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Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*

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Abstract *Nitrosomonas eutropha*, an obligately lithoautotrophic bacterium, was able to nitrify and denitrify simultaneously under anoxic conditions when gaseous nitrogen dioxide (NO_2) was supplemented to the atmosphere. In the presence of gaseous NO_2, ammonia was oxidized, nitrite and nitric oxide (NO) were formed, and hydroxylamine occurred as an intermediate. Between 40 and 60% of the produced nitrite was denitrified to dinitrogen (N_2). Nitrous oxide (N_2O) was shown to be an intermediate of denitrification. Under an N_2 atmosphere supplemented with 25 ppm NO_2 and 300 ppm CO_2, the amount of cell protein increased by 0.87 mg protein per mmol ammonia oxidized, and the cell number of *N. eutropha* increased by 5.8×10^9 cells per mmol ammonia oxidized. In addition, the ATP and NADH content increased by 4.3 μmol ATP (g protein)$^{-1}$ and 6.3 μmol NADH (g protein)$^{-1}$ and was about the same in both anaerobically and aerobically grown cells. Without NO_2, the ATP content decreased by 0.7 μmol (g protein)$^{-1}$, and the NADH content decreased by 1.2 μmol (g protein)$^{-1}$. NO was shown to inhibit anaerobic ammonia oxidation.

Key words *Nitrosomonas* · Anaerobic ammonia oxidation · Anaerobic cell growth · Nitrogen dioxide · Nitric oxide · Nitrous oxide · Dinitrogen

Introduction

Lithoautotrophic ammonia-oxidizing bacteria oxidize ammonia to nitrite in a two-step reaction (Suzuki 1974; Drozd 1976). The first step is the oxidation of ammonia to hydroxylamine (Eq. 1; Hollocher et al. 1981) catalyzed by ammonia mono-oxygenase (AMO) (Rees and Nason 1966; Dua et al. 1979; Wood 1986). In this reaction two reducing equivalents are consumed.

$$NH_3 + 2\,H^+ + 2e^- + O_2 \rightarrow NH_2OH + H_2O \quad \text{(Eq. 1)}$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5\,H^+ + 4e^- \quad \text{(Eq. 2)}$$

The second step, the oxidation of hydroxylamine, gains reducing equivalents (Eq. 2) and is catalyzed by hydroxylamine oxidoreductase (HAO) (Hooper and Terry 1979). Water is the source of bound oxygen that appears in nitrite (Anderson and Hooper 1983; Hooper et al. 1984). During the oxidation of hydroxylamine, four electrons are released. In order to activate oxygen, two electrons are transferred to the AMO (Wood 1986), while the remaining two electrons are fed into the respiratory chain, which is described to be the energy-conserving process in the cells. To confirm the mechanism described above, ammonia oxidation has been investigated using isotope techniques and inhibitor studies (Dua et al. 1979; Anderson and Hooper 1983; Hyman and Wood 1985; Hyman et al. 1990). A gene coding for the AMO has been cloned (*amoA*). The gene product, a 32-kDa polypeptide, has been identified by an acetylene-binding domain. Within the same operon another gene (*amoB*), which codes for a 43-kDa polypeptide, has been sequenced. Neither the expressed polypeptides of *amoA* and *amoB* nor the purified proteins from *Nitrosomonas* cell homogenates are able to oxidize ammonia to hydroxylamine (Hyman and Wood 1985; Hyman et al. 1990).

As shown by Bock et al. (1995), the obligately lithoautotrophic *Nitrosomonas eutropha* is also a denitrifying organism that uses hydrogen as electron donor and nitrite as electron acceptor. Under anoxic and oxic conditions, *N. eutropha* is able to produce nitric oxide (NO) and nitrous oxide (N_2O). It is not clear whether these gaseous compounds are formed in the course of the biological reduction of nitrite or by chemical decomposition of hydroxylamine (Ritchie and Nicholas 1972; Poth and Focht 1985; Remde and Conrad 1990). Moreover, in pure cultures of *N. eutropha*, nitrogen losses of up to 60% due to denitrification have been determined. N_2 has been detected as the main product (Bock et al. 1995; Mulder et al. 1995). It has

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been shown that obligately chemolithotrophic, ammonia-oxidizing bacteria of the genus *Nitrosomonas* are able to carry out nitrification and denitrification simultaneously under oxygen limitation. Under anoxic conditions, ammonia is oxidized with nitrite as electron acceptor; cell growth, however, has not been observed (Bock et al. 1995).

The purpose of this study is to demonstrate anaerobic cell growth of *N. eutropha* with ammonia as electron donor and NO_2 as electron acceptor. Furthermore, the simultaneous denitrification of nitrite to dinitrogen is demonstrated. Nitrogen dioxide (NO_2) is shown to be essential for anaerobic ammonia oxidation.

Materials and methods

Organism

The experiments were performed with the obligate lithoautotroph *Nitrosomonas eutropha* strain N904, which was isolated from cattle manure. The strain is kept in the culture collection of the Institut für Allgemeine Botanik (University of Hamburg).

Medium and growth conditions

The mineral medium consisted of the following components per liter of distilled water: NH_4Cl (535 mg), NaCl (585 mg), $CaCl_2 \times 2\ H_2O$ (147 mg), KCl (74 mg), KH_2PO_4 (54 mg), $MgSO_4 \times 7\ H_2O$ (49 mg), Hepes (12 g) and 1 ml trace element solution containing 0.02 M HCl (1 l), $FeSO_4 \times 7\ H_2O$ (973 mg), H_3BO_3 (49 mg), $ZnSO_4 \times 7\ H_2O$ (43 mg), $(NH_4)_6Mo_7\ O_{24} \times 4\ H_2O$ (37 mg), $MnSO_4 \times H_2O$ (34 mg), $CuSO_4$ (16 mg). The medium was adjusted to pH 7.0.

Cells of *N. eutropha* for the anoxic experiments were grown aerobically in 1-l Erlenmeyer flasks containing 600 ml mineral medium. The cultures were grown in the dark at 28° C without stirring or shaking.

Experimental design

All experiments were carried out in the absence of oxygen using Clark-type Oxygen Electrode Units (DW1; Bachofer, Reutlingen, Germany) as reaction vessels. Two electrode units were installed in a gas-tight incubation chamber (acrylic box) with a volume of 3 l (Bachofer, Reutlingen, Germany) equipped with a gas flow-through system that was flushed with oxygen-free nitrogen or helium gas to exclude oxygen from the whole system. The gas contained a maximum of 100 ppb oxygen (gas cylinder from Messer Griesheim, Germany). The gas flow was kept constant at a flow rate of 400 ml/h, resulting in an overpressure of about 10^4 Pa in the incubation chamber. Depending on the experimental conditions, NO_2 (10–100 ppm) and CO_2 (300 ppm) were added using a gas diluter (Tecan, München, Germany). Furthermore, the used gas contained traces of NO (0.1–0.5 ppm) in the final gas mixture. Since NO_2 and NO are in equilibrium with N_2O_3, also the latter was expected to be present in the atmosphere. Upon dissolution of NO_2 and N_2O_3 in the medium, nitrite and nitrate were formed chemically. In sterile control experiments, the chemical formation of nitrite and nitrate was determined and taken into account for further investigations and calculations. A nitrite formation rate of up to 15 μmol l^{-1} h^{-1} and a nitrate formation rate of up to 2 μmol l^{-1} h^{-1} were measured in sterile control experiments. Chemical oxidation of ammonia could not be detected. The values from the control without cells were subtracted under the assumption that the rate of chemical reaction is the same with and without cells, viz. that the cells do not influence the chemical reactions. Using a helium atmosphere, the NO_2 consumption and the NO and N_2 production could be calculated on the basis of the different concentrations in the gas inlet and gas outlet.

Exponentially growing cells of *N. eutropha* cultivated in 1-l Erlenmeyer flasks were harvested by centrifugation, washed, and resuspended in mineral medium. Both reaction vessels were filled with 3 ml cell suspension, and subsequently air was replaced by an oxygen-free atmosphere. Most of the experiments were performed at 24° C with suspensions of 8×10^9 cells/ml, which is equivalent to about 800 μg protein/ml. Liquid and gas samples were taken with a gas-tight syringe through a rubber stopper in the top of the incubation chamber.

Analytical procedures

Ammonia, nitrite, and nitrate were measured by HPLC (Stüven et al. 1992); hydroxylamine was measured by a modified method according to Verstraete and Alexander (1972). NO and NO_2 were determined with the aid of a NO/NO_x Analyzer (Tecan, München, Germany). N_2, N_2O, and CO_2 were quantified by gas chromatography with a thermal conductivity detector (Model 9001; Chrompack, Frankfurt a.M., Germany) using a Poraplot Q and a molecular sieve column (5 Å, 60/80 mesh). Helium served as the carrier gas.

Glycerol was measured with the aid of a test kit (Boehringer Mannheim, Germany).

Cell numbers were determined by light microscopy with the aid of a Helber chamber. The protein content was measured according to Bradford (1976). Using cells that were disintegrated for 30 min at 90° C in a 0.9% NaCl/0.4 N NaOH solution. The intracellular pool of ATP was determined by a method according to Strehler and Trotter (1952); the pool of NADH was determined according to Slater and Sawyer (1962).

Results

Anaerobic ammonia oxidation

Anaerobic ammonia oxidation and cell growth of *N. eutropha* were performed in a helium atmosphere supplied with 0–50 ppm NO_2 and 300 ppm CO_2. Pure cultures of *N. eutropha* oxidized ammonia anaerobically and produced nitrite (Fig. 1). Simultaneous consumption of NO_2 and production of NO were detectable (Fig. 2). NO_2 consumption and NO production were closely linked to the decrease of ammonia. A small and almost constant quantity of 10 μM hydroxylamine was measured (data not shown). Since a constant gas stream was passing through the incubation chamber, the activity of the cells was proportional to the difference between the NO_2 and NO concentrations in the gas inlet and gas outlet. Hence, the NO_2 consumption and NO production in Fig. 2 show the changes of the concentrations between the gas inlet and gas outlet. Approximately 47% of the nitrite derived from ammonia was converted into gaseous N compounds by denitrification. The main gaseous products of the anaerobic metabolism were dinitrogen and NO (Fig. 2), and traces of N_2O were formed (data not shown). Moreover, after incubation for 70 h, up to 54 μg glycerol/l had been excreted by cells of *N. eutropha* into the bulk medium (not shown).

To reveal the stoichiometry of the anaerobic ammonia oxidation, the activities of consumption and production of N compounds by *N. eutropha* were calculated. The NO_2 consumption and NO production were calculated from the flow rate and the difference between the concentrations in the gas inlet and gas outlet. The mean values of ten ex-

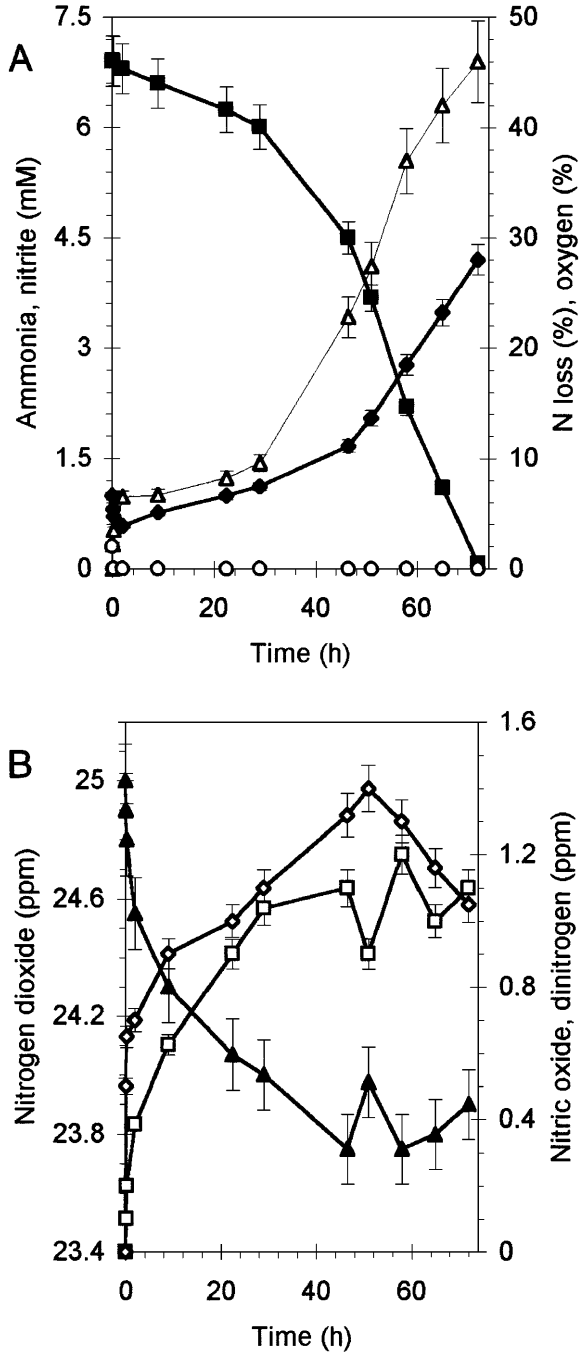

Fig. 1A, B Anaerobic oxidation of ammonia by *Nitrosomonas eutropha* when incubated in an He atmosphere supplemented with 25 ppm NO_2 and 300 ppm CO_2. **A** Ammonia consumption (■), nitrite production (◆), the N loss (△), and oxygen concentration (○) in the medium and in the headspace of the reaction chamber. **B** NO_2 consumption (▲) in the incubation chamber and the net NO (□) and N_2 production (◇) during the 72 h of the experiment

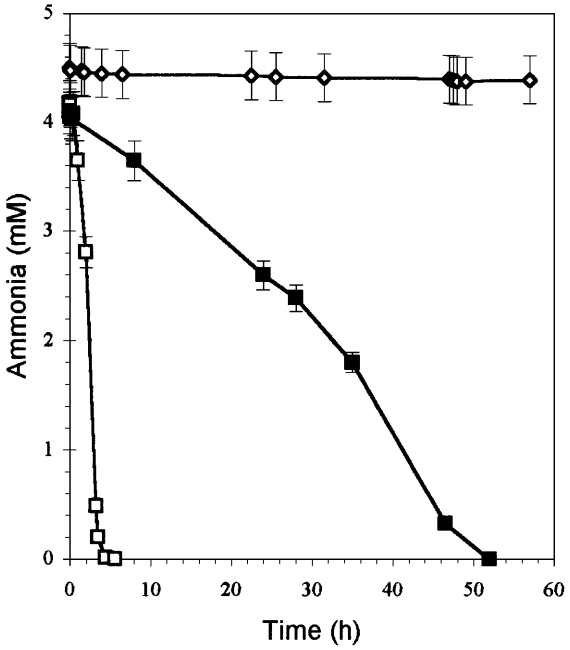

Fig. 2 Comparison of the rate of ammonia oxidation by *Nitrosomonas eutropha* kept under an atmosphere of N_2 (◇), air (□), and N_2 supplemented with 25 ppm NO_2 and 300 ppm CO_2 (■)

Table 1 Anaerobic ammonia oxidation of *Nitrosomonas eutropha* with NO_2 as oxidant. The rates are presented as consumption (–) and production (+) of N compounds in a He atmosphere with 25 ppm NO_2 and 300 ppm CO_2. The rates are determined between the 50th and 60th hour of the experiment

N compounds	Activity [μmol (g protein)$^{-1}$ h^{-1}]
NH_4^+	–134.4 ± 6.7
NO_2	–150.0 ± 10.5
NO_2^-	+87.5 ± 4.4
NO	+125.8 ± 8.3
N_2	+30 ± 1.0
N_2O	+0.11 ± 0.05

periments are given. The activities were calculated between the 50th and 60th hour of the experiments (Figs. 1, 2).

From the data presented in Table 1, it is evident that the consumption rates of ammonia and NO_2 were approximately equivalent to the production rates of NO, nitrite, and the denitrification products N_2 and N_2O. In sterile control experiments, neither ammonia consumption nor N_2 formation or N_2O formation were detectable, but nitrite and nitrate were formed chemically by the reaction of NO_2 and N_2O_3 with water. The values from the control experiments without cells were subtracted under the assumption that the rate of chemical reaction was the same with and without cells.

To assess the significance of the capacity for anaerobic ammonia oxidation, activities of cells from the same preculture were measured under an anoxic atmosphere with NO_2 and under air. The activities were calculated on the basis of the highest ammonia-oxidizing rates, which were measured under the different gas phases (Fig. 3). The anaerobic ammonia-oxidizing activity in an N_2 atmosphere supplied with 25 ppm NO_2 and 300 ppm CO_2 was about tenfold lower than the activity under an atmosphere of air ($N_2/CO_2/NO_2$: 134.4 μmol ammonia (g protein)$^{-1}$ h^{-1}; air: 1377.6 μmol ammonia (g protein)$^{-1}$ h^{-1}). In a pure N_2 atmosphere, almost no activity could be detected (Fig. 3).

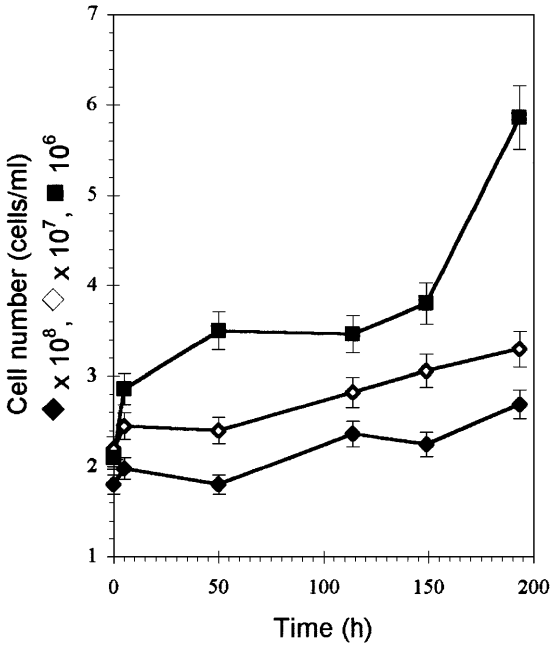

Fig. 3 Growth of *Nitrosomonas eutropha* in an N_2 atmosphere supplemented with 25 ppm NO_2 and 300 ppm CO_2 with different initial cell numbers of 2×10^8 (◆ cell number $\times 10^8$), 2×10^7 (◇ cell number $\times 10^7$), and 2×10^6 (■ cell number $\times 10^6$) cells/ml. After an incubation time of 190 h, 6.7 mM ammonia was consumed

To ascertain the optimal composition of the gas atmosphere for the anaerobic ammonia oxidation of *N. eutropha*, different NO_2 concentrations were tested (Table 2).

The highest anaerobic ammonia-oxidizing activity was achieved at 25 ppm NO_2 in the headspace. The NADH content increased during incubation from 2.2 to 8.5 μmol (g protein)$^{-1}$, the ATP content from 2.0 to 6.3 μmol (g protein)$^{-1}$. High anaerobic ammonia-oxidizing rates were accompanied with high N losses. In the absence of NO_2, only a small amount of ammonia was oxidized (Table 2). The N loss was low, and the NADH and ATP content of the cells decreased.

Anaerobic cell growth

For the determination of cell growth in an N_2 atmosphere supplemented with 300 ppm CO_2 and 25 ppm NO_2, the cell density was adjusted to between 2×10^6 and 2×10^8 cells/ml (equivalent to a protein concentration of between 0.5 and 50 μg protein/ml). Within 190 h of incubation, 6.7 mM ammonia was consumed in each experiment. As shown in Fig. 4, an almost constant increase of cell numbers was detected.

The protein concentration of the cell suspension increased parallel to the increase of cell density. The protein concentrations at the beginning (t_0) and at the end of the experiment (t_1) are documented (Table 3). In all cases, the cell yield was nearly the same.

Influence of NO on anaerobic ammonia oxidation

Anaerobic ammonia oxidation in the presence of NO_2 led to NO formation. In the experiments described above, NO concentration in the gas phases ranged between 0.1 and 0.5 ppm. In sterile control experiments, no anaerobic ammonia oxidation was found. Here increasing NO_2 and NO

Table 2 Anaerobic and aerobic ammonia oxidation rates, N loss, NADH increase and ATP increase (+) or decrease (–) depending on the concentration of gaseous NO_2 in the headspace of the reaction chamber. The cell suspension of *Nitrosomonas eutropha* contained 8×10^9 cells/ml. The *shaded area* denotes the highest specific activity; *initial* and *final* concentrations mark the period of highest activity between the 50th and 60th hour of the experiment

	NO_2 (ppm)	Ammonia oxidation rate [μmol (g protein)$^{-1}$ h^{-1}]	N loss (%)	NADH concentration [μmol (g protein)$^{-1}$]		ATP concentration [μmol (g protein)$^{-1}$]	
				initial	final	initial	final
Anoxic conditions	0	5.1	< 4	2.1	0.9	1.9	1.2
	10	15.5	17	2.1	3.2	2.1	2.0
	25	134.4	47	2.2	8.5	2.0	6.3
	50	85.8	42	2.1	7.2	2.0	5.8
Oxic conditions	0	1,377.6	< 6	2.3	21.2	2.2	6.9

Table 3 Increase of protein concentrations and cell yield in a *Nitrosomonas eutropha* cell suspension incubated under strict anoxic conditions in an N_2 atmosphere supplemented with 25 ppm NO_2 and 300 ppm CO_2. In each experiment, 6.7 mM ammonia was consumed after 190 h of incubation.(t_0 Protein concentration at the beginning of the experiment, t_1 protein concentration at the end of the experiment)

Protein concentration at t_0 (μg protein ml^{-1})	Protein concentration at t_1 (μg protein ml^{-1})	Cell yield (mg protein mmol ammonia^{-1})
0.5± 0.05	6.3 ± 0.63	0.87 ± 0.09
5 ± 0.5	9.8 ± 0.98	0.72 ± 0.08
50 ± 5	56 ± 0.56	0.89 ± 0.09

Table 4 Effect of NO_2 and NO concentrations in the headspace of the reaction chamber on the ammonia oxidation rates under anoxic conditions. The ammonia oxidation rate is given in μmol μg protein)$^{-1}$ h^{-1}. The *shaded area* denotes the highest anaerobic ammonia oxidation rate. Duration of the experiment = 70 h

NO_2 (ppm)	NO (ppm)		
	0	25	50
0	5.1	3.5	0.5
10	15.5	12.8	1.1
25	134.4	125.6	35.8
50	85.8	65.0	53.7
100	25.2	15.2	5.1

concentrations in the gas atmosphere resulted in increasing nitrite and nitrate concentrations in the medium. A nitrite formation rate of up to 25 μmol nitrite l^{-1} h^{-1} and a nitrate formation rate of up to 2 μmol nitrate l^{-1} h^{-1} were detected. To determine the effect of NO on anaerobic ammonia oxidation by *N. eutropha*, NO was added to the gas inlet in concentrations of between 0 and 50 ppm, and NO_2 in concentrations of between 0 and 100 ppm. An increase of NO concentration resulted in decreasing ammonia-oxidizing activities (Table 4). Cells kept in a NO_2-free atmosphere showed almost no anaerobic ammonia-oxidizing activity (Table 4). After an incubation period of 5 days, aerobic nitrifying activity was extremely low (data not shown). Cell suspensions supplied with NO_2 showed not only a high anaerobic ammonia-oxidizing activity (Table 4) but also a high nitrifying activity when subsequently incubated under oxic conditions (data not shown). If NO was added to the atmosphere, the anaerobic oxidation of ammonia was inhibited. Subsequently, no nitrifying activity was detectable when the cells were incubated aerobically.

Discussion

Ammonia-oxidizing bacteria of the genus *Nitrosomonas* are representatives of strictly aerobic, obligately chemolithotrophic micro-organisms. The results presented here provide the first evidence that ammonia oxidation is also possible under anoxic conditions if NO_2 is present. A rough calculation of the stoichiometry of the production and consumption of N compounds was based on rate measurements because the turnover of the gases NO_2 and NO could only be measured in a flow-through system.

Under anoxic conditions, ammonia and NO_2 were consumed by *Nitrosomonas eutropha* in a ratio of approximately 1:1. Concurrently, an equivalent amount of NO was produced, but the nitrite concentration was lower than expected from the ammonia consumption. As shown before, nitrite was consumed by denitrification, leading to the formation of N_2 and – to a lesser extent – of N_2O. However, the amount of nitrite plus dinitrogen was almost equivalent to the amount of consumed ammonium. Furthermore, hydroxylamine, a typical intermediate of aerobic ammonia oxidation, was detected.

Therefore, ammonia seems to be oxidized to hydroxylamine under anoxic and under oxic conditions. Hydroxylamine is subsequently oxidized to nitrite by the hydroxylamine oxidoreductase. During this reaction, four reducing equivalents are formed (Hooper and Terry 1979).

NO was released into the headspace of the reaction chamber in amounts equimolar to the NO_2 consumed. The NO production of *N. eutropha* could be due to denitrification, but this amount of NO produced by denitrification was far too little to give evidence (Remde and Conrad 1990). Therefore, it seems obvious that *N. eutropha* is able to reduce NO_2 to NO.

It could be argued that at O_2 partial pressure near zero, normal nitrification might have occurred. But the oxygen concentration used in the gas atmosphere was extremely low (up to a maximum of 100 ppb). Aerobic nitrification with such a small amount of oxygen has never been reported. Nevertheless, if the amount of oxygen had been used for nitrification, the calculated decrease of 0.02 μmol ammonia h^{-1} would be negligible.

Under oxic conditions, up to 97% of the oxygen of hydroxylamine is derived from $^{18}O_2$ (Dua et al. 1979). Under anoxic conditions, the oxygen source is unknown. In the experiments described above, nitrite, NO_2, and NO could theoretically have been used as the oxygen source in a heretofore unknown type of reaction. Direct incorporation of oxygen from NO_2 and, especially, from NO seems thermodynamically improbable.

N. eutropha has been shown to transfer reducing equivalents to nitrite as the terminal electron acceptor, resulting in a high denitrification activity (N losses of about 50%). The increasing pool sizes of intracellular ATP and NADH indicated energy conservation in the absence of oxygen. Under these conditions, reducing equivalents are also used for the reduction of CO_2, resulting in cell growth and excretion of extracellular organic compounds (e.g., glycerol) into the medium. This "overflow metabolism" has been described for different species of bacteria (Russell and Cook 1995).

The trace gases NO and NO_2 (NO_x) are toxic to microorganisms even at low concentrations (Zumft 1993). It is, therefore, interesting that NO_2 concentrations of up to 50 ppm have no toxic effects on the anaerobic ammonia oxidation of *N. eutropha*. However, NO at concentrations higher than 25 ppm was shown to have inhibitory effects on the ammonia oxidation. For many chemo-organotrophic micro-organisms, antimicrobial activity of NO has been observed at concentrations as low as 1 ppm. Increasing NO_2 concentrations appears to reduce the toxic effect of NO (Zumft 1993). The antimicrobial activity of NO is due to the formation of metal-nitrosyl complexes with heme, copper, and iron-sulfur proteins (Henry et al. 1991). However, the toxic effect of NO among bacteria varies strongly (Zumft 1993).

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