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Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants

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The phenotypes of three different *Nitrosomonas europaea* strains – wild-type, nitrite reductase (NirK)-deficient and nitric oxide reductase (NorB)-deficient strains – were characterized in chemostat cell cultures, and the effect of nitric oxide (NO) on metabolic activities was evaluated. All strains revealed similar aerobic ammonia oxidation activities, but the growth rates and yields of the knock-out mutants were significantly reduced. Dinitrogen (N_2) was the main gaseous product of the wild-type, produced via its denitrification activity. The mutants were unable to reduce nitrite to N_2, but excreted more hydroxylamine leading to the formation of almost equal amounts of NO, nitrous oxide (N_2O) and N_2 by chemical auto-oxidation and chemodenitrification of hydroxylamine. Under anoxic conditions *Nsm. europaea* wild-type gains energy for growth via nitrogen dioxide (NO_2)-dependent ammonia oxidation or hydrogen-dependent denitrification using nitrite as electron acceptor. The mutant strains were restricted to NO and/or N_2O as electron acceptor and consequently their growth rates and yields were much lower compared with the wild-type. When cells were transferred from anoxic (denitrification) to oxic conditions, the wild-type strain endogenously produced NO and recovered ammonia oxidation within 8 h. In contrast, the mutant strains remained inactive. For recovery of ammonia oxidation activity the NO concentration had to be adjusted to about 10 p.p.m. in the aeration gas.

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INTRODUCTION

Nitrosomonas europaea obtains its energy for growth from aerobic and anaerobic ammonia oxidation or hydrogen-dependent denitrification (Rees & Nason, 1966; Abeliovich & Vonshak, 1992; Bock *et al.*, 1995; Schmidt & Bock, 1997). The anaerobic ammonia oxidation is an NO_2-dependent process coupled to cell growth (Schmidt & Bock, 1997). Nitrogen oxides (NO_2 and NO) were also shown to be involved in the aerobic ammonia oxidation (NO_x cycle, Schmidt *et al.*, 2001a–c). Under anoxic conditions in the presence of hydrogen, *Nitrosomonas* species are able to grow via denitrification (Bock *et al.*, 1995). Nitrite, but not nitrate, is used as the terminal electron acceptor. Nitric oxide, nitrous oxide and dinitrogen are well-documented gaseous products of the metabolic activity of *Nitrosomonas* (Hooper, 1968; Poth & Focht, 1985; Poth, 1986; Abeliovich & Vonshak, 1992; Bock *et al.*, 1995; Bodelier *et al.*, 1996; Kester *et al.*, 1997; Beaumont *et al.*, 2002). Under anoxic conditions nitrite is the only electron acceptor available, and energy generation depends on the denitrification activity. Under oxic conditions, the importance of denitrification is less obvious. Denitrification activity is induced by lowering the oxygen partial pressure (Miller & Nicholas, 1985). High denitrification rates at high oxygen concentrations (more than 4 mg l^{-1}) have been observed in the presence of NO (more than 20 p.p.m. in the aeration gas), indicating that NO has a regulatory effect on the denitrification activity of ammonia oxidizers (Goreau *et al.*, 1980; Miller & Nicholas, 1985; Zart & Bock, 1998; Zart *et al.*, 2000; Schmidt *et al.*, 2004). Genes encoding the denitrification enzymes nitrite reductase (Nir) and nitric oxide reductase (Nor) are present in the genome of *Nsm. europaea* (Chain *et al.*, 2003). The mechanism of N_2 formation observed in ammonia oxidizers has yet to be elucidated. Homologous genes encoding a nitrous oxide reductase (*nosRZDFYL*) are not present in *Nsm. europaea* genome (Chain *et al.*, 2003).

The *nirK* gene cluster in the genome of *Nsm. europaea*

Abbreviations: DMPS, 2,3-dimercapto-1-propane-sulfonic acid; DO, dissolved oxygen.

contains three further ORFs. These genes code for a soluble blue copper oxidase and two periplasmic *c*-haem-containing polypeptides (Whittaker *et al.*, 2000; Beaumont *et al.*, 2002). The *nor* genes *norC*, *norB*, *norQ* and *norD* are encoded in one operon. The putative *nirK* gene (Beaumont *et al.*, 2002) and the *norB* gene (Beaumont *et al.*, 2004) of *Nsm. europaea* were disrupted, and the phenotype of the NirK-deficient strain was characterized in aerobic batch cultures. This mutant had a lower tolerance against nitrite than the wild-type cells. The denitrification activity of ammonia oxidizers has already been discussed to serve as a protection mechanism against negative effects of high nitrite concentrations (Poth & Focht, 1985; Stein & Arp, 1998). Surprisingly, the NirK-deficient strain produced more N_2O than the wild-type strain. Since the denitrification pathway was inactivated (NirK deficiency), it was speculated that the hydroxylamine oxidoreductase (HAO) might be responsible for the emission of nitrogen oxides (Beaumont *et al.*, 2002).

The present study aimed to investigate and characterize the phenotype of the *Nsm. europaea* wild-type, the NirK-deficient and the NorB-deficient strain with regard to their combined aerobic and anaerobic ammonia oxidation and denitrification capabilities. Furthermore, the high N_2O and NO production of the NirK- and NorB-deficient strains were examined in detail.

METHODS

Organism. Cultures of *Nsm. europaea* (ATCC 19718), the *Nsm. europaea* NirK-deficient strain (Beaumont *et al.*, 2002) and the *Nsm. europaea* NorB-deficient strain (Beaumont *et al.* 2004) were grown aerobically in 1 l Erlenmeyer flasks containing 400 ml mineral medium (Schmidt & Bock, 1997). The medium for both mutants was supplemented with 20 mg kanamycin l^{-1}. The cultures were grown for 1–2 weeks in the dark at 28 °C without stirring or shaking.

Experimental design (chemostat). All strains were grown in 5 l laboratory scale reactors with 3·5 l medium. To maintain oxygen concentrations between 0 and 5 mg l^{-1} the reactor was aerated (0·1–2 l min^{-1}) with variable mixtures of oxygen, carbon dioxide and argon using mass-flow controllers. The NO concentration in the off-gas (outlet) was permanently measured, and the N_2O and N_2 concentration was measured offline via gas chromatography (GC). Medium level, temperature, dissolved oxygen (DO) and pH value were continuously measured and controlled. The medium contained 20 mM NH_4^+ (nitrification) or 1 mM nitrite (denitrification) and the medium for the mutants was supplemented with 20 mg kanamycin l^{-1}. Temperature was maintained at 28 °C. The pH value was kept at 7·4 by means of a 20 % Na_2CO_3 solution. Samples for offline determination of ammonium (NH_4^+), hydroxylamine (NH_2OH), nitrite (NO_2^-), nitrate (NO_3^-) and cell numbers were taken regularly. The reactor was inoculated with 400 ml of a *Nsm. europaea* cell suspension. The phenotypes of the three *Nsm. europaea* strains (wild-type, NirK- and NorB-deficient strain) were characterized under three growth conditions: (i) Cells were grown with ammonia as energy source under oxic conditions. (ii) They were grown with ammonia as energy source under anoxic conditions with NO_2 (N_2O_4) as oxidizing agent. Under these conditions, nitrite is used as terminal electron acceptor (Schmidt & Bock, 1997). (iii) Cells were grown under anoxic conditions with hydrogen (gas atmosphere with 80 % H_2 and 20 % CO_2) as electron donor and nitrite (medium contained 1 mM nitrite), NO (1000 p.p.m. in the gas atmosphere) or N_2O (1000 p.p.m. in the gas atmosphere) as terminal electron acceptor. The redox potential was adjusted between −300 and −200 mV by adding sodium sulfide (Na_2S) or titanium(III) chloride ($TiCl_3$) (Bock *et al.*, 1995).

Analytical procedures. Ammonium was measured according to Schmidt & Bock (1998), hydroxylamine according to a modified method by Verstraete & Alexander (1972), and nitrite and nitrate according to van de Graaf *et al.* (1996). Nitric oxide (NO) and nitrogen dioxide (NO_2) concentrations were measured online with an NO_x analyser (chemiluminescence) and the N_2O and N_2 concentration by gas chromatography with a thermal conductivity detector (TCD) using a Poraplot Q and a molecular sieve column (5 Å, 60/80 mesh). Helium served as the carrier gas. The protein concentrations were determined according to Bradford (1976) and the cell numbers by light microscopy using a Helber chamber (SD 5 %). The intracellular pool of ATP was determined by a method according to Strehler & Trotter (1952) and the pool of NADH according to Slater & Sawyer (1962). The ^{15}N analysis was performed by isotope-ratio mass spectrometry. The ^{15}N-labelled ammonium and nitrite were analysed after conversion to N_2 with hypobromite or urea, respectively (Risgaard-Petersen *et al.*, 1995).

RESULTS

Characterization of the phenotype of *Nsm. europaea* wild-type, NirK-deficient and NorB-deficient strain

The experiments were designed to investigate the nitrification/denitrification activities and growth parameters of *Nsm. europaea* wild-type and the NirK- and NorB-deficient strains under defined growth conditions in a chemostat culture. Before the experiments were started all strains were cultured for 3 weeks under oxic conditions without NO_x-supplementation at a DO of 2 mg l^{-1} (dilution rate 0·1, 20 mM ammonium), and the cell number stabilized at about 5×10^8 cells ml^{-1} (preconditioning). After these 3 weeks, the growth parameters were changed according to the needs of the different metabolic activities: First, the strains were examined during aerobic ammonia oxidation without supplementing NO or NO_2 (DO, 5 mg l^{-1}); second, during anaerobic NO_2-dependent ammonia oxidation (NO_2 concentration 150 p.p.m.). The dilution rate of the reactor system was reduced to 0·013 h^{-1} to compensate for the lower growth rates during anaerobic ammonia oxidation; third, during anaerobic denitrification. The dilution rate of the ammonium-free medium was 0·013 h^{-1}. The cells were allowed to adapt to these new conditions for five volume changes before data acquisition was started.

Aerobic ammonia oxidation

The ammonia oxidation activity and the nitrogen loss were not significantly different in *Nsm. europaea* wild-type, the NirK- or the NorB-deficient strains (Table 1). However, clear differences were detectable in the amount of hydroxylamine released and the composition of the nitrogen gases. The hydroxylamine concentration in the medium of

Table 1. Characterization of the phenotype of *Nsm. europaea* wild-type, NirK- and NorB-deficient strain during aerobic ammonia oxidation

N-loss: share of ammonia converted via nitrite into nitrogen gases (NO, N_2O and N_2) in %. Values are means ± SD.

Parameter	Wild-type	NirK-deficient	NorB-deficient
Ammonia oxidation*	1378 ± 68	1357 ± 75	1399 ± 103
NH_2OH concn. (μM)	11 ± 4	82 ± 15	77 ± 8
N-loss (%)	12 ± 2·8	9 ± 3·6	11 ± 3·1
N_2†	89 ± 8·7	30 ± 3·3	25 ± 2·9
NO†	6 ± 0·5	37 ± 2·2	44 ± 3·6
N_2O†	5 ± 0·4	33 ± 1·8	31 ± 3·9
ATP concn.‡	6·9 ± 0·6	4·5 ± 1·8	5·3 ± 1·3
NADH concn.‡	9·8 ± 1·2	7·4 ± 1·3	6·9 ± 0·9
Growth yield§‖	8·5 ± 0·009	6·3 ± 0·011	5·8 ± 0·008
Growth rate (h^{-1})‖	0·125 ± 0·002	0·1 ± 0·0015	0·09 ± 0·0013
Cell number¶	6×10^8	3×10^8	3×10^8

*Specific activity in μmol (g protein)$^{-1}$ h^{-1}. The differences are not significant (error rate of 0·05, Mann–Whitney U-Test).

†The total N-loss was set to 100. The data given for N_2, NO and N_2O represent the proportionate contribution to the total N-loss.

‡Concentrations in the cells in μmol (g protein)$^{-1}$. The differences between the wild-type and the mutants are significant (error rate of 0·1, Mann–Whitney U-Test).

§Growth yield in mg protein (g NH_4^+)$^{-1}$.

‖The differences between the wild-type and the mutants are significant (error rate of 0·05, Mann–Whitney U-Test).

¶Cell number after five volume changes in cells ml^{-1}. Standard deviation (SD), 5 %. The cell number of both mutants decreased, because the dilution rate (0·1 h^{-1}) was slightly higher than their growth rates.

the two mutant strains was about seven times higher compared with the wild-type (Table 1). Further significant differences between the wild-type and the mutants were detectable by analysing the growth rates and yields. In both cases, the values for the wild-type were higher than those for the mutants (Table 1). These results were reflected by the ATP and NADH contents of the different strains. The mean values for ATP and NADH were higher in *Nsm. europaea* wild-type cells.

The main product of the denitrification activity of the wild-type was N_2. Only small amounts of NO and N_2O were formed. In contrast, both mutants released almost equal amounts of NO, N_2O and N_2 (Table 1). Since in both mutants the denitrification pathway was interrupted (nitrite reductase or nitric oxide reductase), it can be speculated that the nitrogen gases might have been released by auto-oxidation and chemodenitrification of hydroxylamine (Chalk & Smith, 1983). ^{15}N-labelling experiments were performed to evaluate the production pathway of the nitrogen gases. The experiments were started in the chemostat cultures by adding ^{15}N- instead of ^{14}N-ammonium as substrate. In control experiments the ^{14}N-nitrite concentration was adjusted to 50 mM and this ^{14}N-nitrite pool served as a trap for ^{15}N-nitrite produced during ammonia oxidation. The results are shown in Table 2. The gaseous nitrogen compounds in *Nsm. europaea* wild-type were almost completely produced via the denitrification pathway. When the ^{15}N-nitrite produced during ammonia oxidation was trapped in an ^{14}N-nitrite pool, hardly any ^{15}N-gases were produced. In contrast, the ^{14}N-nitrite pool did not influence the production of ^{15}N-gases by both mutants. Experiments with ^{14}N-ammonium and a ^{15}N-nitrite pool resulted in an NO, N_2O and N_2 production by the wild-type, but not by the mutants (Table 2).

Control experiments with heat-inactivated *Nsm. europaea* biomass in a medium with 80 μM hydroxylamine (concentration of hydroxylamine in experiments with the mutants, Table 1) were performed (data not shown). Here, similar amounts of NO, N_2O and N_2 were released compared with the growth experiments, with both mutants.

Anaerobic NO_2-dependent ammonia oxidation

The anaerobic ammonia oxidation activity was lower than the aerobic activity (Table 3, Schmidt & Bock, 1997).

During anaerobic ammonia oxidation nitrite was used as terminal electron acceptor by the wild-type (Table 3). About 53 % of the produced nitrite was immediately reduced (indicated by the apparently low nitrite-production

Table 2. ^{15}N-labelling experiments during aerobic ammonia oxidation of *Nsm. europaea* wild-type, NirK- and NorB-deficient strain

The specific ^{15}N-NO_2^-, -NO, -N_2O and -N_2 production rates are given in μmol (g protein)$^{-1}$ h^{-1}. SD, 4 %.

Added N-compounds	Wild-type				NirK-deficient				NorB-deficient			
	NO_2^-	NO	N_2O	N_2	NO_2^-	NO	N_2O	N_2	NO_2^-	NO	N_2O	N_2
$^{15}NH_4^+$*	1212	8·2	9·6	147	1161	57	48	37	1185	85	43	29
$^{15}NH_4^+ + {}^{14}NO_2^-$†	1351	0·8	0·6	2·5	1234	56	49	36	1262	84	42	24
$^{14}NH_4^+ + {}^{15}NO_2^-$†	1150	11	10	143	1074	ND	ND	ND	1123	ND	ND	ND

*10 mM NH_4^+ added. Up to 9 mM NO_2^- produced.
†10 mM NH_4^+ and 50 mM NO_2^- added. ND, production rate not detectable.

rate), leading to an N-loss of 47 %. Interestingly, the NirK-deficient strain was able to oxidize ammonia and to grow under anoxic conditions (Table 3), although this strain is not able to use nitrite as terminal electron acceptor (Tables 1 and 2). The ratio of the ammonia oxidation rate and the nitrite production rate was about 1 : 1 (Table 3). In contrast to the wild-type, significantly less NO was released by the NirK-deficient strain. For further evaluation, the NO-binding compound 2,3-dimercapto-1-propanesulfonic acid (DMPS) was added. As a consequence, the NO produced during anaerobic ammonia oxidation was immediately removed from the system. *Nsm. europaea* wild-type was not affected by DMPS, but ammonia oxidation and growth of the NirK-deficient strain were completely inhibited (not shown). In further control experiments with the NirK-deficient strain, ^{15}N-NO was added, and immediately after the ammonia oxidation started, ^{15}N-N_2O and ^{15}N-N_2 were detectable. The NorB-deficient strain remained inactive under anoxic conditions (Table 3).

Denitrification by *Nsm. europaea* wild-type and the mutants

Nsm. europaea wild-type cells immediately switched their metabolic activity from aerobic ammonia oxidation to anaerobic denitrification (Table 4) when the redox potential was adjusted between −300 and −200 mV, the gas atmosphere was changed to H_2/CO_2 (80/20 %) and nitrite (1 mM) was added as electron acceptor. Both mutants remained inactive throughout the 16 days of the experiment (five volume changes) and were washed out. In further experiments, NO or N_2O were added as electron acceptor. The wild-type strain was able to denitrify with both electron acceptors, but the activities were reduced and the

Table 3. Characterization of the phenotype of *Nsm. europaea* wild-type, NirK- and NorB-deficient strains during anaerobic NO_2-dependent ammonia oxidation

The experiments were gassed with 150 p.p.m. NO_2 in argon. Values are means ± SD.

Parameter	Wild-type	NirK-deficient	NorB-deficient
Ammonia oxidation*	149 ± 16	117 ± 11	ND
Nitrite production*	68 ± 14	114 ± 8	ND
NO production*	298 ± 13	94 ± 9	ND
NH_2OH concn.†	11 ± 4	9 ± 6	ND
N-loss (%)	47 ± 7·2	2·6 ± 2·0	ND
ATP concn.‡	4·3 ± 0·8	2·6 ± 0·3	ND
NADH concn.‡	6·3 ± 1·3	4·9 ± 0·4	ND
Growth yield§	5·3 ± 0·01	4·3 ± 0·008	ND
Growth rate (h^{-1})	0·011 ± 0·004	0·009 ± 0·004	ND
Cell number‖	2×10^8	9×10^7	1×10^7

*Specific activity in μmol (g protein)$^{-1}$ h^{-1}.
†NH_2OH concentration in the medium in μM.
‡Concentrations in the cells in μmol (g protein)$^{-1}$.
§Growth yield in mg protein (g NH_4^+)$^{-1}$.
‖Cell number after five volume changes in cells ml^{-1}. SD, 5 %. ND (activity), not detectable. The cell number of all strains decreased, because the dilution rate (0·013 h^{-1}) was higher than the growth rate.

Table 4. Characterization of the phenotype of *Nsm. europaea* wild-type, NirK- and NorB-deficient strains during anaerobic hydrogen-dependent denitrification

The experiments were performed with nitrite, NO or N_2O as electron acceptor. Values are means ± SD.

Parameter	Wild-type			NirK-deficient		NorB-deficient
NO_2^- consumption*	120 ± 35			ND		ND
NO consumption*	41 ± 14			44 ± 6		ND
N_2O consumption*	23 ± 1			21 ± 1		25 ± 1
Growth yield†	11·6‡	8·6§	3·2‖	7·1§	2·5‖	3·5‖
Growth rate (h^{-1})¶	0·019‡	0·005§	0·002‖	0·007§	0·003‖	0·003‖

*Specific activity in μmol (g protein)$^{-1}$ h^{-1}.
†Growth yield in mg protein (g NO_2^-, NO or N_2O)$^{-1}$. SD, 12 %.
‡Nitrite as electron acceptor.
§NO as electron acceptor.
‖N_2O as electron acceptor.
¶SD, 15 %. ND, activity not detectable.

growth rates and yields were low. Interestingly, the NirK-deficient strain was able to grow with NO or N_2O, reaching a growth yield similar to that of the wild-type. The NorB-deficient strain was only able to grow with N_2O as electron acceptor (Table 4). The main product of the denitrification activity was always N_2.

Recovery of ammonia oxidation activity

Further experiments were performed to evaluate the capability of the three strains to recover ammonia oxidation activity after they were grown via hydrogen-dependent denitrification. Therefore, the strains were grown with nitrite (wild-type), NO (NirK-deficient strain) or N_2O (NorB-deficient strain) as electron acceptors for 4 weeks in a chemostat. By adding ammonium (20 mM) and oxygen (1 mg l^{-1}) the recovery of ammonia oxidation was initiated. *Nsm. europaea* wild-type cells first recovered ammonia oxidation activity within 8 h (Fig. 1), and within 40 to 48 h they reached the maximum activity of 1380 μmol (g protein)$^{-1}$ h^{-1}. Schmidt *et al.* (2001c) showed that the recovery of an ammonia oxidation activity depends on the presence of NO or NO_2. Here, throughout the recovery process an NO concentration between 5 and 8 p.p.m. was detectable in the off-gas (Fig. 1). When NO was removed by the addition of DMPS, *Nsm. europaea* wild-type cell did not recover ammonia oxidation activity.

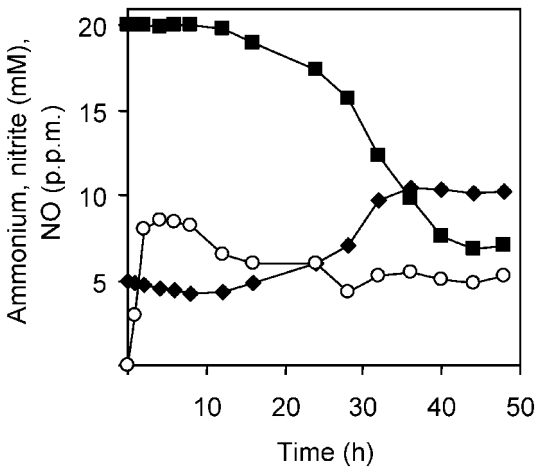

Fig. 1. Recovery of ammonia oxidation by *Nsm. europaea* wild-type in continuous culture. Experiments (three replicates) were performed in chemostats. At time 0, the ammonium concentration was adjusted to 20 mM and the oxygen concentration to 1 mg l^{-1} (not shown). During the experiment fresh medium (ammonium concentration 20 mM) was added continuously (dilution rate 0·07 h^{-1}), and the oxygen concentration was kept constant. The NO concentration was measured in the off-gas. Ammonium (■), nitrite (◆) and NO (○).

The NirK- and the NorB-deficient strains were not able to recover any ammonia oxidation activity under oxic conditions within 6 weeks. The NirK-deficient strain was unable to produce NO and remained inactive. In contrast, the NorB-deficient strain released NO leading to an NO concentration of about 210 ± 43 p.p.m. in the headspace within 8 h. After 8–10 h the NO production stopped and the cells became inactive. When the NO concentration was adjusted to a concentration of 10 p.p.m. (NO addition to the NirK-deficient strain, NO removal from the NorB-deficient strain) both strains recovered ammonia oxidation within 10–14 h.

DISCUSSION

The role of the denitrification activity by ammonia oxidizers has evolved from being recognized as a side aspect of metabolism to a process of high importance for anaerobic growth (Poth & Focht, 1985; Bock *et al.*, 1995; Schmidt *et al.*, 2001a) as well as a pathway supplying the cells with NO (NO_x cycle) (Schmidt *et al.*, 2001a, b, 2004). Furthermore,

the denitrification activity seems to be important for energy generation under oxic conditions (Table 1). Although the ammonia oxidation activities were similar in the wild-type and the NirK- and NorB-deficient strains, the ATP- and NADH-content, as well as the growth rates and yields of the mutants, were significantly lower. Both mutants released more hydroxylamine, the electron-providing substrate, into the medium (Table 1). The increased loss of hydroxylamine by the mutants caused a loss of electrons and finally energy, leading to reduced growth rates and yields.

Aerobic denitrification might supply the NO necessary for ammonia oxidation (NO_x cycle). Evidence that NO activates nitrification was given in this study and by Schmidt *et al.* (2001c). Denitrifying *Nsm. europaea* wild-type cells were unable to recover ammonia oxidation if the NO produced was removed from the system. As a key element of the NO_x cycle and ammonia oxidation, NO might be necessary to start-up this metabolic function. Whether NO further acts as a transcription factor has yet to be examined. The NirK-deficient strain was unable to denitrify and to produce NO and had to be supplied with a catalytic amount of NO to recover its ammonia oxidation. In contrast, the NorB-deficient strain produced high amounts of NO. The nitrite reductase in this strain might be active, leading to the formation of NO, but the cells were not able to further reduce NO. As a consequence NO accumulated and, most probably, the toxicity of NO caused cell damage (Wink & Mitchell, 1998) and inactivated the recovery process. When the NO concentration was lowered to a non-toxic level of about 10 p.p.m., the NorB-deficient cells recovered an ammonia oxidation activity almost as quickly as the *Nsm. europaea* wild-type.

Beaumont *et al.* (2002) characterized the NirK-deficient strain during aerobic ammonia oxidation in batch culture. The high N_2O production by this strain, which was three to four times higher than in *Nsm. europaea* wild-type, was confirmed in this study in chemostat cultures (Table 1). The wild-type mainly produced N_2, while the mutants released almost equal amounts of N_2, NO and N_2O. Experiments with ^{15}N-ammonium and a ^{14}N-nitrite pool (trap for ^{15}N-nitrite produced by ^{15}N-ammonia oxidation) demonstrated that in *Nsm. europaea* wild-type, ammonia is converted via nitrite, NO and N_2O to N_2 (Table 2). Here, denitrification is the major source of gaseous N-compounds. In contrast, the two mutants did not produce N-gases via denitrification: First, when ^{15}N-nitrite was added during ammonia oxidation, the cells did not produce ^{15}N-gases (Table 2). Second, a ^{14}N-nitrite pool did not prevent the formation of ^{15}N-gases. These results do not only demonstrate that the mutants lack a denitrification activity, but also show that they release NO and N_2O during the oxidation of ammonia to nitrite. Most probably, the auto-oxidation and chemodenitrification of hydroxylamine is responsible for the emission of nitrogen gases. The NorB-deficient strain should be able to produce NO with its nitrite reductase, leading to an increased NO production (Fig. 2), but the

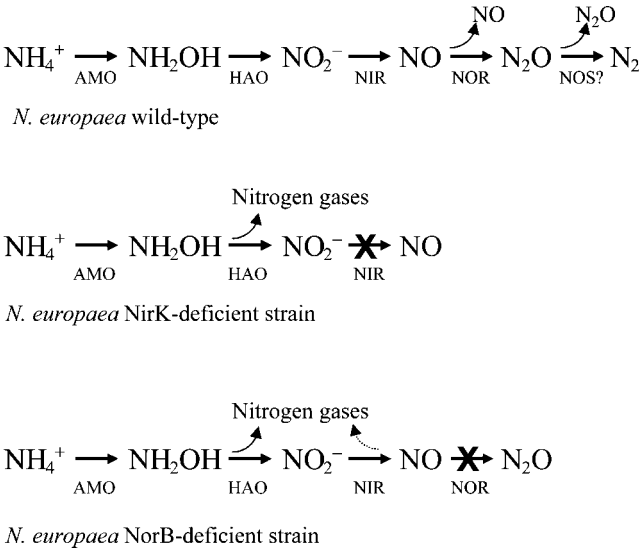

Fig. 2. Pathways of NO and N_2O production by *Nsm. europaea* wild-type and the NirK- and NorB-deficient strains during aerobic ammonia oxidation and denitrification. While the wild-type released NO/N_2O during denitrification, both mutants released high quantities of hydroxylamine into the medium which subsequently converted into nitrogen gases (auto-oxidation/chemodenitrification). AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS?, not yet identified nitrous oxide reductase.

results presented in Table 2 did not indicate NO formation from nitrite. Why the nitrite reductase remained inactive in these experiments although other experiments indicate an active nitrite reductase (high NO concentration during the recovery of ammonia oxidation) has yet to be determined.

During anaerobic NO_2-dependent ammonia oxidation only nitrite is available as the electron acceptor (Schmidt & Bock, 1997). The wild-type strain oxidized ammonia via hydroxylamine to nitrite, and about 47 % of this nitrite was reduced, mainly to N_2 (Table 3). Since anaerobic ammonia oxidation depends on nitrite as the electron acceptor, both mutants were supposed to be inactive under anoxic conditions. True to this prediction, the NorB-deficient strain was unable to convert ammonia and grow (Fig. 3), although nitrite might serve as a potential electron acceptor (reduction to NO). In contrast, the NirK-deficient strain was active. The ammonia oxidation activity, growth rate and growth yield were about 25 % lower compared with the wild-type (Table 3). The mutant was unable to reduce nitrite (ammonia consumption : nitrite production, about 1 : 1), but interestingly, the apparent NO production was low (ammonia consumption : NO production, about 1 : 0·8). When ^{15}N-NO was added the NirK-deficient strain converted it into ^{15}N-N_2, but in the absence of NO (addition of NO-binding DMPS) the cells remained inactive. These

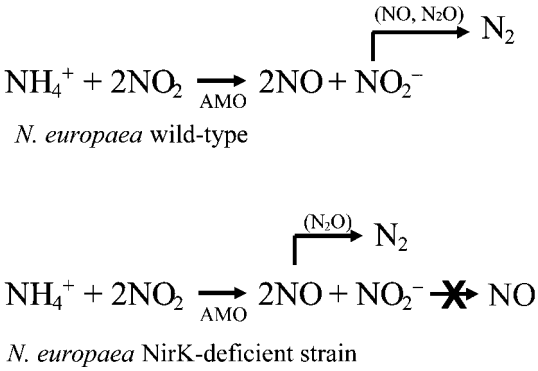

Fig. 3. Anaerobic NO_2-dependent ammonia oxidation of *Nsm. europaea* wild-type and NirK-deficient strain. The wild-type was using nitrite, and the NirK-deficient strain NO, as terminal electron acceptors. The intermediates are shown in parentheses.

results gave evidence that the NirK-deficient strain used the NO produced during anaerobic ammonia oxidation as a terminal electron acceptor (Fig. 3).

The third energy-generating metabolic activity of *Nsm. europaea* is hydrogen-dependent denitrification (Table 4). It is interesting to note that *Nsm. europaea* has the capability to utilize hydrogen as an electron donor, as reported for *Nitrosomonas eutropha* (Bock *et al.*, 1995). A key element to activate this metabolic activity is a low redox potential between −300 and −200 mV. At higher redox potentials a hydrogen-dependent denitrification activity of *Nsm. europaea* was not observed (Uemoto & Saiki, 2000).

Denitrification of *Nsm. europaea* wild-type strain was detectable with nitrite, NO or N_2O as electron acceptors, although the growth yields and rates differed significantly. The mutant strains were dependent on the addition of electron acceptors that compensate for the lack of nitrite reductase and nitric oxide reductase, respectively. Hence, the NirK-deficient strain could only grow with NO and N_2O and the NorB-deficient strain was restricted to N_2O as an electron acceptor. The mechanism of N_2 formation by ammonia oxidizers has yet to be elucidated since the genes encoding a nitrous oxide reductase are missing in the genome of *Nsm. europaea* (Chain *et al.*, 2003). It might be speculated whether a novel NOS is active in *Nitrosomonas*.

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