4. Denitrification by Obligate and Facultative Autotrophs

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Introduction

The Organisms

Denitrification is the use of nitrate, nitrite or the other nitrogen oxides as terminal electron acceptors in bacterial respiration. The end product is generally N_2, although a few species appear to terminate at N_2O (Gayon & Dupetit 1882;. Payne 1981; Stouthamer 1988a; 1988b). Some organisms can only convert nitrate to nitrite, a process known as nitrate respiration, although many (e.g. *Escherichia coli* and *Proteus mirablis*) can then reduce the nitrite to ammonia (Cole 1987; Cole & Brown 1980). This last is generally termed dissimilatory nitrate reduction in order to distinguish it from denitrification, where nitrogen is actually lost. In many organisms, the primary role of dissimilatory nitrate reduction is to serve as an additional sink for electrons during fermentative metabolism.

The denitrifiers form one of the most diverse groups of bacteria, with representatives of most physiological types being included. For example, there are obligate autotrophs and heterotrophs, methylotrophs and phototrophs, extreme halophiles and even nitrogen-fixing strains which denitrify. One of the most unusual denitrifying bacteria may be *Vibrio succinogenes* which reduces nitrate and nitrite to ammonia (as might be expected with a species from this genus) but which can also grow anaerobically with N_2O as the terminal electron acceptor, producing N_2. The resemblance of the responsible enzyme in this fermentative species with that of the true denitrifiers was emphasized by the fact that N_2O reduction by *V. succinogenes* was also inhibited by acetylene (Yoshinari 1980; Stouthamer 1988a). For a broad overview of most known denitrifying species, the reader should consult Payne (1981).

Recent work in our laboratory has concentrated on a denitrifier, *Thiosphaera pantotropha*, which combines a number of unusual properties. As will be discussed below, this organism is a constitutive denitrifier which is able to denitrify under fully aerobic conditions. It is also a heterotrophic nitrifier, being able to oxidize ammonia to nitrite in the presence of an organic

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substrate (for a review of heterotrophic nitrification, see Verstraete 1975). In addition, *Tsa. pantotropha* is a facultative autotroph capable of growth on hydrogen and reduced sulphur compounds under both aerobic and anaerobic (denitrifying) conditions. The combination of all of these properties prompted us to survey the literature on denitrifying autotrophs, and this is the subject of the present review. It was, of course, not a surprise that this cross-section through the microbial world involves a very heterogenous group of organisms, encompassing both obligately and facultatively autotrophic organisms. As will be discussed below, some facultative autotrophs appear to become obligately heterotrophic while denitrifying. Table 1 shows examples of obligate and facultative autotrophs which denitrify. Those species which only reduce nitrate to nitrite (e.g. *Thiobacillus thioparus* or *T. tepidarius*) or which use the dissimilatory nitrate reduction pathway to ammonium (e.g. *Escherichia coli* and *Proteus mirablis*) have been omitted. This paper concentrates on the consequences, some of them dramatic, which a change in electron acceptors (i.e. from oxygen to nitrate, nitrite or N_2O) can have on the physiology of this group of organisms, particularly with reference to our recent findings with *Tsa. pantotropha.*

Table 1. Examples of bacteria capable of chemolithotrophic growth which can also denitrify. obl = obligately autotrophic; fac = facultatively autotrophic.

Genus	Species	Obl/fac	End product	Substrates which support denitrification
Thiobacillus	*denitrificans*	obl	N_2	S^{2-} $S_2O_3^{2-}$ S^0
	versutus	fac	N_2	organic compounds
Thiomicrospira	*denitrificans*	obl	N_2	S^{2-} $S_2O_3^{2-}$ S^0
Thiosphaera	*pantotropha*	fac	N_2	S^{2-} $S_2O_3^{2-}$ H_2 organics
Paracoccus	*denitrificans*	fac	N_2	H_2 organics
Thermothrix	*thiopara*	fac	N_2	organic compounds
Alcaligenes	*eutrophus*	fac	N_2	H_2 organics
Pseudomonas	*saccharophilia*	fac	N_2	H_2 organics
	pseudoflava	fac	N_2	H_2 organics
Nitrosomonas	*europaea*	obl	N_2O	NH_4^+
Nitrosomonas	sp.	obl	N_2	NH_4^+
Nitrobacter	*hamburgensis*	fac	N_2	organics

Of the many inorganic compounds which support aerobic growth, only hydrogen, reduced sulphur compounds and (recently) ammonia have been shown to support denitrification. As can be seen below, the standard free energy charges ($\Delta G'$) per mol of substrate for denitrification are, in many cases, not much lower than those for oxygen respiration.

	$\Delta G_0'$ (kj per mol)
$2H_2 + O_2 \rightarrow 2H_2O$	-237
$5H_2 + 2H^+ + 2NO_3^- \rightarrow N_2 + 6H_2O$	-224
$S^{2-} + 2O_2 \rightarrow SO_4^{2-}$	-831
$5S^{2-} + 8NO_3^- + 8H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O$	-781

Of course, $\Delta G'$ values are extremely useful for showing how much energy can be derived from a given reaction from a thermodynamic point of view, and thus pin-pointing which reactions are possible. However, they can only give a very rough indication of the amount of ATP which may be generated in a physiological reaction because this is determined by a range of factors including the enzymes and cytochromes involved, as well as environmental parameters.

The Denitrifying Pathway and Enzymes

The enzymes and energetics of denitrification have been recently reviewed (Stouthamer 1988a; 1988b) and will not be dealt with in any great detail here. Consideration of the enzymes involved, however, reveals that despite the wide range of energy generating and carbon assimilating systems, those denitrifying enzymes which have been studied appear to be similar, even though they were isolated from widely different species. Indeed, even the nitrate reductase involved in dissimilatory nitrate reduction appears to be similar to that found in various denitrifying bacteria. Two different types of denitrifying nitrite reductase have been observed in different strains. One of these, known as cytochrome cd, has been identified in a number of bacteria (e.g. *Paracoccus denitrificans, Thiobacillus denitrificans*) and has been relatively well studied (e.g. Boogerd 1984; Alefounder & Ferguson 1981; Stouthamer 1980). A second nitrite reductase is copper-based rather than a cytochrome-based enzyme, and has been found in strains such as *Rhodopseudomonas sphaeroides var denitrificans* and *Achromobacter cycloclastes* (Michalski & Nicholas 1985; Iwasaki et al. 1975). Perhaps because of the limited number of strains in which it has thus far been found, this second nitrite reductase has not yet been studied to the same extent as cytochrome cd. The next step in the denitrification pathway, from nitrite to NO, remains a subject of debate. NO may be a free intermediate (with a separate reductase) in some strains, but not in others. Four different pathways which aim to describe the involvement of NO and N_2O have been proposed (Stouthamer 1988b). At least one of these postulates that NO remains enzyme-bound before being reduced to N_2. This might explain the apparent lack of free NO production by most intact bacteria under 'normal' growth conditions, although isolated enzymes do so (e.g. that of *Ps. perfectomarinus;* Payne 1981). The examination of N_2O reductase has been hampered by its instability. However, it appears, like one of the nitrite

reductases, to be a copper protein (Zumft & Matsubara 1982; Zumft et al. 1987; Michalski et al. 1986).

Autotrophy and Denitrification

The first evidence that chemolithotrophs could denitrify was published by Beijerinck in 1904. He used full, tightly-stoppered bottles with sulphur, carbonate and nitrate, and showed that a bacterial population which oxidized the sulphur to sulphate appeared. This was the first mention of *Thiobacillus denitrificans*. In 1910, Beijerink & Minkman described the growth of bacteria on hydrogen and N_2O. By means of tall glass columns filled with a mineral salts/thiosulphate medium and with an oxygen gradient from top to bottom, Lieske (1912) was able to obtain bands of bacterial growth at different redox levels. From the lowest band, he obtained a cultures of a denitrifying autotroph with a requirement for reduced sulphur compounds. In contrast to the strain of *T. denitrificans* described by Beijerinck, this isolate could not tolerate dissolved oxygen concentrations above 20% air and is thus more reminiscent of *Thiomicrosopira denitrificans*, which will be discussed below. Table 2 aims to place these discoveries related to autotrophy and denitrification within their historical context.

Table 2. Autotrophy and autotrophic denitrification in their historical context. Data from van Iterson (1902), Beijerinck & Minkman (1910), Lieske (1912) and Payne (1981).

Phenomenon	Authors/Year	Comment
'Knallgas' oxidation (H_2/air)	de Saussure (1939) Niklewski (1907)	Small, motile, slime-producing rods *B. saussurei*
Nitrate disappearance during putrefaction of animal tissue and from soil.	Davy (1814) Pelouse (1857) Boussingault (1858) Schloesing (1873)	
Microbial responsibility for nitrate disappearance noted	Gayon & Dupetit (1882) Gayon & Dupetit (1886)	'Denitrification' used to describe nitrate reduction
Oxygen-dependent ammonia oxidation	Schloesing (1873) Winogradsky (1890)	*Nitrosomonas* and *Nitrobacter*
Oxidation of $S_2O_3^{2-}$ and S^{2-}	Natanssohn (1902)	Small motile rods
Oxidation of tetrathionate	Beijerinck & Minkman (1902)	*T. thioparus*
Oxidation of sulphur	Jacobsen (1908)	
Denitrification with $S_2O_3^{2-}$	Beijerinck & Minkman (1902) Lieske (1912)	*T. denitrificans*
H_2 oxidation with N_2O	Beijerinck & Minkman (1910)	
CH_4 production from H_2 and CO_2	Söhngen (1906)	

As already mentioned, even when the field is limited to bacteria able to grow autotrophically, the denitrifiers are a diverse group. Some species have been studied in greater detail than others, and different aspects (e.g. energetics, genetics) of denitrification have been emphasized in the various studies. This review will concentrate on representative species from the various physiological groups, and will aim to highlight some of the, sometimes unexpected, consequences of changing electron acceptor.

The Obligate Autotrophs

a. The Hydrogen Oxidizing Bacteria

It is not clear whether the dominance of facultatively autotrophic, hydrogen-utilizing bacteria in the literature is a true reflection of nature, or due to problems associated with the growth of obligately autotrophic species. Two obligately autotrophic strains, *Calderobacterium hydrogenophimum* and *Hydrogenobacter thermophilus* have been described (Kryukov et al. 1983; Kawasumi et al. 1984). In the descriptions of these new species, *H. thermophilus* is described as being 'nitrate reduction positive' and *C. hydrogenophilum* is reported as being able to reduce nitrate to nitrite, but in neither case is it clear whether this nitrate reduction is associated with the assimilatory or dissimilatory pathways. The question of denitrification by obligately autotrophic hydrogen bacteria must therefore remain open.

Among the autotrophic denitrifiers, the most extensively studied are probably the colourless sulphur bacteria and, very recently, the nitrifiers.

b. The Colourless Sulphur Bacteria

Two obligately autotrophic members of this group can grow anaerobically while reducing nitrate or nitrite to nitrogen. These are *Thiobacillus (T.) denitrificans* and *Thiomicrospira (Tms.) denitrificans* (Kelly 1989a; 1989b; Kuenen & Tuovinen 1981; Kuenen & Robertson 1989a). Although superficially similar in that they both grow autotrophically on reduced sulphur compounds, they are morphologically different. *T. denitrificans* is a rod which may be motile by means of a polar flagellum, whereas currently available strains of the spiral-shaped *Tms. denitrificans* are non-motile. Chemostat cultures of *T. denitrificans* can be grown at a range of dissolved oxygen concentrations from air saturation (211 μM) to anaerobic (Justin & Kelly 1978a). The growth of *Tms. denitrificans* is inhibited by oxygen (even at μmolar concentrations), and aerobic growth has only been achieved under oxygen limitation (Timmer ten Hoor 1975; 1977). Unlike *T. denitrificans, Tms. denitrificans* resembles the facultatively autotrophic *Thiosphaera (Tsa.) pantrotropha* (which will be discussed below), in that its denitrifying enzymes are constitutive.

As with other denitrifiers, both *T. denitrificans* and *Tms. denitrificans* gave

lower yields when denitrifying than when using oxygen (Table 3). The aerobic biomass yields reported for *T. denitrificans* are, like those of *T. tepidarius*, approximately double those found for other autotrophic sulphur bacteria such as *T. thioparus* or *T. neopolitanus*. Kelly (1989b) has suggested that these different yields are related to the coupling of reduced sulphur compound oxidation to either cytochrome b (higher yields) or cytochrome c (lower yields). If the oxidation of reduced sulphur compounds is also coupled to cytochrome c in *Tms. denitrificans* and *Tsa. pantotropha* (both of which give 'low' yields) their cytochrome chains must be substantially different from those published (e.g. Stouthamer 1988a) for either *T. denitrificans* or *Paracoccus denitrificans* (another strain which gives 'low' aerobic yields on thiosulphate), otherwise *Tms. denitrificans* and *Tsa. pantotropha* would not be able to denitrify on thiosulphate and nitrate. It is believed that in strains such as *Pa. denitrificans*, the branch point by which electrons flow to nitrate occurs 'upstream' of cytochrome c, and thus before the electrons from thiosulphate enter the cytochrome chain (Stouthamer 1988a; 1988b).

Table 3. Comparison of aerobic and anaerobic yields obtained with obligately autotrophic, colourless sulphur bacteria (data from Timmer ten Hoor 1977). Yields expressed as g dry weight per mol electron donor.

Species	Electron donor	Electron acceptor	Yield
T. denitrificans	$S_2O_3^{2-}$	NO_3^-	9.3
T. denitrificans	$S_2O_3^{2-}$	O_2	13.2
Tms. denitrificans	$S_2O_3^{2-}$	NO_3^-	5.2
Tms. denitrificans	$S_2O_3^{2-}$	O_2	7.7

T. denitrificans cultures grown at 12 μM O_2 transiently accumulated nitrite when switched to anaerobic conditions, but had fully adjusted after 4 hours. It has been reported that *T. denitrificans* can be switched between aerobic and denitrifying growth with relative ease (Justin & Kelly 1978a). Interestingly, it was shown that *T. denitrificans* gave higher biomass yields at low dissolved oxygen concentrations (12μM). The yield decreased as the dissolved oxygen increased, indicating that at higher dissolved oxygen concentrations, oxygen can act as a metabolic inhibitor (Justin & Kelly 1978b).

At first glance, the existence of physiologically similar species in the same habitat seems puzzling. However, research by Timmer ten Hoor (1975; 1977) shed some light on the probable ecological niches of the two species when she found that the presence (or absence) of even very low concentrations of dissolved oxygen determined which species occurred. In sulphide-dependent enrichment cultures grown in chemostats from which oxygen had been rigorously excluded, *Tms denitrificans* tended to dominate. However, if oxygen was not completely excluded, *T. denitrificans* appeared. It was psoposed by the author that the predominant selective pressure determining the outcome of these experiments was the redox of the cultures. Thus *Tms. denitrificans* would

tend to be favoured in deep, low-redox, sulphide-rich sediments, whereas *T. denitrificans* would fit an ecological niche nearer the surface, where conditions might fluctuate between aerobiosis, oxygen limitation and anaerobiosis.

c. The Nitrifiers

Broda (1977) proposed that denitrifying bacteria which obtain their energy for growth from the oxidation of ammonium to nitrite ($\Delta G' = -360$ kj with nitrite as electron acceptor and N_2 as the end product) should exist, and termed them one of the 'lithotrophs missing in nature'. However, *Nitrosomonas* species have long been known to be able to produce N_2O during aerobic growth with ammonium as their energy source. Many papers about N_2O production by nitrifiers have been published, and it is not possible to cover more than a representative selection here. For a more extensive overview, the reader is referred to Bremner & Blackmer (1981). Figure 1 shows a compilation of the aerobic and anaerobic reactions which have been proposed by various authors.

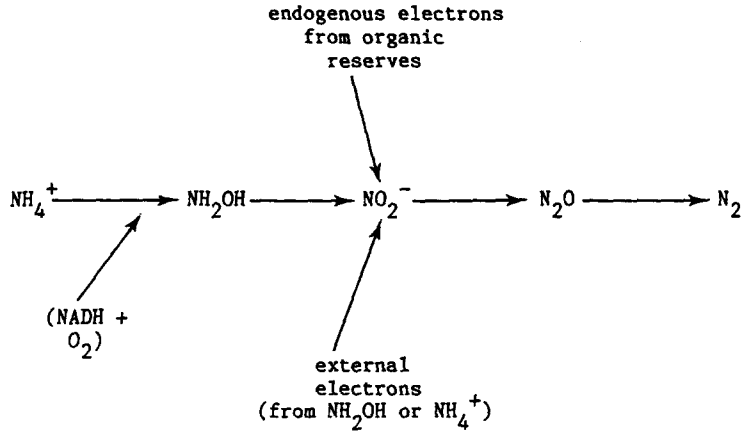

Fig. 1. Hypothetical scheme compiling the postulated pathways for electron transport during nitrite production and reduction by ammonia oxidizers.

Hooper (1968) described the isolation of a strictly aerobic enzyme from *N. europaea* which reduced NO_2^- to N_2O and NO with NH_2OH as the electron donor. Since then, it has become widely accepted that *N. europaea* can produce N_2O during nitrification, but it was assumed that this was a result of the breakdown of an unstable nitrification intermediate (Poth & Focht 1985). However, Ritchie & Nicholas (1972) showed that under anaerobic conditions, resting *N. europaea* cells provided with NH_4^+ and NO_2^-, or NO_2^- alone, produced twice as much N_2O as aerobic cells with NH_4^+ (83-84 and 38 nmol per mg protein per hour, respectively). Subsequent experiments with $^{15}NH_4^+$ and $^{15}NO_2^-$ confirmed that the bulk of the N_2O was being derived from NO_2^-.

Hynes & Knowles (1984) also showed that the rate of N_2O production by *N. europaea* was five times higher under anaerobic conditions than aerobically. The highest anaerobic rate of N_2O production (36 nmol per hour per culture flask) was reached with a mixture of NH_4^+ and NO_2^-, but rates approximately half this were found with NO_2^- alone. Acetylene inhibited (aerobically incubated) or partially inhibited (anaerobically incubated) N_2O production from NH_4^+, but not from NO_2^- (Fig. 1). Aerobic acetylene-treated cells supplied with NH_4^+ and NO_2^- gave N_2O production rates similar to those achieved with only NO_2^-. Aerobic NO_2^- production from NH_2OH was not affected by acetylene. As acetylene inhibits ammonia monooxygenase as well as nitrous oxide reductase, this appears to indicate that although NH_4^+ can contribute to NO_2^- reduction, it is not essential and the reaction can proceed on the basis of endogenous energy. That N_2O could be generated from NO_2^- in the absence of NH_4^+ indicates that the enzyme responsible is not hydroxylamine oxidoreductase, which requires NH_2OH as well as NO_2^- (Hooper 1984). Poth & Focht (1985) used ^{15}N-labelled compounds to confirm that NO_2^- (but not NO_3^-) is reduced to N_2O under oxygen stress. They postulated that by using NO_2^- as terminal electron acceptor, the organism was able to conserve the limited amount of available oxygen for nitrification. Poth (1986) followed this work with a later paper in which a *Nitrosomonas* sp. which could reduce NO_2^- to N_2 was described. Again, ^{15}N-labelled compounds were used to confirm the source of the N_2O, and the reactions occurred under oxygen limitation while the strain derived energy for growth from NH_4^+ oxidation. Poth observed in this paper that the demonstration of anaerobic, autotrophic denitrification by this strain would have to await the finding of a suitable artificial energy source because ammonia monooxygenase requires O_2 and NH_2OH is toxic, even in aerobic cultures. However, if substrate-limited chemostat cultures are used, rather than batch cultures, it is relatively easy to grow bacteria on toxic substrates, e.g. sulphide (Timmer ten Hoor 1975), methyl sulphides (Suylen 1988) and NH_2OH (Robertson 1988). If it is physiologically possible, it should therefore be feasible to show anaerobic, hydroxylamine-dependent denitrification by this strain in chemostat cultures. Finally, *Nitrosomonas* cells rich in organic reserve materials should be able to denitrify anaerobically.

The denitrifying reactions carried out by autotrophic, ammonia-oxidizing bacteria may not be so much a way of generating energy for growth, since the rates are very low, but may rather be a mechanism for surviving periods of anaerobiosis at the expense of internal (organic) energy sources. However, resolution of the role of denitrification by these bacteria must await experiments to determine whether or not they gain energy from the reactions, probably in chemostat studies. Bremner & Blackmer (1981) have observed that it is likely that most, of not all, ammonia-oxidizing bacteria are able to carry out this reaction under suitable conditions.

The Facultative Autotrophs

Membership of the physiological group known as the facultative autotrophs is steadily rising, and it appears that many strains are unrecognised as belonging to this group for lack of testing, rather than for lack of physiological ability. This is especially true for the ability to grow at the expense of reduced sulphur compounds as this property is not generally included in routine taxonomic tests (Kelly 1989a; 1989b; Mason & Kelly 1988; Robertson et al. 1989a). Friedrich & Mitrenga (1981) showed that many strains known to be facultatively autotrophic on hydrogen (including *Pa. denitrificans* and *Hydrogenobacter* strains) were also capable of autotrophic growth on thiosulphate, and Suylen & Kuenen (1986) were able to grow a pink methylotroph on thiosulphate. It is clear that the ability to grow autotrophically on a given class of inorganic substrates is an insufficiently precise criterion for taxonomic classification among the facultative autotrophs (Kuenen & Tuovinen 1981; Kelly 1989a).

In order to complete the picture, the chemolithoheterotrophs and 'incidental lithotrophs' should be briefly considered. The chemolithoheterotrophs are capable of generating energy from the oxidation of inorganic compounds, but cannot fix CO_2. In this group are many hydrogen- and sulphur-oxidizing bacteria (see, for example, Suylen, 1988). The 'incidental lithotrophs' are heterotrophs which can oxidize inorganic compounds but seem to be unable to gain metabolically useful energy from the reaction. Some examples are able to oxidize reduced sulphur compounds (Kelly 1989a), and it might be considered that the heterotrophic nitrifiers also fall within this category. As yet, facultatively autotrophic ammonia oxidizers have not been found, and ammonia oxidation by those heterotrophs known to be capable of doing it requires an organic substrate to drive the reaction (Verstraete 1975). As heterotrophic nitrification is frequently found in combination with denitrification, it will be discussed in more detail below.

Because many of the facultative autotrophs are capable of autotrophic growth on more than one type of compound (e.g. hydrogen, thiosulphate, etc), they cannot be so clearly separated in terms of substrate as most of the obligate autotrophs, and many strains cannot be allocated to either (e.g. *Tsa. pantotropha, Pa. denitrificans*). Thus, although the strains are discussed below in terms of their classically-recognised groups, for the sake of convenience, the reader should bear in mind that many strains have only been tested on either hydrogen or reduced sulphur compounds, and have been named on the basis of incomplete taxonomic tests. Where screening for the use of both has been done, for example in the study of thiosulphate metabolism by hydrogen-utilizing bacteria carried out by Friedrich & Mitrenga (1981), it rapidly becomes clear that the division is artificial. The nitrite-oxidizing strains form, perhaps, the only well-defined group among the facultative autotrophs.

As seen in Table 1, most strains which are capable of mixotrophic and autotrophic growth under aerobic conditions lose this ability when they denitrify. Among these are most strains of *T. versutus* (Wood & Kelly 1983), the

thermophilic colourless sulphur bacterium, *Thermothrix (Tx.) thiopara* (Brannan & Caldwell 1980) and *Nitrobacter hamburgensis* (Bock et al. 1986). *Paracoccus denitrificans* loses its ability to oxidize reduced sulphur compounds when denitrifying, but is able to grow anaerobically on hydrogen. In contrast, *Tsa. pantotropha* (Robertson & Kuenen 1983a; Kuenen & Robertson 1989b) retains its autotrophic potential on reduced sulphur compounds as well as on hydrogen while denitrifying. However, its μ_{max} while denitrifying on thiosulphate is at least a factor 10 lower than that of aerobic cultures.

For obvious reasons, most of the studies on denitrification by facultative autotrophs have been undertaken with heterotrophically-grown cultures. *Pa. denitrificans* has been one of the favourite experimental species for denitrification experiments in various laboratories (e.g. Kucera & Dadak 1983; Kucera et al. 1984; Alefounder et al 1983; 1984; Stouthamer 1980), and the work has been extensively reviewed (Stouthamer 1988a; 1988b). This section will therefore concentrate on facets of (generally heterotrophic) denitrification by other species which can oxidize hydrogen or reduced sulphur compounds, or which nitrify.

a. The Hydrogen-Oxidizing Bacteria

Hydrogen-oxidizing heterotrophs include species from many genera including *Pseudomonas, Alcaligenes* (including the former *Hydrogenomonas*), *Bacillus, Xanthobacter* and *Nocardia*. Their aerobic hydrogen metabolism was extensively reviewed by Bowien & Schlegel in 1981. Of course, not all of these species denitrify. Of those which do, perhaps the best known are *Paracoccus denitrificans* and *Alcaligenes eutrophus*. Some *Alcaligenes* strains are remarkable in that they lack a denitrifying nitrate reductase and can generally only denitrify if supplied with nitrite or if they are grown in ammonium-deficient medium when the assimilatory nitrate reductase can generate nitrite for denitrification (Pfitzner & Schlegel 1973). Much of the work on hydrogen metabolism by these organisms has been on their genetics, and is thus not appropriately treated here.

N_2-fixing bacteria which also denitrify embody both extremes of the nitrogen cycle. Species from various genera, including *Azospirillum, Bradyrhizobium, Rhizobium* and *Rhodopseudomonas (Rhodobacter)* are able to do this. Batch culture experiments on such an organism, which can also oxidize hydrogen, were described by Chan (1985). *Pseudomonas sp.* H8 grew in complex media at the expense of nitrate, nitrite and N_2O. Moreover, denitrifying growth was observed with hydrogen as the sole source of energy. Hydrogen oxidation was slower while denitrifying than when 10% or 21% oxygen was supplied (50%, 100% and 66% of the initial hydrogen after 22 hours incubation, respectively). This change in substrate oxidation rates is reflected in the lower growth rates shown by hydrogen-oxidizing *Paracoccus denitrificans* when denitrifying (Nokhal & Schlegel 1983).

b. The Colourless Sulphur Bacteria

Thiobacillus versutus presents an excellent example of a species which undergoes drastic alterations in its metabolism as a result of changing from aerobic to anaerobic, denitrifying growth. Firstly, *T. versutus* can only denitrify heterotrophically. Cultures grown aerobically in the presence of thiosulphate (and which therefore have the appropriate enzymes induced) have been shown to be capable of thiosulphate-dependent N_2 production in short-term anaerobic experiments with resting cells (Robertson & Kuenen 1983a), but there is no evidence that this reaction occurs in anaerobically growing mixotrophic cultures, or that it provides energy. It is more likely fortuitous. This failure to denitrify with reduced sulphur compounds as electron donors is probably due to the electrons from thiosulphate being fed into the cytochrome chain at cytochrome c. Electron flow to the denitrifying enzymes (to nitrate reductase 'upstream' of cytochrome c in the electron transport chain, and to nitrite and nitrous oxide reductase directly from cytochrome c) would therefore not generate any energy.

In addition to the loss of its ability to generate energy from the oxidation of reduced sulphur compounds on transfer to denitrifying conditions, the heterotrophic enzymology of *T. versutus* presents another example of the dramatic alterations in microbial physiology which can occur when the electron acceptor is changed. *T. versutus* has, for a long time, been studied as a member of a group of baceria which grow on acetate by means of an unknown pathway which does not involve the first enzyme of the glyoxylate cycle, isocitrate lyase (Gottschal & Kuenen 1980; Claassen et al. 1986). However, when denitrifying cells were examined, a significant isocitrate lyase activity was found (Table 4). This activity was not present in aerobically-grown cells, or in biomass grown aerobically or anaerobically on succinate (Claassen & Zehnder 1986). The authors used a range of techniques including enzyme assays, ^{13}C NMR spectroscopy and mass spectrometry to confirm that the enzyme indeed converted isocitrate to glyoxylate and succinate (i.e. it really was isocitrate lyase). The isocitrate lyase-negative pathway used during aerobic growth remains, as yet, unknown.

Table 4. The appearance of isocitrate lyase in denitrifying *T. versutus* cultures (data from Claassen & Zehnder, 1986).

Substrate	Electron acceptor	Isocitrate lyase
	nmol min^{-1} mg^{-1}	
Acetate	oxygen	0
Acetate	nitrate	52
Succinate	oxygen	1
Succinate	nitrate	1

c. The Ammonia-Oxidizers

Thiosphaera pantotropha is especially appropriate for inclusion in this review as it is capable of aerobic and denitrifying growth on thiosulphate, sulphide and hydrogen as well as a range of organic compounds including acetone (Robertson & Kuenen 1983; Bonnet-Smits et al. 1988). As will be discussed below, it can also oxidize ammonia, a phenomenon which, combined with its other properties, earns it a place in all three of the groups delineated by the definitions used among the obligate autotrophs. Ammonia oxidation by means of heterotrophic nitrification appears to be inseparable from denitrification in *Tsa. pantotropha*, and the two phenomena will therefore be discussed together.

During the comparison of its denitrifying potential with those of related organisms such as *T. versutus,* it was found that the denitrifying enzymes of *Ts. pantotropha* appeared to be constitutive. Aerobically grown biomass was able to produce nitrogen immediately it was supplied with nitrate and substrate (Robertsen & Kuenen 1984a), in contrast to *T. versutus* and *Pa. denitrification*, both of which required 3-4 hours induction period before they were able to denitrify. As the existence of aerobic denitrification was, at the time, somewhat controversial, experiments were carried out to discover whether or not the constitutive denitrification enzymes of *Tsa. pantotropha* were active under aerobic conditions.

Aerobic (>80% air saturation) batch cultures of *Tsa. pantotropha* grew more rapidly on organic substrates when provided with two electron acceptors

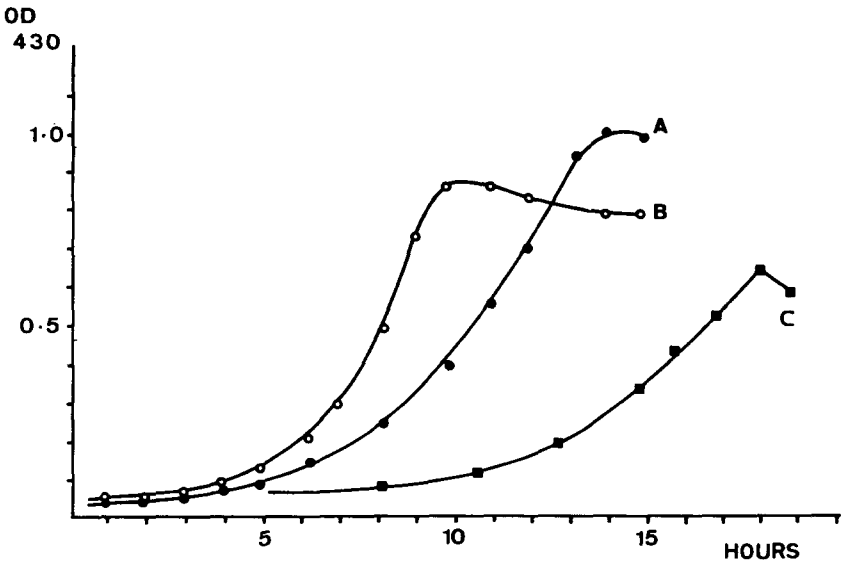

Fig. 2. Growth curves obtained with batch cultures of *Tsa. pantotropha* provided with acetate and ammonium. A: aerobic (>80% air). B: aerobic (>80% air) with nitrate. C: anaerobic with nitrate (Robertson & Kuenen 1984a).

(O_2 and NO_3^-) than with only one (Fig. 2). Analysis showed that about half of the respiration of the culture was taking place via denitrification. Protein measurements confirmed the impression give by the optical density readings that the yields were, as might be expected, intermediate between those obtained with either of the single electron acceptors (Robertson & Kuenen 1984a; 1984b). Oxygen and nitrate electrodes were used to show that aerobically-grown cell suspensions simultaneously utilized nitrate and oxygen, even when the only nitrogen compound supplied in the original growth medium was ammonium (Robertson et al. 1986). During experiments to test whether nitrite had the same effect on the aerobic growth rate as nitrate, it was found that the nitrite concentration in the cultures increased for a time, before disappearing (Fig. 3). This only happened in aerobic cultures in the presence of an organic substrate such as acetate, and is therefore heterotrophic rather than autotrophic nitrification (Verstraete 1975).

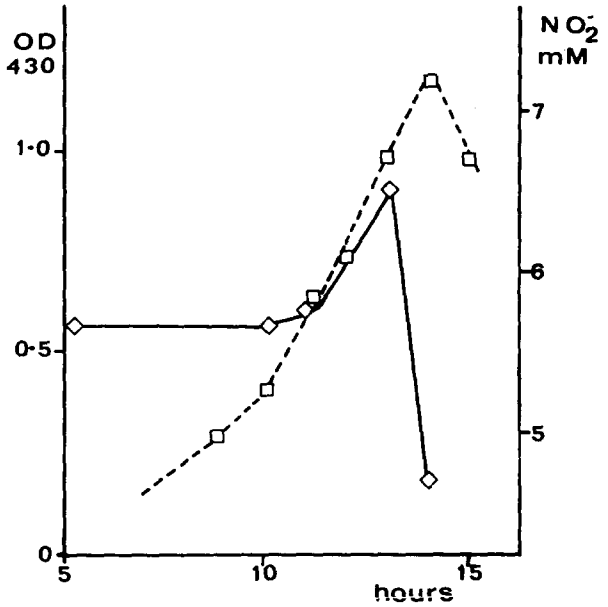

Fig. 3. Growth and nitrite concentration in aerobic (>80% air) batch cultures of *Tsa. pantotropha* provided with acetate, nitrite and ammonium. solid line = optical density at 430 nm, broken line = nitrite concentration.

Chemostat studies showed that aerobic denitrification and heterotrophic nitrification are intimately linked in *Tsa. pantotropha* (Robertson et al. 1988). As nitrite is common to both pathways (Fig. 4) and inhibits heterotrophic nitrification (Robertson & Kuenen 1988), it is not unlikely that the concentration of nitrite and nitrite reductase are controlling factors in determining the relative raltes of nitrification and denitrification in these cultures.

The nitrification and denitrification rates found in acetate-limited chemostat

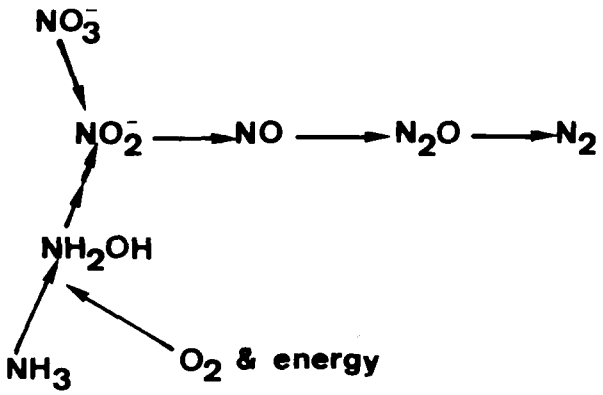

Fig. 4. Pathways of nitrification and denitrification as they appear to occur in *Tsa. pantotropha.*

cultures increased as the growth rate (dilution rate) increased, and also as the dissolved oxygen fell (Robertson et al. 1988). The provision of nitrate or nitrite in the medium resulted in lower nitrification rates (Table 5). Moreover, cultures grown mixotrophically on acetate and thiosulphate showed steadily decreasing nitrification and aerobic denitrification rates as the amount of thiosulphate in the medium increased (Robertson et al. 1988). Neither phenomenon occurred in mixotrophic cultures where the thiosulphate and acetate concentrations were roughly equivalent, or where the thiosulphate concentration exceeded that of the acetate (Nanninga et al. 1988).

Table 5. Correlation of chemostat yields (mg/L) with nitrification and denitrification rates (nmol^{-1} min^{-1} mg protein^{-1}). All cultures were substrate limited, and supplied with NH_4^+. Dissolved O_2 = 80% air saturation. D = 0.04 h^{-1}. * indicates yield lower than expected. (Data from Robertson et al., 1988).

Additive	Nitrification	Denitrification	Yield
–	43	43	81*
NO_3^-	12	107	103
NO_2^- (limiting)	48	85	80*
NO_2^- (saturating)	25	98	115
NH_2OH	45	45	75*
$S_2O_2^{2-}$	21	21	145
$S_2O_3^- / NO_3^-$	6	36	120

It has always been believed that heterotrophic nitrifiers do not gain energy from nitrification, in contrast to the autotrophic nitrifiers. The results obtained with the chemostat experiments indicated that not only is energy not gained, but that it actually appears to be lost during heterotrophic nitrification by *Tsa. pantotropha*, despite the resemblance of its nitrifying pathway to that found in autotrophic ammonia oxidizers (Robertson & Kuenen 1988). Cultures exhibiting high nitrification rates gave protein yields which were only about

60% of those expected. Denitrifying and mixotrophic cultures (with lower nitrification rates) both gave biomass yields in the range expected (Table 5).

A model based on physiological data and preliminary cytochrome experiments has been developed in an effort to explain the appearance of heterotrophic nitrification and aerobic denitrification in *Tsa. pantotropha*, and their relationship to other physiological phenomena such as autotrophy and NO_x assimilation. The basic assumption is that there is a bottleneck on the flow of electrons along the cytochrome chain to oxygen via cytochrome aa_3. Allowing electrons to flow to the denitrification pathway and oxygen simultaneously would thus permit a faster flow of electrons through the main part of the cytochrome chain, and therefore a faster reoxidation of NAD(P)H. If nitrogen oxides are not available, and denitrification cannot proceed, NAD(P)H can be reoxidized during nitrification as it is required by the ammonia monooxygenase (Robertson & Kuenen 1988). This hypothetical flow is outlined in Fig. 5. During the transition from heterotrophic metabolism to mixotrophic and then autotrophic growth, additional cytochromes are induced (Robertson 1988), and these would also be a means of overcoming any bottleneck.

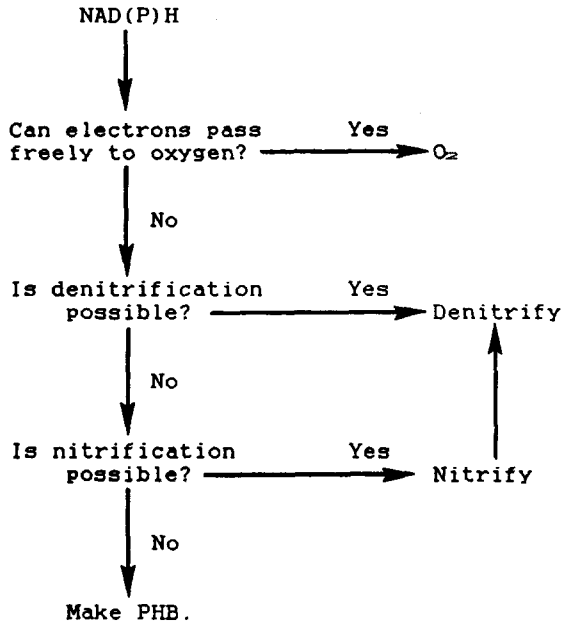

Fig. 5. Flow chart to show, schematically, the working hypothesis developed to explain the control of heterotrophic nitrification and aerobic denitrification in *Tsa. pantotropha* (Robertson et al. 1988).

Having established that *Tsa. pantotropha* was simultaneously nitrifying and denitrifying, and that it would not have been identified as a heterotrophic nitrifier by the classically-employed methods (which involve the measurement

of nitrite accumulation), the next interesting question was whether other strains also possessed the combined pathway. One example from this screening programme is a strain which was formerly known, together with other, unrelated strains, as *'Pseudomonas denitrificans'* (Douderoff et al. 1974; JCSB, 1982). Aerobic, heterotrophic chemostat cultures of this strain also nitrified and denitrified simultaneously (Robertson et al. 1989b), but they differed from *Tsa. pantotropha* in that the denitrifying nitrate reductase was not constitutive, although the rest of the denitrification pathway was. As thiosulphate inhibited nitrification by *Tsa. pantotropha* (Kuenen & Robertson 1987; Robertson & Kuenen 1988; Robertson et al. 1988; Nanninga et al. 1988), it seemed useful to test its effect on heterotrophic nitrification using a strain which was believed to be an obligate heterotroph. During acetate-limited chemostat experiments in which 5 mM thiosulphate was added to the medium, the nitrification rate fell by almost half, and the yield rose by almost a third. That this increase in yield was, however, not an indication of the amount of energy 'lost' during nitrification became clear when it was found that the strain had induced a thiosulphate-oxidizing capacity (615 nmol min^{-1} mg protein $^{-1}$). CO_2 was, of course, being fixed by means of the Calvin cycle (Robertson et al. 1989a; 1989b). Although the strain resembled *Tsa. pantotropha* in its simultaneous nitrification and denitrification and in its facultative autotrophy, it proved unable to use reduced sulphur compounds anaerobically. It has not yet been tested for the ability to oxidize hydrogen.

Having shown that at least two species were simultaneously nitrifying and denitrifying, it became important to establish how widespread this combination of the two phenomena is. Castignetti & Hollocher (1984) described their finding that many common denitrifying bacteria from soil were able to nitrify oximes or hydroxylamine heterotrophically. However, as with other nitrification studies, the nitrification rates were evaluated in terms of nitrite accumulation and it seemed that the nitrification rates were relatively insignificant. As *Tsa. pantotropha* and *'Ps. denitrificans'* would not have been detected as nitrifiers by this method because they simultaneously denitrify the nitrite produced, other bacterial strains were screened for the combined heterotrophic nitrification and aerobic denitrification pathway. Table 6 shows some of the results obtained with batch cultures. It was found that all of the heterotrophic nitrifiers tested were also capable of aerobic denitrification, and their growth rates were stimulated by the provision of nitrate in the medium. However, the dissolved oxygen concentration above which denitrification began to be inhibited was different in the various strains. For example, chemostat cultures of an *Alcaligenes* species nitrified and denitrified efficiently at dissolved oxygen concentrations below 50% air, but began to accumulate intermediates such as nitrite and hydroxylamine of the dissolved oxygen was above this level (Kuenen & Robertson 1987; van Niel et al. 1988).

The combination of heterotrophic nitrification and aerobic denitrification thus appears to be fairly widespread, and the physiological and ecological implications of a pathway which is apparently so wasteful of energy pose

Table 6. 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. Adapted from Robertson et al., 1989A.

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

nd = not determined

interesting questions. Even if it has evolved as a means of overcoming redox problems in the cytochrome chain, the possession of a constitutively active denitrifying system has obvious advantages in situations where the dissolved oxygen concentration fluctuates. This is clearly shown by a comparison of the results from experiments where the response of chemostat cultures of *Tsa. pantotropha* and a *Pa. denitrificians* strain (which is not an aerobic denitrifier) to a sudden shift from steady-state aerobic conditions to anaerobiosis was monitored (Fig. 6). The *Tsa. pantotropha* culture showed a small drop in optical density, which might be expected as the additional energy obtainable from oxygen respiration was lost, but soon stabilized under the new conditions. In contrast, *Pa. denitrificans* washed out at a rate in line with the dilution rate. Moreover, because of the amount of nitrite produced during its anaerobic phase (>10mM), *Pa. denitrificans* failed to recover when the air supply was

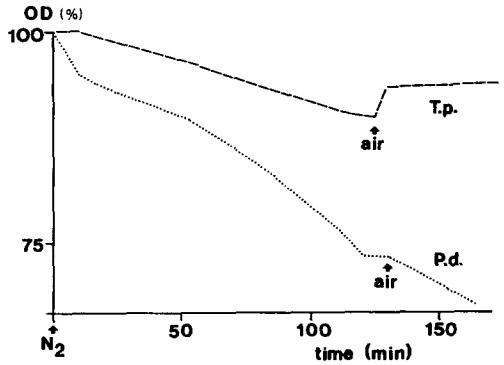

Fig. 6. The outcome of switching steady-state, acetate-limited chemostat cultures of *Tsa. pantotropha* (T.p.) and *Pa. denitrificans* (P.d.) from aerobiosis (80% air saturation) to anaerobiosis (Robertson 1988).

restored, and continued to wash out (Robertson & Kuenen, in preparation).

Situations where the dissolved oxygen is either low, or fluctuating occur at aerobic/anaerobic interfaces where the products of anaerobic metabolic activity such as reduced sulphur compounds and ammonia can also be expected to occur. With the results obtained with *'Ps. denitrificans'* in mind, other heterotrophic nitrifiers (including *Pseudomonas* and *Alcaligenes* strains) were also screened for the ability to oxidize reduced sulphur compounds. Of 7 strains tested, only 2 failed to induce a thiosulphate-oxidizing capacity when grown in batch cultures with 5 mM acetate and 10 mM thiosulphate (Robertson et al. 1989a). Whether or not these nitrifier/denitrifiers are able to grow autotrophically (aerobically or anaerobically) on reduced sulphur compounds remains to be tested. However, the fact that these strains combine the abilities to oxidize reduced sulphur compounds and ammonia indicates that a wider screening programme which tests putative heterotrophs for the ability to oxidize reduced sulphur compounds and ammonia in combination with denitrification may reveal whether a link between these properties is only superficial, or deserves further investigation.

d. The Nitrite-Oxidizing Bacteria

Recent work has shown that members of the genus *Nitrobacter* are facultative autotrophs. The pattern of mixotrophic growth in three species described by Bock et al. (1986) differs from that of the colourless sulphur bacteria in that *N. hamburgensis* and *N. winogradskyi* utilized organic compounds and nitrite simultaneously, even in batch culture when diauxy might be expected. Another *Nitrobacter* species exhibited diauxic growth, but still differs from the colourless sulphur bacteria in that it consumed the inorganic substrate first, rather than the organic one (Bock et al. 1986).

Recent studies (Freitag et al. 1987; Bock et al. 1988) have shown that various strains of *Nitrobacter* are able to reduce nitrate to nitrite, ammonia and to nitrogen gases, especially N_2O. However, in common with *Pa. denitrificans* and *Tx. thiopara*, they required the presence of an organic substrate (e.g. pyruvate) in order to denitrify. Approximately 40% of the nitrogen content of the medium was lost from the anaerobic cultures. In addition to their production of ammonia (generally associated with dissimilatory nitrate reduction) as well as N_2O, these organisms differed from all of the other species discussed in this review in one major respect – their protein yield after anaerobic growth (56 mg/l) in a mixotrophic (pyruvate and nitrite) medium was approximately 3 times higher than that obtained with similar, aerobic cultures (Freitag et al. 1987), but the large amounts of poly β-hydroxybutyrate (PHB) synthesised under mixotrophic and heterotrophic conditions should, perhaps, be taken into account.

Anaerobically-grown *Nitrobacter* cells are morphologically very different from aerobic, autotrophically grown cells (Freitag et al. 1987). They lose most, or all of their carboxysomes and synthesize substantial amounts of poly

β-hydroxybutyrate. This was clearly shown in experiments where an oxygen-permeable silicon tube was suspended in the culture medium and a biofilm formed on the surface of the silicon (Freitag et al. 1987). Examination of the biolayer with an electron microscope revealed that two morphologically distinct forms were appearing. At the bottom of the biolayer, near the silicon surface, the cells contained carboxysomes and were probably growing autotrophically on nitrite and oxygen. Closer to the surface of the biofilm, as the oxygen became depleted, the cells contained poly β-hydroxybutyrate and were morphologically similar to those grown in suspension at the expense of pyruvate and nitrate.

Concluding Remarks

If the hydrogen or sulphur-oxidizing denitrifiers and the denitrifying nitrifiers are indeed especially suited to life at aerobic/anaerobic interfaces where sulphide and nitrate might both be expected to occur, is this not also true of methanotrophs? Denitrification on methanol is known to occur in a limited number of strains, notably the Hyphomicrobia, but although there have been claims for methane-dependent denitrification by consortia (Hamer & Meschner 1984), a methane-oxidizing denitrifier remains to isolated. As with the obligately autotrophic hydrogen oxidizing bacteria mentioned above, it is not clear whether this lack is due to the difficulty of growing the bacteria, or reflects the true picture, perhaps because of the requirement for methane monooxygenase. It should be remembered that bacteria utilizing other substrates which were previously believed to require the use of a monooxygenase (e.g. acetone) have now been shown to be capable of denitrification.

At first glance, denitrification is a good illustration of the 'Unity in diversity' concept first voiced by Kluyver in 1924. As mentioned in the introduction, the pathway and enzymes involved are remarkably similar when the wide range of physiological types which denitrify are considered. However, on closer examination, 'Diversity in unity' might be a more appropriate viewpoint. One of the most unifying factors among the denitrifiers is also the most divisive, that the switch from O_2 to NO_3^- or NO_2^- as electron acceptor causes dramatic changes in other physiological factors. These range from the drop in biomass yield under anaerobic conditions, resulting from alterations in the path of electron flow along the cytochrome chain, to the gain and/or loss of pathways, as evinced by the loss of autotrophic potential in some of the facultative autotrophs and the acquisition of isocitrate lyase by anaerobically grown *T. versutus*. As the recent work with the autotrophic nitrifiers has shown, much remains to be discovered before we can claim that we fully understand denitrification.

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