	+	+	+	+	−	−
Mannose	+	+	+	+	−	−
Dulcitol	−	−	+	+	+	+
Glycogen	−	−	+	+	+	+
Formate	−	−	+	+	+	−

was not observed in any of the cultures. None of the organisms grew on sulphite at the levels tested. An anaerobic batch culture of strain GB17 on thiosulphate gave a protein yield of 2·67 g (mol substrate)$^{-1}$. This is equivalent to approximately 4·9 g dry wt per mol thiosulphate consumed assuming a biomass protein content of 50%.

Aerobically grown strain GB17 was capable of denitrifying on acetate, thiosulphate, and mixtures of the two (Figs 4 and 5). However, in the presence of sulphide, gas production was lower (not shown). This is essentially in agreement with the results obtained with *Pseudomonas perfectomarinus* (Sørenson *et al.*, 1980). Nitrogen release began immediately after the addition of nitrate and substrate, and continued until either the single energy source or the nitrate, when mixed substrates were involved, was exhausted. In contrast, *Thiobacillus* A2 required a 4 to 5 h lag phase before gas production began. Again, nitrogen release in the heterotrophic and mixotrophic vessels continued until the acetate or nitrate became exhausted, but little or no nitrogen was produced by the cells receiving thiosulphate. The rate of gas production by *Thiobacillus* A2 was much lower than that by similar preparations of strain GB17, requiring up to 20 h for completion. The only strain GB17 preparation requiring this length of time was that incubated with thiosulphate. In all cases the results obtained depended on the history of the culture, the influence of thiosulphate only being evident in those cultures which had been grown in the presence of thiosulphate or sulphide (Fig. 4).

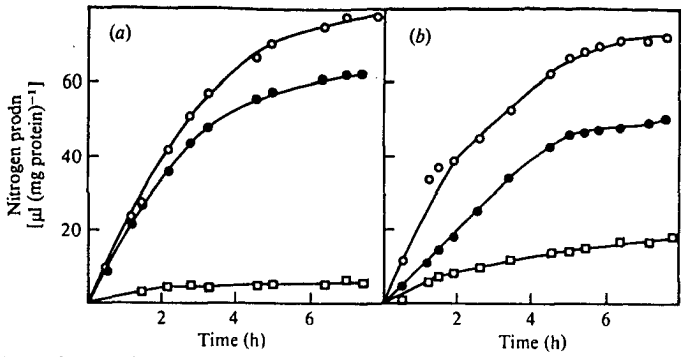

Fig. 4. Typical gas production curves obtained with *Thiosphaera pantotropha* (strain GB17) pregrown aerobically in the presence of nitrate. (*a*) Cells grown on acetate; (*b*) cells grown on a mixture of acetate and thiosulphate. Gas production from acetate (●), thiosulphate (□) and both acetate and thiosulphate (○) supplied as substrate.

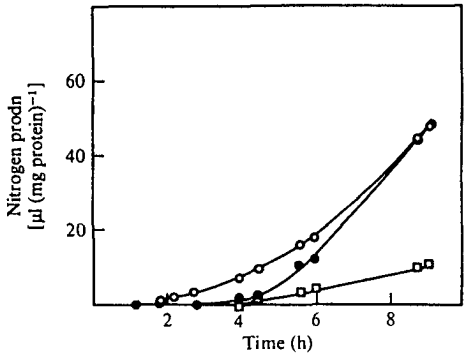

Fig. 5. Gas production obtained with *Thiobacillus* A2, pregrown aerobically with nitrate, acetate and thiosulphate. Symbols as in Fig. 4.

Table 3. *Anaerobic consumption of thiosulphate by Thiobacillus A2 under mixotrophic conditions showing that thiosulphate is consumed with an increase in biomass and elimination of excess nitrate in the cultures*

Organic substrate (mM)	Thiosulphate consumed (mM)	Nitrate provided (mM)	Nitrate required* (mM)	Increase in OD over single substrate	Detectable nitrate remaining	Nitrite produced (concn)
Succinate† 4	—	20	7·3 (35%)		+	1·7 mM
Succinate 4	7·5	20	19·3 (35%)	29%	−	<10 µM
Acetate† 20	—	20	20·8 (35%)		+	0·4 mM
Acetate 20	2·5	20	24·8 (35%)	32%	−	<10 µM
Formate† 75	—	40	27 (10%)		+	1 mM
Formate 75	8·5	40	40·6 (10%)	33%	−	<10 µM

* The nitrate requirement was based on the total substrate consumption and was corrected for carbon assimilation. The assumed values for carbon assimilation are given in parentheses.

† Thiosulphate not provided.

The theoretical balances for total oxidation of these substrates using nitrate are:

$$5(CH_3COO^-)_2 + 14NO_3^- \rightarrow 7N_2 + 20CO_2 + 14OH^- + H_2O$$

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10CO_2 + 8OH^- + 6H_2O$$

$$5HCOOH + 2NO_3^- \rightarrow 5CO_2 + N_2 + 2OH^- + 4H_2O$$

$$5S_2O_3^{2-} + 8NO_3^- + H_2O \rightarrow 9SO_4^{2-} + H_2SO_4 + 4N_2$$

Table 4. *RuBPcase activities found in extracts from Thiosphaera pantotropha (strain GB17) grown under different conditions*

Growth conditions	Growth phase	Substrate and concn (mM)	RuBPcase activity [nmol CO_2 fixed min^{-1} (mg protein)$^{-1}$]
Aerobic	Exponential	Thiosulphate, 20	99
Anaerobic	Exponential	Thiosulphate, 10	83
Anaerobic	Exponential	Sulphide, 8·5	78
Anaerobic	Late-stationary	Thiosulphate, 10	5
Aerobic	End-exponential	Acetate, 10	ND
Anaerobic	End-exponential	Acetate, 10	ND

ND, Not detectable.

The utilization of thiosulphate during the denitrification on mixed substrates by *Thiobacillus* A2 was supported by results obtained in the substrate screening programme when mixotrophic cultures showed a 30% increase in OD over acetate-, fructose- or succinate-grown cultures, together with thiosulphate consumption (Table 3). Sulphur was not observed in these cultures.

The amounts of RuBPcase found in strain GB17 cells batch-grown under different conditions are shown in Table 4.

DISCUSSION

The new isolate, strain GB17, is able to utilize both organic substrates and reduced sulphur compounds under aerobic and anaerobic conditions. The amounts of RuBPcase found in cells grown anaerobically in batch culture are about half those obtained from aerobic cells, and sufficient to support growth. The very low amount of RuBPcase found in cells from late-stationary phase cultures shows that the stage of growth of the cells when harvested is critical, and confirms similar observations by McFadden & Denend (1972). The yields of protein obtained with strain GB17 under anaerobic conditions compare favourably with the yields from batch (Loya, 1979) and chemostat (Kuenen, 1979) cultures of *Thiobacillus* A2 grown aerobically. They are also in the same order of magnitude as yields of the aerobic obligate chemolithotrophs or the autotrophic denitrifyer, *Thiomicrospira denitrificans*, although half that obtained with *T. denitrificans* under anaerobic conditions (Timmer ten Hoor, 1977). These results clearly show that strain GB17 is a facultatively anaerobic, facultative autotroph of the type predicted in the Introduction. However, the importance of organisms of this metabolic type in the effluent-treatment system remains to be established. Attempts to isolate an obligate autotroph have so far failed.

During comparisons with strain GB17, it was found that, in contrast to previous reports (Taylor & Hoare, 1969), the strain of *Thiobacillus* A2 used in these experiments can also use thiosulphate, at least mixotrophically, under anaerobic conditions. However, contrasting results have been obtained by A. P. Wood & D. P. Kelly (personal communication) with another strain, and further studies to provide clarification are in progress. The ability of *P. denitrificans* to grow aerobically on thiosulphate has only recently become known (Friedrich & Mitrenga, 1981). Although we have been able to confirm this observation, attempts to culture this organism anaerobically on reduced sulphur compounds have so far failed.

The contrast in the times required before gas production appears after aerobic culture suggests that strain GB17 is better adapted for anaerobic growth in a variable environment than is *Thiobacillus* A2. Preliminary studies indicate that its denitrifying properties are constitutive as similarly reported for *Thiomicrospira denitrificans* (Timmer ten Hoor, 1975). Since the effluent-treatment system is normally an anaerobic environment which is occasionally contaminated with oxygen, the ecological advantage would be taken by the species whose nitrate reductase synthesis is not repressed by the presence of oxygen. Should the enzyme prove to be constitutive, strain GB17 might more accurately be termed a facultative aerobe than a facultative anaerobe.

One interesting point that arose during substrate screening was the ability of all three species to utilize acetone as their sole source of carbon and energy under anaerobic conditions. It had previously been considered that the initial step of acetone metabolism requires an oxygenase reaction (Taylor *et al.*, 1980). Since all three species tested had this property, it would appear that the ability to utilize acetone anaerobically may be widespread, although not universal since we confirmed that the organism used by Taylor *et al.* (1980), bacterium A1, grew anaerobically on acetate but not acetone (unpublished results). The pathway of anaerobic acetone metabolism in these organisms is currently under investigation in this laboratory.

A comparison between strain GB17, *Thiobacillus* A2 and *P. denitrificans* showed that although all three are very versatile in their substrate metabolism, and a fair degree of overlap in their substrate range was to be expected, significant differences do occur. The fact that strain GB17 is a coccus means that it falls outside the genus description for *Thiobacillus*. The many differences between strain GB17 and *P. denitrificans*, including anaerobic sulphur metabolism and use of fumarate as an electron acceptor argue against classification of the new isolate as a *Paracoccus*. Moreover, the ability to denitrify with hydrogen as an energy source has also been reported for a third, totally different species, *Alcaligenes eutrophus* (Pfitzner & Schlegel, 1973). Although Friedrich & Mitrenga (1981) have commented that the ability to oxidize thiosulphate may be more widespread than previously thought, and therefore not selective enough a property to permit classification of a group of organisms, insufficient evidence is currently available to make a judgement. We therefore propose to form a new genus, *Thiosphaera*, to accommodate isolate GB17 in recognition of its sulphur metabolism. In view of its versatility, it has been given the species name *pantotropha*. A culture has been deposited in the Delft Culture Collection under this name, and has been designated the type species of the genus. It has the collection number LMD 82.5.

Description of the genus Thiosphaera

Thi.o.sphae'ra Gr. noun *thion* sulphur; Gr. noun *sphaera* a sphere. *Thiosphaera* a sulphur sphere.

Cells coccoid, occurring singly, in pairs and in chains. Usually non-motile. Gram-negative. Metabolism respiratory, able to use oxygen, nitrate, nitrite or a nitrogen oxide as the terminal electron acceptor. Able to oxidize reduced sulphur compunds as an energy source for growth. CO_2 can serve as the sole carbon source. Oxidase- and catalase-positive.

Type species *Thiosphaera pantotropha* LMD 82.5 (Delft Culture Collection) *pan.to'troph.a* Gr. prefix *panto* all; Gr. noun *trophos* feeder; M.L. adj. *pantotrophus* omnivorus. Description as in the above paper.

The authors would like to thank Mrs W. H. Batenburg-van der Vegte for doing the electron microscopy, Miss H. van Eyk and Ir J. van der Toorn for taxonomical asistance, and Drs G. M. H. Scheulderman-Suylen for constructive discussion. Additionally, thanks are due to Gist Brocades N.V., Delft, for making samples from their pilot plant effluent treatment system available to us.

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Archives of Microbiology

Aerobic denitrification: a controversy revived*

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

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

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

It has commonly been accepted that denitrification requires completely anoxic conditions (Tiedje et al. 1982; Payne 1981) because some well studied bacteria completely shut down their denitrifying capacity upon exposure to oxygen. However, there have been periodic reports of aerobic denitrification (Marshall et al. 1953; von Meschner and Wuhrmann 1963; Krul 1976). These have often been dismissed since the dissolved oxygen concentration was often not monitored and may have been limiting (Watahiki et al. 1983). In other cases the inhomogeneity of the culture, for example because of clumping by the bacteria, has been held responsible for the creation of anaerobic microniches which would allow denitrification. However, in more recent experiments the dissolved oxygen was measured, and the cultivation conditions were such that dissolved oxygen was present in homogenously suspended bacterial cultures at concentrations ranging from 10% to twice air saturation. The results of these experiments clearly indicate that aerobic denitrification does indeed occur (Krul and Veeningen 1977; Meiberg et al. 1980).

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

Materials and methods

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

Cultures were grown in Kluyver flasks which incorporated an oxygen electrode. Aerobic cultures were sparged with air, and the dissolved oxygen did not fall below 80% of air saturation. Anaerobic cultures were sparged with nitrogen. The mineral salts medium contained (g l^{-1}); $Na_2HPO_4 \cdot 7H_2O$, 7.9; K_2HPO_4, 1.5; NH_4Cl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; and 2 ml of a trace element solution (Vishniac and Santer 1957). All cultures were provided with 10 mM sodium acetate. 20 mM KNO_3 was provided when required. *Tsa. pantotropha* was grown at 37°C and *T. versutus* at 30°C.

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

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

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

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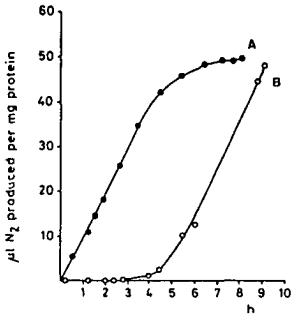

Fig. 1. Gas production by aerobically grown *Thiosphaera pantotropha* and *Thiobacillus versutus* cultures in anaerobic manometric experiments. (Data from Robertson and Kuenen 1983) Curve *A: Tsa. pantotropha*, curve *B: T. versutus*

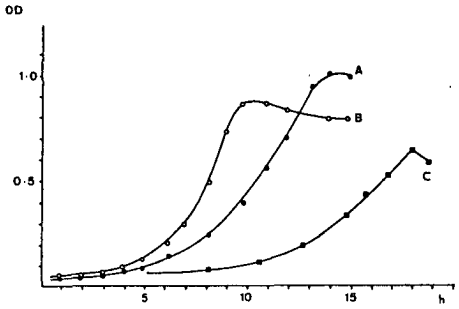

Fig. 2. Growth curves from *Thiosphaera pantotropha* cultures grown: *A* aerobically with acetate; *B* aerobically with acetate and nitrate; *C* anaerobically with acetate and nitrate. *OD*, Optical density at 430 nm. Acetate and nitrate concentrations 10 and 20 mM, respectively

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

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

Results and discussion

As has been reported previously (Robertson and Kuenen 1983), it was found that although aerobically-grown *T. versutus* required the expected 4–5 h induction period before gas production from nitrate began, *Tsa. pantotropha* began releasing gas immediately (Fig. 1). That the enzymes of denitrification were not only synthesized under aerobic as well as anaerobic conditions (i.e. constitutive), but also were active was indicated by the results of the growth experiments. From Fig. 2 it can be seen that the acetate culture provided with both nitrate and oxygen grew faster than the cultures receiving either, and that the final optical density of this culture lay between those of the other two. The specific growth rates and the protein yields obtained with these cultures (Table 1) confirmed this. As expected, aerobic growth of *T. versutus* was unaffected by the presence of nitrate ($\mu = 0.12\ h^{-1}$, results not shown). The upward flow of gas in the Kluyver flasks maintained the culture as a homogenous suspension in which clumps did not occur. 5.5 mM nitrate disappeared from the aerobic, nitrate containing, culture. As the assimilatory nitrite reductase was not present (< 5 nmol min^{-1} mg^{-1} protein), and as the ammonium in the medium was depleted but not exhausted at the end of the experiment, it was concluded that this disappearance was not due to nitrate assimilation. Only trace amounts of nitrite were present, and it was therefore concluded that the nitrate had been reduced to nitrogen gas. From the biomass yield it can be calculated that 65% of the acetate was oxidized. Of this, about one half must have been oxidized through nitrate, the remainder presumably through oxygen. If only the oxygen respiration produced respiratory energy, a yield of half that obtained from the aerobic, nitrate-free culture could be expected. As the actual yield was considerably higher, it was concluded that respiration with nitrate as the terminal electron acceptor also contributed to the energy budget of the cells. The lower yield obtained under anaerobic conditions confirms that, as with other species, denitrification provides less energy than oxygen-linked respiration (Stouthamer 1980).

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

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

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

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

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

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

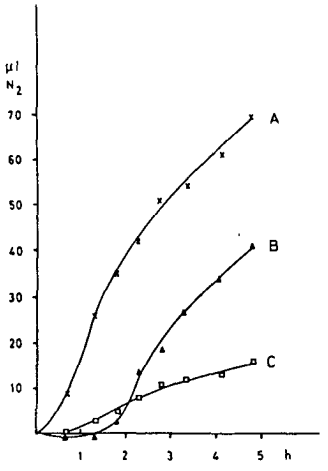

Fig. 3. Gas production by aerobically grown *Thiosphaera pantotropha* cells in anaerobic manometric experiments with denitrification inhibitors: *A* no inhibitor; *B* 10 μM cyanide: *C* antimycin a

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

In a second experiment involving the Biological Oxygen Monitor, nitrite formation from nitrate in the presence of antimycin a, an inhibitor of nitrite rather than nitrate (Stouthamer 1980), was followed while oxygen was also monitored. It was found that even cells which had been grown aerobically in the absence of nitrate produced significant amounts of nitrite (7.2 nmol $min^{-1} mg^{-1}$ protein) while oxygen uptake continued (249 nmol min^{-1} mg^{-1} protein). This level of nitrite production corresponds well with the amount of nitrate reductase found in the cells.

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

Acknowledgements. The authors would like to acknowledge the technical assistance of Miss Annemiek Laanen.

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Received March 3, 1984/Accepted April 22, 1984

Journal of Microbiological Methods 5 (1986) 237–242
Elsevier

JMM 00170

A microcomputer-based method for semi-continuous monitoring of biological activities

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(Received 7 February 1986) (Revised version received 12 May 1986) (Accepted 20 May 1986)

Summary

A method by which a microcomputer can be used to monitor bacterial physiology by means of various electrodes (including oxygen, redox and ion-specific electrodes) is described and an outline of the software being used is given. A sample experiment, taken from the authors' studies on aerobic denitrification, is shown.

Key words: *Aerobic denitrification – BBC Computer – Ion-specific electrodes*

Introduction

During studies on *Thiosphaera pantotropha*, a facultatively chemolithotrophic sulphide oxidizing bacterium [1], it was found that this species was an aerobic denitrifier [2, 3]. In the early stages of the work, single parameters (e.g., oxygen consumption, nitrate reduction) were measured in separate experiments. However, in view of the controversy surrounding the actual occurrence of aerobic denitrification, it became important that simultaneous oxygen and nitrate reduction be shown in the same sample. It was therefore decided to develop a simple system for combining and monitoring various electrodes. The combined use of nitrate and oxygen electrodes for measuring the activity of *Pseudomonas aeruginosa* was described by van Kessel [4]. However the system required separate meters and recorders for each electrode, which is costly both in terms of space and money, and would also not permit the convenient changing of the types of electrode in use. This paper describes the modification of van Kessel's original equipment for easy use with various combinations of electrodes, and the use of a microcomputer both to control the reading of redox and ion electrodes via a single meter, and as an electronic multi-point recorder.

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Materials and Methods

Hardware

The microcomputer was a BBC B (Acorn, OS 1.2) fitted with BASIC 2, the Acorn Disc Filing System and the Watford Dumpout ROM. Interaction with the electrodes was via an interface (type 532.001) manufactured by UNILAB (U.K.). The redox and ion electrodes were plugged into the computer-controlled switches on the interface. The output from the switches was connected via a single plug to the electrode socket on the meter, and the recorder output from the meter to the first of the analogue input ports on the interface. When electrodes with independent amplification were involved, they were connected to the other three analogue ports. The electrodes used with this system came from various manufacturers and included oxygen (Yellow Springs Instruments Co., U.S.A.), nitrate, redox and their calomel reference (Radiometer, Denmark), and ammonia (Orion Research, U.S.A.). As will be seen, the diameter of the electrode is almost as important as its sensitivity. For amplification of the electrode signals, where necessary, Radiometer (Copenhagen) PHM85 and Corning-Eel digital research meters have been used in this work, but any meter with a high impedance input which is suitable for use with ion electrodes and which has an output for a recorder would be suitable. The program has been written for use with the Gemini Star printer but will also function with most Epson printers or the Brother HR5. Only one line needs to be changed to allow the use of another screen dump ROM, or alternatively to call a printing procedure.

Because each electrode has different storage requirements when not in use, it has proved most convenient to mount each electrode in an individual holder. This obviates the necessity for removing the electrodes from the holder at the end of an experiment, and reduces the chance of damaging them. Each holder makes up a quarter of a cylinder so that any four can be combined for an experiment. Should fewer than four electrodes be required, solid quarter cylinders can be used to replace superfluous electrode holders. As the reference electrode was most likely to be used at all times, a small hole was drilled through its holder to allow both the escape of air from the test vessel and the addition of solutions to the experimental mixture once the electrodes were in place. The holder for the oxygen electrode was also modified by the provision of two tubes which would allow the test mixture to be pumped rapidly out of the test vessel, through a sparging chamber (e.g., for extra oxygen or nitrous oxide) and back into the test vessel again. For use, four holders are slotted together to form a cylinder (with a small perspex block in the centre to prevent slipping) and held firmly by two O-rings.

Experiments are done in a round, straight-sided glass vessel in which the electrode cylinder fits precisely. The working volume in the vessel currently in use is 30 ml. A constant temperature is maintained by means of a water jacket. The whole unit is mounted on a magnetic stirrer.

Software

Flow charts for the program are shown in Fig. 1A and B. In addition to providing information for the running of the program, the start-up procedure allows time for the electrodes to adjust to the temperature and ionic levels in the sample. The internal

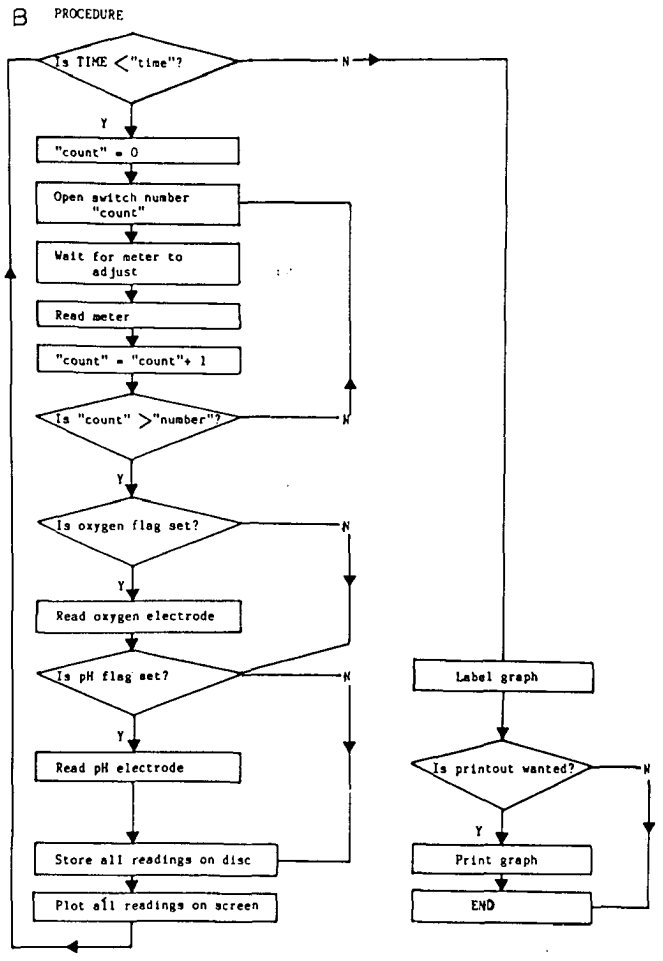

Fig. 1B. Flow sheet for the experimental section of the program.

signals are individually amplified. The program has been written using the facility in BBC Basic for the use of Procedures within a structured programme. This means that the addition of a new electrode to the system only requires the addition of its name to the start-up menu and the addition of a Procedure to handle any special sensitivity or plotting requirements. A print-out of the program is available on request, together with a short program for recovering the data from disc. A program which will allow the calculation of user-determined areas of slope on the screen is under development.

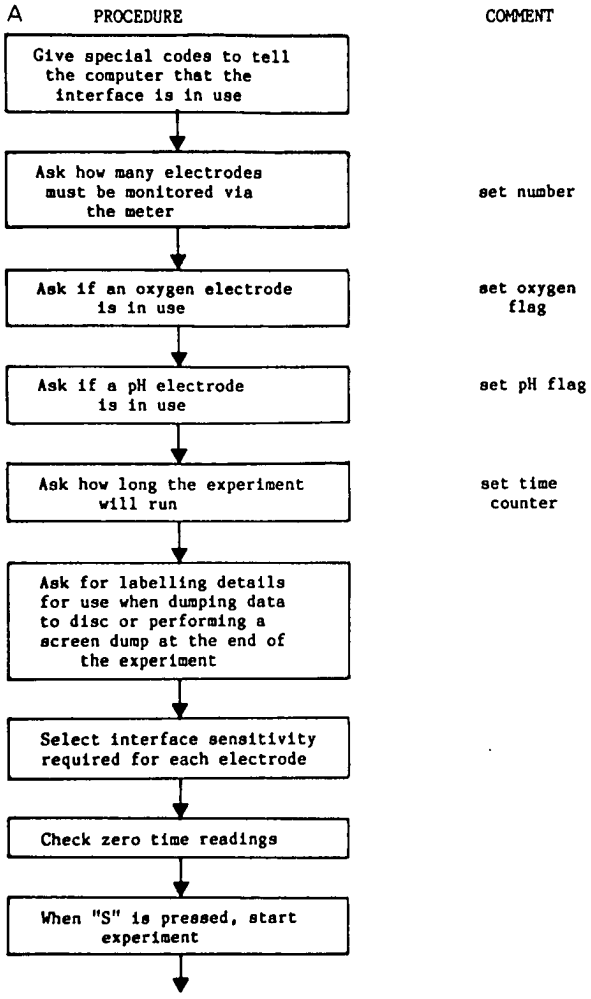

Fig. 1A. Flow sheet for the start-up procedure in the program.

clock of the computer is used in the plotting of the data and to control the length of the experiment. The graphics MODE 1 of the BBC is used for the screen display during the experiment as this allows high resolution graphics in four colours (the background and three others). By using dotted and solid lines, the lines from six electrodes can easily be displayed at any one time. If more lines were required, the sixteen colours of the BBC's MODE 2 could be used but this would mean sacrificing resolution. The oxygen and pH readings are handled separately as these electrode

Miscellaneous methods

Both *Thiosphaera pantotropha* and the media used have already been described [1]. The cells used for the sample experiment shown in the results section were grown in an acetate-limited chemostat with ammonium as the sole nitrogen source, and with the dissolved oxygen controlled at 30% of air saturation at 37 °C. The culture had not been exposed to nitrate before the start of the experiment. Acetate was added to the 30 ml sample at the start of the experiment, and nitrate was then added at the indicated intervals.

Results and Discussion

Figure 2 shows a sample printout from an experiment combining nitrate, oxygen and redox electrodes. Previous attempts to show that oxygen and nitrate can be simultaneously used by this organism had involved the measurement of oxygen utilization in one suspension while nitrate reduction was measured in a duplicate. The computer/electrode combination has made it possible to do the whole experiment in a much more direct way. Also, before cultures could be screened for denitrifying ability it was previously necessary to set up a respirometry experiment, and the physiological state of the test organisms could very well change during the

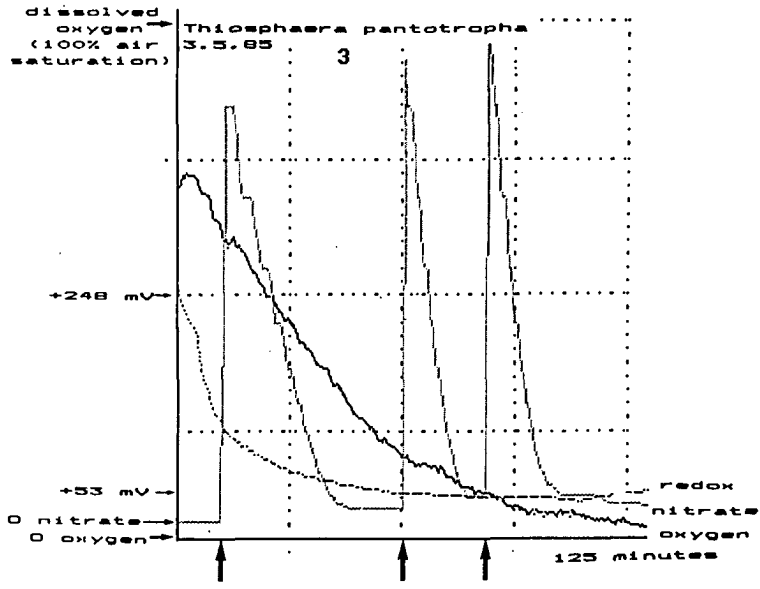

Fig. 2. Sample printout from an experiment where the use of oxygen and nitrate by aerobically grown *Thiosphaera pantotropha*, together with redox changes in the culture, were monitored. The arrows indicate the addition of nitrate (50 μM). Redox readings were corrected for the reference electrode after calibration with quinhydrone solutions at different pH values.

45 min gassing period needed to make the equipment anaerobic for the measurement of nitrogen production. With the new system, cultures can be sampled and tested immediately.

This technique has now successfully been used for the screening of new isolates for constitutive nitrate reducing systems, and is currently in use for the monitoring of heterotrophic nitrification/denitrification by *T. pantotropha*. The method is fully adaptable, and could be used for the monitoring of any metabolite for which there is a suitable electrode available. The main limitation lies in the requirements of individual electrodes. For example, the nitrite electrode available from Orion can only be used below pH 2.0 and could not therefore be directly combined with, for example, the ammonia electrode from the same manufacturers as this requires alkaline conditions. It has proved possible to read nitrite in these experiments only by fitting the electrode with a flow-through cell into which test culture and an acid solution are slowly pumped through converging tubes. This necessitates the use of duplicate cultures – one for the other electrodes and one for nitrite. Again, although the use of the ammonia electrode with living organisms is limited to those cells which are active above pH 9.0, it can be very useful in combination with the oxygen electrode for the measurement of nitrifying enzymes in cell extracts, an assay normally done at pH 10.0.

Acknowledgements

The authors are grateful to Gerard van der Toolen, Peter Vetter and Rob Suijkerbuijk of the Delft University of Technology workshops for converting sometimes vague drawings into usable hardware.

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Simultaneous nitrification and denitrification in aerobic chemostat cultures of *Thiosphaera pantotropha*.

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Applied and Environmental Microbiology IN PRESS,

ABSTRACT.

Thiosphaera pantotropha is capable of simultaneous heterotrophic nitrification (oxidizing ammonia to nitrite) and denitrification in aerobic batch and chemostat cultures. Consequently, its nitrification potential could not be judged from nitrite accumulation (the traditional means of evaluating heterotrophic nitrifiers), but was estimated from complete nitrogen balances. The maximum rate of nitrification obtained during these experiments was 93.9 nmol min^{-1} mg $protein^{-1}$. The nitrification rate could be reduced, or almost eliminated, by the provision of nitrate, nitrite or thiosulphate in the culture medium. Both nitrification and denitrification by this organism increased as the dissolved oxygen concentration fell, until a critical level was reached at approximately 25% of air saturation. At this point, the rate of (aerobic) denitrification was maximal (i.e. equivalent to the anaerobic rate). At this dissolved oxygen concentration, the combined nitrification and denitrification was such that cultures receiving ammonium as their sole source of nitrogen appeared to become oxygen limited and the nitrification rate became lower. It appeared that under carbon and energy limited conditions, a high nitrification rate was correlated with a reduced biomass yield.

In order to facilitate experimental design, a working hypothesis for the mechanism behind nitrification and denitrification by *Thiosphaera pantotropha* has been formulated. This hypothesis involves the basic assumption that this species has a "bottleneck" in its cytochrome chain to oxygen, and that denitrification and nitrification are used to overcome this.

The nitrification potential of other known heterotrophic nitrifiers has been reconsidered in the light of the results obtained with *Thiosphaera pantotropha*, and it appears that several species previously considered to be "poor" nitrifiers are, in fact, also simultaneously nitrifying and denitrifying, thus giving a falsely low impression of their nitrification potential.

INTRODUCTION

Most of the detailed investigations into nitrification and denitrification have involved a very limited group of specialized bacteria. This has resulted in a view that denitrification can only occur under completely anaerobic conditions and that successful nitrification requires autotrophic nitrifying bacteria (17). However, it has been shown that denitrification can occur in fully aerobic conditions with a wide range of bacteria (10, 15, 20, 21). Moreover, many heterotrophic bacteria and fungi are able to nitrify (some at significant rates) provided that they

are supplied with a source of energy (9, 29). Many of the common denitrifying bacteria in soil are also heterotrophic nitrifiers (2, 13) and, indeed, it has been proposed that nitrification by heterotrophic bacteria may be responsible for all nitrate and nitrite production in those types of soil where conditions (e.g. low pH, low pO_2) do not favour autotrophic nitrification (9, 27).

The physiological reasons for heterotrophic nitrification are not yet fully understood. It does not appear that energy is obtained from the reaction (see, for example, 9). In some cases it is possible that a competitive advantage might be gained from the production of toxic chemicals such as nitrite (29). An alternative explanation is that the production of nitrite or nitrate is a by-product or by-pass of a pathway used for the production of hydroxamic acids which serve as chelators when iron is limiting (29).

Evidence has been published to support the view that in some bacteria and fungi organic nitrogen compounds are involved in the heterotrophic nitrification pathway (29). Indeed, in the fungi and in some bacteria this may be the only pathway (9). However, studies with extracts of a heterotrophic nitrifier, *Thiosphaera pantotropha* (21a) have indicated that the enzymes involved in this species are in many respects similar to those found in the chemolithotrophic ammonia oxidizing bacteria. Various organisms may, therefore, operate inorganic or organic nitrification pathways, or even combinations involving inorganic and organic steps (29).

In the study of bacterial heterotrophic nitrification, the emphasis has so far been on the accumulation of oxidation products such as nitrite or nitrate in batch culture, and the efficiency of the nitrifier has been judged accordingly. However, during studies on *T. pantotropha* (19), it was found that this organism is not only a heterotrophic nitrifier, but also an aerobic denitrifier. Nitrite only accumulated in batch cultures when its reduction by the denitrifying enzymes was inhibited (12, 20, 21). If this property is not unique to this species, it indicates the existence of a previously unstudied group of heterotrophic nitrifiers (13, 28). In view of their possible role in the nitrogen cycle, and in particular their effects on soil fertility as well as their potential use in waste water treatment, these organisms merit detailed study, and for this reason research on *T. pantotropha* was extended to include the controlled environment provided by the chemostat.

This paper will describe batch and chemostat experiments to study simultaneous heterotrophic nitrification and aerobic denitrification (or, more accurately, co-respiration of oxygen and nitrate or nitrite) in *T. pantotropha* as a representative of the nitrifying/denitrifying group of bacteria.

MATERIALS AND METHODS

Organisms

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide oxidizing wastewater treatment system (19). *Paracoccus denitrificans* LMD 22.21 was obtained from the Delft Culture Collection, and is the strain isolated by Beijerinck (1).

Batch cultures

Batch cultures were made in Kluyver flasks (21) which incorporated an oxygen electrode. Anaerobic batch cultures were made by sparging cultures in Kluyver flasks with oxygen-free

argon or nitrogen. *T. pantotropha* was grown at 37 C and *P. denitrificans* at 30 C.

The medium described for the growth of *Thiobacillus versutus* (formerly *Thiobacillus* A2) by Taylor & Hoare (26) 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; 2ml 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. Unless otherwise stated, the initial concentration of the carbon sources in the batch cultures was 10mM. Succinate was used at a concentration of 5mM. KNO_3 and KNO_2 were supplied at concentrations of 20mM and 5mM, respectively.

The trace element solution (31) 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.

Continuous Cultures

Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 37 C (*T. pantotropha*) or 30 C (*P. denitrificans*) and the pH at 8.0.

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. 20 mM acetate was supplied as substrate unless otherwise stated. Thiosulphate, when used, was supplied at the concentrations shown in the text. Unless otherwise stated, when KNO_3 and KNO_2 were used their concentrations were 40mM and 20mM, respectively.

Respiratory measurements

Oxygen uptake was measured using a Clark-type electrode mounted in a thermostatically controlled cell which is closed except for a small hole through which additions may be made. Simultaneous use of oxygen and nitrate was measured using a computer-controlled switching and monitoring system (22).

Biomass analysis

Protein was measured spectrophotometrically, by means of the Micro-Biuret method (6). The presence of poly-β-hydroxybutyrate (PHB) was detected by examining thin sections under the electron microscope, and measured as crotonic acid by means of the method described by Law & Slepecky (14).

Biomass obtained under various conditions was analyzed for its carbon, hydrogen and nitrogen content. The generalized formula for bacterial biomass often quoted in the literature is C_1 H_2 $O_{0.5}$ $N_{0.25}$ (23) which gives a C:N ratio of 4. The general formula for PHB is $(C_4$ H_2 $O_3)_n$.

Except when PHB was present, the results of the element analysis correlated well with those of the protein determination. Because of the formation of PHB under some growth conditions, both dry weight and total organic carbon measurements would have given an artificially high yield. Yield estimates for the nitrogen balances were therefore based on the protein determinations. When grown in the chemostat under carbon and energy limitation, 50% of the dry weight of *T. pantotropha* is protein (L.A. Robertson, unpublished data). The protein concentrations were therefore doubled (to give total biomass) and the amount of nitrogen calculated from the general formula quoted above.

Analysis of medium

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer). Thiosulphate was measured

colourimetrically, using the method described by Sörbo (25).

Nitrite was measured colourimetrically, with the Griess-Romijn reagent (8) or by means of an HPLC fitted with a ionosphor-TMA column (Chrompak) and a Walters RI detector. Nitrate was also measured colourimetrically, using diphenylamine sulphonic acid chromogene (Szechrome NAS reagent, Polysciences Inc.). 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 (11).

Hydroxylamine was determined colourimetrically by means of the method described by Frear & Burrell (5). Ammonia was determined by following the oxidation of NADH in the presence of a-ketoglutarate and L-glutamate dehydrogenase using 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 in steady states a maximum ammonia loss of 0.3mM (or 6 μmol min^{-1} $litre^{-1}$) could be expected from stripping.

RESULTS.

Aerobic denitrification

Using computer-monitored electrodes, it was possible to show that well-mixed suspensions of aerobic, chemostat-grown *T. pantotropha* simultaneously used nitrate and oxygen (22). However, it was found that that the rates of nitrate reduction at dissolved oxygen concentrations above 30% of air saturation were half those below 30% (e.g. with 100 μmol acetate, the rates were 0.8 and 1.6 μmol min^{-1} mg protein $^{-1}$, respectively). The oxygen uptake rate was lower when the dissolved oxygen was below 30% of air saturation (22). Experiments of this type have confirmed the previous finding that *T. pantotropha* is able to co-respire nitrogen oxides and oxygen (20, 21).

Nitrogen balances

As already mentioned, *T. pantotropha* has the potential to nitrify and denitrify simultaneously, and its actual rate and amount of nitrification therefore cannot be estimated from nitrite accumulation data alone. It was thus necessary to make nitrogen balances for all of the cultures in order to quantify the nitrification and denitrification taking place in the cultures.

From the nitrogen balances (Table 1), it became apparent that nitrogen disappearance was dependent on the nitrogen compounds provided, and also on the dilution rate. N_2O was not detected, and it has therefore not been included in the table. Ammonia losses from the cultures with 5 mM nitrite and the ammonia/oxygen culture were highest. The relationship between the growth rate and the amount of ammonia disappearing from the cultures supplied with nitrate and nitrite was similar in that less appeared to be lost as the dilution rate increased. In contrast, when ammonia was the sole nitrogen compound present, ammonia losses were slightly higher at the higher dilution rates. Nitrite could not be detected in the ammonia/oxygen and ammonia/oxygen/5 mM nitrite cultures, but a proportion of the

added nitrate or nitrite (as appropriate) remained present in the others.

Although the actual concentration of ammonia which disappeared from most of the cultures was highest at low dilution rates (Table 1), calculation of the specific nitrification rates (expressed as nmol NH_3 oxidized min^{-1} mg $protein^{-1}$) showed that, in fact, the rates increased with increasing dilution rate (Table 2). The total denitrification rates (i.e., ammonia oxidized and then reduced plus nitrate or nitrite reduced) also increased as the growth rate increased. The presence of nitrate or 20 mM nitrite in the medium led to lower nitrification rates than those found in the other heterotrophic cultures. Mixotrophic (acetate and thiosulphate) cultures displayed lower nitrification rates than the heterotrophic cultures (not shown in the tables). For example, at a dilution rate of 0.056 h^{-1}, a mixotrophic culture with ammonia as the sole nitrogen source had a nitrification rate of 20.5 nmol min^{-1} mg protein $^{-1}$. When nitrate was included, the nitrification rate in an acetate/thiosulphate culture fell to only 6.1 nmol min^{-1} mg protein $^{-1}$.

TABLE 1. The results obtained from the nitrogen balances mad for aerobic (>80% air), steady state cultures.

D (h^{-1})	N-compounds	NH_3[a] mmol/l	NO_3^- mmol/l	NO_2^- mmol/l
0.02	NH_3	-4.3[b]	0.0	0.0
0.05		-4.6	0.0	0.0
0.10		-5.1	0.0	0.0
0.02	NH_3 / NO_3^-	-2.3	-8.6[b]	+0.1
0.04		-1.7	-13.4	+0.1
0.09		-1.3	-15.7	+0.2
0.14		-1.0	-16.6	+0.2
0.17		-1.0	-17.3	+1.5
0.03	NH_3 /5mM NO_2^-	-5.6	0.0	-5.0
0.06		-4.7	0.0	-5.0
0.02	NH_3 /20mM NO_2^-	-1.7	0.0	-1.0
0.03		-1.8	0.0	-3.0
0.04		-2.6	0.0	-4.1
0.06		-1.5	0.0	-9.7
0.12		-0.4	0.0	-9.8

[a] The ammonia disappearance has been corrected for assimilated nitrogen and for ammonia lost because of sparging.
[b] A negative number indicates disappearance, a positive number indicates production.

Table 2 thus shows that *T. pantotropha* was capable of a significant level of nitrification under a variety of growth conditions. Since the autotrophic ammonia oxidizers derive energy from this reaction, the next step was to compare yields from cultures with high and low nitrification activity.

Yields

In batch cultures maintained at a dissolved oxygen concentration of 80% air saturation on acetate or succinate, the co-respiration of oxygen and nitrate resulted in a higher maximum

specific growth rate and yielded less protein (Table 3).

TABLE 2. Nitrification and denitrification rates obtained with chemostat cultures of *T. pantotropha* with various media.

Substrate (mM)	N-compounds	D h^{-1}	nitrif. rate [a]	denitrif. rate [b]
Acetate (20)	NH_3	0.017	12.7	12.7
		0.046	43.3	43.3
		0.100	93.9	93.9
Acetate (20)	NH_3, NO_3^-	0.018	7.9	38.1
		0.044	11.9	107.3
		0.086	17.2	233.6
		0.137	21.1	389.1
		0.175	26.4	506.9
Acetate (20)	NH_3, 5mM NO_2^-	0.034	48.2	84.5
		0.060	77.4	145.0
Acetate (20)	NH_3, 20mM NO_2^-	0.017	9.6	12.9
		0.036	20.9	40.9
		0.059	24.7	98.1
		0.117	33.8	191.8
		0.165	43.5	177.2

[a] The nitrification rates are expressed as nmol NH_3 oxidized min^{-1} mg protein $^{-1}$.
[b] The denitrification rates are also expressed as nmol min^{-1} mg $protein^{-1}$ and include nitrate and nitrite supplied in the medium together with nitrite produced by the ammonia oxidation.

This has been interpreted as demonstrating that the organism is able to grow more rapidly because of relief from electron acceptor limitation, but must pay for this with a lower yield because of the denitrification (20). Replacement of the nitrate with nitrite gave similar results (not shown). However, when *T. pantotropha* was grown at sub-maximal rates in acetate limited chemostats with ammonia as the only nitrogen source, the protein yield of *T. pantotropha* cultures with oxygen as the sole electron acceptor was considerably lower than expected from the batch results (Fig. 1). This was true at low and high dilution rates. The protein yields were even lower than those obtained from chemostat cultures respiring both oxygen and nitrate (Fig. 1).

Further comparisons were made between aerobic chemostat cultures with various medium supplements, as shown in Table 4. Cultures supplied with 20 mM nitrite gave yields somewhat higher (Table 4, 3b) than those obtained with nitrate as the second electron acceptor (Table 4, 1b). In contrast, the yields from cultures supplied with only 5 mM nitrite (Table 4, 3a) were as low as those where oxygen was the sole electron acceptor (Table 4, 1a). Protein yields from anaerobic, nitrate-containing chemostat cultures were, as expected, the lowest of them all (Fig. 1; Table 4, 1c). Succinate limitation produced a similar pattern (results not shown). Cultures of *P. denitrificans* were, however, unaffected by the presence or absence of nitrate (Fig. 1). In contrast, protein yields obtained from mixotrophic (acetate/thiosulphate) cultures and from cultures fed with ammonia-free media (Table 4, 2a) were not unexpectedly low.

TABLE 3. Maximum specific growth rates and protein yields obtained with batch cultures of Thiosphaera pantotropha grown in Kluyver flasks at (with the exception of the anaerobic culture) 80% of air saturation *.

Growth conditions	μmax h^{-1}	protein mg/l	%
succinate, NH_3, O_2	0.45	178	127
succinate, NH_3, O_2, NO_3^-	0.58	140	100
succinate, O_2, NO_3^-	0.49	105	75
acetate, NH_3, O_2	0.28	81	135
acetate, NH_3, O_2, NO_3^-	0.34	60	100
acetate, NH_3, NO_3^-	0.25	40	67

* 5 mM succinate or 10 mM acetate were provided. The inorganic nitrogen compounds were present in excess in all cultures. To facilitate comparison with the chemostat data, the yield on O_2, NH_3 and NO_3^- was assumed to be 100%. (Acetate data from (22)).

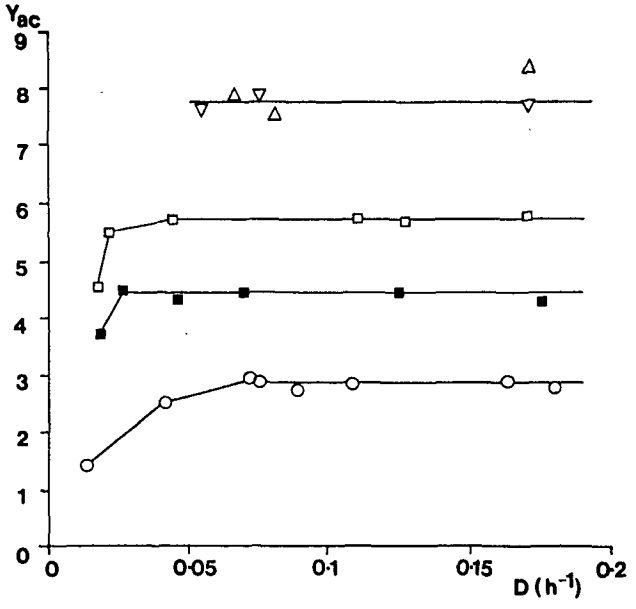

FIGURE 1. Protein yields (as grams per mol acetate, Yac) from acetate limited chemostat cultures of *T. pantotropha* and *P. denitrificans* as a function of the dilution rate (D) with different electron acceptors. For the cultural conditions and concentrations of the various components, see Materials and Methods. ○ = *T. pantotropha*, anaerobic, with nitrate. ■ = *T. pantotropha*, aerobic. □ = *T. pantotropha*, aerobic, with nitrate. △ = *P. denitrificans*, aerobic. ▽ = *P. denitrificans*, aerobic with nitrate.

The yields shown in Tables 3 and 4 were expressed as percentages of their respective O_2/NO_3^- cultures. In Table 4, the yields of cultures 1 - 3 were calculated relative to 1b as 100%, and the % yields of the mixotrophic cultures were based on 4b as 100%. If these percentages are compared, it can be seen that the anaerobic cultures were 30%, or more, lower than the aerobic cultures in all cases. In line with the observations made with the batch cultures (Table 3), the mixotrophic chemostat cultures with oxygen as the only electron acceptor (Table 4, 4a) were at least 20% higher. In addition, the cultures provided with 20 mM nitrite (Table 4, 3b) also showed a relatively high yield. In contrast, heterotrophic chemostat cultures whose sole electron acceptor was oxygen (Table 4, 1a) and the O_2/5 mM NO_2^- cultures (Table 4, 3a) gave relatively low yields.

TABLE 4. Comparison of yields from various chemostat cultures growing on a medium feed with 20 mM acetate at a dilution rate of 0.075 h^{-1}.

	energy and nitrogen source		electron acceptors *	yield ˣ (mg protein per litre)	percentageʸ of NO_3^-/ O_2 cultures
1	acetate + ammonia	a	O_2	81	79
		b	O_2/ NO_3^-	103	100
		c	NO_3^-	50	49
2	acetate + nitrate	a	O_2/NO_3^-	110	107
3	acetate + ammonia	a	O_2/ 5mM NO_2^-	76	74
		b	O_2/ 20mM NO_2^-	135	131
4	acetate + thiosulphate + ammonia	a	O_2	145	121
		b	O_2/ NO_3^-	120	100
		c	NO_3^-	69	58

ˣ Except in the anaerobic cultures, the dissolved oxygen concentration was maintained at 80% of air saturation.
ʸ The yields are shown as mg per litre to allow comparison with the mixotrophic cultures (which received 5 mM thiosulphate in addition to the 20 mM acetate).
* The percentages for the cultures in groups 1, 2 and 3 were calculated with reference to culture 1b, and those in group 4 are based on culture 4b.

Effect of dissolved oxygen concentration

As already mentioned at the beginning of this results section, denitrification by *T. pantotropha* seems to proceed at two different speeds, depending on whether the dissolved oxygen was above or below approximately 30% of air saturation. Some autotrophic nitrifiers perform less efficiently at reduced oxygen tensions (27), and it was therefore appropriate to determine the effect of the dissolved oxygen concentration on both nitrification and denitrification by *T. pantotropha*.

Until a "critical" dissolved oxygen concentration of around 25% of air was reached, both nitrification and denitrification increased with decreasing dissolved oxygen (Fig.2). At lower dissolved oxygen concentrations, both rates increased sharply in those cultures supplemented with nitrate. This is in agreement

with the trend in nitrate reduction rates found with "resting" cells and shown in Fig. 1. Carbon and nitrogen balances showed that at the lower concentrations of oxygen (<30%), the amount of denitrification in the nitrate-supplemented cultures (from nitrate supplied and nitrite produced from nitrification) was sufficient to have supplied all of the required electron acceptor for the cells. For example, it could be calculated from the carbon balance that a culture growing at a dissolved oxygen concentration of 5% air saturation dissimilated 16.3 mM acetate and would therefore have required 26.1 mM nitrate for its complete oxidation to CO_2 (assuming that N_2 was the end product of denitrification). The measured denitrification involved 27.0 mM nitrate (supplied) and 2.5 mM nitrite (generated from ammonia oxidation), which is equivalent to 17.8 mM acetate.

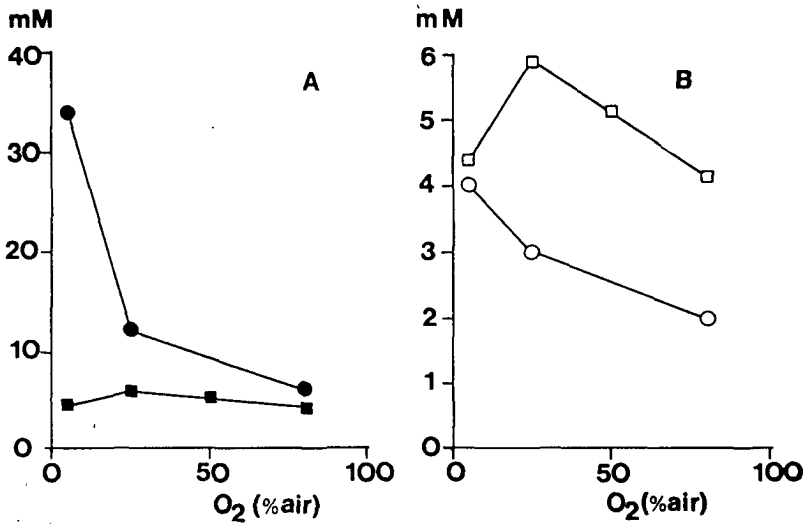

FIGURE 2. A: Total denitrification (i.e. from NO_3^- provided and NO_2^- produced by nitrification) and B: Nitrification by chemostat cultures of *T. pantotropha* as a function of the dissolved oxygen. The cultures were acetate-limited and were provided with ammonia as the nitrogen source. □,■ = NH_3 only, ○,● = NH_3 + NO_3^-.

The nitrification rate found in the ammonia/oxygen cultures fell at lower dissolved oxygen concentrations, and they appeared to become oxygen limited. The protein content of the biomass fell (from 87 mg/l at 50% air to 61 mg/l at 5% air), and PHB was made in large enough quantities to be seen under the phase contrast microscope. Its identity was confirmed spectrophotometrically (see Materials and Methods). Element analysis showed that the C:N ratios of PHB synthesizing cells had risen from 4.0 - 4.3 (found with other *T. pantotropha* cultures and with *P. denitrificans*) to 7.0 - 7.5, indicating that as much as half the dry weight of the biomass in these samples might be made up by the polymer. In general, PHB formation was found in those cultures in which nitrification was partially or wholly prevented (e.g. by low oxygen or the presence of hydroxylamine; Robertson & Kuenen,

1988) and which, in addition, were not supplied with nitrate or nitrite so that they could denitrify without first nitrifying. In the mixotrophic cultures, where the nitrification rates were among the lowest, PHB was not observed and the C:N ratio approached the theoretical 4.0.

DISCUSSION

This paper gives a phenomenological description of the environmental conditions which control nitrification and denitrification in *T. pantotropha*. In view of the controversy which surrounds phenomena such as heterotrophic nitrification and aerobic denitrification, it seemed appropriate to quantitatively document these processes even though they are not yet fully understood. It has been possible to formulate a working hypothesis which can be used to design experiments to establish a fuller understanding of these phemomena. This will be discussed below.

At μmax (i.e. in batch culture), the co-respiration of nitrate (or nitrite) and oxygen appears to confer a higher growth rate, but is paid for by the lower yield due to the fact that denitrification makes a significant contribution to the respiratory activity of the cell (Table 3; (20)). However, in chemostat culture, the yields obtained from the acetate or succinate-limited cultures which were co-respiring oxygen and nitrate or nitrite were higher than those obtained when oxygen was the sole electron acceptor, although lower than those from similar cultures of *Pa. denitrificans* (Table 4, Fig. 1). The following observations indicate that these unexpectedly low yields were associated with the degree of heterotrophic nitrification in *T. pantotropha*:

1: The presence of nitrate or nitrite (in "non-limiting" amounts) decreased the rate of heterotrophic nitrification in a culture, compared with the activity found in cultures where ammonia was the sole nitrogen source (Table 1, Table 2). In contrast to the ammonia/oxygen cultures with their higher nitrification rates, the protein yields from the nitrate-and nitrite containing cultures were not unexpectedly low.

2: The provision of thiosulphate in the culture medium also reduced the amount of nitrification taking place. The increase in protein yield found for the mixotrophic NH_4/O_2 cultures (Table 4) was far too high to be due only to energy derived from thiosulphate metabolism (e.g. an increase of 64 mg protein from only 5 mM thiosulphate). This implies that at least some of the yield increase is due to another factor, possibly the reduced nitrification.

3: The cultures provided with only 5mM nitrite had reduced all of it, and the ambient nitrite concentration was therefore 0. The nitrification rate in these cultures was as high, if not higher, than that found for the ammonia/oxygen cultures, and the yield was also low (Table 2, Table 4).

4: The aerobic cultures supplied with nitrate as their N-source rather than ammonia, and which were therefore denitrifying but not nitrifying, gave protein yields comparable with the ammonia/nitrate/oxygen cultures (Table 4).

Consideration of these four points clearly suggests that there is a relationship between nitrification rate and protein

yield, with the lowest nitrification rates coinciding with the highest yields. The factor which is common to those cultures yielding unexpectedly low amounts of protein is a relatively high rate of nitrification. This observation has been confirmed by experiments with other bacteria which are also capable of combined nitrification/denitrification (L.A. Robertson, R. Cornelisse & J.G. Kuenen, unpublished data).

There appears to be an inverse relationship between the dissolved oxygen concentration and the rates of nitrification (provided that oxygen is not limiting) and denitrification (Fig. 2). As pointed out in the results section, below a critical oxygen concentration (approx. 30% air saturation), all acetate respiration appeared to be proceeding via denitrification, and oxygen uptake was apparently being used exclusively for nitrification. This suggests that the respiratory and nitrifying systems compete for oxygen, with priority being given to nitrification. The rate of denitrification would thus be controlled by the outcome of this competition.

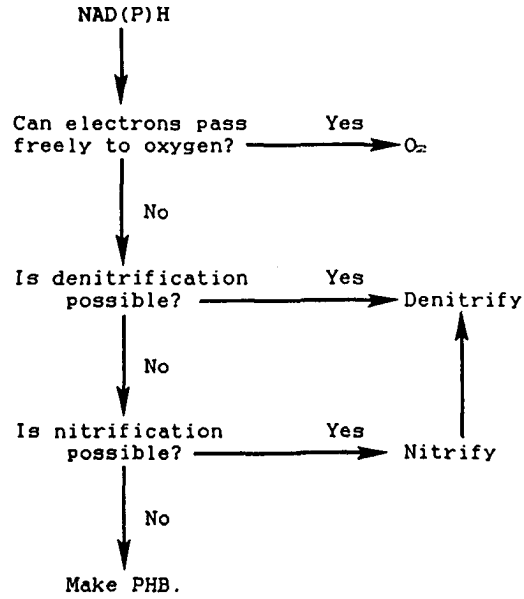

FIGURE 3. A flow chart to show, diagrammatically, the working hypothesis to explain the control of heterotrophic nitrification and denitrification in *T. pantotropha*.

On the basis of the increased growth rates shown by batch cultures given nitrate and oxygen when compared with those supplied with only one of the electron acceptors, it was suggested (20) that nitrate/oxygen co-respiration might be a mechanism by which a "bottleneck" in the electron transport chain between cytochrome c and cytochrome aa3 could be overcome by

allowing electrons to flow to the denitrifying enzymes as well as to oxygen. However, the results presented here indicate that the situation is somewhat more complex, possibly involving a branched cytochrome chain where the utilization of nitrate, nitrite or thiosulphate would permit the induction and/or use of additional cytochromes. This would allow *T. pantotropha* to overcome the postulated "bottleneck" by using more than one branch of the cytochrome chain at the same time. Preliminary results indicate that *T. pantotropha* has 3 - 4 different cytochromes c which vary depending on the growth conditions (Chapter 7). Figure 3 shows a flow chart which aims to provide a simplified model of this system as it would work for heterotrophic growth. In addition to causing redox problems in the cytochrome chain, the hypothetical "bottleneck", if not by-passed, could result in an insufficiency of NAD(P)H oxidizing power. Heterotrophic nitrification may provide a means of overcoming this. It is known that *in vitro* the ammonia oxygenase from *Tsa. pantotropha* can use NADPH (21a). The production of PHB by cells whose ammonia oxidation has been partially inhibited (eg by oxygen or hydroxylamine) would then also support the "bottleneck" hypothesis.

The high nitrification rate observed in the presence of 5 mM nitrite (Table 1, Table 2) requires further investigation. It is possible to speculate that the presence of a "limiting" amount of nitrite in the medium supply is sufficient to induce additional nitrite reductase formation, but not to saturate the enzyme. Nitrite would thus be reduced as fast as it was formed and any inhibitory effect on the nitrification caused by higher steady state nitrite levels would be lost.

TABLE 5A. Nitrification rates calculated from published results[a] from batch culture experiments.

Organism	Activity[b]	N-compound used	Reference
Ps. aeruginosa	12-28	hydroxamate	18
Ps. aeruginosa	70-90	hydroxylamine	18
Ps. denitrificans	2.6	pyruvic oxime	2
Ps. aureofaciens	2.8	pyruvic oxime	2
Al. faecalis	11.9	pyruvic oxime	2
Alcaligines sp.	33	pyruvic oxime	3
Arthrobacter sp.	0.8	ammonia	30
T. pantotropha	35.4	ammonia	28
N. europaea	50-100	ammonia	4
Nitrosomonas sp.	590-2300	ammonia	7

[a] For ease of comparison, where other nitrogen compounds were used the results have been recalculated as though for ammonia.
[b] The activity is expressed as nmol NH_4/min/mg dry weight.

In addition to the physiological implications for *T. pantotropha*, a second point which must be considered in the light of the results presented here is the ecological significance of heterotrophic nitrifiers. It has long been assumed that heterotrophic nitrification is too slow and generates insufficient nitrite or nitrate to be of major significance, except in situations where autotrophic nitrifiers cannot prosper (eg acid soils). Evidence that fungal nitrification might be of importance in forest soils has recently been presented (9), but while the efficiency of nitrification by heterotrophs was judged

by the amount of nitrogen oxides they accumulated, their activity was not considered to be significant. The discovery that some heterotrophic nitrifiers may appear to be "poor" nitrifiers because they reduce any nitrite or nitrate produced during nitrification directly to gaseous products rather than allowing them to accumulate, demands a re-examination of the nitrification potential of other heterotrophic species. Indeed, ammonium oxidation and nitrite reduction in aerobic cultures of *T. pantotropha* are normally so well-balanced that nitrite does not accumulate, and this species, therefore, was not recognised as a heterotrophic nitrifier until nitrogen balances were made for the cultures. Some published data was therefore used to recalculate nitrification rates for other heterotrophic nitrifiers on the basis of ammonium disappearance.

TABLE 5B. Nitrification rates calculated from published results from chemostat culture experiments[a].

Organism	Activity[b]	N-compound used	Reference[c]
T. pantotropha	6-47	ammonia	x
N. europaea	670-835	ammonia	4
N. europaea	130-1550	ammonia	24
N. europaea	1385-5290	ammonia	16
N. europaea	400-1020	ammonia	z

[a] For ease of comparison, where other nitrogen compounds were used the results have been recalculated as for ammonia.
[b] The activity is expressed as nmol NH_3/min/mg dry weight.
[c] x = This paper; z = E.W.J. van Niel, unpublished results.

When these are compared with the rates reported for the *Nitrosomonas* species (Tables 5a and 5b), it can be seen that in some cases the heterotrophic nitrification rates are only a factor ten lower than those for the autotrophs. When it is also considered that the growth rates of heterotrophs tend to be higher than those of the autotrophs (the μ^{max} for *N.europaea* is about 0.03-0.05 h^{-1}, and that of *T. pantotropha* can be as high as 0.4 h^{-1} under some growth conditions), and that heterotrophs tend to be present in many environments in considerably higher numbers, it is apparent that the ecological significance of heterotrophic nitrification requires reassessment. Experiments are now underway to determine which environmental conditions, if any, will favour nitrification by this new group of heterotrophs rather than by the autotrophic bacteria.

ACKNOWLEDGMENT.

The authors are grateful to Jan Koppejan, Annemiek Laanen, and Arjan de Wit for excellent experimental assistance, to GertJan Medema for providing some of the mixotrophic samples and to Hans van Dijken for his constructive comments on the manuscript.

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APPENDIX TO CHAPTER 5

The results from various chemostat experiments, while insufficient to merit a separate chapter, were considered pertinent to the central argument of this thesis. They have therefore been added as an appendix to Chapter 5, as this contains the bulk of the chemostat data.

All of the methods used for the experiments described in this appendix as the same as those outlined in the Materials and Methods of Chapter 5.

1: The effect of hydroxylamine, thiosulphate and allyl thiourea on heterotrophic nitrification.

Hydroxylamine

Hydroxylamine is an intermediate between ammonia and nitrite in the nitrifying pathway of *Tsa. pantotropha*. Moreover, ammonia oxidation is inhibited by the presence of hydroxylamine (Robertson & Kuenen, 1988; Chapter 5). Its effect on growth in batch and chemostat cultures was therefore tested. *Tsa. pantotropha* grew in batch cultures with hydroxylamine as the sole source of nitrogen (μ max = 0.3 h^{-1}) and with a combination of ammonia and hydroxylamine. However, it did not grow when nitrate and hydroxylamine were provided. 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}, Table 1). However, it must be assumed 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 nitrogen balances from these experiments indicated that the amount of ammonia which was being lost from the cultures was considerably lower than when ammonia was the sole nitrogen source. In common with cultures in which ammonia was the sole source of nitrogen, the concentration of ammonia oxidized increased, somewhat, as the dilution rate was increased. All of the hydroxylamine was being oxidized (Table 1).

TABLE 1. Nitrogen balances for acetate limited (20 mM) chemostat cultures of *Tsa. pantotropha* in a medium containing 7.5 mM NH_4Cl and varying amounts of hydroxylamine.

D h^{-1}	NH_2OH supplied mM	NH_3 used mM	NH_2OH used mM	NO_2^- accumulating mM	protein (g/mol) acetate
0.02	0.0	4.3	-	0	3.85
0.01	0.8	0.8	0.8	0.2	3.8
0.016	0.8	1.3	0.8	0.2	4.2
0.016	2.2	2.1	2.2	0.3	4.0
0.032	2.2	3.1	2.2	0.3	4.0
0.016	4.0	2.8	3.6	0.4	3.8

Hydroxylamine inhibits the ammonia oxygenase in *Tsa. pantotropha* (Robertson & Kuenen, 1988), and this inhibition is reflected in the nitrification rates based on ammonia

disappearance (Table 2). However, when the rates were recalculated on the basis of ammonia and hydroxylamine oxidation, together, they became comparable with those obtained from the NH_4/O_2 cultures (Table 2). It has already been pointed out that high nitrification rates appear to be associated with low yields in *Tsa. pantotropha* (Chapter 5), and the yields obtained with these hydroxylamine/ammonia cultures were also low (Table 1), compared with those obtained when nitrate was also present (100 mg/L; Chapter 5). In this case, the nitrification rate can be separated into ammonia and total oxidation rates. Because the ammonia oxidation rates were low in the presence of hydroxylamine, and most of the nitrification was due to hydroxylamine oxidation, it is unlikely that the low yields are associated with loss of reducing equivalents in the oxygenase reaction, but may be related to hydroxylamine oxidation, the step which generates energy for growth in the autotrophic nitrifiers. Alternatively, the loss may be due to the end product of nitrification, nitrite, which is known to inhibit cytochrome aa_3, the terminal oxidase to oxygen in these bacteria (Kučera & Dadák, 1983; Kučera et al., 1984). The reasons for this must await the elucidation of the cytochrome chain in *Tsa. pantotropha*.

TABLE 2. Nitrification rates obtained with acetate-limited chemostat cultures of *Tsa. pantotropha* provided with hydroxylamine. Nitrification is expressed as nmol NH_4 oxidized min^{-1} mg $protein^{-1}$ (ammonia) and nmol NH_4 and NH_2OH oxidized min^{-1} mg $protein^{-1}$ (total). NH_4 was present in all cultures. The rates were calculated from the data in Table 1.

hydroxylamine concentration (mM)	D h^{-1}	nitrification (ammonia) rate	nitrification (total) rate
0	0.017	12.7	12.7
0.8	0.010	1.9	3.7
0.8	0.016	4.2	6.8
2.2	0.016	6.8	14.0
2.2	0.032	20.9	35.4
4.0	0.016	9.8	22.5

Electron microscopy showed that hydroxylamine-grown cells were storing poly β-hydroxybutyrate (PHB), and that the amount in the cells increased as the hydroxylamine concentration was increased. This was confirmed by analysis (results not shown; see Chapter 5). Element analysis indicated that as much as 50% of the biomass grown on hydroxylamine was PHB. As the protein content of these cultures was not significantly different from that of culture supplied with ammonia as their sole source of carbon and energy, it is assumed that this PHB is a measure of the NAD(P)H which *Tsa. pantotropha* was unable to "dump" by means of the ammonia oxidation.

Another feature of these cultures was the appearance of mesosome-like structures. Thin sections of *Tsa. pantotropha* characteristically show loops of membrane which appear to be invaginations of the cytoplasmic membrane, and seem to be a form of "over-production" of the membrane loops. Although it remains possible that these structures are artefacts generated during sample preparation, they have been shown using two different staining techniques. Moreover, their appearance could be

consistently predicted to occur when the ammonia oxidation step of the nitrification pathway was in some way inhibited (e.g. by inclusion of hydroxylamine in the medium, or because of low oxygen concentrations (< 25% air saturation)). Similar structures have been found in *Escherichia coli*. These are known to contain elevated amounts of NADH oxidase (Greenawalt, 1974; Brunson et al., 1978; Wiegand et al., 1970). Should this also be the case for *Tsa. pantotropha*, their appearance would fit the hypothesis that heterotrophic nitrification is an "overflow" mechanism which is used to overcome a bottleneck in the flow of electrons to oxygen via cytochrome aa_3 (Robertson & Kuenen, 1984a; 1984b; Chapter 5a; Chapter 7).

Thiosulphate

Thiosulphate inhibits autotrophic nitrification (Sharma & Ahlert, 1977) and has also been found to inhibit nitrification in heterotrophically grown *Tsa. pantotropha* (Robertson & Kuenen, 1988). When *Tsa. pantotropha* was grown mixotrophically on acetate and a small amount of thiosulphate, it was noticed that the nitrification rates were much lower than those obtained with similar cultures which did not receive thiosulphate (Chapter 5). Nitrification was therefore checked in chemostat cultures receiving a range of acetate:thiosulphate ratios. It was found that the amount 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. At a ratio of 4.0:1, nitrification had ceased (H.J. Nanninga, unpublished data).

The effect of thiosulphate is complex. In cultures which do not have induced thiosulphate oxidizing enzymes, its effect must be simply that of an inhibitor, but once thiosulphate metabolism is induced other factors come into play. *Tsa. pantotropha* is known to have a branched cytochrome chain, and it is likely that the simultaneous use of autotrophic and heterotrophic modes of growth permits the use of different electron transport pathways (see Chapter 7).

Allylthiourea

Allylthiourea (ATU) is one of the classical inhibitors of autotrophic nitrification which is commonly used to test whether nitrification is due to heterotrophs or autotrophs, depending on whether or not inhibition takes place. As nitrification by *Tsa. pantotropha* shares a sensitivity to thiosulphate with the autotrophs, and since the nitrification enzymes present in *Tsa. pantotropha* appear to be similar to those of the autotrophs, ATU was tested for activity against nitrification by *Tsa. pantotropha*. Acetate-dependent oxygen uptake by resting cells in phosphate buffer and in the presence of 10µM 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 ATU, however, was only 90% of this (104 nmol- min^{-1} ml^{-1}). In the absence of an inhibitor, stimulation by NH_3 of around 120-140% could be expected from these cells (Robertson & Kuenen, 1988). It is therefore clear that ATU is not a specific inhibitor for autotrophic nitrification, but can affect at least one heterotroph. Possibly, the determining factor is whether the

inorganic or organic pathways of nitrification are in use (Robertson & Kuenen, 1988).

2: Growth with nitrate as the sole source of nitrogen.

TABLE 3. Nitrogen balances from *Tsa. pantotropha* cultures growing on acetate with nitrate (7.5 mM) as the sole source of nitrogen. $D = 0.04\ h^{-1}$. a = PHB synthesized; b = ammonia (7.5 mM) + nitrate (40 mM) cultures shown for comparison.

Dissolved oxygen (% air)	Nitrate lost (mM)	Nitrite formed (mM)	Protein (g mol^{-1} acetate)
80	1.3	0.03	5.9
50	0.8	0.05	4.3
33	1.8	0.45	3.6
23	6.3	0.02	2.7[a]
80	0.5	0.03	5.5
40	3.5	0.05	5.5
20	14.7	0.10	2.8
80[b]	13.4	0.02	5.2
0[b]	24.0	0.10	2.5

Chemostat cultures of *Tsa. pantotropha* in which nitrate was the sole source of nitrogen denitrified less than similar cultures in which ammonia was also present. Table 3 shows the nitrogen balances for cultures receiving nitrate at a concentration equivalent to the usual ammonia concentration in the medium (approx. 7.5 mM), and cultures receiving the usual amount of nitrate (approx. 40 mM).

Comparison of these figures with the levels of denitrification found when ammonia was also present showed that aerobic denitrification was lower when nitrate was the sole nitrogen source. As with the NH_3/ NO_3^- cultures, the amount of denitrification increased as the dissolved oxygen fell, to the extent that the "low nitrate" cultures grown below 30% air saturation became nitrogen limited and synthesized PHB.

3: Heterotrophic nitrification and aerobic denitrification in a mutant of *Tsa. pantotropha* which lacks a functional nitrite reductase.

Although it could reduce nitrate to nitrite, little or no gas was produced by *Tsa. pantotropha* TP43 during anaerobic respirometry experiments. This confirmed that nitrite reductase is either missing or inactive in this mutant (Chandra & Friedrich, 1986). Cultures were tested at the end of chemostat experiments in order to confirm that they had not reverted to the wild type.

Oxygen uptake by aerobically grown *Tsa. pantotropha* TP43 showed little stimulation by ammonia (103-106% of that without ammonia). This was in agreement with results from similarly grown *Tsa. pantotropha* (110%). The greatest amount of stimulation by ammonia has been found with anaerobically grown *Tsa. pantotropha* cells (Robertson & Kuenen, 1988) and *Tsa. pantotropha* TP43 does

not grow anaerobically. This small amount of stimulation was not, therefore, an indication of whether or not the nitrification pathway was operative. Nitrite was not found to accumulate in batch cultures of the mutant, and it was therefore assumed that the organism was not nitrifying. However, it was found that oxygen uptake by both the wild and mutant strains of *Tsa. pantotropha* was equally resistant to hydroxylamine, as compared with *Pa. denitrificans*. For example, when 1 mM hydroxylamine was used, the rate obtained with the wild type was 77% of that obtained with hydroxylamine-free cells. The equivalent rates for the mutant and *Pa. denitrificans* were 80% and 46%, respectively). It has been suggested that the greater sensitivity of *Pa. denitrificans* to hydroxylamine is due to its lack of hydroxylamine oxidoreductase (Robertson & Kuenen, 1988). The insensitivity of *Tsa. pantotropha* TP43 to hydroxylamine would therefore indicate that its hydroxylamine oxidizing enzyme, at least, was operative.

TABLE 4. Nitrogen balances and nitrification rates obtained with *Thiosphaera pantotropha* TP43 in acetate-limited chemostat culture. The *Tsa. pantotropha* wild type data which have been added for comparison are from Robertson et al., (1988). Nitrification rates are given as nmol min^{-1} mg $protein^{-1}$.

Dissolved O_2 (%)	D (h^{-1})	Y_{max} (g/mol)	Biomass N mM	N-loss mM	Nitrification rate
80	0.05	4.52	1.73	1.47	13.6
	0.07	4.53	1.90	1.03	13.3
	0.10	4.88	2.11	1.82	31.1
50	0.07	5.04	1.95	1.90	22.0
	0.10	5.32	2.29	2.77	43.4
30	0.06	5.31	1.70	3.07	30.1
	0.10	6.02	2.25	2.47	34.2
15	0.07	5.38	1.88	3.47	37.6
Thiosphaera pantotropha wild type					
80	0.05	4.05	1.60	4.63	43.3
	0.10	4.10	1.76	5.07	93.9

Nitrite inhibits heterotrophic nitrification in *Tsa. pantotropha* (Robertson & Kuenen, 1988; Chapter 5). It might therefore be expected that the failure of *Tsa. pantotropha* TP43 to reduce nitrite would result in the inactivation of the nitrification pathway, even if all of the nitrifying enzymes were present. To test this, *Tsa. pantotropha* TP43 was grown in the chemostat under acetate limitation, with ammonia as its sole source of nitrogen. The nitrogen balances for various dilution rates and dissolved oxygen concentrations are shown in Table 4. Ammonia was still disappearing from the culture in quantities greater than could be expected to be due to sparging at the dilution rates and pH used. It must therefore be concluded that the cultures were still nitrifying. However, despite the inability of *Tsa. pantotropha* TP43 to reduce nitrite to gaseous products anaerobically, little or no nitrite accumulated. Only trace amounts of hydroxylamine were occasionally detected. It is known that the hydroxylamine oxidoreductase of *Nitrosomonas* species can reduce equimolar amounts of NH_2OH and NO_2^-, producing

N_2O (Hooper, 1984). The hydroxylamine oxidoreductase of *Tsa. pantotropha* has been found to be similar to that of *Nitrosomonas europaea* in other respects (Robertson & Kuenen, 1988) and it may well be that it can also perform in this reductive fashion when necessary. The nitrification rate of the *Tsa. pantotropha* TP43 cultures increased with decreasing dissolved oxygen concentration, indicating that the reductive function of the enzyme was operating more efficiently when less oxygen was available.

The yields obtained from these *Tsa. pantotropha* TP43 cultures were higher than those obtained with similar cultures of the wild type *Tsa. pantotropha* (Chapter 5), although still lower than those obtained with *Pa. denitrificans*. As pointed out above, low yields of *Tsa. pantotropha* are associated with high nitrification rates, and particularly high hydroxylamine oxidation rates. The hypothetical oxidizing/reducing behaviour of the hydroxylamine oxidoreductase would mean that only half of the ammonia-N undergoing nitrification would be oxidized from hydroxylamine to nitrite, the remainder being directly reduced to gaseous products. Higher yield could therefore be expected, and their occurrence provides further evidence to support the hypothesis that *Tsa. pantotropha* TP43 remains able to denitrify despite its lack of nitrite reductase by utilizing the double function of its hydroxylamine oxidoreductase. *Tsa. pantotropha* TP43 cultures resembled those of the wild type in which ammonia oxidation had been blocked in that the bacteria contained PHB and mesosome-like structures.

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Journal of General Microbiology (1988), **134**, 857–863. *Printed in Great Britain*

Heterotrophic Nitrification in *Thiosphaera pantotropha*: Oxygen Uptake and Enzyme Studies

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(*Received 22 July 1987; revised 7 December 1987*)

Thiosphaera pantotropha is a heterotrophic nitrifying bacterium which reduces nitrite produced from ammonia to nitrogen gas, regardless of the ambient dissolved O_2 concentration. Under certain growth conditions, nitrous oxide may be produced. The ammonia oxygenase showed a number of similarities with that of autotrophic nitrifiers [e.g. light sensitivity, Mg^{2+} requirement, NAD(P)H utilization], as did the hydroxylamine oxidoreductase (cytochrome *c* oxidation, hydrazine inhibition). However, there were also differences (e.g. hydroxylamine inhibition of ammonia oxidation) and this apparent similarity may be superficial. Control experiments with a strain of *Paracoccus denitrificans* (which does not nitrify) did not show the presence of either enzyme.

INTRODUCTION

Heterotrophic nitrification is still a poorly understood microbiological process. Killham (1986) has shown that in fungi the oxidation of reduced nitrogen compounds proceeds, at least in part, via organic compounds. However, Verstraete (1975) reported that in some heterotrophic bacteria, the oxidation of ammonia and related compounds to nitrite is a bypass of a pathway for the synthesis of chelating agents when iron is limiting. Many denitrifying bacteria also nitrify heterotrophically (Castignetti & Hollocher, 1984). *Thiosphaera pantotropha* is a heterotrophic nitrifying bacterium which co-respires nitrate and oxygen according to the scheme shown in Fig. 1 (Robertson & Kuenen, 1983, 1984*a*, *b*). Implicit in this scheme is the concept that this bacterium actively converts ammonia to nitrogen gas under aerobic conditions without the accumulation of intermediates such as hydroxylamine or nitrite. Thus, in contrast to other heterotrophic nitrifiers, organisms of this type cannot be recognized by nitrite accumulation in the culture medium. Current studies in this laboratory have demonstrated that heterotrophic nitrification and 'aerobic denitrification' (co-respiration of nitrate or nitrite and oxygen) are not restricted to *T. pantotropha*, but occur also in a hitherto unrecognized group of heterotrophic nitrifiers which do not accumulate nitrite. To appreciate the importance of these bacteria in the natural environment and waste-water treatment systems, it is essential to understand the mechanisms by which the two processes occur. This paper describes experiments performed to reveal the nature of the enzymes and pathway involved in heterotrophic nitrification in *T. pantotropha*. *Paracoccus denitrificans* was included for comparison as it does not nitrify and only denitrifies under anaerobic conditions (Alefounder *et al.*, 1985; L. A. Robertson, E. W. J. van Niel, R. Torremans & J. G. Kuenen, unpublished results).

METHODS

Organisms and growth. Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide-oxidizing waste-water treatment system (Robertson & Kuenen, 1983). *Paracoccus denitrificans* LMD 22.21 was obtained from the Delft Culture Collection, and is the strain isolated by Beijerinck (1910).

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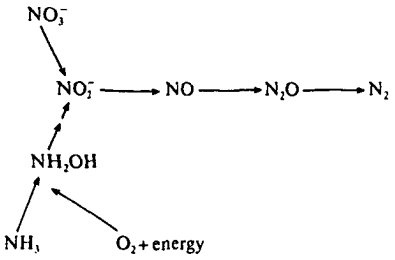

Fig. 1. Schematic representation of the interlinking pathways of heterotrophic nitrification and denitrification as they probably occur in *T. pantotropha*.

The batch culture medium (Robertson & Kuenen, 1983) contained (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; and 2 ml trace element solution l^{-1} (Vishniac & Santer, 1957). The medium supplied to the chemostat 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 trace element solution. Acetate (sodium salt; 10 mM and 20 mM) was supplied as substrate to batch and chemostat cultures, respectively.

Mixotrophic cultures were grown in the chemostat with 20 mM-acetate and 5 mM-sodium thiosulphate. KNO_3 (40 mM) was added in some experiments. Batch cultures were grown in Kluyver flasks (culture vol., 1 l; headspace, 1 l) (Robertson & Kuenen, 1984*b*) which incorporated an oxygen electrode. When needed, cells were grown anaerobically by sparging either with Ar or with O_2-free nitrogen. *T. pantotropha* was grown at 37 °C and *P. denitrificans* at 30 °C. Continuous cultures were grown in chemostats (culture vol., 1·5 l; headspace, 1 l) fitted with dissolved O_2 and pH control. The temperature was maintained at 30 or 37 °C, the dissolved oxygen at 80% air saturation and the pH at 8·0. The dilution rates ranged between 0·04 and 0·06 h^{-1}.

Preparation of cell extracts. Biomass for the preparation of extracts was harvested from the chemostats by centrifugation (40000 *g*, 20 min), washed twice in 0·05 M-phosphate buffer (pH 8·0) and frozen as pellets at −20 °C. Immediately before use, the cells were disrupted ultrasonically in the presence of 50% (w/v) Ballotini beads (diameter 0·11 mm) and then centrifuged (10000 *g*, 5 min) to remove remaining whole cells and debris. During and after disruption, the extracts were kept at 0–4 °C in the dark.

Buffers. The HEPES buffer used during experiments with the extracts contained 100 mM-HEPES, 15 mM-$MgCl_2$ and 4 mM-EDTA. The pH was adjusted to 7·0 with KOH. A 0·05 M-potassium phosphate buffer, pH 8·0, was used as specified.

O_2 uptake experiments. O_2 uptake was measured using a Clark-type electrode mounted in a thermostatically controlled cell which was closed except for a small hole through which additions could be made. Nitrous oxide was detected by the oxygen electrode under anaerobic conditions created by the addition of baker's yeast (Kucera *et al.*, 1984). The effect of thiosulphate on O_2 uptake was determined in heterotrophically grown cultures which were not induced for thiosulphate oxidation. In all cases, the accuracy of the results was better than ±5%.

Chemical analyses. Protein was measured by the micro-biuret method (Goa, 1953). Nitrate was measured with the Griess-Romijn reagent (Griess-Romijn van Eck, 1966). Ammonia was determined by following the oxidation of NADH in the presence of 2-oxoglutarate and L-glutamate dehydrogenase using a test kit (Sigma). Since both ammonia and ammonium would be present at the pH values used, the term ammonia will be used to cover both the protonated and unprotonated forms. Hydroxylamine was determined as described by Frear & Burrell (1955).

RESULTS AND DISCUSSION

Ammonia disappearance

Nitrogen balances for aerobic chemostat cultures showed that ammonia was being lost. Since control experiments in an uninoculated chemostat indicated that the maximum ammonia loss due to sparging was less than 0·006 μmol l^{-1} h^{-1} this disappearance was assumed to be a consequence of nitrification/denitrification. Up to 71 μmol ammonia l^{-1} h^{-1} was lost from cultures grown with acetate and ammonia. In the presence of nitrate (20 mM in the influent), nitrite (10 or 20 mM), thiosulphate (5 mM), thiosulphate and nitrate (5 and 20 mM, respectively) and hydroxylamine (1–4 mM), 25, 65, 41, 16 and 33 μmol ammonia l^{-1} h^{-1}, respectively, was nitrified and subsequently denitrified. These losses represent *in vivo* specific activities ranging from 5–43 nmol ammonia oxidized min^{-1} (mg protein)$^{-1}$.

Heterotrophic nitrification in T. pantotropha

Table 1. *Effect of pH on ammonia-dependent O_2 uptake by cell-free extracts of T. pantotropha*

Extracts in HEPES buffer were incubated with 5 mM-Mg^{2+} and 0·63 mM-NADPH. The cells had been grown in an acetate-limited chemostat at a dissolved O_2 level of 40% air saturation, with ammonia as the sole source of nitrogen.

	O_2 uptake rate [nmol (mg protein)$^{-1}$ min^{-1}]	
pH	5 mM-NH_4^+	10 mM-NH_4^+
8·7	1·9	2·5
8·7*	0	0
9·0	2·9	3·4
10·5	0·5	0·9

* Inactivated extract.

Ammonia oxygenase

Ammonia oxidation, measured as O_2 uptake, could be demonstrated in cell-free extracts of *T. pantotropha* in the presence of NADPH and Mg^{2+} provided that light was excluded. Trace amounts of hydroxylamine (< 3 μM), the expected product of this oxidation, appeared during the first few minutes of the experiment. However, the reaction stopped before the hydroxylamine concentration reached a level at which it could be accurately estimated (about 5 μM). Initially, it appeared that ammonia oxidation was optimal at pH values > 10, but this was shown to be an artefact due to the masking effect of NADPH oxidase. When this was taken into account, the highest rates were recorded at pH 9·0 (Table 1). The reaction rate increased with the concentration of NADPH [e.g. at pH 10·5, with 5 mM-NH_4^+, 0·3 mM or 1·0 mM-NADPH gave O_2 uptake rates of 0·2 and 1·0 nmol min^{-1} (mg protein)$^{-1}$, respectively]. Doubling the amount of extract doubled the rate of O_2 uptake. Boiled extract, and cell extracts which had been exposed to normal laboratory lighting for several hours, did not give a reaction and little or no activity was detected if Mg^{2+} was omitted. These data indicate that it was unlikely that ammonium ions were acting as an uncoupling agent of NAD(P)H respiration by membrane vesicles in the cell-free extracts. At pH 8·7 and pH 10·0, NADH-dependent activity could not be detected using 5 mM-NH_4^+. When 10 mM-NH_4^+ was used, a very low level of activity [0·14 nmol O_2 (mg protein)$^{-1}$ min^{-1}] was detected. At pH 10·5, NADH-dependent activity was approximately 50% of the NADPH-dependent activity (with 0·63 mM-NADPH). We were unable to block the NADH oxidase activity (e.g. with cyanide) without also affecting the ammonia oxidation rate, except by raising the pH. Inhibitors of NADH oxidase such as cyanide also inhibit the cytochrome activity of the cytochrome chain and, since nitrification is probably linked to the cytochrome chain, nitrification will also be inhibited. NADH oxidase has a pH optimum around 7·0, and becomes less active at higher pH values. Raising the pH therefore appears to be the only way to selectively inhibit the NADH oxidase without affecting nitrification. At low NADH concentrations, the NADH oxidase activity is lower and does not mask completely the effect of ammonia oxidation. Therefore it was possible to work only with NADH concentrations (0·31 mM) equivalent to the lower concentrations of NADPH used.

Hooper (1981) reviewed the enzymology of ammonia oxidation by chemolithotrophic ammonia-oxidizing bacteria and reported that the ammonia oxygenase requires activation by several factors including Mg^{2+}, and is light sensitive. The rates of ammonia oxidation recorded with cell extracts are generally insufficient to explain the *in vivo* rates, and this was attributed to the complexity of the enzyme system involved (Hooper, 1981). Ammonia oxidation by extracts of *Nitrosomonas europaea* was stimulated by NADH [22 nmol O_2 consumed min^{-1} (mg protein)$^{-1}$; the value was 0 with NH_4^+ alone], whereas whole cells of *N. europaea* oxidized 100–200 (batch-grown cells) to 260–10000 (chemostat-grown cells) nmol ammonia min^{-1} (mg protein)$^{-1}$ (Suzuki *et al.*, 1976), showing that rates recorded with cell-free extracts are at least a factor of 10 too low. The ammonia oxygenase of *T. pantotropha* is similar in some respects [e.g. light sensitivity, use of NAD(P)H] and, in addition, the O_2 uptake rates *in vitro* were 10–20 times

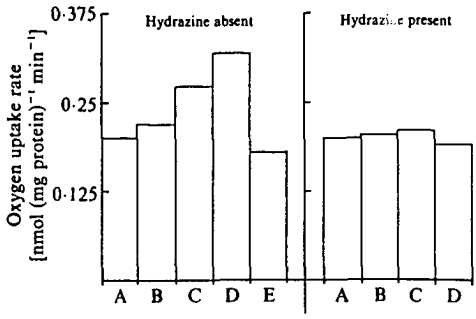

Fig. 2. Oxidation of hydroxylamine (as measured by O_2 uptake) by extracts of *T. pantotropha* in HEPES buffer, in the absence and presence of 3 mM-hydroxylamine. An experiment where hydroxylamine was added before cytochrome *c* gave essentially similar results. A, endogenous; B, 1·5 μM-cytochrome *c* added; C, 0·05 mM-hydroxylamine added; D, a further 0·05 mM-hydroxylamine added; E, 0·25 mM-nitrite added.

lower than those in whole cells grown in chemostat culture (L. A. Robertson, E. W. J. van Niel, R. Torremans & J. G. Kuenen, unpublished results). However, ammonia oxidation by *N. europaea* extracts was also stimulated by hydroxylamine [27 nmol min^{-1} (mg protein)$^{-1}$; the value was 0 with NH_4^+ alone], and Mg^{2+} could replace NADH (Suzuki *et al.*, 1976). The apparent inhibition of ammonia oxidation by extracts of *T. pantotropha* after a small amount of hydroxylamine had accumulated indicates that hydroxylamine is inhibitory and implies a greater sensitivity to hydroxylamine than that reported for *N. europaea* (Dua *et al.*, 1979). When hydroxylamine was added to the reaction mixture after ammonia oxidation had started, there was an increase in the O_2 uptake rate, but the rate was the same as that obtained without cell extract. This apparent stimulation must therefore be chemical in nature (see below). The inhibition of ammonia oxidation in *T. pantotropha* by hydroxylamine has been confirmed in chemostat cultures which were supplied with both ammonia and hydroxylamine (L. A. Robertson, E. W. J. van Niel, R. Torremans & J. G. Kuenen, unpublished results). It is possible that the *in vitro* assay for ammonia oxygenase in *T. pantotropha* worked best at alkaline pH because hydroxylamine is less stable at these pH values (see below) and thus might be kept below the critical inhibitory concentration. An alternative hypothesis is that free NH_3 is required by the enzyme and is more available in alkaline reaction mixtures, as has been reported for the autotrophs (see, e.g., Suzuki *et al.*, 1981).

Chemical oxidation of hydroxylamine

Since hydroxylamine was apparently an intermediate of ammonia oxidation to nitrite by *T. pantotropha*, its oxidation was studied using whole cells and cell extracts. Although hydroxylamine was stable in the reaction mixture up to pH 8·5, at pH 10·0, there was a significant amount of non-biological hydroxylamine-dependent O_2 uptake (7–9 nmol min^{-1} ml^{-1}), and nitrite was formed (1–2 nmol min^{-1} ml^{-1}). Similar results were obtained with and without boiled cell extract and with various concentrations of NADPH.

Hydroxylamine oxidoreductase

Biological hydroxylamine-dependent O_2 uptake could be shown at pH 7·0–7·5 with cell-free extracts in the presence of 1·5 μM-cytochrome *c* (Fig. 2) provided that HEPES buffer was used. Little or no activity was found using phosphate buffer. Hydrazine (3, 6 and 12 mM) inhibited hydroxylamine-dependent O_2 uptake, as in *N. europaea* (Dua *et al.*, 1979), although endogenous hydroxylamine-independent O_2 uptake was not affected by the concentrations of hydrazine used. Nitrite was also inhibitory (Fig. 2). Whole cells oxidized hydroxylamine slowly. Hydroxylamine oxidation rates were stimulated by the redox mediator phenazine methosulphate (0·075 μM). Again, it was necessary to use HEPES buffer. The low activity obtained with phosphate buffer was similar to that found with killed cells in both buffers and

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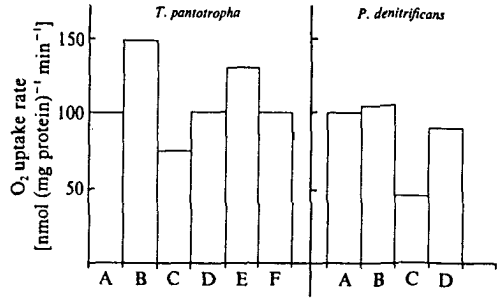

Fig. 3. Effect of NH_4^+, hydroxylamine and nitrite on the rate of acetate-dependent O_2 uptake by washed cells of *T. pantotropha* and *P. denitrificans*. The effect of thiosulphate on *T. pantotropha* is also shown. The cultures had been grown, anaerobically, to the end of the exponential phase in batch culture with acetate as the carbon and energy source and nitrate as the electron acceptor. A, control; B, 7·5 mM-NH_4^+; C, 1·0 mM-hydroxylamine; D, 5·0 mM-nitrite; E, 7·5 mM-NH_4^+ + 5 mM-thiosulphate; F, 7·5 mM-NH_4^+ + 10 mM-thiosulphate.

was presumably due to chemical oxidation reactions. Nitrite was produced from hydroxylamine by cell extracts and whole cells. Nitrous oxide production was only observed when nitrite and hydroxylamine were supplied simultaneously. A similar enzyme activity was observed in extracts of *Arthrobacter globiformis*, and the enzyme responsible was purified (Kurokawa *et al.*, 1985). Activity was measured by following cytochrome *c* reduction in Tris buffer; the optimum pH for hydroxylamine oxidation was 9·0, the reaction required iron and was inhibited by EDTA.

Using the same buffers, pH values and substrate concentrations, we found no evidence for the presence of either ammonia oxygenase or hydroxylamine oxidoreductase in *P. denitrificans*.

O_2 uptake experiments

To characterize further the process of heterotrophic nitrification and to investigate possible influences of growth conditions, the effect of ammonia on the maximum rate of O_2 uptake by *T. pantotropha* and *P. denitrificans* was compared. Both species were grown on acetate or succinate under various regimes. The cells were then harvested, washed and resuspended in phosphate buffer. Since hydroxylamine and nitrite were an intermediate and end-product, respectively, in heterotrophic nitrification by *T. pantotropha*, they were included in the respiration experiments. Similar results were obtained with acetate and succinate, and therefore only the acetate data are shown. It was anticipated that ammonia would stimulate O_2 uptake by *T. pantotropha*, but not by *P. denitrificans*, and that *T. pantotropha*, as a nitrifier, should be able to oxidize hydroxylamine under aerobic conditions and thus might suffer less from its toxic effects than *P. denitrificans*. The responses of anaerobic, batch-grown cultures of *T. pantotropha* and *P. denitrificans* were indeed very different (Fig. 3). Ammonia stimulated the rate of acetate-dependent O_2 uptake by *T. pantotropha* (149% of that without ammonia), whereas *P. denitrificans* was only slightly stimulated (5%). The presence of nitrite in the *T. pantotropha* reaction mixture was shown qualitatively. Both cultures were, to some extent, inhibited by hydroxylamine, but *P. denitrificans* was most sensitive. Nitrite alone had little effect on either culture. Ammonia stimulated the O_2 uptake rate of *T. pantotropha*, but not of *P. denitrificans* (Table 2). Furthermore, all *T. pantotropha* cultures, irrespective of growth conditions, were affected by ammonia, implying that at least the ammonia oxygenase activity is constitutive. The increased stimulation of the O_2 uptake rate of cells which were grown without being able to nitrify (i.e. without O_2 or without ammonia, Table 2) remains, as yet, unexplained. The relative rate of O_2 uptake (110%) by cells grown in the presence of O_2 and ammonia (and thus actively nitrifying during growth) increased to between 140 and 150% after 3 d starvation in nitrogen-free medium (although the O_2 uptake rates with acetate alone were little changed), whereas starvation in the

Table 2. *Effect of ammonia and nitrite on the maximum acetate-dependent rate of O_2 uptake by whole cells of T. pantotropha and P. denitrificans*

Cells were grown aerobically or anaerobically in batch culture with acetate as carbon and energy source, and harvested at the end of the exponential phase. For ease of comparison, the results are shown as a percentage of the O_2 uptake rate in phosphate buffer alone. As the experiments were done at pH 8·0, NH_3 should be considered to represent a mixture of ammonia and ammonium. The values for O_2 uptake [nmol (mg protein)$^{-1}$ min^{-1}] equivalent to 100% were as follows. *T. pantotropha* aerobically grown cells, 240–250; anaerobic cells, 380. *P. denitrificans*: aerobic cells, 140–160; anaerobic cells, 280.

Growth conditions	Compound added: NH_3 (10 mM)	NH_2OH (1 mM)	NO_3^- (10 mM)
T. pantotropha			
NH_3/O_2	110	85	108
NH_3/NO_3^-	149	75	105
NO_3^-/O_2	142	75	90
P. denitrificans			
NH_3/O_2	105	48	92
NH_3/NO_3^-	105	50	105

Table 3. *Effect of increasing hydroxylamine concentration on the rate of O_2 uptake by whole cells of T. pantotropha*

Cells were grown aerobically with nitrate or ammonium as the sole source of nitrogen. The value for O_2 uptake equivalent to 100% was 240–250 nmol (mg protein)$^{-1}$ min^{-1}. ND, Not determined.

NH_2OH concn (μM)	O_2 uptake (percentage of rate in NH_2OH-free sample): NO_3^-	NH_3
0	100	100
1	85	100
3	75	ND
5	70	ND
8	ND	75
12	ND	70
16	ND	65
24	ND	60

presence of ammonia did not affect ammonia-stimulated respiration. This might indicate that cells which had been grown and/or maintained in the presence of ammonia contained reserves of ammonia or other metabolic intermediates.

The greater sensitivity of *P. denitrificans* to hydroxylamine (Fig. 3), indicated that *T. pantotropha*, but not *P. denitrificans*, has a mechanism for detoxifying this compound. That this resistance was increased by active nitrification during the growth of the cells (implying the presence of higher concentrations of a functional hydroxylamine oxidoreductase) was also indicated by the observation that cells grown solely on ammonia/O_2 and thus nitrifying the most, were far less sensitive than those grown on nitrate (Table 3).

Since reduced sulphur compounds inhibit nitrification in some autotrophs (Sharma & Ahlert, 1977; Heijnen, 1984), the effect of thiosulphate was tested on heterotrophically grown cultures of *T. pantotropha* and *P. denitrificans*. With *T. pantotropha*, ammonia had less effect on the rate of O_2 uptake as the thiosulphate concentration increased (Fig. 3). Thiosulphate had no effect on O_2 uptake by *T. pantotropha* or *P. denitrificans* in the absence of ammonia (results not shown). These results are in agreement with the lower amounts of ammonia nitrified in mixotrophically grown (acetate/thiosulphate) chemostat cultures (L. A. Robertson, E. W. J. van Niel, R. Torremans & J. G. Kuenen, unpublished results).

In conclusion, the results suggest that the enzymes of nitrification in *T. pantotropha* resemble those of the chemolithotrophic nitrifiers only in some respects. There is also some similarity to the NADH-dependent oxidation of ammonia and cytochrome-*c*-linked oxidation of hydroxylamine by extracts of *Methylococcus* species reported by Dalton (1977) and Drozd *et al.* (1978). This similarity is probably superficial. The enzyme involved in ammonia oxidation by *M. capsulatus* is methane monooxygenase, and *T. pantotropha* can neither oxidize nor grow on methane (Robertson & Kuenen, 1983). Current studies on the cytochrome chain of *T. pantotropha* may explain why the autotrophs generate sufficient energy for growth from the reaction whereas *T. pantotropha* cannot.

The authors are grateful to Jaap Twisk and Rob Torremans for excellent experimental assistance, and to Dr Paul Wood and Ir Hans Frank for their constructive comments on the manuscript.

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Electron transport during denitrification, nitrification and oxygen respiration by *Thiosphaera pantotropha*: A working model.

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ABSTRACT

A model which attempts to describe electron transport in *Thiosphaera pantotropha* during aerobic denitrification and heterotrophic nitrification has been developed on the basis of cytochrome spectra and physiological data. Various results indicate that *T. pantotropha* has an electron transport which is basically similar to that described for *Paracoccus denitrificans* in that it includes cytochromes b, c, aa_3 and o. However, there appears to be a "bottleneck" in the flow of electrons via cytochrome aa_3 to oxygen. Another essential difference between *T. pantotropha* and P. denitrificans lies in the induction and probable location of cytochrome o in the membrane. It appears that electrons from both heterotrophic nitrification and thiosulphate oxidation pass to oxygen via cytochrome o. In addition, a correlation has been found between the possession of the copper-containing nitrite reductase and the capacity for aerobic denitrification.

INTRODUCTION.

Aerobic denitrification is a phenomenon which occurs in some species (e.g. *Thiosphaera pantotropha*, "*Pseudomonas denitrificans*"), but not in others (e.g. *Paracoccus denitrificans*) (Robertson & Kuenen, 1984A; 1984B; 1988; Kuenen & Robertson, 1987; Stouthamer, 1988; Kučera et al., 1984; Alefounder et al., 1981). Moreover, all of the aerobic denitrifiers so far tested have also been heterotrophic nitrifiers, and the two phemomena appear to be inextricably linked in these organisms (Kuenen & Robertson, 1987). The enzymes of heterotrophic nitrification in *T. pantotropha* are, at least superficially, similar to those of the autotrophic ammonia oxidizers, but the reaction does not yield energy for growth, as it does in the autotrophs. Cultures displaying a high nitrification rate, in fact, give protein yields much lower (approx. 60%) than expected (Robertson & Kuenen, 1988; Robertson et al., 1988).

It has been suggested that the simultaneous utilization of oxygen and nitrate as terminal electron acceptors allows *T. pantotropha* to overcome a "bottleneck" in the flow of electrons to oxygen via cytochrome aa_3 (Robertson & Kuenen, 1984A; Robertson et al., 1988). In addition, it seemed likely that the apparent lack of energy generation by nitrification in *T. pantotropha* could also only be explained by the specific properties and/or location of the cytochromes involved in this reaction. It was therefore decided to investigate the electron transport chain of this organism.

This paper describes the first results from the study of the cytochromes of *T. pantotropha*, and postulates a model which aims

to explain the various pathways of electron flow under different growth conditions, and the consequences for the (energy) metabolism of the organism. As with all of the other experiments in this series investigating aerobic denitrification and heterotrophic nitrification in *T. pantotropha*, *P. denitrificans* has been used as a reference organism because it only denitrifies under essentially anaerobic conditions, and does not nitrify to any significant extent.

MATERIALS AND METHODS

Organisms

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide oxidizing wastewater treatment system (Robertson & Kuenen, 1983). *Paracoccus denitrificans* LMD 22.21 was obtained from the Delft Culture Collection, and is the strain isolated by Beijerinck (Beijerinck, 1910). The growth and analysis of the biomass used in these experiments, together with medium analyses and element balances, are described elsewhere (Robertson et al., 1988).

Continuous Cultures

Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 37 C (*T. pantotropha*) or 30 C (*P. denitrificans*) and the pH at 8.0.

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. 20 mM acetate was supplied as substrate unless otherwise stated. Thiosulphate, when used, was supplied at the concentrations shown in the text. Unless otherwise stated, when KNO_3 and KNO_2 were used their concentrations were 40 mM and 20 mM, respectively.

The trace element solution (Vishniac & Santer, 1957) 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.

Respiratory measurements

Oxygen uptake was measured using a Clark-type electrode mounted in a thermostatically controlled cell which is closed except for a small hole through which additions may be made.

Gas production from denitrification was measured with standard manometric techniques.

Cytochrome spectra

Cytochrome spectra were recorded at room temperature and at 77 K in a DW-2a spectrophotometer (American Instruments Co.) equipped with a magnetic stirrer. Gas inputs allowed the cell suspensions to be kept under argon or CO atmospheres. For spectrum deconvolution and other data handling, the methods and software described by van Wielink et al. (1983) were used. To release CO-liganding, flashes of light from a photographic flashlight (Braun) were used.

Separation of soluble cytochromes.

Cells were disrupted ultrasonically, or by means of the french press. Membrane fragments were removed from crude supernatants (obtained by centrifuging at 10,000 g for 5 minutes) by precipitation with 0.5 M ammonium sulphate. The cytochrome content of the supernatant was then concentrated on a phenyl-sepharose column in the presence of 1.5 M $(NH_4)_2SO_4$ and eluted with 0.05 M phosphate buffer, pH 7.0.

The various soluble cytochromes were separated with a 30

minute (0.5 ml/min) linear ammonium sulphate gradient on a FPLC phenyl-sepharose column (Pharmacia S/S). The eluting peaks were monitored with a HP 1040A photo-diode array detector (Hewlett-Packard) which permits the aquisition of full spectra during elution. Fractions were collected, and their reduced (dithionite) spectra measured in a HP 8452A spectrophotometer (Hewlett-Packard).

Miscellaneous

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.

RESULTS AND DISCUSSION

The results presented here are essentially qualitative. However, by correlating changes in the cytochrome content of cells with their growth conditions and physiological behaviour, it has been possible to construct a working model which aims to describe electron transport within *T. pantotropha*, and will promote the design of further experiments.

First the results on which the model is based will be presented and discussed. The model will then be described and, finally, the correlation between the observed behaviour of *T. pantotropha* and that predicted from the model will be discussed.

a: Experimental results

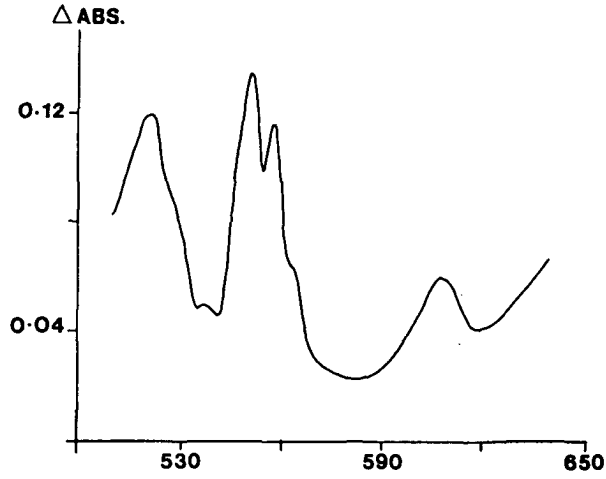

FIGURE 1. Scan from 500 to 650 nm of reduced (argon + dithionite), frozen (77 K) *T. pantotropha* which had been grown at 80% air saturation in an acetate-limited chemostat with NH_4^+ as the sole nitrogen source.

The presence of cytochromes b, c and aa_3 in whole *T. pantotropha* cells could be shown by scanning reduced (dithionite/argon atmosphere) cell suspensions at 77K (Figure 1). Deconvolution of these spectra showed that peaks due to at least two b-type (peaks at 557 and 563 nm) and two c-type (peaks at 546 and 551 nm) cytochromes were present (Figure 2). The

relative contribution of each of these peaks to the spectrum varied with the growth conditions, but they were all present in all samples. Possession of multiple cytochromes b and c is, of course, not unusual among bacteria (e.g. Stouthamer, 1980; van Verseveld & Bosma, 1987). Even anaerobically grown cells (cultivated under H_2 in the presence of a catalyst to ensure strict anaerobiosis) contained the terminal oxidase, cytochrome aa3 (peak at 605 nm). However, as with anaerobically grown *P. denitrificans*, it was present in considerably lower (<20%) amounts than in aerobically grown cultures.

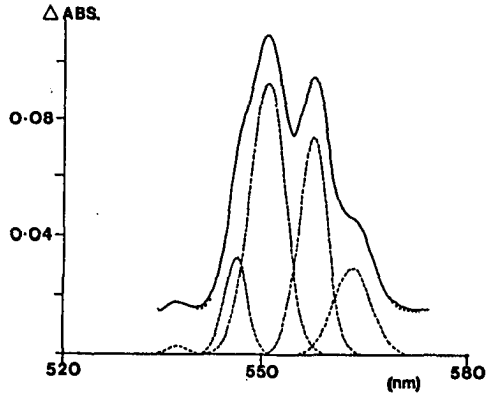

FIGURE 2. Deconvolution of the α band from the spectrum shown in figure 1. The solid line shows the actual spectrum, the broken line shows the calculated peaks, and the dots show where the calculated spectrum does not fit the actual spectrum.

The presence of a second terminal oxidase, cytochrome o, was revealed by the use of CO. As can be seen in Figure 3a, the shape of the reduced cytochrome b peak at 563 nm changed when CO was added, indicating liganding with the CO. CO-argon reduced difference spectra (Figure 3). CO-argon difference spectra clearly showed the presence of the peaks (at 418, 539 and 571 nm) and trough (at 557 nm) attributed to cytochrome o (Figure 3B). Also visible were the peaks (at 430 and 593) and troughs (at 445 and 605 nm) attributed to cytochrome aa_3.

Further confirmation of the presence of both cytochromes was obtained by making CO-argon reduced difference spectra at 77 K and then exposing the frozen samples to flashes of light in order to partially release the ligand (Figure 4). The peaks and troughs then disappeared and the difference spectrum flattened out (Figure 4, dotted line). Spectra made using membrane vesicles with and without CO showed that, as with other species, cytochrome o is membrane bound in *T. pantotropha*. *P. denitrificans* seems to depend most on cytochrome o when growing on rich media or at low oxygen tensions (i.e. when its cytochrome chain could be expected to be fairly reduced) and little is produced when this species is cultured in aerobic, mineral media (Cox et al., 1978). It is therefore worthy of note that it was the *T. pantotropha* cells grown at the higher oxygen concentration

with ammonia as the sole nitrogen compound present which contained most cytochrome o, and it was barely detectable in the cells grown with ammonia and nitrite at the lower oxygen tension.

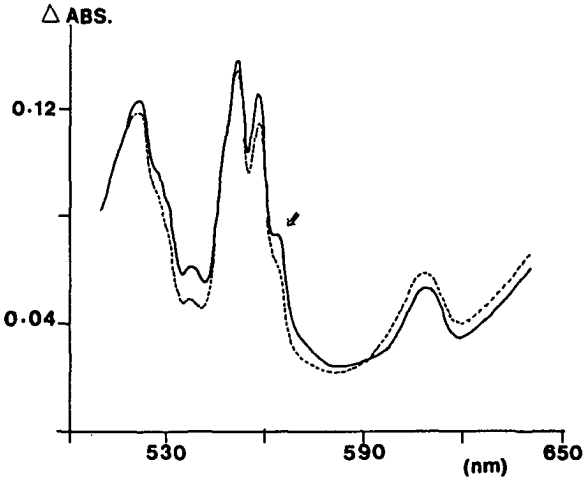

FIGURE 3A. The change in the 77 K spectrum obtained with *T. pantotropha* cells exposed to CO after reduction with argon and dithionite. The cells had been grown at 80% air saturation in an acetate-limited chemostat with NH_3 as the sole nitrogen source. Dotted line = argon/dithionite; solid line = CO. Arrow indicates the changing peak at 563.

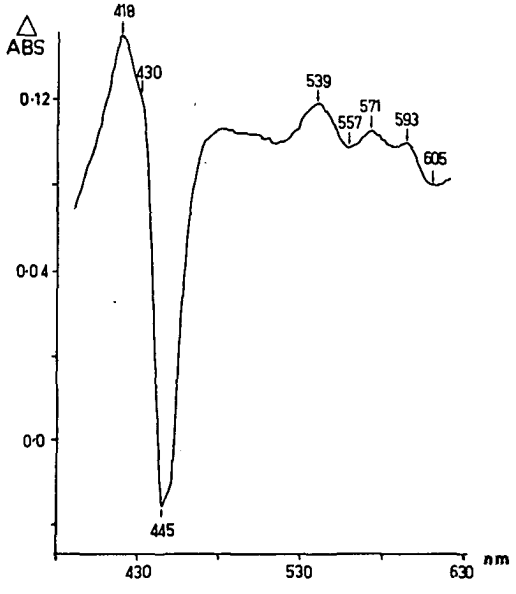

FIGURE 3B: Difference spectrum for the curves shown in figure 3A, obtained by subtracting the argon/dithionite spectrum from the CO spectrum.

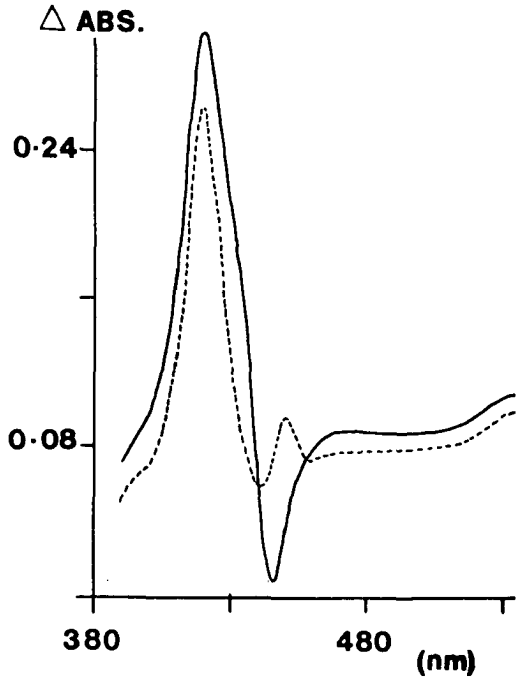

FIGURE 4. Difference spectra obtained by subtracting the spectrum obtained at 77 K with argon-reduced cells from that obtained with CO treated cells. Solid line = before and dotted line = after 2 flashes of white light.

It is known (Stouthamer, 1980) that cytochrome o is much less sensitive to cyanide than cytochrome aa_3. The sensitivity of oxygen uptake by acetate-respiring *T. pantotropha* to cyanide was therefore tested. *T. pantotropha* was grown in a chemostat at 80% air saturation with ammonia as the sole nitrogen source, washed and resuspended in phosphate buffer, pH 8.0. The effect of different concentrations of cyanide on the acetate-dependent respiration rates of cells in the presence and absence of ammonia was then measured. 50% inhibition was obtained with 280 μM cyanide when the cells were provided with ammonia (and thus nitrifying) while those without ammonia required only 70 μM for the same effect.This could indicate that cytochrome o is involved in nitrification, rather than cytochrome aa_3.

TABLE 1. Nitrification rates, carbon assimilation and PHB formation by the cells from which the cytochrome spectra and soluble fractions were prepared. N-comps. = nitrogen compounds supplied; Nitrif. Rate = nitrification rate as nmol min^{-1} mg $protein^{-1}$ nd = not determined.

	Substrate	O_2 % air	N-comps.	Nitrif. Rate	protein (mg/l)	PHB
I	acetate	80	NH_3	56.3	90	–
II	acetate	30	NH_3	52.0	92	+
III	acetate	80	NH_3/NO_2^-	17.4	121	–
IV	acetate	30	NH_3/NO_2^-	19.7	135	–
V	acetate + thiosulphate	80	NH_3	<0.1	nd	–

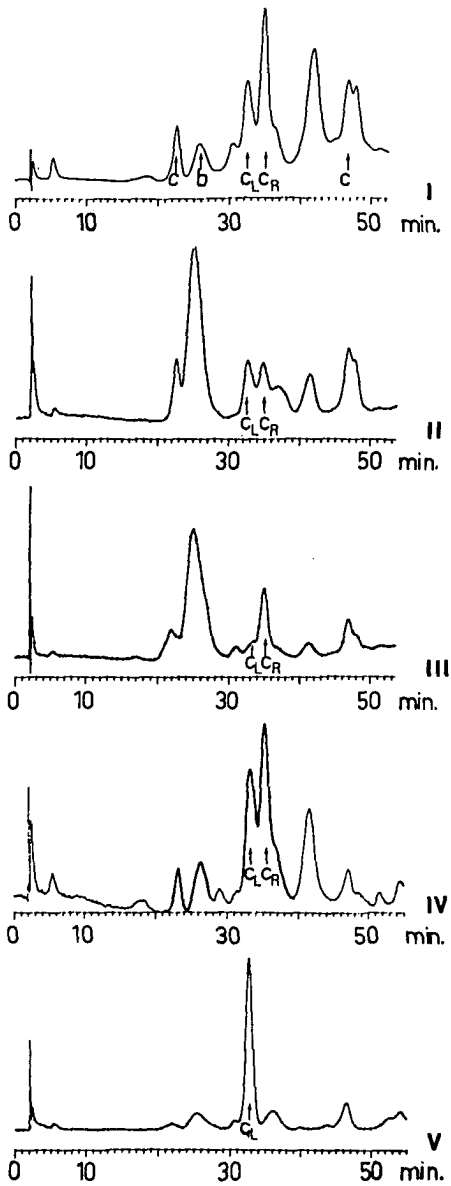

FIGURE 5. Continuous monitoring at 410 nm of the eluent from a phenyl sepharose column through which the soluble fraction from *T. pantotropha* extracts was passing on an ammonium sulphate gradient. The growth conditions (indicated by I to V) are shown in Table 1).

Spectra of the soluble fraction from disrupted cells revealed that cytochrome c made up the bulk of the soluble cytochromes. Separation by means of hydrophobic interaction chromatography revealed the presence of at least 5 different cytochromes c of which two had absorption maxima at 552 nm (Figure 5). For convenience these have been designated R(ight) and L(eft) for their positions relative to each other in Figure 5. There was also a small peak due to cytochrome b. The ratios of these cytochromes to each other varied with the growth conditions (Figure 5). The ratio between the two cytochromes c_{552} correlated with the yield (and thus the amount of acetate being oxidized to CO_2), and the nitrification rate (Table 1).

Of the two cytochromes c_{552}, cytochrome c_{552}R dominated in acetate-grown cells when the nitrification rate was low (Table 1; Robertson et al., 1988). This is especially to be seen in sample III (Figure 5) in which the growing cultures were provided with both NO_2^- and O_2. Higher nitrification rates in the cultures corresponded with an increase in the importance of cytochrome c_{552}L (I), and in the samples where PHB was being synthesized *from some of the acetate, cytochrome* c_{552}L *dominated (II). When* thiosulphate was provided as an additional source of energy at a concentration at which all of the acetate was used for biosynthesis (Gommers et al., 1988; H.J. Nanninga unpublished data), cytochrome c_{552}R had disappeared completely (V). The hydroxylamine oxidoreductase of *Nitrosomonas* sp. contains cytochrome c_{552} haems (Hooper, 1984). It is, therefore, not unlikely that some of the 5 cytochromes c apparent from these experiments are also part of such complexes.

An additional cytochrome peak (at 591 nm) was observed in cells grown aerobically or anaerobically in the presence of nitrate. The 590 nm absorption band has formerly been attributed to to the alternative cytochrome oxidase, cytochrome a_1. However, it has been shown that in some species, a form of cytochrome b also gives a peak at this wavelength (Poole et al., 1984; 1985). For example, the 595 nm band found in *Escherichia coli* has been shown to be partially due to cytochrome b, and partially to its cytochrome d-containing oxidase (Poole et al., 1984). It seems unlikely that the appearance or concentration of a terminal oxidase would be related to the presence or absence of nitrate in the growth medium, and an alternative explanation for the appearance of this peak has been sought. *P. denitrificans,* when grown anaerobically in nitrate-containing medium, was found to contain "cytochrome a_1". On switching to aerobiosis (and a medium which did not contain nitrate), this peak gradually disappeared (van Verseveld et al., 1983). The peak at 590 is also clearly visible on spectra made with *P. denitrificans* grown in the presence of nitrate and azide by Calder et al. (1980). The peak is much larger and better-defined in the azide-grown cells which also contained three times as much nitrate reductase as those grown on nitrate. Spectra made for a mutant which lacked nitrate reductase do not show the peak at 590 nm. Ballard and Ferguson (1988) have recently shown that nitrate reductase from *P. denitrificans* contains two b-haems. The association of "cytochrome a_1" with nitrate reduction has also been found in *Haemophilus parainfluenzae*. Maximal amounts were synthesized during anaerobic growth with nitrate and, moreover, nitrate could be shown to oxidize the reduced cytochrome. (Sinclair & White, 1970; White, 1962). It is therefore likely that "cytochrome a_1" in *T. pantotropha* is also linked to electron transport to nitrate.

Cytochrome cd was not observed in any of the *T. pantotropha* cultures. Azide and DDC were therefore used to test whether *T. pantotropha* has the "alternative", copper-based nitrite reductase according to the method described by Shapleigh & Payne (1985). It was found that, in contrast to *P. denitrificans* which has cytochrome cd, denitrification by *T. pantotropha* was sensitive to DDC and not to azide (Figure 6), indicating that copper was an important part of its nitrite reductase. It has recently been found that other bacteria capable of aerobic denitrification (e.g. *Alcaligenes* sp., "*Pseudomonas denitrificans*" and *A. faecalis* S6) also have the copper-based nitrite reductase (Chapter 9). The demarcation line between bacteria capable and not capable of co-respiring oxygen and nitrate at significant oxygen concentrations may be partially dependent on the type of nitrite reductase present in the culture.

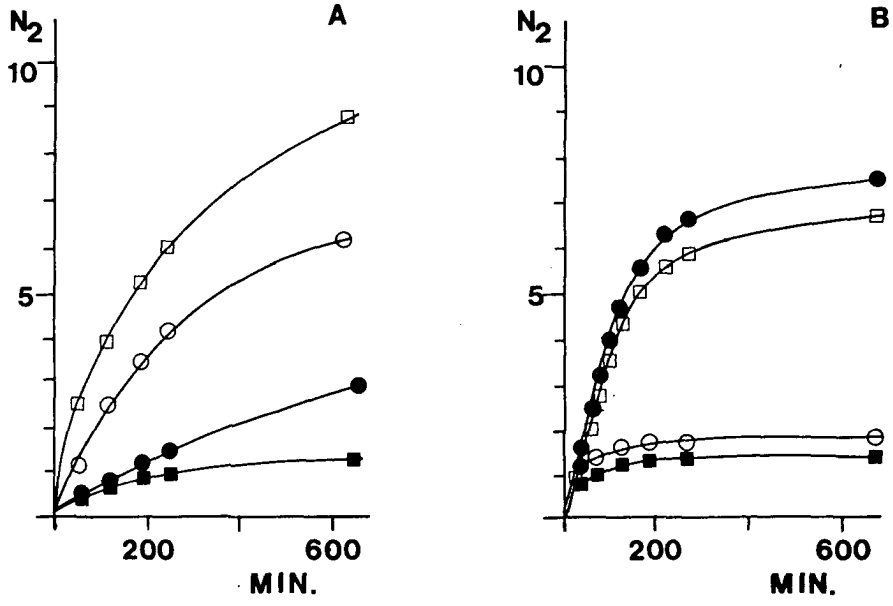

FIGURE 6. Comparison of the effect of azide (closed circles) and DDC (open circles) on acetate dependent nitrogen production by *P. denitrificans* (A) and *T. pantotropha* (B). Open squares = acetate present, no inhibitor; closed squares = endogenous, no inhibitor.

b: The model.

The model discussed here is based on the hypothesis that *T. pantotropha* has a "bottleneck" in the electron flow to oxygen via cytochrome c and cytochrome aa_3. Passage of electrons to the denitrification or nitrification pathways would reduce the "overload", allowing a more rapid oxidation of NAD(P)H. This

hypothesis is supported by the appearance of cytochrome o (an indicator of a reduced cytochrome chain) in cultures dependent on O_2 as their sole electron acceptor.

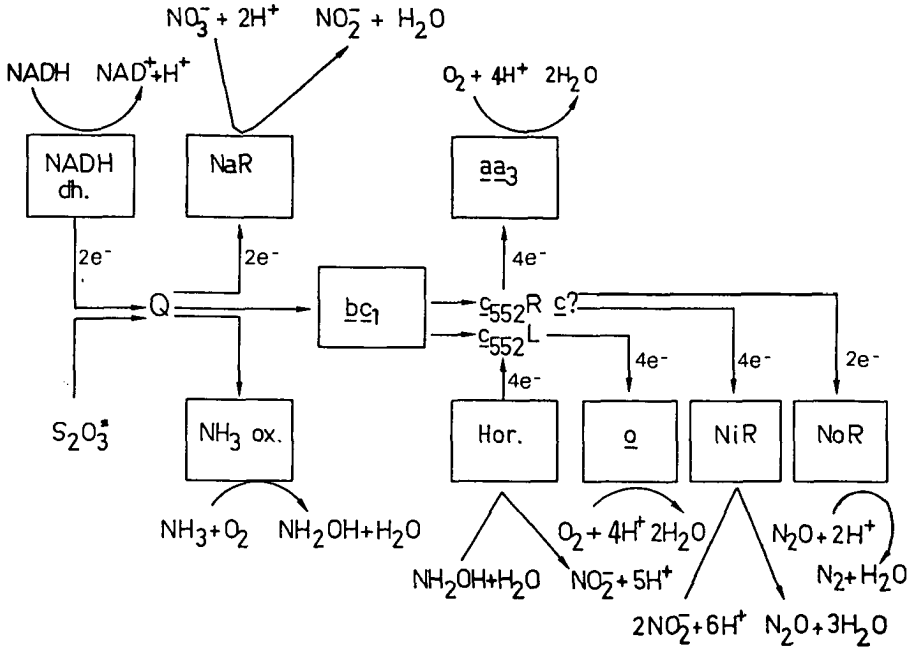

FIGURE 7. Schematic representation of the model used to describe the various potential routes for electron transport in *T. pantotropha*. Cytochromes are underlined; NADH dh. = dehydrogenase; NaR = nitrate reductase; NH_3 ox. = NH_3 monooxygenase; Hor. = Hydroxylamine oxidoreductase; NiR = nitrite reductase; NoR = Nitrous oxide reductase; c? indicates one or more additional cytochromes c; e = electrons; $S_2O_3^=$ = thiosulphate oxidizing pathway.

A diagram of the model in terms of postulated paths of electron flow under different growth conditions is shown in figure 7. The reasoning behind the construction of this model is presented in the following paragraphs.

The basic assumption which must be made is that the sequence of cytochromes in the chain is substantially similar to that in *P. denitrificans* (ie. UQ to cytochrome b to cytochrome c to cytochrome aa_3). The results presented above suggest that the cytochrome c involved in this direct chain is cytochrome c_{552}(R).

In *P. denitrificans*, nitrate reductase receives its electrons from ubiquinone and the nitrite and nitrous oxide reductases are supplied with electrons by one or more cytochromes c (Stouthamer, 1980; van Verseveld & Bosma, 1987). The electron transport chains from NADH to oxygen and from NADH to nitrate, nitrite and N_2O in *T. pantotropha* may be organised in a similar way to those of *P. denitrificans*. However, if the model is correct, one major difference must be that the active site of cytochrome o in *T. pantotropha* must be on the periplasmic side of the cytoplasmic membrane because electron transport from hydroxylamine to oxygen in this organism should not give rise to charge separation over the cytoplasmic membrane. As already discussed, "cytochrome a_1" appears to be associated with electron transport to nitrate in *T. pantotropha*. *T. pantotropha* has the copper nitrite reductase rather than cytochrome cd, but for the purposes of the model, it has been assumed that both nitrite reductases gain their electrons from cytochrome c. Published evidence is conflicting on this subject. It has been claimed that cytochrome c_{552} is involved with the copper nitrite reductase of "*Ps. denitrificans*" (Myata & Mori, 1969) and cytochrome c_2 appears to be linked to nitrite reduction in *Rhodopseudomonas* sphaeroides var *denitrificans* (Sawada et al., 1978). However, it has been shown that neither the copper nitrite reductase nor its associated blue copper protein from *Achromobacter cycloclastes* accept electrons from cytochrome c (Liu et al., 1986), and the question remains open. Experimental evidence (Robertson & Kuenen, 1988) has shown that, in vitro, hydroxylamine oxidoreductase can deliver electrons to cytochrome c, and the results presented here suggest that this could be cytochrome c_{552}(L). Both ammonia oxidation and hydroxylamine oxidation in *Nitrosomonas* species have been shown to involve cytochrome c (Hooper, 1984). The apparent correlation between high nitrification rates and cytochrome o levels may be coincidental, but the use of cytochrome o as the terminal oxidase for nitrification would explain the relative resistance of nitrifying cells to cyanide inhibition.

Electron flow from thiosulphate as shown in figure 7 is hypothetical, and has little experimental evidence to support it. Cytochrome c_{552}(L) is clearly related to thiosulphate metabolism (figure 5). However, we assume that thiosulphate delivers electrons to ubiquinone because if electrons from thiosulphate were passed directly into cytochrome c, *T. pantotropha* would be unable to grow anaerobically on reduced sulphur compounds while denitrifying nitrate. As the organism is able to do so (Robertson & Kuenen, 1983), it is evident that electrons from thiosulphate must enter the cytochrome chain "upstream" of cytochrome c. As yet, there is no evidence that *T.. pantotropha* contains cytochrome P460, which has been shown to be involved in autotrophic hydroxylamine oxidation.

c: Correlation of the working model with physiological data

It must be emphasized that the situation discussed here is simplified. Whenever nitrification takes place in *T. pantotropha*, denitrification also occurs. Nitrification also proceeds in

almost all cultures and "inhibits" in this case is a relative term, indicating a reduction rather than a cessation of a phenomenon (Robertson et al., 1988).

1: During aerobic denitrification, the flow of electrons to the denitrification pathway, as shown in the model, would overcome the postulated "bottleneck" in the transport of electrons to oxygen via cytochrome aa_3, thus preventing the cytochrome chain from becoming sufficiently reduced to cause the accumulation of NAD(P)H. Cultures provided with excess nitrate or nitrite had low nitrification rates (Robertson et al., 1988). Because denitrification yields less energy than respiration of oxygen, protein yields from these cultures were somewhat lower than might be expected (Robertson et al., 1988). If, for any reason, electrons could not flow to the denitrification pathway, then the cytochrome chain would become more reduced, NAD(P)H would not be oxidized, and it would become necessary to "dump" the reducing power by nitrifying.

2: High nitrification rates correspond with lower, rather than higher, protein yields (Robertson et al., 1988). It was difficult to see why this should be so if the electrons from hydroxylamine passed to oxygen via cytochrome aa_3 as the oxidation of hydroxylamine generates sufficient energy for the autotrophic ammonia oxidizers to grow. However, if hydroxylamine oxidoreductase passes its electrons to cytochrome o, and if cytochrome o is located, like hydroxylamine oxidoreductase, on the periplasmic side of the membrane, electron transport between the two would not involve proton translocation. The proposed route would thus explain why nitrification does not generate energy. The low yields would be caused by a combination of factors including the cost (in terms of NAD(P)H) of oxidizing ammonia and the need to denitrify the nitrite generated.

3: The yields obtained with *T. pantotropha* when growing autotrophically on thiosulphate are lower than those found for other mixotrophs (e.g. Gottschal & Kuenen, 1980) and for other denitrifiers such as *Thiobacillus denitrificans* which are thought to have proton translocating sites between ubiquinone and cytochrome c, and between cytochrome c and oxygen (Timmer ten Hoor, 1977). In *P. denitrificans*, the use of cytochrome o as the terminal electron acceptor, rather than cytochrome aa_3, appears to involve one less proton translocating loop and thus generates less energy (Stouthamer, 1980). Thus, electron transport from thiosulphate to oxygen through cytochrome o rather than cytochrome aa_3 could provide an explanation for the low protein yields.

Thiosulphate inhibits nitrification even when it is present in limiting amounts, and its concentration is very low (<50 uM) (Table 1; Robertson et al., 1988). This inhibitory effect is unlikely, therefore, to be purely that of a chemical inhibitor. If the nitrification and thiosulphate oxidation systems share a common cytochrome (e.g. cytochrome c_{552}(L)), the passage of electrons from thiosulphate would make the cytochrome too reduced to accept electrons from hydroxylamine. A small amount of hydroxylamine would then accumulate and inhibit ammonia oxidation (Robertson & Kuenen, 1988). Thiosulphate would thus inhibit nitrification even though its effective concentration was close to zero.

A further, indirect piece of evidence which supports the view that thiosulphate metabolism and heterotrophic nitrification are linked in some way is that six other known

heterotrophic nitrifiers which have so far been tested have also all been able to oxidize thiosulphate (Chapter 9; Chapter 10).

4: If neither denitrification nor ammonia oxidation are possible, then the organism would make poly β-hydroxybutyrate in order to dispose of excess reducing power.

ACKNOWLEDGMENT

The authors are grateful to Willem Reijnders, Rob Torremans & Adele van Houwellingen for experimental assistance, and to Henk Nanninga for providing the mixotrophically grown cells.

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The effect of electron acceptor variations on the behaviour of *Thiosphaera pantotropha* and *Paracoccus denitrificans* in pure and mixed cultures.

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ABSTRACT

The competitive advantages provided by a capacity for aerobic denitrification have been tested by comparing *Thiosphaera pantotropha* (which denitrifies aerobically and anaerobically), with a strain of *Paracoccus denitrificans* (which only denitrifies under anaerobic conditions) in acetate-limited chemostats. A comparison of μ-C_s curves based on K_s and μ_{max} measurements indicated that *Pa. denitrificans* could be expected to dominate mixtures of the two species at high growth rates when the dissolved oxygen was above 80% of air saturation and NH_4 was the sole source of nitrogen. The comparison also suggested that at lower growth rates, lower dissolved oxygen tensions, and/or in the presence of nitrate, *Tsa. pantotropha* should have the competitive advantage. Chemostat experiments with mixtures of the two species showed that *Tsa. pantotropha* did, indeed, dominate the population when expected. However, when *Pa. denitrificans* was expected to dominate, only a small increase in the *Pa. denitrificans* numbers was possible before *Tsa. pantotropha* formed a biofilm on the walls of the chemostat instead of washing out, and was again able to out-compete *Pa. denitrificans* for acetate.

Experiments with axenic chemostat cultures subjected to aerobic/anaerobic switches showed that *Tsa. pantotropha*, with its constitutive denitrifying system, was able to adjust smoothly to the changing environmental conditions and thus continued to grow. *Pa. denitrificans* does not have constitutive denitrifying enzymes, and could consequently not adjust its metabolism to the lack of oxygen rapidly enough. It therefore washed out at a rate equivalent to the dilution rate.

INTRODUCTION.

The postulated ecological niche for aerobic denitrifiers such as *Thiosphaera pantotropha* lies in situations where oxygen is limiting, or when the environment goes through relatively short cycles of aerobiosis and anaerobiosis (Robertson & Kuenen, 1984A; 1984B). *Tsa. pantotropha* is, of course, also a heterotrophic nitrifier (Robertson & Kuenen, 1988; Kuenen & Robertson, 1987). Both nitrification and denitrification are of importance in soil fertility (where they are undesirable) and waste water treatment (where both phenomena play an important part in nitrogen removal). An understanding of the factors which will select for bacteria which can simultaneously nitrify and denitrify is thus eminently desirable. Because of the complexity of experiments to test the combined phenomena and the number of variables involved, it was decided that their ecological importance should be investigated separately.

Experiments were therefore set up in order to discover

whether, under what conditions, its abilities as an aerobic denitrifier give *Tsa. pantotropha* a selective advantage over *Paracoccus denitrificans*, a species which only denitrifies under anaerobic conditions.

The problem was approached in two ways. Firstly, the outcome of competition between the two species in different acetate-limited chemostat cultures was checked. Secondly, the effect of aerobic/anaerobic switches on axenic cultures of *Tsa. pantotropha* and *Pa. denitrificans* was measured in order to test their adaptability.

This paper reports the preliminary results from these experiments.

MATERIALS AND METHODS

Organisms

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide oxidizing wastewater treatment system (Robertson & Kuenen, 1983). *Paracoccus denitrificans* LMD 22.21 was obtained from the culture collection of this Laboratory, and is the strain isolated by Beijerinck (1910).

Media

The medium for 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. Sodium acetate (20 mM) was supplied as the limiting substrate. When appropriate, 32 mM KNO_3 was used.

The medium described for the growth of *Thiobacillus versutus* (formerly *Thiobacillus* A2) by Taylor & Hoare (1969) was used for batch cultures. 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; 2ml of trace element solution. The $MgSO_4$, trace element solution, substrates and KNO_3 (20 mM) were added as needed. The initial concentration of the sodium acetate in the batch cultures was 10 mM.

The trace element solution (Vishniac & Santer, 1957) used with all 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.

For colony counting, 2% (w/v) Difco bacto agar was added to the batch culture medium. The substrate in these plates was a mixture of 0.3 g l^{-1} yeast extract, 0.1 g l^{-1} fructose and 0.1 g^{-1} sodium acetate. As *Tsa. pantotropha* is more sensitive to ampicillin than *Pa. denitrificans*, plates with and without 0.15 mg l^{-1} ampicillin were used to obtain *Pa. denitrificans* and total counts, respectively.

Continuous Cultures

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. For the competition experiments, *Tsa. pantotropha* and *Pa. denitrificans* were grown axenically to steady state at the appropriate dilution rate and dissolved oxygen concentration (80 % of air unless otherwise specified). Once the steady state had been confirmed by analysis of the medium and biomass, the two cultures were mixed. To avoid potential contamination problems during the mixing, the chemostats had been linked before sterilization by tubes through which the cultures could be pumped. In a control experiment, it was confirmed that each of the test species grew well in filter-sterilized medium in which the other had previously been grown, indicating that neither produced excretion products which would be inhibitory to the other.

The aerobic (80% of air saturation) and anaerobic cycles in the cultures were achieved by sparging with air or ultra-pure nitrogen. The gas flow was regulated by timer-controlled valves.

Biomass analysis

Protein was measured spectrophotometrically, by means of the Micro-Biuret method (Goa, 1953). Total organic carbon was measured using a Beckman Tocamaster Model 150B.

Because *Tsa. pantotropha* tends to make poly-β hydroxybutyrate (PHB) under some growth conditions, both dry weight and total organic carbon measurements could give an artificially high yield. The yield estimates for the nitrogen balances were therefore based on the protein determinations, as previously described (Robertson et. al., 1988).

Analysis of culture medium

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer).

Nitrite was measured colourimetrically, with the Griess-Romijn reagent (Griess-Romijn van Eck, 1966) or by means of an HPLC fitted with a ionosphor-TMA column (Chrompak) and a Walters RI detector. Nitrate was also measured with the HPLC.

Hydroxylamine and ammonia were determined colourimetrically by means of the methods described by Frear & Burrell (1955) and Fawcett & Scott (1960), respectively. 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.

Miscellaneous

The maximum specific growth rates for the two species under each set of growth conditions were determined during the batch culture start-up phase of each chemostat experiment.

Oxygen uptake was measured using a Clark-type electrode mounted in a thermostatically controlled cell which is closed except for a small hole through which additions may be made.

K_s values for acetate were determined using direct linear plots (Williams, 1984) of the oxygen uptake dependant on various acetate concentrations. Washed cells were resuspended in 0.05 M phosphate buffer, pH 8.0, and the measurements were done in the presence or absence of ammonia and nitrate.

Immuno-fluorescent staining was carried out using antibodies raised against *Tsa. pantotropha* by the method described by Muyzer et. al., (1987).

RESULTS AND DISCUSSION

a: Competition studies

Using the K_s values for acetate and the μ_{max} of each species for the conditions under test (see Materials and Methods), μ/C_s curves were constructed according to the Monod formula (Monod, 1942). When the curves for the two species were superimposed, it was found that *Pa. denitrificans* could be expected to dominate a mixed culture of the two species at high growth rates in the aerobic cultures in which ammonia was the sole source of nitrogen (figure 1a), and *Tsa. pantotropha* would dominate at lower dilution rates. When nitrate was also present in the medium, the increase in the μ_{max} of *Tsa. pantotropha* was such that it should outcompete *Pa. denitrificans* for acetate at all growth rates (figure 1b), even when the dissolved oxygen was very high. At lower dissolved oxygen concentrations, *Tsa. pantotropha* also increased its growth rate, and *Pa. denitrificans* should, again, lose the competition (figure 1c).

Experimental testing of the model shown in figure 1a at the lower growth rates showed that *Tsa. pantotropha* did, indeed, dominate and *Pa. denitrificans* amounted to less than 1% of the population after more than 10 volume changes irrespective of the ratios of the two species in the original culture (Table 1). It was confirmed that this majority population was *Tsa. pantotropha*, rather than an ampicillin-sensitive revertant of *Pa. denitrificans* by means of immuno-fluorescent staining. Further evidence that *Tsa. pantotropha* was dominant came from the following observations. The yield in the cultures which contained 90% *Pa. denitrificans* immediately after mixing, fell (as the proportion of *Pa. denitrificans* fell) to a value similar to that found in the *Tsa. pantotropha* cultures (from 8.1–8.7 g protein mol acetate $^{-1}$ to 6.2–6.6 g protein mol acetate^{-1}). Moreover, the ammonia loss from the axenic *Pa. denitrificans* cultures, which had been very low (equivalent to a nitrification rate of 6 nmol min^{-1} mg protein^{-1} at a dilution rate of 0.07 h^{-1}), rose after mixing to values equivalent to those in the *Tsa. pantotropha* cultures (e.g. 18 nmol^{-1} min^{-1} mg protein^{-1} at a dilution rate of 0.07 h^{-1}).

TABLE 1. Competition between *Tsa. pantotropha* and *Pa. denitrificans* in the chemostat under different growth regimes (corresponding to those shown in Figures 1a and 1b). a = only 4 volume changes. b = before formation of biofilm.

D (h^{-1})	N-compound	%*Tsa. pantotropha* Start	End	%*Pa. denitrificans* Start	End	see Fig
0.06	NH_3	10	98	90	2	1a
0.07	NH_3	10	98	90	2	1a
0.2	NH_3	10	>99	90	<1	1a
0.2	NH_3	90	98	10	2	1a
0.4	NH_3	99	87[b]	1	13[b]	1a
0.4	NH_3	99	92[b]	1	8[b]	1a
0.06	NH_3/ NO_3^-	90	99	10	1	1b
0.06	NH_3/ NO_3^-	10	21[a]	90	79[a]	1b

Once the dilution rate was increased to a value above the cross point of the curves shown in figure 1a, the population of *Pa. denitrificans* began, as expected to recover, reaching 13% of the community after 5 volume changes. Unfortunately, at this point, a rapidly thickening biofilm became apparent on the wall of the chemostats, and the suspended *Pa. denitrificans* population fell back to below 1%. Immunofluorescent staining showed that this biofilm consisted almost entirely of *Tsa. pantotropha*. The formation of a biofilm under unfavourable conditions is understandable when it is considered that *Tsa. pantotropha* was originally isolated from a fluidized bed column where the biomass was attached to sand. One of the main selective pressures in such a system is the ability to attach to surfaces when the dilution rate exceeds the μ_{max} of the organism (Heijnen, 1984). As *Tsa. pantotropha* has a much lower K_s for acetate than *Pa. denitrificans* (28 and 63 µM, respectively), the attached *Tsa. pantotropha* was able to build a population sufficiently large to successfully compete for substrate even though the *Pa. denitrificans* remained in suspension.

As expected, when the model shown in Figure 1bwas tested, *Tsa. pantotropha*, dominated one of the cultures and was steadily increasing in the second before technical problems brought a premature end to the experiment. Because of wall growth, the model shown in Figure 1c was not tested.

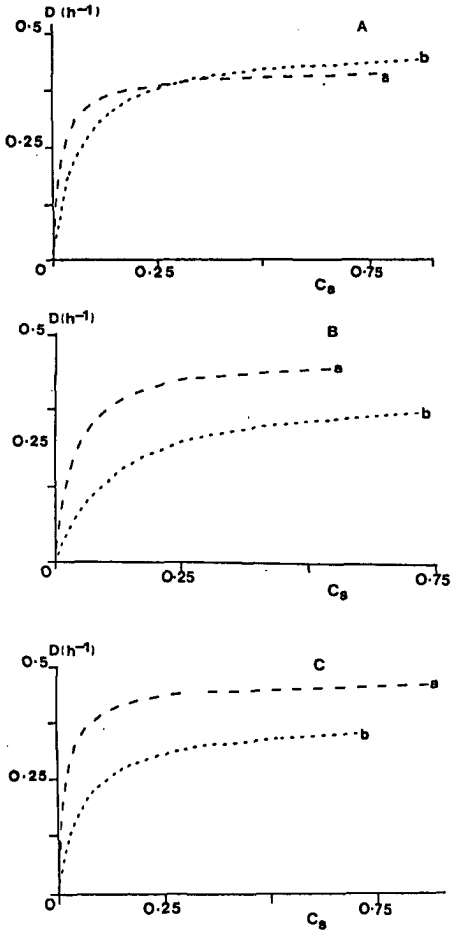

FIGURE 1. μ/C_s curves showing the theoretical outcome of competition for acetate between *Tsa. pantotropha* (a) and *Pa. denitrificans* (b) in the chemostat. 1A = 80% air saturation, NH_3 as sole nitrogen source; 1B = 95% air saturation, NH_3 and NO_3^- both supplied; 1C = 25% air saturation, NH_3 as sole nitrogen source. D = dilution rate (= μ).

b: Aerobic/anaerobic cycles.

In order to test the prediction that *Tsa. pantotropha* (as an aerobic denitrifier) would have an advantage over *Pa. denitrificans* (as a specialist which only denitrifies anaerobically) in situations where the oxygen supply fluctuated, cultures were grown in chemostats undergoing alternate cycles of aerobiosis and anaerobiosis. In order to allow the study of the behaviour of *Pa. denitrificans* under this regime, without the masking effect caused by potential *Tsa. pantotropha* biofilm formation, the experiments were run with pure cultures. The behaviour of the cultures was monitored with continuous, on-line optical density measurements. After aerobic (80% of air) growth to steady state on media containing ammonia and nitrate, the cultures were made anaerobic by switching the sparging from air to nitrogen.

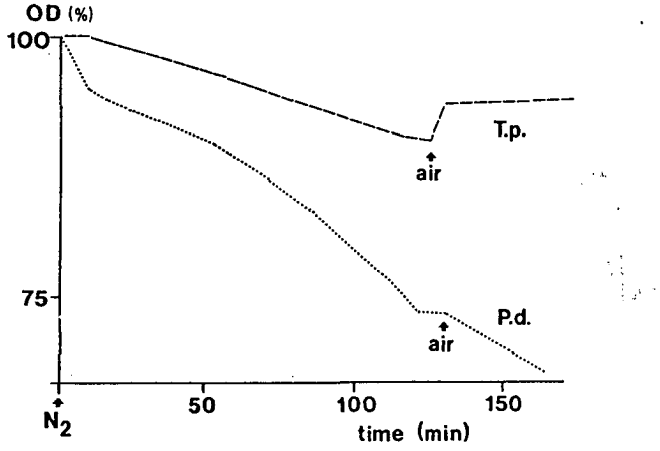

FIGURE 2. The outcome of switching steady-state, acetate-limited, chemostat cultures of *Tsa. pantotropha* (T.p.) and *Pa. denitrificans* (P.d.) from aerobiosis (80% air saturation) to anaerobiosis.

Tsa. pantotropha was not significantly affected by 3 hours anaerobiosis, and showed little or no response when aerobiosis was restored (Figure 2). It did not attach to the chemostat. In contrast, after the change to anaerobic conditions, *Pa. denitrificans* washed out at a rate proportional to the dilution rate (0.25 h^{-1}). After about 3 hours, the population appeared to stabilize, but resumed the washout when the air flow was reconnected (Figure 2). Lower dilution rates (0.125 h^{-1}), and shorter periods of anaerobiosis did not improve the recovery of the *Pa. denitrificans* cultures. That this failure to recover after the restoration of aerobiosis was not due to repression of cytochrome oxidases was apparent because the rate of acetate-dependent oxygen uptake by washed cells of *Pa. denitrificans* sampled after different periods of anaerobiosis did not change significantly. It therefore seemed likely that the

problem was due to the medium. Analysis showed that the level of nitrite in the culture rose from 0.2 mM to 1.2 mM during the 3 hour anaerobic period. A similar, transient (3 hours) accumulation of nitrite, accompanied by a fall-off in cytochrome oxidase activity, was observed with batch cultures of *Pa. denitrificans* which had been switched from aerobic to oxygen-limited growth in the presence of nitrate (Kučera et al., 1984). Nitrite is known to inhibit oxygen uptake by *Pa. denitrificans* (McCarthy & Ferguson, 1981; Robertson & Kuenen, 1988). Furthermore, nitrate reductase in *Pa. denitrificans* is much less sensitive to oxygen than nitrite reductase (Lam & Nicholas, 1969; Alefounder et al., 1981). If oxygen uptake was inhibited to some extent by the high nitrite concentration, the cytochrome chain would become more reduced. Electrons would then be able to flow to nitrate, generating more nitrite and creating a "vicious circle". Presumably, if permitted a longer period of anaerobiosis in order to generate its complete denitrifying pathway, *Pa. denitrificans* would be able to reduce the nitrite concentration, and the problem would not arise again until after the next aerobic/anaerobic switch.

CONCLUSIONS

1: As judged by the theoretical predictions shown in figure 1, *Tsa. pantotropha* is at a disadvantage at high growth rates if ammonia is the sole nitrogen source. When aerobic denitrification can take place (because of the provision of nitrate in the growth medium), the μ_{max} of *Tsa. pantotropha* becomes greater than that of *Pa. denitrificans* and it will out-compete *Pa. denitrificans* for acetate at all dilution rates. At lower oxygen tensions, *Tsa. pantotropha* exhibited a higher μ_{max} than *Pa. denitrificans*, even when ammonia was the sole source of nitrogen. This, again, allowed *Tsa. pantotropha* to dominate the population (fig 1c). Under steady state anaerobiosis in the presence of nitrate, *Pa. denitrificans* had a higher μ_{max} than *Tsa. pantotropha*, and therefore a clear advantage.

2: The competition experiments for growth-limiting acetate thus essentially confirmed the predictions made from the relative u-C_s curves of *Tsa. pantotropha* and *Pa. denitrificans* (Figure 1). However, at high dilution rates, *Tsa. pantotropha* displayed a strong tendency to stick to the available surfaces in the culture vessel. This behaviour counteracted the initial, predicted washout of this species at high dilution rates. In future competition experiments to show the relative positions of the μ-C_s curves of the two metabolic types (aerobic and specialist denitrifiers), care should be taken to use strains which do not have such a strong ability to attach to surfaces under unfavourable conditions. An alternative would be to test an analogous hypothesis with two attaching species.

3: The results of the aerobic/anaerobic switching demonstrate that our strain of *Pa. denitrificans* does not respond well to fluctuations, but that *Tsa. pantotropha* remains unaffected. Of course, if nitrate or nitrite were not provided, *Tsa. pantotropha* would not be able to survive long periods of anaerobiosis, either. It appears that *Tsa. pantotropha* is, as predicted, well suited to life under oxygen fluctuation, whereas this strain of *Pa. denitrificans* is better suited to steady conditions.

ACKNOWLEDGMENT

The authors are grateful to Anke de Bruyn for help with the immunofluorescent staining, and to Brigit van Elk and Harro Slobbe for experimental assistance.

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Heterotrophic Nitrification and Aerobic Denitrification in Common Soil Bacteria.

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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 a 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_4^+ 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*" was found to possess the copper nitrite reductase.

As with *T. pantotropha*, thiosulphate inhibited nitrification by "*Ps. denitrificans*", and the strain proved capable of mixotrophic growth on acetate and thiosulphate in the chemostat.

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. 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 *Tsa. 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.

Culture techniques

Batch cultures were made in Kluyver flasks (Robertson & Kuenen 1984A) which incorporated 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 mineral salts media have already been described (Robertson & Kuenen 1983, 1988). Heterotrophic batch and chemostat cultures were supplied with 10 and 20 mM acetate, respectively. 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 a ionosphor-TMA column (Chrompak) and a Walters RI detector.

Nitrite was measured colourimetrically, with the Griess-Romijn reagent (Griess Romijn van Eck, 1966) or by means of the HPLC. Nitrate was also measured colourimetrically, using diphenylamine sulphonic acid chromogene (Szechrome NAS reagent, Polysciences Inc.), or with the HPLC. 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 umol min^{-1} $litre^{-1}$) could be expected from stripping. Analysis of the effluent gas using acid wash bottles confirmed that 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, 1984B; Robertson et al., 1988). *Paracoccus denitrificans* (LMD 22.21), which denitrifies only under anaerobic conditions and does not nitrify, 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 heterotrophic 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 are consistent with 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).

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)		Δ 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

A first indication 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*.

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	$+NH_3$ 7.5 mM	$+NO_3^-$ 10 mM	$+NO_2^-$ 5 mM	$+NH_2OH$ 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

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 (Douderoff et al., 1974; ICSB, 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 immediately if it had been grown (aerobically or anaerobically) in the presence of nitrate. Cultures grown with ammonia as the sole source of nitrogen required an induction period. Gas production from nitrite was immediate in all cases, indicating that the remainder of the denitrification pathway was constitutive.

The copper chelator, diethyldithiocarbamate (DDC), inhibited acetate-dependant gas production completely and azide had little or no effect. Azide inhibits cytochrome cd and DDC inhibits the copper nitrite reductase (Shapleigh & Payne, 1985). It therefore appears that, like *T. pantotropha* (Chapter 7), *Pseudomonas* sp. contains the copper nitrite reductase rather than cytochrome cd. Acetate-dependant oxygen uptake in the presence of ammonia and DDC was 95% of the control, whereas when the DDC was omitted the oxygen uptake was slightly stimulated (108%). The cells were 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, again 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*, and appears to be associated with high nitrification rates (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*.

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_4 L 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

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 dilution rate of 0.05 h^{-1}, these were 43 and 12 nmol min^{-1} mg $protein^{-1}$, respectively; 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 in the cultures where both ammonia and nitrate were present 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.

Nitrogen balances for chemostat experiments with a strain of *Alcaligenes* essentially confirmed that this organism was also a combined nitrifier/denitrifier, but with a lower oxygen tolerance. Some of these results have been reported recently (Kuenen & Robertson, 1987), and further details will be published elsewhere (E.W.J. van Niel & K. Braber, unpublished data).

Thiosulphate as an inhibitor.

In order to evaluate the effect that aerobic denitrification has on cultures without the added complication of the nitrification, inhibitor studies are required. However, most inhibitors effective against nitrification (e.g. DDC, azide, cyanide) are also effective against denitrification. Thiosulphate is known to inhibit heterotrophic nitrification in *T. pantotropha* (Robertson & Kuenen, 1988). However, *T. pantotropha* is a colourless sulphur bacterium and studies using thiosulphate are therefore complicated by the fact that the organism can use thiosulphate as a substrate. Only short-term experiments with heterotrophically grown *T. pantotropha* were possible (Robertson & Kuenen, 1988) as, on prolonged exposure to thiosulphate, the organism will induce its thiosulphate oxidizing pathway (Robertson & Kuenen, 1983; Robertson et al., 1988). As with *T. pantotropha*, thiosulphate eliminated the stimulation of the oxygen uptake rate by heterotrophically grown cells caused by ammonia (Robertson & Kuenen, 1988). It was therefore decided to study the effect of thiosulphate on *Pseudomonas* sp. in the hope of inhibiting nitrification and thus measuring the effect of aerobic denitrification without the complication of mixotrophic growth.

The addition of 5 mM thiosulphate to the medium caused an immediate increase in the yields obtained from acetate-limited, aerobic cultures (from 127 to 173 mg protein $litre^{-1}$ at a dilution rate of 0.06 h^{-1}) and the nitrification rates fell by 50%. As already mentioned, heterotrophic nitrification is associated with low yields (Robertson et al., 1988), and an increase in the yield was to be expected if nitrification was inhibited. However, cells from these cultures were found to have a thiosulphate oxidizing capacity of 615 nmol oxygen min^{-1} mg $protein^{-1}$ which was not present in biomass grown without thiosulphate. Analysis of the effluent medium showed that all of the thiosulphate had been oxidized to sulphate. It was clear that the increase in yield was due to mixotrophic growth. Subsequent results, which will be described in detail elsewhere (Chapter 10), showed that the *Pseudomonas* sp. was, in fact, a facultatively chemolithotrophic, sulphur oxidizing bacterium.

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 (mentioned above) obtained with the strain of *Alcaligenes* which has been shown to be capable of efficient aerobic denitrification only at dissolved oxygen concentrations below 50% of air saturation (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.

ACKNOWLEDGEMENT

The authors are grateful to Marlou Verdurmen and Adele van Houwellingen for experimental assistance.

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Thiosulphate oxidation by heterotrophic nitrifiers.

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ABSTRACT

A strain of "*Pseudomonas denitrificans*" has been shown to be able to grow mixotrophically (with acetate) and autotrophically in a chemostat with thiosulphate as the energy source. Preliminary taxonomic tests have shown that this train is different from any of the known facultatively chemolithotrophic, colourless sulphur bacteria.

As this strain shares with *Thiosphaera pantotropha* the ability to simultaneously nitrify (heterotrophically) and denitrify (aerobically), other heterotrophs were also tested and found to be nitrifiers which were also able to oxidize thiosulphate. In mixotrophic batch cultures provided with acetate as well as thiosulphate, tetrathionate (rather than sulphate) was formed from the oxidation.

INTRODUCTION

During studies of its potential as a heterotrophic nitrifiers and aerobic denitrifier, it was found that a *Pseudomonas* sp. (formerly one of the heterogenous group known as *Ps. denitrificans*; Douderoff et al., 1974; ICSB, 1982) was able to induce a thiosulphate-oxidizing potential, and grow mixotrophically on acetate and thiosulphate (Robertson et al., 1988A). The strain was therefore tested for autotrophic growth on thiosulphate. In subsequent experiments, other known heterotrophic nitrifiers were tested for the ability to oxidize thiosulphate.

MATERIALS AND METHODS

Organisms

The strains used, together with their collection numbers, are shown in the text. They were all obtained from the Delft Culture Collection.

Culture techniques

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.

The mineral salts media have already been described (Robertson & Kuenen, 1983, 1988). Heterotrophic batch and chemostat cultures were supplied with 10 and 20 mM acetate, respectively. The mixotrophic batch cultures were supplied with 5 mM acetate and 10 mM thiosulphate. Autotrophic cultures were supplied with 10 mM thiosulphate.

Respiratory measurements

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

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 ionosphor-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. Nitrate was also measured with the HPLC. Hydroxylamine was determined colourimetrically by means of the method described by Frear & Burrell (1955). Ammonia was determined using 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 µmol min^{-1} $litre^{-1}$) could be expected from stripping (Robertson et al. 1988B).

RESULTS AND DISCUSSION

Pseudomonas sp. - Thiosulphate metabolism

The balances of substrates and products from mixotrophic cultures of *Pseudomonas* sp. are shown in Table 1. Results from heterotrophic *Pseudomonas* sp. cultures and mixotrophic *Tsa. pantotropha* cultures are also shown for comparison. It can be seen that, as with *Tsa. pantotropha*, 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. All of the thiosulphate which had been oxidized was recovered as sulphate.

TABLE 1. Ammonia, thiosulphate and sulphate balances, together with the protein yields from mixotrophic cultures of *Pseudomonas* sp. Data from heterotrophic cultures, added for comparison, from Robertson et al., 1988A. *Tsa. pantotropha* data from Robertson et al., 1988B. 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)
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
acetate/thiosulphate	20/5	0.18	-2.9	-4.9	+10.0	178

Pseudomonas sp. 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 those found for *Tsa. pantotropha* (1.4 g/mol; H.J. Nanninga, unpublished data). All of the thiosulphate was oxidized to sulphate. Nitrification did not take place in the autotrophic cultures. CO_2 fixation rates around 16.5 nmol min^{-1} mg $protein^{-1}$ were obtained using cell free extracts. 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 $protein^{-1}$). Oxygen uptake experiments showed that the thiosulphate oxidation potential was not present in heterotrophically grown cultures and was at its highest in the autotrophically grown cultures. The acetate oxidizing capacity of the mixotrophically grown cells was slightly lower than in the heterotrophically and mixotrophically grown cultures (Table 2).

TABLE 2. 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)	$S_2O_3^{=}$ (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

Taxonomic status

Pseudomonas sp. is gram negative, and is most often seen under substrate-limited growth in the chemostat as cocci 1.05 x 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 3). It also differed from the 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 *Tsa. 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., 1988A).

Other organisms

In view of these results, and the fact that *Thiosphaera pantotropha* is also a facultative chemolithotroph, it was decided to screen other heterotrophic nitrifiers for their ability to oxidize thiosulphate. The strains used, and their collection numbers, are shown in Table 4.

TABLE 3. Comparison of the reactions shown by *Pseudomonas* sp. and the three known denitrifying facultative colourless sulphur bacteria when screened with the API20B system.

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

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

Abbreviations: GEL, gelatine liquefaction; NIT, nitrate reduction; ONPG, B-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; MOT, motility; v, variable.

Their nitrifying activities were first confirmed in heterotrophic cultures growing on acetate. Between 3-4 mM ammonia (corrected for biomass and sparging losses) appeared to have been nitrified by many of the cultures (Table 4). As only trace amounts (µmolar) of nitrite were found, it must be assumed that this had been simultaneously reduced to gaseous products. Other cultures had lost smaller amounts of ammonia (Table 4).

TABLE 4. Ammonia disappearance and thiosulphate oxidation rates obtained with aerobic batch cultures of various heterotrophic bacteria. All cultures were grown with NH_4 as the sole source of nitrogen, and with 5 mM acetate and 10 mM thiosulphate. The NH_4 disappearance figures have been corrected for assimilated nitrogen. Oxygen uptake rates obtained with chemostat-grown *Pseudomonas* sp. (20 mM acetate and 5 mM thiosulphate) have been included for comparison. * = N-limited, nd = not determined.

Organism	NH_4 (mM)	Oxygen uptake rate (nmol/min/mg protein
Pseudomonas sp. LMD 84.60	nd	615
A.faecalis LMD 84.59	-3.6*	598
A.faecalis S6 LMD 60.48	-2.7	0
Ps.aureofaciens LMD 37.26	-2.9	652
Ps.fluorescens LMD 60.46	-4.6	109
Ps.fluorescens LMD 60.44	-3.3	128
Ps.fluorescens LMD 60.48	0	0
Ps.fluorescens LMD 60.71	-1.2	0

Batch cultures were then grown mixotrophically, and tested for thiosulphate-dependant oxygen uptake. The results fell into

three groups, as shown in Table 4. Oxygen uptake rates from chemostat-grown *Pseudomonas* sp. have been included for comparison. The first group achieved rates comparable with *Tsa. pantotropha* and *Pseudomonas* sp.. A second group oxidized thiosulphate much more slowly, and a few strains did not appear to be able to oxidize it at all. Amounts of thiosulphate ranging between 2.5 and 8.2 mM had disappeared from the cultures of the four strains able to oxidize it, but only one, *A. faecalis*, produced a trace (2 % of expected) of sulphate. The remainder of the thiosulphate had been oxidized to tetrathionate. Whether these species resemble *Tsa. pantotropha* and *Pseudomonas* sp. in their ability to oxidize thiosulphate to sulphate, or to obtain energy from the reaction, must await pH-controlled chemostat studies in order to control the alkalinity (>pH 9.5) due to polythionate formation. There was some, but not complete, correlation between high ammonia losses and thiosulphate oxidation potential.

CONCLUSION

It is obvious that this strain of the former "species" "*Pseudomonas denitrificans*" is 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 also differs from the three known versatile denitrifying species in various physiological reactions (Tables 1 and 2). Because of its coccoid shape, it falls outside the description of the genus *Thiobacillus*. On the basis of the API20B results, and because of its heterotrophic nitrification and aerobic denitrification this strain appears to be least closely related to *Pa. denitrificans*. It is thus most appropriate that the strain be grouped in the genus *Thiosphaera* in recognition of its autotrophic potential on thiosulphate, but not assigned a species name. It will therefore, for the moment, be referred to as *Thiosphaera* AJK.

In view of the growing number of species which are being found to be able to oxidize reduced sulphur compounds (see also Kuenen, 1988; Friedrich & Mitrenga, 1981; Suylen & Kuenen, 1986; Mason & Kelly, 1988), the use of this property as a primary taxonomic criterion will soon require revision, at least for the facultative chemolithotrophs and chemolithoheterotrophs.

It is clear that not all facultatively autotrophic sulphur oxidizing bacteria are heterotrophic nitrifiers. For example, *Pa. denitrificans* can grow autotrophically on thiosulphate (Friedrich & Mitrenga, 1981), but does not nitrify to any significant extent (Robertson et al., 1988B). However, given the fact that the organisms shown in Table 4 were chosen arbitrarily, it appears that many heterotrophic nitrifiers are sulphur-oxidizing bacteria. Whether this is more than simple coincidence must await a detailed investigation of any heterotrophic nitrifiers which do not appear to be able to oxidize thiosulphate, and experiments to discover whether the oxidizing capacities of most of the strains described here are limited to tetrathionate production. As was shown for *Thiobacillus* Q (Gommers & Kuenen, 1988), chemostat experiments with thiosulphate limitation are frequently required before mixotrophic growth can definitely be established for some apparent heterotrophs.

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GENERAL DISCUSSION

This work was originally initiated as an investigation of sulphur oxidizing metabolism in a denitrifying reactor. However, the dominant mixotroph isolated from the system, *Thiosphaera pantotropha*, proved to have several somewhat unusual capabilities (including its combined nitrification and denitrification pathways) and the direction of the work was "diverted" to concentrate on these points. Thus, as so often in research, serendipity has contributed a certain amount to the work.

a: Aerobic denitrification.

The first, cautious, observation in the paper describing *Thiosphaera pantotropha* (Chapter 2) that the organism might have a constitutive denitrifying system has resulted in nearly five years research. We now know that the denitrification pathway of *Tsa. pantotropha* is not only constitutive (Chapter 2) but is active under aerobic conditions in both batch (Chapter 3) and chemostat (Chapter 5) culture, as well as in resting cells (Chapter 4).

The traditional view that bacteria will show biphasic consumption of electron acceptors when given the choice between oxygen and nitrate has dominated the literature for many years. As with the diauxic growth of, for example, *Escherichia coli* on a mixture of two sugars, biphasic growth 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. For example, it is now widely accepted that during competition for limited resources in nature, mixotrophy, metabolic flexibility and reactivity are equally important metabolic parameters in determining the competitive success of a species (see, for example, Beudeker et al., 1982).

In this context, it is apparent that the versatility conferred by these metabolic parameters is no more surprising in electron accepting systems than it is in electron donation. As pointed out in Chapter 1, much of the controversy regarding aerobic denitrification was due to poor experimental design and techniques. However, equipment is improving all the time, and new techniques are rapidly becoming available. The use of sensitive 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. This 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* (Chapter 5), the rate of denitrification by *Z. ramigera* achieved its maximum at an oxygen concentration somewhat lower than 50% of air saturation.

The main focus of our experimental approach in order to prove the existence of, and to quantify aerobic denitrification in *Tsa. pantotropha* has involved the techniques mentioned above. In addition, other modern techniques, especially the use of ^{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 more

detail. Preliminary experiments using this method with labelled and unlabelled inorganic nitrogen compounds have shown that N_2 rather than N_2O is produced by *Tsa. pantotropha* during denitrification. Moreover, cells supplied with $^{15}NH_4Cl$ generated $^{15}N_2$ (E.W.J. van Niel, L.A. Robertson & R.P. Cox, unpublished results). 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 reported in Chapter 5), *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 much higher rates (3.42-5.61 nmol min^{-1} mg $protein^{-1}$). This is, of course, much lower (10-100 times, depending on the conditions) than the denitrification rates found with chemostat cultures of *Tsa. pantotropha* (see chapter 5). All of the strains also produced small amounts of N_2O. 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.

Research in this field has reached the point where, instead of disputing whether aerobic denitrification exists, interest can now focus on the quantitative aspects, as well as determining under what conditions, and why it occurs. Denitrification in both its aspects, as a bane to agriculture and a boon to waste water treatment, is of sufficient importance to demand an understanding of all its facets.

Observations in the field and in natural samples were made by Trevors (1985), Trevors & Starodub (1987) and Rönner & Sörensson (1985). They showed denitrification in the presence of reduced oxygen concentrations in soil samples, freshwater sediments and water from the deeper areas of the Baltic, respectively. In all three cases, denitrification was measured as N_2O production after acetylene treatment. Trevors & Starodub (1987) showed that the initial availability of nitrate was more important for the onset of denitrification than the degree of anoxia. 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. This observation is particularly interesting in view of the results presented in Chapter 9 where it is shown that other *A. faecalis* strains are also 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, and considered that the rate of mineralisation in soil was a more important controlling factor in denitrification than anaerobiosis. 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 from Trevors and Rönner & Sörensson, mentioned above.

On the other side of the coin, a recent review (Tiedje, 1988) has claimed that denitrification in the presence of significant amounts of oxygen has not been measured in nature. Furthermore, although Christensen & Tiedje (1988) state that denitrification is an exclusively anaerobic process, they do not raise any arguments against aerobic denitrification. Unfortunately, many of the methods used to measure denitrification in the field rely on acetylene inhibition of nitrous oxide reductase, with subsequent measurement of the N_2O produced. This technique may not always be entirely reliable for several reasons. Firstly, it is not yet clear how universal the sensitivity to acetylene is. Moreover, nitrifiers are also sensitive to acetylene and experiments which rely on naturally-produced nitrate or nitrite may thus give negative results in tests for denitrification for lack of nitrogen oxides. Obviously, the wealth of positive results in the literature shows that acetylene can be profitably used in many situations, but negative results should, perhaps, be treated with a degree of suspicion until checked by another method. Another problem which especially affects long-term checks for aerobic denitrification in soil was revealed by Topp & Germon (1986) who showed that certain *Nocardia* and *Arthrobacter* species (both common in soil) can metabolise acetylene, resulting in reduced inhibition because of acetylene concentrations which are lower than desired. The amount of denitrification taking place in long term experiments with soils or enrichment cultures could thus be underestimated because N_2 rather than N_2O was being produced, and not detected.

All in all, it can be concluded that the weight of evidence from quantitative laboratory and field experiments supports the contention that aerobic denitrification is a widespread phenomenon. It is likely that, rather than a universal "all or nothing" rule, a spectrum of electron acceptor utilization exists. As with other physiological phenomena, the bacteria best adapted for the predominant environmental and nutritive conditions will occur in any given situation.

b: Heterotrophic nitrification.

The observation that *Tsa. pantotropha* was able to oxidize ammonia to nitrite in batch culture (Robertson & Kuenen, 1984A) led to the finding that this and other species were simultaneously nitrifying and denitrifying, and that heterotrophic nitrification is probably more widespread and of greater significance than previously thought (Chapter 9). Preliminary results have indicated that even *Pa. denitrificans* may nitrify at a very low level (Chapter 8). Furthermore, in their chemostat study of *Z. ramigera*, Strand et al.. (1988) also measured ammonia levels in their cultures and it appears from the data provided that ammonia was disappearing from their cultures at a rate about half that found with similar cultures of *Tsa. pantotropha* (Chapter 5).

Once the existence of aerobic denitrification has been accepted, it immediately becomes clear that nitrification rates

obtained with mixed populations, especially in soil, require fresh evaluation as many were obtained from product (i.e. nitrate or nitrite) accumulation data and may be underestimates. Of course, where soil samples and other complex populations are concerned, it is not necessary that one organism carries out both reactions (e.g. an autotrophic bacterium or a fungus could be nitrifying and a heterotrophic bacterium denitrifying under aerobic conditions). In view of the combined nitrifying/denitrifying activities of *Tsa. pantotropha* and the other bacteria described in this thesis, the significance of heterotrophically nitrifying bacteria, especially, should be reconsidered. Neither *Tsa. pantotropha* or our strain of "*Ps. denitrificans*" would have been identified as heterotrophic nitrifiers if nitrite accumulation had been the only criterion under investigation. Of course, the maximum nitrification rates obtained with *Tsa. pantotropha* (around 240 nmol min^{-1} mg $protein^{-1}$) are still one or two orders of magnitude lower than those achieved by the autotrophs.

One of the methods popular in environmental work for distinguishing between heterotrophic and autotrophic nitrifying bacteria is the use of inhibitors. However, this may be only partially successful because *Tsa. pantotropha* has proved sensitive to chemicals (e.g. allyl thiourea, nitrapyrin, thiosulphate; Chapter 5A) which are classically regarded as inhibitors of autotrophic nitrification (Kuenen & Robertson, 1987). This is not surprising in view of the finding that the enzyme pathway for nitrification in *Tsa. pantotropha* is similar to that of the autotrophic nitrifiers (Chapter 6). The existence of this pathway in *Tsa. pantotropha* is also supported by the results of experiments with $^{18}O_2$ (D.J.D. Nicholas, personal communication). Given these facts, many of the field studies in which these inhibitors were used to discriminate between autotrophic and heterotrophic nitrifiers may have included inhibitor-sensitive heterotrophs in the autotrophic population, and thus may have underestimated their activity. It must therefore be concluded that the inhibitory effect of different reagents may depend on the type of nitrification pathway involved (i.e. organic or inorganic), rather than the autotrophic or heterotrophic nature of the bacteria involved. Experiments using $^{14}CO_2$ fixation as a measure of autotrophic activity (MacFarlane & Herbert, 1984) might give more reliable results provided that autotrophic growth on hydrogen or reduced sulphur compounds is excluded.

The results obtained with *Tsa. pantotropha*, "*Ps. denitrificans*" and other strains (Chapters 5 and 9) serve to emphasize that the activity of heterotrophic nitrifiers cannot be easily dismissed, and that their position requires reevaluation.

c: Simultaneous nitrification and denitrification - mechanisms and control.

Once the existence of heterotrophic nitrification, aerobic denitrification and a combination of the two phenomena in one organism has been established, it is obvious that both biochemical/physiological and ecological explanations must be provided. This section will focus on the biochemical and physiological aspects. In the following section, the ecological implications will be addressed.

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

1: 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 results presented in Chapter 6, where it is shown that the nitrification pathway of *Tsa. pantotropha* bears similarities to that of the autotrophic nitrifiers, have only added to this puzzle.
2: Why are the denitrifying enzymes of some bacteria sensitive to oxygen, and others not?

The model to describe electron transport in *Tsa. pantotropha* (Chapter 7) has aimed to provide at least partial answers to both of these questions. The model 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 (Chapter 5; H.J. Nanninga unpublished data).

Figure 1 outlines the various options *Tsa. pantotropha* has for producing and reoxidizing NAD(P)H. They will be discussed in a clock-wise sequence.

1: Under normal growth conditions, the organism must strike a balance between biomass production and NAD(P)H generation. In heterotrophic chemostat cultures, approximately 25% of the acetate is assimilated (Chapter 5), 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.
2: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".
3: NADPH is required by the ammonia monooxygenase of the nitrification pathway (Chapter 6). Nitrification, therefore, is also a means of reoxidizing NAD(P)H.
4: 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 (Chapter 7, Chapter 9, Kuenen & Robertson, 1987), and it might be postulated that the cytochrome cd-linked nitrite reducing system is more sensitive to oxygen than the copper nitrite reductase. However, the induction of the nitrate reductase-linked cytochrome b_{559} in aerobic as well as anaerobic cultures (Chapter 7) indicates that at least one other factor plays an important part. This induction may be possible even under aerobic conditions if, in contrast to other denitrifiers, the cells remain permeable to nitrate even under aerobic conditions (Alefounder et al., 1983).

5: The final option for the reoxidation of NAD(P)H by *Tsa. pantotropha* could be defined as "If all else fails, make PHB".

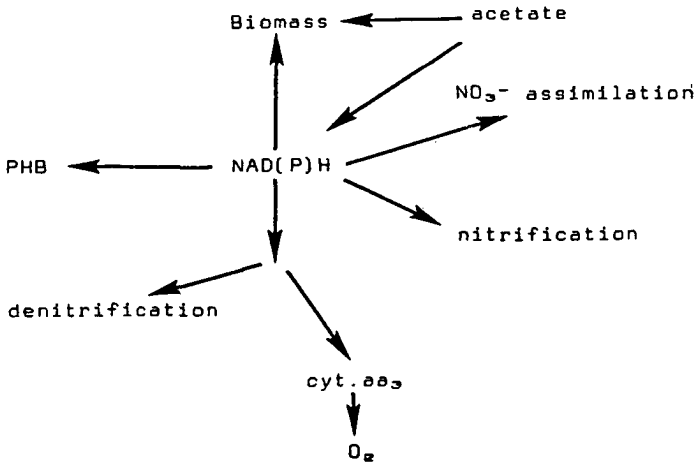

FIGURE 1. Simplified scheme showing the various possible options for NAD(P)H utilization available to *Tsa. pantotropha*.

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. *Tsa. pantotropha* strain TP43 lacks the denitrifying nitrite reductase and yet is still able to nitrify (to a lesser extent than the wild type) without accumulating nitrite (Chapter 5A). Hydroxylamine oxidoreductase from autotrophic NH_3 oxidizing bacteria has been shown to not only carry out its appropriate physiological function of oxidizing hydroxylamine to nitrite, but also to possess a second, catalytic ability in combining hydroxylamine and nitrite to make N_2O (Hooper, 1984). It may be speculated that the hydroxylamine oxidoreductase of *Tsa. pantotropha* also has this double function, permitting the mutant to continue to nitrify/denitrify, albeit at a lower rate. As the second catalytic function of the enzyme is obviously limited, this would mean that ammonia oxidation is, to some extent, inhibited. In line with this assumption, it appears that even *Tsa. pantotropha* TP43 cells growing aerobically on acetate and ammonia contained PHB (Chapter 5A). Although the low nitrification rates obtained with some *Pa. denitrificans* cultures were a little lower than those obtained with *Tsa. pantotropha*

TP43 (Chapter 8). PHB formation was not observed, indicating that the process is not so metabolically significant in *Pa. denitrificans*.

d: Ecological implications.

From the evidence presented here, it is obvious that a number of facultatively anaerobic denitrifiers are capable of the simultaneous utilization of oxygen and nitrate (or another nitrogen oxide). The observation that "an important aspect of bacterial metabolism that seems to be little appreciated is the fact that an organism may use two alternative respiratory mechanisms at the same time, provided that conditions are satisfactory for both" (Skerman et al., 1951) is still true today. In this sense, most denitrifying bacteria are able to do so "aerobically". The important difference is the concentration of oxygen at which the denitrification capacity is reduced or shut down completely, a concentration which can vary enormously between different organisms. From a physiological point of view the important question is whether oxygen is saturating the terminal cytochrome oxidase or not. This generally occurs at concentrations in the order of 1-2% of air saturation (2-4 micromoles/litre). In instances where the level of oxygen is above this threshold, as was the case with many of the experiments described here, it appears to be justified to speak of "aerobic denitrification". From an ecological or environmental point of view, aerobic denitrification means that the process occurs at detectable levels of air saturation (in common practice, around 5% of of air saturation).

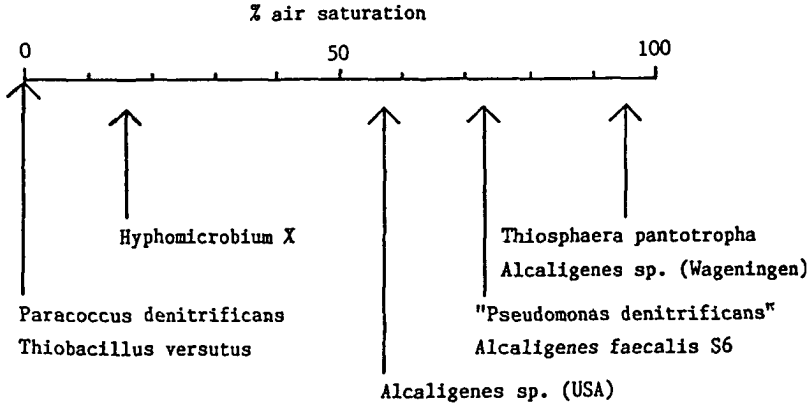

FIGURE 2.The different sensitivities of the denitrification systems of various bacteria to the dissolved oxygen concentration (from Kuenen & Robertson, 1987).

The finding that various denitrifying bacteria can tolerate different levels of dissolved oxygen before shutting down their denitrifying pathways (Kuenen & Robertson, 1987; Figure 2) has complicated the discussion of the ecological niche of these

bacteria from two different points of view. Firstly, variable nitrate disappearance and/or nitrite accumulation will be found with cultures in which the dissolved oxygen is not controlled, as this will depend on the reproducibility of the shaking/sparging and the oxygen sensitivity of the culture. Secondly, since these bacteria appear to have different "cutoff" oxygen concentrations, a range of oxygen concentrations must be tested before the behaviour of any particular nitrifier/denitrifier can be evaluated.

Can aerobic denitrification be rationalized, or even justified, in ecological terms? The obvious conceptual problem is that, because of the lower yield, it seems inefficient for an organism to co-respire oxygen and nitrate. In biochemical terms this might indeed be a disadvantage and, at first glance, it would appear that evolutionary pressure would act against the maintenance of such a trait in a species. The same could be said of heterotrophic nitrification, where a high nitrification rate is linked to a low yield (Chapter 5). This reasoning depends on the implicit assumption that these species exist in the same niche in which aerobic and anaerobic conditions are constant or alternate in a time span favourable for efficient growth leading to an optimal yield. However, not all niches are the same and growth yield is not the only property important for survival. The specific growth rate under the relevant conditions and the ability of the organism to respond efficiently and rapidly to a changing environment (i.e. the reactivity of the organism (Kuenen & Beudeker, 1982; Leegwater, 1983)) are also of great importance. A number of recent ecophysiological studies have addressed this type of question with regard to electron donor specificity and/or alternating mixed substrate utilization (Rittenberg, 1969; Gottschal & Kuenen, 1980; Kuenen & Gottschal, 1982; Kuenen & Robertson, 1984A; 1984B).

It seems likely that some parallels can be drawn with respect to oxygen versus nitrate respiration. Certainly, a species which did not need to reinduce its denitrifying system after exposure to oxygen, or which could continue to denitrify while taking advantage of the extra energy provided by limiting amounts of oxygen, would be able to out-compete other species not possessed of these properties. The washout of aerobically-grown *Pa. denitrificans* from a chemostat culture after a switch to anaerobiosis, while *Tsa. pantotropha* made a smooth transition to anaerobic growth (Chapter 8) emphasizes the advantages which flexibility with regard to electron acceptors can confer under changing conditions. Equally, a denitrifier living in an environment where long periods of anaerobic conditions are interspersed by short periods of aerobiosis should not inactivate its denitrifying system too readily since this would result in insufficient or no denitrifying capacity when anaerobic conditions were restored. *Tsa. pantotropha* was isolated from a waste water treatment system receiving nitrate as well as intermittent amounts of oxygen. As pointed out in Chapter 1, other aerobic (i.e. constitutive) denitrifiers have been isolated, frequently from wastewater treatment systems (e.g. Krul, 1976; Krul & Veeningen, 1977; Meiberg et al., 1980; Robertson & Kuenen, 1983; Chapter 9). Their activities may account for some of the nitrogen losses from aerobic treatment plants, and may even explain the occurrence of levels of denitrifying enzymes in well aerated soils (Tiedje et al., 1982).

Other types of bacteria are being found to operate more than one sort of electron acceptor. The simultaneous use of

nitrate and sulphate by a strain of *Ps. stutzeri* var. *denitrificans* (as well as the simultaneous use of oxygen and nitrate) seems apparent in results published by Ilyaletdinov et al.. (1982).Whether those sulphate reducers capable of nitrate reduction use the two electron acceptors simultaneously remains to be seen. As mentioned in Chapter 1, some fermentative species can obtain respiratory energy from the reduction of nitrate to nitrite. At least one of these, a species of *Klebsiella*, has been shown to use oxygen and nitrate simultaneously if grown at reduced dissolved oxygen tensions (Dunn et al., 1979). Another species of *Klebsiella* from the same source as *Tsa. pantotropha* also utilized nitrate at dissolved oxygen concentrations up to 80% of air saturation (L.A. Robertson & J.G. Kuenen, unpublished results), and it is clear that the phenomenon of co-respiration is not limited to the true denitrifiers. It is not unlikely that mixed electron acceptor utilization occurs in many different forms, of which aerobic denitrification is only one. However, rigorously quantitative experiments will be required to establish whether or not this is true.

Consideration of the ecological niche occupied by *Tsa. pantotropha* and other physiologically similar species is somewhat complicated by the fact that these bacteria must be viewed in relation to the nitrifiers as well as the denitrifiers. However, although it might at first be thought that competition for ammonia might be an important selective factor, the autotrophs are alone in gaining energy from the reaction, and the K_s values of *Tsa. pantotropha* for ammonia are such that direct competition for ammonia should not be a factor under normal conditions. Furthermore, it should be remembered that *Tsa. pantotropha* can use the nitrite or nitrate generated by the autotrophs as its nitrogen source as well as an electron acceptor, and thus competition for nitrogen is not really relevant. Other factors must determine whether autotrophic or heterotrophic nitrifiers occur in a given situation. The environmental impact of aerobic denitrifiers on the autotrophic nitrifiers may actually be mutualistic as potentially inhibitory nitrite and nitrate would be removed.

As mentioned in Chapter 1, there has been some controversy over the relative importance and activity of heterotrophic nitrifiers in nature. The suggestion that heterotrophic nitrifiers might be important in acid soils (see Chapter 1; Kuenen & Robertson, 1987) does not hold true for *Tsa. pantotropha*, "*Ps. denitrificans*" or any of the other nitrifiers described here (Chapter 9) as none of them grow at low pH. Also, "*Ps. denitrificans*" has been shown to nitrify more at pH 8.0 than at pH 7.0 (Chapter 9). It is likely that fungi are responsible for most of the nitrification taking place at low pH. Killham (1986) showed that autotrophic nitrifiers predominated in cultivated soil which had been ploughed and fertilized. Heterotrophic nitrifiers were more important in undisturbed forest soil. In situations such as these, the availability of organic or inorganic substrates would play a central role in determining whether the turnover of ammonia is due to the action of autotrophs or heterotrophs. The dissolved oxygen concentration might also be an important factor. Helder & de Vries (1983) showed that some autotrophic nitrifiers were inhibited by low dissolved oxygen concentrations. In a series of experiments, they showed that once the concentration of oxygen in a mixed culture of *Nitrosomonas* and *Nitrobacter* species fell below 85 µmol/litre, nitrite began to accumulate in the supernatant. Once the

dissolved oxygen dropped to below 32 μmol/litre, ammonia also accumulated. The K_m for oxygen for *Tsa. pantotropha* while using acetate is between 3.5 and 4.5 μM (L.A. Robertson, unpublished data), while that of *N. europaea*, while utilizing ammonia, is around 18 uM (E.W.J. van Niel, unpublished data). *Tsa. pantotropha* thus remains able to use oxygen at much lower concentrations than *N. europaea*. Also, it appears to use all of its oxygen uptake at these low concentrations for nitrification, with the denitrification pathway carrying the entire respiratory load (Chapter 5). Moreover, *Tsa. pantotropha* increases its nitrification rate at lower dissolved oxygen concentrations (Chapter 5). It thus seems reasonable to suggest that much of the nitrification taking place at reduced oxygen concentrations where the activity of most autotrophic nitrifiers (excepting any with a high affinity for oxygen) would be reduced or absent (e.g. in undisturbed soil and near aerobic/anaerobic interfaces) is due to the activity of heterotrophically nitrifying bacteria.

Concluding remarks

Thiosphaera pantotropha is a good example of a bacterium well adapted to life at variable interfaces where opportunism would be a major survival force. Both its extreme flexibility with regard to electron acceptors and its versatility in substrate utilization (especially its ability to use reduced sulphur compounds) demonstrate that this species is well suited to the conditions surrounding aerobic/anaerobic interfaces.

As mentioned in Chapter 1, the story of aerobic denitrification began almost 100 years ago with the claim by Bréal (1892) that his test species were denitrifying aerobically. The possibility of simultaneous nitrification and nitrate reduction was originally put forward by Léone in 1887. He introduced the concept that some bacteria could have both an oxidative and a reductive function, depending on the conditions. Truly there is "nothing new under the sun" (Geoffrey Chaucer, "The Knight's Tale", 1387 (approx.)).

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SUMMARY

Nitrogen removal from wastewater is becoming increasingly important as we become aware of the harm which ammonia, nitrite and other nitrogen compounds can do to people, livestock and the environment, in general. In addition, millions are spent each year on nitrogenous fertilizers for the agricultural industry. In order to keep nitrogen compounds where they are wanted (on the fields) and away from where they are not wanted (e.g. in natural waters), it is necessary that denitrification (by which fixed nitrogen is returned to the atmosphere as N_2) and nitrification (where reduced nitrogen compounds are oxidized, making them available for denitrification) are fully understood (Chapter 1). Many basic microbiological principles are most easily derived from quantitative experiments using pure cultures or simple mixtures of known bacteria. Data gathered in this way can then be used to make models of the different processes and predict microbial behaviour in more complex situations (Chapter 1).

We are currently investigating the microbiology of denitrification in a recently developed wastewater treatment system which employs fluidized bed reactors. One of the bacteria isolated from this denitrifying, sulphide oxidizing wastewater treatment reactor was found to be the first species of a new genus. This denitrifying, facultative chemolithoautotroph was given the name *Thiosphaera pantotropha* in recognition of its morphology and ability to grow on reduced sulphur compounds, hydrogen and a wide range of organic substrates (Chapter 2). *T. pantotropha* differed from other known colourless sulphur bacteria in various physiological traits, one of the most important being that it retained its sulphur oxidizing potential while growing under anaerobic conditions, with nitrate or nitrite as its electron acceptor.

During studies on its denitrifying abilities, it was found that the denitrifying enzymes of *T. pantotropha* were not only constitutive (Chapter 2), but were actually active in resting cells (Chapter 4) as well as batch and chemostat cultures, even at dissolved oxygen concentrations 80-95% of air saturation (Chapters 3 and 5, respectively). Simultaneous nitrate and oxygen uptake could be shown be means of electrodes (Chapter 4). Aerobic denitrification (i.e. the simultaneous use of oxygen and denitrification when oxygen is not limiting) has been a subject of controversy for about 100 years. Among the reasons for this controversy have been poor experimental design and monitoring, the fact that denitrification is energetically less favourable than oxygen utilization, and the concentration of denitrification research on relatively few strains. For this reason, it was felt necessary to carry out detailed, quantitative experiments in order to establish whether or not aerobic denitrification is really an artefact.

In batch cultures when the dissolved concentration was 80% that of air saturation, sufficient nitrate was used to account for half of the respiration taking place (Chapter 3). In the chemostat, the rate of denitrification varied with the dilution (growth) rate. For example, at a dilution rate of 0.02 h^{-1}, the denitrification rate in a fully aerobic, acetate-limited culture growing in a medium which contained NH_3 and NO_3^- was about 40 nmol min^{-1} mg $protein^{-1}$. When the dilution rate was increased to 0.14 h^{-1}, the denitrification rate rose about ten-fold, to 389 nmol min^{-1} mg $protein^{-1}$ (Chapter 5). Similar results, with slightly lower rates, were obtained in aerobic chemostat cultures

supplied with nitrite (Chapter 5). Reducing the dissolved oxygen also caused an increase in the amount of denitrification taking place. This reached its maximum at a dissolved oxygen concentration around 30% of air saturation.

It seemed that the ability to use oxygen and nitrate simultaneously allowed *T. pantotropha* to grow more rapidly than it could when growing on either electron acceptor, alone (Chapter 3). This increase of its aerobic maximum specific growth rate (μ_{max}) in the presence of nitrate allowed *T. pantotropha* to dominate in mixed cultures with a strain of *Paracoccus denitrificans* which does not denitrify aerobically, even at growth rates at which *P. denitrificans* would have had an advantage when nitrate was absent (Chapter 8). Its constitutively active denitrifying system also meant that *T. pantotropha* was relatively unaffected by aerobic/anaerobic switches, whereas aerobically-grown *P. denitrificans* washed out when switched to anaerobic conditions, because it was unable to synthesize its denitrification enzymes rapidly enough (Chapter 8).

T. pantotropha also proved to be a heterotrophic nitrifier, oxidizing both ammonia and hydroxylamine, when provided in the medium, to nitrite in the presence of an external energy source such as acetate or succinate (Chapters 5 and 6). The rates achieved depended on the growth rate but could be as high as 240 nmol min^{-1} mg $protein^{-1}$ (only 1–2 orders of magnitude lower than those obtained with the autotrophic ammonia oxidizers). That *T. pantotropha* does not gain energy from ammonia oxidation was shown by the correlation between high nitrification rates and low protein yields obtained from acetate limited chemostats (60% of expected values). When nitrification was inhibitedin various ways (e.g. by the addition of thiosulphate or nitrate to the culture medium), the yields more nearly approached their expected values (Chapter 5).

The enzymes used for nitrification by *T. pantotropha* were physiologically similar to those reported for the autotrophs. The ammonia monooxygenase required NAD(P)H and Mg^{2+}, and was light sensitive. Hydroxylamine oxidoreductase activity could be shown in vitro using cytochrome c, and could be inhibited by hydrazine (Chapter 6). Nitrification by *T. pantotropha* could be inhibited by chemicals classically regarded as specific inhibitors of autotrophic nitrification (e.g. nitrapyrin, allylthiourea). It seems logical that response to inhibitors is determined by the type of pathway (i.e. enzymes and intermediates) involved, rather than whether the organism is an autotroph or a heterotroph.

It became clear that nitrification and denitrification by *T. pantotropha* were very closely linked, and possibly shared a common cause or function. A model based on experimental cytochrome measurements and physiological data was therefore developed in an attempt to explain the two phenomena (Chapter 7). It seems likely that heterotrophically growing *T. pantotropha* has problems reoxidizing NAD(P)H, possibly because of a "bottleneck" in the pathway of electron flow to oxygen via cytochrome aa_3. Simultaneous denitrification and oxygen utilization would allow electrons to pass more rapidly along the cytochrome chain, but would generate less energy, thus sacrificing "efficiency" for speed. If denitrification was not possible, the requirement for NAD(P)H of the ammonia monooxygenase would also provide a means of regenerating $NAD(P)^+$ (Chapter 7). When unable to nitrify or denitrify, *T. pantotropha* made poly β-hydroxybutyrate (Chapter 5), another means of reoxidizing NAD(P)H. Aerobic cultures in which nitrate was the sole source of nitrogen only denitrified at

about 10% of the rates found with similar cultures which received ammonia and nitrate. As the assimilatory nitrite reductase also requires NAD(P)H, the use of this enzyme would also reduce any potential excess of NAD(P)H.

Of course, *T. pantotropha* would not have been recognised as a heterotrophic nitrifier by the traditional methods of detection as these involve the measurement of nitrite or nitrate accumulating in the culture medium. Because of the aerobic denitrification, this does not occur (Chapters 3 and 5). A survey of other heterotrophs which had been identified either as aerobic denitrifiers or as "poor" heterotrophic nitrifiers showed that they were also simultaneously nitrifying and denitrifying, although the concentration of oxygen which affected their denitrification systems varied (Chapter 9). Another indication that they do not all behave in an identical fashion came from chemostat experiments with "*Pseudomonas denitrificans*" which showed that while it has constitutive nitrite and nitrous oxide reductases, its nitrate reductase must be induced by the presence of nitrate. However, one factor which all of the aerobic denitrifiers so far tested have in common is that they all have the copper nitrite reductase rather than cytochrome cd (Chapters 7 and 9). Given the fact that cytochrome cd is known to be inhibited by very low concentrations of oxygen, perhaps because it can also serve as a terminal oxidase, this might be an indication that the copper-based nitrite reductase is less oxygen sensitive. It is clear that combined nitrification and denitrification is relatively widespread, and that the ammonia oxidizing activity of heterotrophic nitrifiers can only be evaluated from full nitrogen balances. In retrospect, many cases may have been undervalued.

In common with the autotrophic ammonia oxidizers, nitrification by *T. pantotropha* and "*Ps. denitrificans*" was inhibited by thiosulphate (Chapters 5 and 9). As both species have also been shown to grow autotrophically on it, other heterotrophic nitrifiers were tested for the ability to oxidize thiosulphate. Most of the strains tested oxidized thiosulphate to tetrathionate in batch culture (Chapter 10). A full evaluation of their thiosulphate-utilizing potential must await chemostat studies. However, it seems that the use of an ability to oxidize reduced sulphur compounds as a taxonomic criterion for facultative autotrophs may require reconsideration. While it is possible that all heterotrophic nitrifiers can oxidize thiosulphate, it is unlikely that all thiosulphate oxidizers nitrify (Chapter 10).

As a bacterium which can adjust to changes in its substrate supply by inducing the appropriate enzymes and growing autotrophically, heterotrophically or mixotrophically, and which can also cope with sudden changes in its electron acceptor supply, *T. pantotropha* has taken the concept of versatility to extremes. This type of physiology is clearly well suited to life under the varying regimes which would be found in wastewater treatment systems, as well as at the aerobic/anaerobic interfaces which occur in soil and sediments.

SAMENVATTING

Een beter inzicht in diverse deelprocessen van de stikstofcyclus is essentieel omdat stikstofverbindingen in het milieu tal van problemen kunnen veroorzaken en de verwijdering van stikstofverbindingen uit afvalstromen van groot belang is. Hoewel toevoeging van stikstofverbindingen voor de bodemvruchtbaarheid noodzakelijk is, vormt overbemesting via uitspoeling tot een ongewenste belasting van oppervlakte- en grondwater met ammonia en nitraat. Ammonia in het oppervlaktewater kan tot eutrofiering leiden en is, afhankelijk van de pH, ecotoxisch. Te veel nitraat kan ook tot algenbloei aanleiding geven. Omdat grondwater vaak als bron van drinkwater fungeert is met name nitraat daarin ongewenst. In het menselijk lichaam wordt het nitraat immers door de darmflora in het bijzonder schadelijke nitriet omgezet. In dit proefschrift richt de aandacht zich vooral op de biologische denitrificatie en nitrificatie, deelprocessen die in combinatie in de landbouw tot ongewenste stikstofverliezen in de bodem leiden, maar die tevens in afvalwaterzuiveringssystemen benut kunnen worden om vervuiling van oppervlaktewater met respectievelijk nitraat en ammonia te beperken of te voorkomen.

Veel microbiologische basisprincipes kunnen afgeleid worden uit kwantitatieve experimenten met *reincultures* of eenvoudige mengsels van bekende bacterien. De gegevens, die op deze wijze worden verkregen, kunnen worden gebruikt om modellen te maken van de verschillende deelprocessen waarmee vervolgens de complexe processen in (semi) natuurlijke systemen voorspeld kunnen worden (Hoofdstuk 1).

Ons onderzoek heeft zich gericht op de microbiologie van denitrificatie in een recent ontwikkeld afvalwaterzuiveringssysteem, waarbij gebruik wordt gemaakt van fluide-bedreactoren. Tijdens dit onderzoek kwamen een tweetal onverwachte microbiologische processen aan het licht, die een nadere studie vereisten: het vermogen van bepaalde bacterien om onder aerobe omstandigheden te denitrificeeren en het vermogen om dit proces te koppelen met gelijktijdige heterotrofe nitrificatie. Een van de dominante bacterien uit het denitrificerende, sulfide-oxyderende, afvalwaterzuiveringssysteem werd in detail bestudeerd om dit proces nader te analyseren. Dit organisme was een denitrificerende, facultatief chemolithoautotrofe bacterie, die niet in een bekend bacteriegeslacht kon worden ondergebracht. Het kreeg de naam *Thiosphaera pantotropha* vanwege zijn morfologie en het vermogen om naast gereduceerde zwavelverbindingen en waterstof op een grote verscheidenheid aan organische verbindingen te kunnen groeien (Hoofdstuk 2). Fysiologisch verschilt *Tsa. pantotropha* in verschillende opzichten van andere kleurloze zwavelbacterien. Een van de belangrijkste hiervan is dat het vermogen om zwavel te oxyderen gehandhaafd blijft bij groei onder anaerobe condities met nitriet of nitraat als electronenacceptor.

Tijdens de bestudering van het denitrificerende vermogen van *Tsa. pantotropha* bleek dat de denitrificerende enzymen niet alleen constitutief aanwezig waren (Hoofdstuk 2) maar ook nog steeds actief waren in rustende cellen (Hoofdstuk 4), zowel in batch- als in chemostaat-cultures, zelfs bij opgeloste zuurstofconcentraties van 80-95% luchtverzadiging (respectievelijk Hoofdstukken 3 en 5). Met behulp van electroden kon de gelijktijdige opname van nitraat en zuurstof worden aangetoond (Hoofdstuk 4). Aerobe denitrificatie (d.w.z. het

simultane gebruik van zuurstof en nitraat (of nitriet), wanneer zuurstof niet beperkend is) is al 100 jaar een controversieel onderwerp. Het bestaan van deze controverse is o.a. te wijten aan (i) slecht opgezette experimenten en ontoereikende meetmethoden, (ii) het gegeven dat denitrificatie energetisch minder gunstig is dan het gebruik van zuurstof, en (iii) de beperking van het onderzoek aan denitrificatie tot relatief weinig stammen. Daarom werd de behoefte gevoeld gedetailleerde en kwantitatieve experimenten uit te voeren ten einde vast te stellen of aerobe denitrificatie nu wel of niet een artefact is.

In batch-cultures, waarin de opgeloste zuurstofconcentratie 80% luchtverzadiging was, bleek nitraatverademing de helft van de totale ademhaling voor zijn rekening te nemen (Hoofdstuk 3). In de chemostaat varieerde de mate van denitrificatie met de verdunnings(groei)snelheid. Bij een verdunningssnelheid van 0.02 h^{-1} was de denitrificatiesnelheid van een volledig aerobe, acetaat-gelimiteerde cultuur, groeiend in een medium met NH_4^+ en NO_3^-, ongeveer 40 nmol min^{-1} mg $eiwit^{-1}$. Opvoeren van de verdunningssnelheid tot 0.14 h^{-1} leidde tot een bijna 10-voudige toename van de denitrificatie tot 390 nmol min^{-1} mg $eiwit^{-1}$ (Hoofdstuk 5). Vergelijkbare resultaten, zij het met iets lagere snelheden, werden verkregen met aerobe chemostaat-cultures in aanwezigheid van nitriet (Hoofdstuk 5). Vermindering van de opgeloste zuurstofconcentratie veroorzaakte eveneens een toename van de denitrificatie, terwijl het maximale effect werd waargenomen bij 30% verzadiging.

Het vermogen om zuurstof en nitraat gelijktijdig te gebruiken bleek *Tsa. pantotropha* in staat te stellen om sneller te groeien dan op elk van de electronenacceptoren afzonderlijk (Hoofdstuk 3). Deze toename van de aerobe maximale specifieke groeisnelheid (μ_{max}) in aanwezigheid van nitraat stelde *Tsa. pantotropha* in staat te domineren in een mengcultuur met een stam van *Paracoccus denitrificans* (die niet aeroob kan denitrificeren), zelfs bij groeisnelheden waarbij *Pa. denitrificans* in het voordeel geweest zou zijn bij afwezigheid van nitraat (Hoofdstuk 8). Het constitutieve, actieve denitrificerende systeem van *Tsa. pantotropha* betekende voor dit organisme dat het weinig hinder ondervond van aerobe/anaerobe overschakelingen, terwijl aerobe gekweekte *Pa. denitrificans* cellen uitspoelden na overschakeling op anaerobe condities omdat deze niet in staat bleken te zijn om snel genoeg denitrificerende enzymen te synthetiseren (Hoofdstuk 8).

Tsa. pantotropha bleek ook een heterotrofe nitrificeerder te zijn, in staat om het aangeboden ammonia of hydroxylamine tot nitriet te oxyderen in aanwezigheid van een externe energiebron zoals acetaat of succinaat (Hoofdstukken 5 en 6). De snelheid hiervan hing af van de groeisnelheid en kon een waarde van 240 nmol min^{-1} mg $eiwit^{-1}$ bereiken (slechts 1 a 2 orden van grootte kleiner dan die bij autotrofe ammonia-oxydeerders) gevonden zijn. Dat *Tsa. pantotropha* geen energie haalt uit de oxydatie van ammonia bleek uit het verband tussen hoge nitrificatiesnelheden en lage eiwitopbrengsten in acetaat-gelimiteerde chemostaat-cultures (60% van de verwachte waarden). Inhibitie van de nitrificatie (door b.v. thiosulfaat of nitraat aan het kweekmedium toe te voegen) leidde tot eiwitopbrengsten die dichter in het buurt kwamen van de verwachte waarden (Hoofdstuk 5).

De enzymen betrokken bij de nitrificatie door *Tsa. pantotropha* waren fysiologisch vergelijkbaar met die welke beschreven zijn bij de autotrofe organismen. Het

ammonia-monooxygenase had NAD(P)H en Mg^{2+} nodig en was gevoelig voor licht. Met behulp van cytochroom c kon activiteit van het hydroxylamine-oxidoreductase en de remming ervan door hydrazine, in vitro worden aangetoond (Hoofdstuk 6). Nitrificatie door *Tsa. pantotropha* kon geremd worden met chemische verbindingen, die van oudsher bekend staan als specifieke remmers van autotrofe nitrificatie (b.v. nitrapyrine, allylthioureum). Het lijkt logisch dat de gevoeligheid voor remmers eerder wordt bepaald door de betrokken enzymen en intermediairen dan door het autotroof of heterotroof zijn van het organisme.

Het was duidelijk dat nitrificatie en denitrificatie door *Tsa. pantotropha* nauw met elkaar samenhangen en mogelijk een gemeenschappelijke oorzaak of funktie hebben. Om de twee fenomenen beter te kunnen verklaren werd daarom een model ontwikkeld, gebaseerd op zowel experimentele metingen aan cytochromen als op andere fysiologische gegevens (Hoofdstuk 7). Waarschijnlijk heeft de heterotroof groeiende *Tsa. pantotropha* problemen met de heroxydatie van NAD(P)H, mogelijk vanwege een "bottleneck" in de electronentransportketen waarlangs electronen via cytochroom aa$_3$ naar zuurstof vloeien. Het simultaan optreden van denitrificatie en het gebruik van zuurstof zou dan het electronentransport langs de cytochroomketen versnellen, maar minder energie opleveren zodat "efficiency" opgeofferd wordt ten gunste van snelheid. Wanneer ook denitrificatie niet mogelijk zou zijn, dan zou als gevolg van de NAD(P)H-behoefte van het ammonia-monooxygenase, regeneratie van $NAD(P)^+$ mogelijk zijn (Hoofdstuk 7). Wanneer *Tsa. pantotropha* niet in staat was te nitrificeren of te denitrificeren, maakte het organisme poly-β-hydroxybutyraat, hetgeen beschouwd kon worden als nog een alternatief om NAD(P)H te oxyderen (Hoofdstuk 5). Aerobe cultures met nitraat als enige stikstofbron denitrificeerden met een snelheid die slechts 10% was van die in cultures waarin tevens ammonia aanwezig was. Aangezien het bij de assimilatie betrokken nitriet-reductase ook NAD(P)H nodig heeft, zal ook langs deze weg de potentiele overmaat van NAD(P)H verkleind kunnen worden.

Het spreekt vanzelf dat *Tsa. pantotropha* met behulp van de traditionele detectiemethoden niet geidentificeerd zou zijn als een heterotrofe nitrificeerder, omdat deze methoden gebaseerd zijn op het meten van de accumulatie van nitriet of nitraat in het kweekmedium. Als gevolg van de aerobe denitrificatie treedt dit niet op (Hoofdstukken 3 en 5). Een serie proeven met andere heterotrofen, die indertijd geidentificeerd waren als aerobe denitrificeerders, dan wel als "slechte" heterotrofe nitrificeerders, liet zien dat deze ook gelijktijdig tot nitrificatie en denitrificatie in staat waren, alhoewel de zuurstofgevoeligheid van hun denitrificatie-systemen varieerde (Hoofdstuk 9). Een andere aanwijzing dat ze zich niet allemaal op dezelfde wijze gedroegen werd verkregen uit chemostaat-experimenten met "*Pseudomonas denitrificans*", waaruit bleek dat in dit organisme nitriet- en stikstofoxyde-reductases constitutief zijn, terwijl het nitraat-reductase geinduceerd moet worden. Een factor die alle tot dusver geteste aerobe denitrificeerders echter gemeen hebben is dat ze allemaal het koperbevattende nitriet-reductase hebben in plaats van cytochroom cd (Hoofdstukken 7 en 9). Het is duidelijk dat gecombineerde nitrificatie en denitrificatie veel voorkomt en dat de ammonia-oxyderende activiteit van heterotrofe nitrificeerders alleen goed beoordeeld kan worden aan de hand van volledige stikstofbalansen. Uiteindelijk zou kunnen blijken dat het nitrificerende vermogen in veel gevallen ondergewaardeerd is.

Net als bij de autotrofe ammonia-oxydeerders wordt nitrificatie bij *Tsa. pantotropha* en "*Ps. denitrificans*" geremd door thiosulfaat (Hoofdstukken 5 en 9). Omdat beide organismen hierop autotroof konden groeien, werden ook ook andere heterotrofe nitrificeerders getest op hun vermogen thiosulfaat te oxyderen. De meeste stammen oxydeerden in batch-cultures thiosulfaat tot tetrathionaat (Hoofdstuk 10). Een volledige beoordeling van hun vermogen thiosulfaat te gebruiken is pas mogelijk na het uitvoeren van chemostaat-studies, maar het is wel duidelijk dat de oxydatie van gereduceerde zwavelverbindingen meer algemeen vookomt dan vroeger aangenomen werd. Het is mogelijk dat alle heterotrofe nitrificeerders thiosulfaat kunnen oxyderen, maar het omgekeerde, nitrificatie door alle thiosulfaatoxyderende, heterotrofe organismen, lijkt niet waarschijnlijk (Hoofdstuk 10).

Tsa. pantotropha heeft aan het concept van veelzijdigheid een extra dimensie gegeven door naast zijn vermogen om bij verandering van de toevoer van substraten, c.q. electrondonoren, snel de juiste enzymen te induceren en dan zowel autotroof, heterotroof als mixotroof te kunnen groeien, ook in staat te zijn plotselinge veranderingen in de beschikbaarheid van electronenacceptoren goed op te kunnen vangen. Dit soort fysiologie is duidelijk zeer geschikt voor het leven onder de sterk varierende omstandigheden zoals die gevonden worden in afvalwaterzuiverinssystemen en ook op aerobe/anaerobe grensvlakken in de bodem en sedimenten.

AFTERWORD

There can be very little research, these days, which is carried out by a single person working in isolation. There are certainly a great many people to whom I must be grateful for their help and encouragement during the progress of this research.

Firstly, I must thank the various co-authors of the Chapters in this thesis. Without their contributions, much of the work would not have been possible. I am also grateful to Max Diederik, Jos Lispet, Nico Raaphorst and the gentlemen of the Biotechnology Institute workshops for their help in keeping the media flowing and the equipment running. Trudy Suylen, Yde Steensma, Piet Bos and my other colleagues at the Institute for Biotechnology, especially the members of the "Prokaryote-group", provided much needed encouragement during the inevitable crises. Professor Stouthamer kindly allowed us to do most of the work described in Chapter 7 in his Laboratory at the Free University of Amsterdam, and thanks are due to everyone there for making our visits so pleasant. Julian Wimpenny provided the solution to one particularly thorny problem.

I would especially like to thank the many students who participated in the research as a part of their studies (whether as "afstudeerders", "bijvakkers", "stagiaires" or "scriptie" authors). They were:-

Olof Akkerman, Guus Asyee, Leendert Berkhout, Elvis Biekman, Elly Bonnet, Cees Buisman, Bert Christoffels, Ronald Cornelisse, Jos van Daalen, Brigit van Elk, Prashant Gupta, Adele van Houwellingen, Mattijs de Jong, Rene Kleijntjens, Jan Koppejan, Annemiek Laanen, Gerrie Mijnbeek, Ed van Niel, Harro Slobbe, Rob Torremans, Jaap Twisk, Marlou Verdumen, Henk Vernij, Anita van de Vet, Meinte Wildschut, Arjan de Wit.

Ratna Hadioetomo (from Indonesia), Paul de Vos (from Belgium) and Rong Zheng (from China) provided an international flavour during their (too-short) visits.

Finally, of course, there is "the Boss". Thank you, Gijs, for your help and encouragement, for refusing to believe that "aerobic denitrification is impossible" (thermodynamically or otherwise!), and for making this possible.

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