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Robertson, Lesley A; van Niel, Ed; Torremans, Rob A.M.; Kuenen, J. Gijs

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Simultaneous Nitrification and Denitrification in Aerobic Chemostat Cultures of *Thiosphaera pantotropha*

LESLEY A. ROBERTSON,* ED W. J. VAN NIEL, ROB A. M. TORREMANS, AND J. GIJS KUENEN

Laboratory of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67A, 2628BC Delft, The Netherlands

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***Thiosphaera pantotropha* is capable of simultaneous heterotrophic nitrification and aerobic denitrification. Consequently, its nitrification potential could not be judged from nitrite accumulation, but was estimated from complete nitrogen balances. The maximum rate of nitrification obtained during these experiments was 93.9 nmol min^{-1} mg of $protein^{-1}$. The nitrification rate could be reduced by the provision of nitrate, nitrite, or thiosulfate to the culture medium. Both nitrification and denitrification 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 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 fell. It appeared that, under carbon- and energy-limited conditions, a high nitrification rate was correlated with a reduced biomass yield. To facilitate experimental design, a working hypothesis for the mechanism behind nitrification and denitrification by *T. pantotropha* was formulated. This involved 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 heterotrophic nitrifiers has been reconsidered. Several species considered to be "poor" nitrifiers also simultaneously nitrify and denitrify, thus giving a falsely low nitrification potential.**

Most 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 they are supplied with a source of energy (9, 28). Many of the common denitrifying bacteria in soil are also heterotrophic nitrifiers (2, 13); indeed, it has been proposed that nitrification by heterotrophic bacteria may be responsible for all nitrate and nitrate production in those types of soil in which conditions (e.g., low pH, low pO_2) do not favor autotrophic nitrification (9, 31).

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, reference 9). In some cases it is possible that a competitive advantage might be gained from the production of toxic chemicals such as nitrite (28). An alternative explanation is that the production of nitrite or nitrate is a by-product or bypass of a pathway used for the production of hydroxamic acids, which serve as chelators when iron is limiting (28).

Evidence has been published to support the view that in some bacteria and fungi organic nitrogen compounds are involved in the heterotrophic nitrification pathway (28). 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* (22), 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 (28).

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). Thus, current evidence indicates that, under fully aerobic conditions, *T. pantotropha* carries out the following reactions sequentially and simultaneously,

$$NH_4^+ \longrightarrow NH_2OH \longrightarrow NO_2^- \quad (1)$$
$$NO_2^- \longrightarrow N_2O \longrightarrow N_2 \quad (2)$$
$$O_2 \longrightarrow H_2O \quad (3)$$

provided a suitable electron donor (e.g., acetate) is available. This means that, under fully aerobic conditions, the organisms convert ammonia into nitrogen gas without intermediary accumulation of nitrite. Preliminary work with ^{15}N-labeled ammonia has confirmed that N_2 rather than N_2O is the product from ammonia (E. W. J. van Niel, L. A. Robertson, and R. Cox, unpublished data).

If the ability to simultaneously nitrify and denitrify under fully aerobic conditions is not unique to this species, it indicates the existence of a previously unstudied group of heterotrophic nitrifiers (13; E. van Niel, L. A. Robertson, and J. G. Kuenen, Proc. 4th Eur. Congr. Biotechnol. 3:363, 1987). 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 wastewater treatment, these organisms merit detailed study; for this reason, research on *T. pantotropha* was extended to include the controlled environment provided by the chemostat.

* Corresponding author.

This paper describes batch and chemostat experiments to study both qualitative and quantitative aspects of simultaneous heterotrophic nitrification and aerobic denitrification in *T. pantotropha* as a representative of the nitrifying/denitrifying group of bacteria.

MATERIALS AND METHODS

Organisms. *T. pantotropha* LMD 82.5 was originally isolated from a denitrifying, sulfide-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* was grown at 30°C.

The medium described for the growth of *Thiobacillus versutus* (formerly *Thiobacillus* sp. strain A2) by Taylor and Hoare (27) was used for batch culture. It contained the following (in grams per liter): $Na_2HPO_4 \cdot 7H_2O$, 7.9; KH_2PO_4, 1.5; NH_4Cl, 0.3; and $MgSO_4 \cdot 7H_2O$, 0.1; plus 2 ml of trace element solution. The $MgSO_4$, trace element solution, substrates, KNO_3, and KNO_2 were all sterilized separately in a concentrated form and added as needed. Unless otherwise stated, the initial concentration of the carbon sources in the batch cultures was 10 mM. Succinate was used at a concentration of 5 mM. KNO_3 and KNO_2 were supplied at concentrations of 20 and 5 mM, respectively.

The trace element solution (30) used with all batch and chemostat media contained the following (in grams per liter): EDTA, 50; $ZnSO_4$, 2.2; $CaCl_2$, 5.5; $MnCl_2 \cdot 4H_2O$, 5.06; $FeSO_4 \cdot 7H_2O$, 5.0; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.1; $CuSO_4 \cdot 5H_2O$, 1.57; and $CoCl_2 \cdot 6H_2O$, 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 was kept at 8.0.

The medium supplied to the chemostats contained the following (in grams per liter): K_2HPO_4, 0.8; KH_2PO_4, 0.3; NH_4Cl, 0.4; and $MgSO_4 \cdot 7H_2O$, 0.4; plus 2 ml of trace element solution. Acetate (20 mM) was supplied as substrate unless otherwise stated. Thiosulfate, 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 and 20 mM, respectively.

Respiratory measurements. Oxygen uptake was measured with 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 by using a computer-controlled switching and monitoring system (23).

Biomass analysis. Protein was measured spectrophotometrically, by means of a microbiuret method (6). The presence of poly-β-hydroxybutyrate (PHB) was detected by examining thin sections under the electron microscope, and it was measured as crotonic acid by means of the method described by Law and 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 $C_1H_2O_{0.5}N_{0.25}$ (24), which gives a C/N ratio of 4. The general formula for PHB is $(C_1H_2O_{0.75})_n$.

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 *T. pantotropha* is grown in the chemostat under carbon and energy limitation, 50% of its dry weight is protein (L. A. Robertson, unpublished data). The protein concentrations were therefore doubled (to give total biomass), and the amount of nitrogen was calculated from the general formula given above.

Analysis of medium Acetate was determined with acetyl-coenzyme A synthetase, using a test kit (Boehringer GmbH). Thiosulfate was measured colorimetrically, using the method described by Sörbo (26).

Nitrite was measured colorimetrically, with the Griess-Romijn reagent (8), or by using a high-pressure liquid chromatograph fitted with a ionospher-TMA column (Chrompak) and a Walters RI detector. Nitrate was also measured colorimetrically, using diphenylamine sulfonic acid chromogene (Szechrome NAS reagent; Polysciences Inc.). N_2O could be qualitatively determined in solution by means of a Clark-type oxygen electrode provided the test mixture was kept anaerobic by means of a suspension of bakers' yeast (11).

Hydroxylamine was determined colorimetrically by the method described by Frear and Burrell (5). Ammonia was determined by following the oxidation of NADH in the presence of α-ketoglutarate and L-glutamate dehydrogenase, using a test kit supplied by Sigma Chemical Co. As, at the pH values used in these experiments, ammonia and ammonium would both be present, the term *ammonia* is used throughout to indicate both the protonated and the unprotonated forms. Control experiments with 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 0.3 mM (or 6 μmol min^{-1} $liter^{-1}$) could be expected from stripping.

RESULTS

Aerobic denitrification. By using computer-monitored electrodes, it was possible to show that well-mixed suspensions of aerobic, chemostat-grown *T. pantotropha* simultaneously used nitrate and oxygen (23). At dissolved oxygen concentrations of 30 to 80% of air saturation, the rate of acetate (100 μmol)-dependent nitrate reduction was around 800 nmol min^{-1} mg of $protein^{-1}$. When the dissolved oxygen was <30% of air saturation, the rate doubled, to 1.6 μmol min^{-1} mg of $protein^{-1}$. Reciprocally, the oxygen uptake rate appeared to fall when the dissolved oxygen was <30% of air saturation (23). Experiments of this type have confirmed the previous finding that *T. pantotropha* is able to corespire nitrogen oxides and oxygen (20, 21).

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

From the nitrogen balances (Table 1), it became apparent that nitrogen disappearance from aerobic, acetate-limited chemostat cultures was dependent on the nitrogen compounds provided and also on the dilution rate (i.e., specific growth rate). N_2O was not detected, and it has therefore not

TABLE 1. Nitrogen balances from steady-state, aerobic,[a] acetate-limited chemostat cultures

N compounds	D (h^{-1})	mmol liter^{-1}[b]			Nitrification rate (nmol of NH_3 oxidized min^{-1} mg of protein^{-1})	Denitrification rate (nmol min^{-1} mg of protein^{-1})[d]
		NH_3[c]	NO_3^-	NO_2^-		
NH_3	0.02	−4.3	0.0	0.0	12.7	12.7
	0.05	−4.6	0.0	0.0	43.3	43.3
	0.10	−5.1	0.0	0.0	93.9	93.9
NH_3–NO_3^-	0.02	−2.3	−8.6	+0.1	7.9	38.1
	0.09	−1.3	−15.7	+0.2	17.2	233.6
	0.17	−1.0	−17.3	+1.5	26.4	506.9
NH_3–5 mM NO_2^-	0.03	−5.6	0.0	−5.0	48.2	84.5
	0.06	−4.7	0.0	−5.0	77.4	145.0
NH_3–20 mM NO_2^-	0.02	−1.7	0.0	−1.0	9.6	12.9
	0.04	−2.6	0.0	−4.1	24.7	98.1
	0.12	−0.4	0.0	−9.8	43.5	177.2

[a] Dissolved oxygen was at 80% of air saturation (i.e., approximately 250 μmol of O_2 liter^{-1}).
[b] Negative number indicates disappearance; positive number indicates production.
[c] Ammonia disappearance has been corrected for assimilated nitrogen and for ammonia lost because of sparging.
[d] Denitrification rates include nitrate and nitrite supplied in the medium together with nitrite produced by the ammonia oxidation.

been included in Table 1. Ammonia losses from the cultures with 5 mM nitrite and the ammonia-oxygen culture were highest. The relationship between the specific 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.

Rates of nitrification and denitrification. 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 nanomoles of NH_3 oxidized per minute per milligram of protein) showed that, in fact, the rates increased with increasing dilution rate (Table 1). However, at μ_{max}, the nitrification rate was about 50% of what it should have been, had the increase in rate been linear with the increasing growth rate, indicating that nitrification is of less significance when the maximum growth rate has been achieved. 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 cultures (grown under combined acetate and thiosulfate limitation) displayed lower nitrification rates than the heterotrophic cultures (not shown). For example, at a dilution rate of 0.06 h^{-1}, a mixotrophic culture with ammonia as the sole nitrogen source had a nitrification rate of 20.5 nmol min^{-1} mg of protein^{-1}. When nitrate was included, the nitrification rate in an acetate-thiosulfate culture fell to only 6.1 nmol min^{-1} mg of protein^{-1}.

Table 1 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 from batch cultures. In batch cultures maintained at a dissolved oxygen concentration of 80% air saturation on acetate or succinate, the corespiration of oxygen and nitrate resulted in a higher maximum specific growth rate and yielded less protein (Table 2). 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).

Yields from chemostats. When *T. pantotropha* was grown at submaximal 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). Protein yields from anaerobic, nitrate-containing chemostat cultures were, as expected, the lowest of all (Fig. 1). Succinate limitation produced a similar pattern (results not shown). Control experiments with a strain of *P. denitrificans* which does not appear to nitrify or denitrify significantly under aerobic conditions showed that in this organism the yield was unaffected by the presence of nitrate (Fig. 1).

To look for a possible link between the unexpectedly low

TABLE 2. Maximum specific growth rates and protein yields obtained with batch cultures of *T. pantotropha* grown in Kluyver flasks at (except the anaerobic culture) 80% of air saturation[a]

Growth conditions	μ_{max} (h^{-1})	Protein	
		mg liter^{-1}	%
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

[a] 5 mM succinate or 10 mM acetate was provided. The inorganic nitrogen compounds were present in excess in all cultures. To facilitate comparison with the chemostat data, the protein yield on O_2, NH_3, and NO_3^- was assumed to be 100% (acetate data from reference 20).

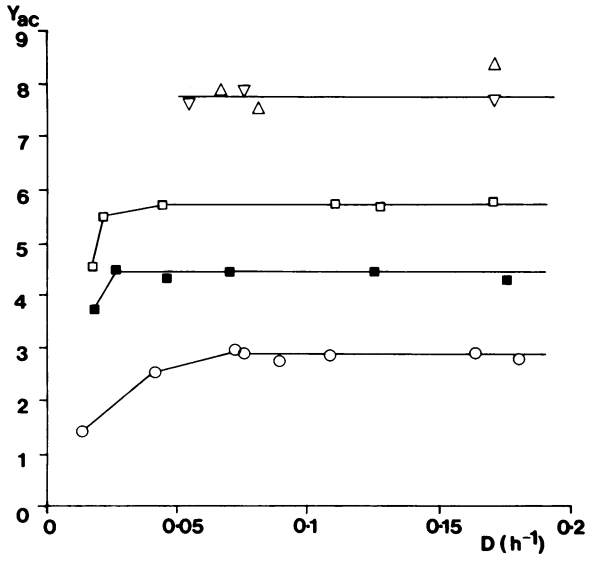

FIG. 1. Protein yields (expressed as grams per mole of acetate, Y_{ac}) from acetate-limited chemostats of *T. pantotropha* and *P. denitrificans* as a function of the dilution rate (*D*) under different electron acceptor regimes. All cultures were provided with ammonia as the nitrogen source, and aerobic cultures were maintained at 80% of air saturation. For concentrations of the various components, see Materials and Methods. Symbols: ○, *T. pantotropha*, anaerobic, with nitrate; ■, *T. pantotropha*, aerobic; □, *T. pantotropha*, aerobic, with nitrate; △, *P. denitrificans*, aerobic; ▽, *P. denitrificans*, aerobic with nitrate.

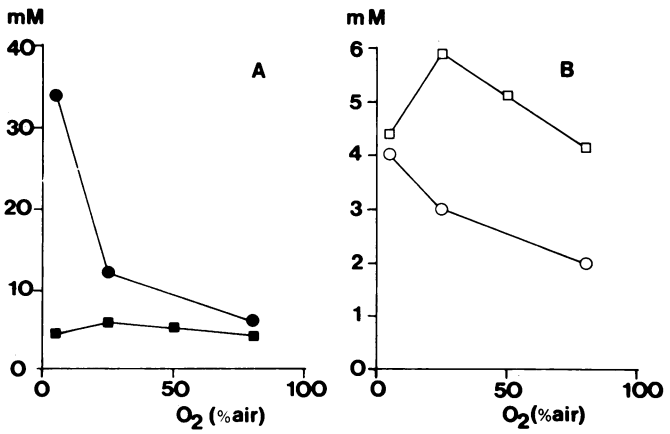

FIG. 2. Total denitrification (i.e., from NO_3^- provided and NO_2^- produced by nitrification) (A) and nitrification (B) by chemostat cultures of *T. pantotropha* as a function of dissolved oxygen. The cultures were acetate limited and were provided with ammonia as the nitrogen source. Symbols: □, ■,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

yields obtained with *T. pantotropha* and the occurrence of nitrification or denitrification or both, further comparisons were made between aerobic chemostat cultures with various medium supplements (Table 3). The yields were expressed as percentages of their respective O_2-NO_3^- cultures. In Table 3, the yields of cultures 1 to 3 were calculated relative to 1b as 100%, and the percent yields of the mixotrophic cultures were based on 4b as 100%. It should be noted that the low yields correspond to the relatively high nitrification rates and not to the denitrification rates. In line with the observations made with the batch cultures (Table 2), the mixotrophic chemostat cultures with oxygen as the only electron acceptor (Table 3, 4a) were at least 20% higher. In addition, the cultures provided with 20 mM nitrate (Table 3, 3b) also showed a relatively high yield. In contrast, heterotrophic chemostat cultures whose sole electron acceptor was oxygen (Table 3, 1a) and the O_2–5 mM NO_2^- cultures (Table 3, 3a) gave relatively low yields.

Effect of dissolved oxygen concentration. As mentioned at the beginning of Results, denitrification by *T. pantotropha* seems to proceed at two 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 (31), 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 mentioned above. Carbon and nitrogen balances showed that, at the lower concentra-

TABLE 3. Comparison of yields from various chemostat cultures growing on a medium fed with 20 mM acetate at a dilution rate of 0.075 h^{-1}

Culture no.	Energy and nitrogen source	Electron acceptors[a]	Yield (mg of protein $litre^{-1}$)[b]	% of NO_3^-–O_2 cultures[c]	Nitrification
1	Acetate + ammonia	(a) O_2	81	79	High
		(b) O_2–NO_3^-	103	100	Low
		(c) NO_3^-	50	49	None
2	Acetate + nitrate	(a) O_2–NO_3^-	110	107	None
3	Acetate + ammonia	(a) O_2–5 mM NO_2^-	76	74	High
		(b) O_2–20 mM NO_2^-	135	131	Low
4	Acetate + thiosulfate + ammonia	(a) O_2	145	121	Low
		(b) O_2–NO_3^-	120	100	Low
		(c) NO_3^-	69	58	None

[a] Except in the anaerobic cultures, the dissolved oxygen concentration was maintained at 80% of air saturation.
[b] Yields are shown in milligrams per liter to allow comparison with the mixotrophic cultures (which received 5 mM thiosulfate in addition to 20 mM acetate).
[c] The percentages for cultures 1, 2, and 3 were calculated with reference to culture 1b, and those for culture 4 are based on culture 4b.

tions (<30%) of oxygen, 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.

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 liter^{-1} at 50% air to 61 mg liter^{-1} 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 to 4.3 (found with other *T. pantotropha* cultures and with *P. denitrificans*) to 7.0 to 7.5, indicating that as much as one-half of 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 [22]) and which, in addition, were not supplied with nitrate or nitrite so that they could denitrify without first nitrifying. In the mixotrophic cultures, in which 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 phenomena. This is discussed below.

Physiological aspects. At μ_{max} (i.e., in batch culture), the corespiration of nitrate (or nitrite) and oxygen appears to confer a higher growth rate, but is paid for by the lower yield because denitrification makes a significant contribution to the respiratory activity of the cell (Table 2) (20). Nitrification at μ_{max} appears to be less significant. However, in chemostat culture, this was not the case, and the cultures in which ammonia was the sole source of nitrogen gave unexpectedly low yields. The following observations indicate that these low yields were associated with the degree of heterotrophic nitrification in *T. pantotropha*.

(i) The presence of nitrate or nitrite (in "nonlimiting" amounts) decreased the rate of heterotrophic nitrification in a culture compared with the activity found in cultures in which ammonia was the sole nitrogen source (Table 1). 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.

(ii) The provision of thiosulfate in the culture medium also reduced the amount of nitrification taking place. The increase in protein yield found for the mixotrophic NH_3-O_2 cultures (Table 3) was far too high to be due only to energy derived from thiosulfate metabolism (e.g., an increase of 64 mg of protein from only 5 mM thiosulfate). This implies that at least some of the yield increase is due to another factor, possibly the reduced nitrification.

(iii) The cultures provided with only 5 mM nitrite had reduced all of it, and the ambient nitrite concentration was therefore 0. The nitrification rate in these cultures was as high as, if not higher than, that found for the ammonia-oxygen cultures, and the yield was also low (Tables 1 and 3).

(iv) 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 to those of the ammonia-nitrate-oxygen cultures (Table 3).

Consideration of these four points clearly suggests that there is a relationship between high nitrification rates and low protein yields. This observation has been confirmed by experiments with other bacteria which are also capable of combined nitrification/denitrification (L. A. Robertson, R. Cornelisse, and J. G. Kuenen, unpublished data).

There appears to be an inverse relationship between the dissolved oxygen concentration and the rates of nitrification (provided oxygen is not limiting) and denitrification (Fig. 2). That denitrification had already reached its maximum rate at 30% air saturation 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.

Working hypothesis. 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 corespiration might be a mechanism by which a "bottleneck" in the electron transport chain between cytochrome *c* and cytochrome aa_3 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

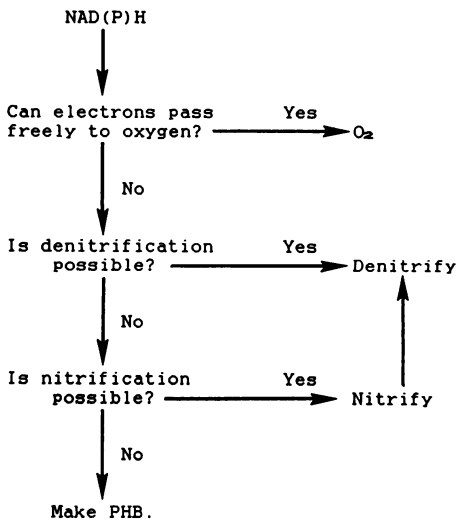

FIG. 3. Flow chart diagrammatically showing the working hypothesis used to explain the control of heterotrophic nitrification and denitrification in *T. pantotropha* growing heterotrophically.

TABLE 4. Nitrification rates calculated from published results[a] of batch culture experiments

Organism	Activity (nmol of NH_3 min^{-1} mg, dry wt^{-1})	N compound used	Reference
Pseudomonas aeruginosa	12–28	Hydroxamate	18
P. aeruginosa	70–90	Hydroxylamine	18
P. denitrificans	2.6	Pyruvic oxime	2
P. aureofaciens	2.8	Pyruvic oxime	2
Alcaligenes faecalis	11.9	Pyruvic oxime	2
Alcaligines sp.	33	Pyruvic oxime	3
Arthrobacter sp.	0.8	Ammonia	29
T. pantotropha	35.4	Ammonia	van Niel et al.[b]
N. europaea	50–100	Ammonia	4
Nitrosomonas sp.	590–2,300	Ammonia	7

[a] For ease of comparison, when other nitrogen compounds were used, the results were recalculated as though for ammonia.
[b] Proc. 4th Eur. Congr. Biotechnol. 3:363, 1987.

thiosulfate would permit the induction 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 three to four different type *c* cytochromes which vary depending on growth conditions (L. A. Robertson, J. E. van Wielink, and J. G. Kuenen, unpublished data). 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 bypassed, 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 *T. pantotropha* can use NADPH (22). The production of PHB by cells whose ammonia oxidation has been partially inhibited (e.g., by oxygen or hydroxylamine) would then also support the bottleneck hypothesis.

Ecological implications. In addition to the physiological implications for *T. pantotropha*, a second point which must be considered in 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 in which autotrophic nitrifiers cannot prosper (e.g., 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 usually so well balanced that nitrite does not accumulate, and this species, therefore, was not recognized as a heterotrophic nitrifier until nitrogen balances were made for the cultures. Some published data were therefore used to recalculate nitrification rates for other heterotrophic nitrifiers on the basis of ammonium disappearance. When these are compared with the rates reported for *Nitrosomonas* species (Tables 4 and 5), it can be seen that in some cases the heterotrophic nitrification rates are only a factor of 10 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 *Nitrosomonas europaea* is about 0.03 to 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 under way to determine which environmental conditions, if any, will favor nitrification by this new group of heterotrophs rather than by the autotrophic bacteria.

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

Organism	Activity (nmol of NH_3 min^{-1} mg, dry $weight^{-1}$)	Reference
T. pantotropha	6–47	This paper
N. europaea	670–835	4
N. europaea	130–1,550	25
N. europaea	1,385–5,290	16
N. europaea	400–1,020	van Niel, unpublished data

[a] For ease of comparison, when other nitrogen compounds were used, the results were recalculated as for ammonia. Ammonia was the N compound in all cases.

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