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The production and utilization of nitric oxide by a new, denitrifying strain of *Pseudomonas aeruginosa*

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Abstract. When a new strain of *Pseudomonas aeruginosa* was grown aerobically and then transferred to anaerobic conditions, cells reduced NO_3^- quantitatively to NO_2^- in NO_3^--respiration. In the absence of nitrate, NO_2^- was immediately reduced to NO or N_2O but not to N_2 indicating that NO_2^--reductase but not N_2O-reductase was active. The formation of the products NO or N_2O depended on the pH in the medium and the concentration of NO_2^- present. When *P. aeruginosa* was grown anaerobically for at least three days N_2O-reductase was also active. Such cells reduced NO to N_2 via N_2O. The new strain generated a H^+-gradient and grew by reducing N_2O to N_2 but not by converting NO to N_2O. For comparison, *Azospirillum brasilense* Sp7 showed the same pattern of NO-reduction. In contrast, *Paracoccus denitrificans* formed 3.5 H^+/NO during the reduction of NO to N_2O in oxidant pulse experiments but could not grow in the presence of NO. Thus the NO-reduction pattern in *P. denitrificans* on one side and *P. aeruginosa* and *A. brasilense* on the other was very different. The mechanistic implications of such differences are discussed.

Key words: Anaerobic respiration – Denitrification – NO_x-formation and utilization – *Pseudomonas aeruginosa* – *Azospirillum brasilense* – *Paracoccus denitrificans*

There is currently much concern about the global fluxes of NO_x from and to the biosphere. NO_x is emitted mainly by anthropogenic processes, but soil is also believed to act as a significant source and sink for NO_x by the activity of microorganisms (Slemr and Seiler 1984; Anderson and Levine 1986; Remde et al. 1989). The biological production of NO and N_2O is due to either nitrification or denitrification. It has been established for denitrification that N_2O is an intermediate but the role of NO is unclear (Payne 1985; Stouthamer 1988; Zumft et al. 1988b; Hochstein and Tomlinson 1988). In intact cells, NO is rarely detected as an intermediate of denitrification (Hochstein and Tomlinson 1988). However, formation of free NO from nitrite by cells has been unequivocally shown by trapping NO extracellularly with hemoglobin (Goretski and Hollocher 1988) or by adding an uncoupler (Kučera 1989). It was stated that NO is never a terminal product of denitrification (Payne 1985).

The utilization of NO by intact cells is also not well characterized. Many bacteria consume exogenous NO. In *P. denitrificans*, the reduction of NO is coupled with the generation of a H^+-gradient (Garber et al. 1982; Shapleigh and Payne 1985). The reduction of NO by vesicles from the cytoplasmic membrane of *P. denitrificans* is sensitive to uncoupling reagents and generates ATP, albeit with low efficiency (Carr et al. 1989). Transposon mutants of *Pseudomonas stutzeri* defective in cytochrome cd_1-dependent nitrite reductase are able to reduce NO unimpaired. Therefore NO- and NO_2^--reductase can be different entities (Zumft et al. 1988a). Homogeneous NO-reductase from *P. stutzeri* has recently been shown to consist of two polypeptides associated with heme b and c (Heiss et al. 1989). Thus all the evidence indicates that microorganisms denitrify by a linear reaction scheme which includes NO. However, growth with NO as the sole respiratory electron acceptor has not been demonstrated as yet. Moreover, mutants unable to utilize NO have not been described. Therefore, it is still possible that exogenous NO is artificially reduced by an enzyme with an entirely different function.

During a screening program for denitrifying soil bacteria associated with plants a bacterium identified as *Pseudomonas aeruginosa* was isolated which was particularly active in the formation and utilization of NO. This paper describes physiological conditions for NO production and consumption by this strain. The energy generation by, and the growth conditions for NO respiration in *P. aeruginosa* are also reported. *A. brasilense* and *P. denitrificans* were used for comparative studies. A more

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detailed account of this investigation is available (Voßwinkel 1990).

Materials and methods

Organisms used

For the present study, 85 isolates from soil samples were checked for their denitrifying capabilities. Out of these one strain (internally coded with E 8/2) displayed particularly active formations and utilizations of NO. It was isolated from wheat straw material which had decomposed for 4–6 months in a loamy soil near Braunschweig, Germany. The isolate was identified by the Deutsche Sammlung von Mikroorganismen in Braunschweig as *Pseudomonas aeruginosa* (DSM deposit no. 6195). The strain utilizes glycolate, which is an unusual substrate for *P. aeruginosa* strains. The bacterium was maintained as stock culture on agar slants containing 0.8% nutrient broth (Difco) and 2% agar. It was transferred to 2 ml liquid medium containing yeast extract (3 g/l), peptone (5 g/l) and KNO_3 (10 mM). After growth for 24 h at 30° C, the cells were grown in malate medium containing in g/l: $MgSO_4 \times 7\ H_2O$ 0.2, NaCl 0.1, $CaCl_2 \times 2\ H_2O$ 0.02, $NaMoO_4 \times 2\ H_2O$ 0.02, $MnSO_4 \times H_2O$ 0.02, KH_2PO_4 0.6, K_2HPO_4 0.78 (phosphates autoclaved separately), $FeSO_4 \times 7\ H_2O$ 13.8 mg/l, EDTA 18.6 mg/ml (Fe-EDTA also autoclaved separately), DL-malate adjusted to pH 7.0 10 mM and KNO_3 10 mM. *Paracoccus denitrificans* (ATCC 19367; DSM 413) was grown in the same medium. The growth conditions for batch cultures of *Azospirillum brasilense* Sp 7 (ATCC 29145; DSM 1690) have been described elsewhere (Zimmer et al. 1984). For some experiments *Pseudomonas aeruginosa* (see Fig. 1) was grown in medium containing per l: NaCl 5 g, tryptone 16 g and yeast extract 10 g (2 × YT medium, Sambrook et al. 1989).

Growth of *P. aeruginosa* which did not form N_2O-reductase (first stage culture): 20 ml ($3-5 \times 10^7$ cells/ml) of a culture grown aerobically in the malate medium were inoculated into 400 ml of fresh malate medium in 1 l flasks. The air was replaced by argon, and the cells were grown at 30° C in a shaking water bath. After 48 h the culture had reached an O.D. at 560 nm of 0.2 and gas content in the headspace of the cultures was analysed.

Growth of *P. aeruginosa* which formed N_2O-reductase (second stage culture): For this, the cells were grown strictly anaerobically for at least three days. Each culture was diluted every 24 h with fresh, O_2-free medium (360 ml malate medium to 40 ml cells in the 1 l flasks), and air admission was strictly avoided during all manipulations. Cells were assayed when the O.D. was 0.3–0.4 at 560 nm. Such cells were active in NO- and N_2O-utilizations.

For the measurements of the formations and utilizations of NO and N_2O, cells were centrifuged ($1 \times 16000 \times g$, $2 \times 8000 \times g$, each time 10 min) and resuspended in nitrogen-free malate medium. The assays were performed with 2 ml bacterial suspensions (protein concentrations indicated in the figures and tables) in 7 ml Fernbach flasks wrapped with aluminum foil and covered with suba seals. Oxygen was removed from the Fernbach flasks by repeatedly evacuating and flushing with argon. The assays were started by adding the electron acceptor (KNO_3 or KNO_2) anaerobically and performed at 30° C in a shaking water bath. To assay NO and N_2O-utilizations by second stage cultures, the 2 ml cells in the 7.0 ml Fernbach flasks were preincubated for 24 h in the absence of any respiratory electron acceptor prior to the addition of NO or N_2O by syringe. The changes in the concentrations of the gases in the headspaces of the flasks were monitored by gas chromatography or in a NO_x-analyzer. After the end of the experiments cells were spun down, and nitrate and nitrite contents of the supernatant were determined colourimetrically.

Analytical methods

For the H^+-translocation determinations with the different electron acceptors, batch cultures in the exponential growth phase were used throughout all experiments. Otherwise the determinations were performed as previously (Danneberg et al. 1989). The calculations of the H^+-extrusions have also been described (Danneberg 1987; Danneberg et al. 1989). In the H^+-translocation experiments with the NO pulses, any O_2 in the NO-solution was critical. Therefore, as controls, the NO in the solutions to be assayed was exchanged for argon after the experiments, and the H^+-translocations by these NO-free solutions were also determined as controls and subtracted from the data. Such H^+-translocations in the controls were due to the presence of NO_2^- previously formed from the reaction of NO with residual O_2 (see Garber et al. 1982; Shapleigh and Payne 1985).

To determine the concentrations of the gases in the headspace of the vessels, samples were taken with a syringe through the sampling septum. N_2- and O_2-concentrations were determined in a Perkin-Elmer model 8500 gas chromatograph equipped with a thermal conductivity detector and a 5 Å molecular sieve column using argon as carrier gas. N_2O and CO_2 were quantitatively measured in a similar gas chromatograph (Perkin-Elmer model Sigma 3B) using a Carbosieve SII column at 180° C and helium as carrier gas. In the initial experiments, NO concentrations had also to be determined in this gas chromatograph using the same column, however, set to 90° C. Later on, an NO_x-analyzer, type CLD 502, (TECAN AG, Hombrechtikon, CH) was available, and the concentration of NO in the gas phase of the samples was determined after their conversion to NO_2 with O_3, and the quantities of photons emitted in this reaction were measured with a chemoluminescence detector. The experimental design and the sampling procedure were similar to that described by Papen et al. (1989). To test NO-utilizations by the bacteria, the gas (> 99.5% pure, O_2-free) was purchased from Linde, Höllriegelskreuth, FRG and transferred by syringe from the tanks to the assay flasks. Nitrate was quantified with salicylic acid

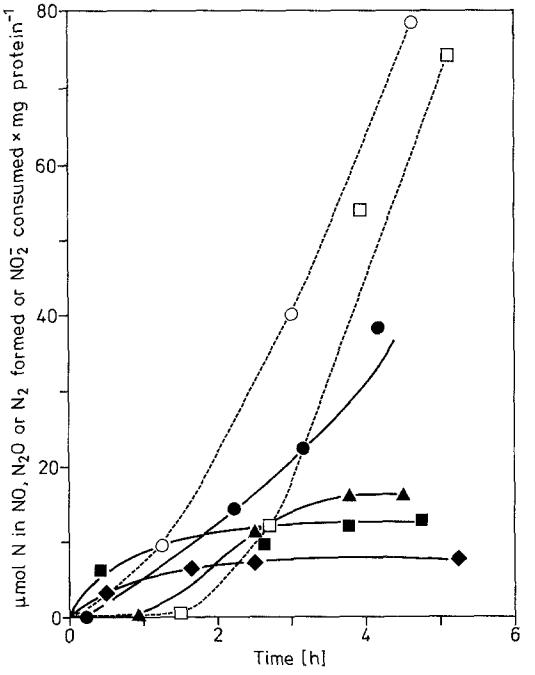


Fig. 1. The formation of nitrogenous oxides from nitrite by *Pseudomonas aeruginosa*. *Solid lines* (first stage cultures): The bacteria were grown aerobically in the 2 × YT-medium (see Materials and Methods). 1 ml of cells (O.D. 560 nm = 1.2) were incubated anaerobically in 40 ml 2 × YT-medium supplemented with 5 mM KNO_2 in 120 ml flasks. ● NO_2^--consumption, ◆ NO-formation, ▲ N_2O-formation, ■ N_2-formation. *Dashed lines* (second stage cultures): The bacteria were grown anaerobically for three days in the 2 × YT-medium (diluted daily with fresh medium) and assayed as for first-stage cultures. These cells formed no NO or N_2O. ○ NO_2^--consumption, □ N_2-formation

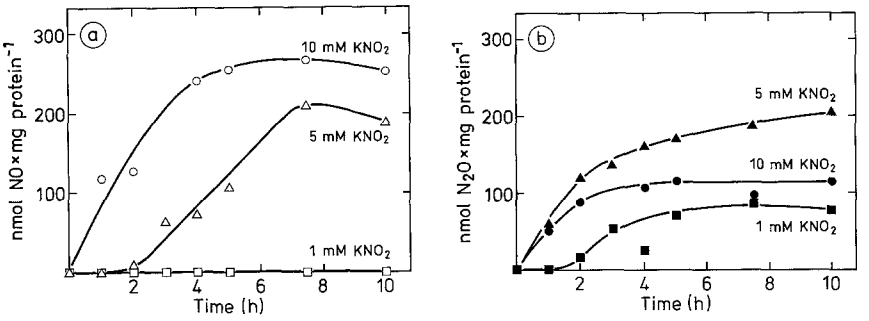


Fig. 2a, b. The dependence of the formation of NO (**a**) and N_2O (**b**) by *Pseudomonas aeruginosa* on the concentration of nitrite in the assay. The assays were performed in 7 ml flasks with 1 ml first stage culture (1 mg protein/ml). The assays were started by injecting 0.1 ml of anaerobic KNO_2 solution (concentration as indicated in the abscissa) through the septum of the rubber stoppers

(Cataldo et al. 1975) and nitrite with Gries-Ilosvay's reagent (Snell and Snell 1949). Nitrate and nitrite concentrations were also determined by their absorbance at 214 nm after separation by HPLC using a Radial-Pak C 18-reverse-phase column and low-UV-PIC A reagent (Kuchnicki et al. 1985). Protein concentrations of intact cells were measured by a modified Lowry method (Herbert et al. 1971). Gases of the highest purities available were purchased from Linde, Unterschleißheim, FRG.

Results

Previous work with other *Pseudomonas aeruginosa* strains had shown that the expression of enzymes involved in denitrification is complex and that the pattern of accumulation of intermediates varies with the culture conditions employed and from strain to strain assayed (Carlson and Ingraham 1983; Bazylinski et al. 1986). In the present study with the new strain, cells were grown aerobically at 30° C for 24 h in a shaking water bath, and this suspension was used as inoculum for measuring growth and production of gases from nitrate under anaerobic conditions. The culture grew for the first 10 h and then entered the stationary phase. It reduced nitrate almost quantitatively to nitrite within 10–22 h. Gas production started as nitrate was depleted and at least 4 mM nitrite had accumulated in the medium. Altogether, the total gas production accounted for less than 10% of the initial amount of nitrate added to the cultures. Gas production was strictly dependent on the nitrite concentration, and the gases produced by these first stage cultures were NO and N_2O and only small amounts of N_2 (Fig. 1). Gas production was not enhanced by C_2H_2, which is an effective inhibitor of all known N_2O-reductases (Knowles 1982). N_2O-reductase was apparently not, or only poorly, synthesized in such first stage cultures.

Nitrogenous oxides could have been formed to detoxify nitrite. The formation of NO increased with increasing concentrations of nitrite (Fig. 2a). In contrast, N_2O-formation was detected at concentrations of NO_2^- as low as 1 mM. It was maximal at 5 mM and lower at the highest amount (10 mM) tested (Fig. 2b). Nitrite at such high concentrations presumably affected NO-reduction activity of the cells. Both NO- and N_2O-formations were not due to chemodenitrification under the assay conditions employed. A similar dependence of NO- and N_2O-productions on the amount of nitrite available had previously been described in *Corynebacterium nephridii* (Renner and Becker 1970).

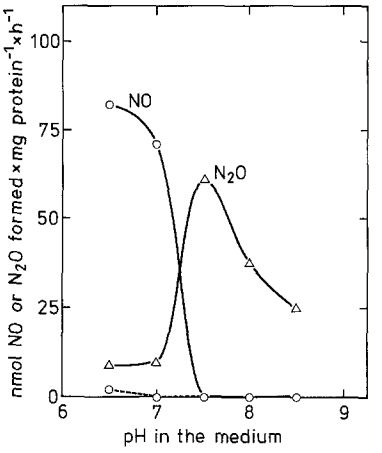


Fig. 3. The dependence of the formations of NO and N_2O in *Pseudomonas aeruginosa* on the pH. Cells from first-stage cultures were threefold centrifuged and washed with N-free medium (pH 7.0). 2 ml of cells with 2.2 mg protein/ml were assayed in 7.0 ml Fernbach flasks, and the pH was adjusted to the values given in the absissa. The assays were started by adding KNO_2 (final concentration in the vessels 10 mM). O——O NO-formation, △——△ N_2O-formation, O----O control, NO-formation by boiled bacteria. No NO-formation with these controls was observed above pH 7.0

The activities of enzymes in denitrification are dependent on the pH in the medium (see Knowles 1982). *P. aeruginosa* produced exclusively N_2O above pH 7.5, whereas NO was the main gas formed between pH 6.5 and 7.0 (Fig. 3). Controls showed that gas production was strictly dependent on nitrite and was a biological, not a chemical reaction in all the experiments. At pH-values < 6.5, NO_2^- was chemically reduced, mainly to NO and NO_2 as described by Perkin et al. (1985). The rate of NO-formation was 71 nmol/h × mg protein at pH 7.0 and 30° C and was 1.6-fold higher than at 20° C. Activities were the same at 20° C and 10° C.

The NO-utilization experiments had to be performed under conditions of strict O_2-exclusion. NO is oxidized to NO_2 by O_2 followed by the formation of nitrite:

$$NO + NO_2 + H_2O \leftrightarrow 2\ HNO_2$$

(Gmelin 1936). This reaction, indeed, occurred in the reaction vessels containing only malate medium. In air, NO was quantitatively converted to NO_2^- within 1 min (Fig. 4). When the vessels were made air-free by repeatedly evacuating and flushing with argon, some residual

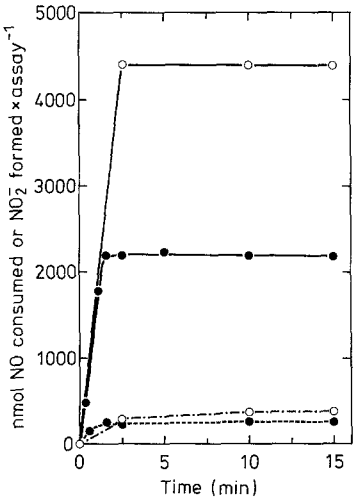


Fig. 4. The chemical conversion of nitric oxide to nitrite. The assay was performed in 7.0 ml Fernbach flasks wrapped with aluminum foil and containing 2 ml malate medium. For the anaerobic assays the flasks were evacuated for 1 h and then gased with argon for 0.5 h. The assays were started by injecting 100 µl NO with a Hamilton syringe aerobic samples: ○——○ NO-consumption, ●——● nitrite formation, anaerobic samples: ○—·—○ NO-consumption, ●----● nitrite formation

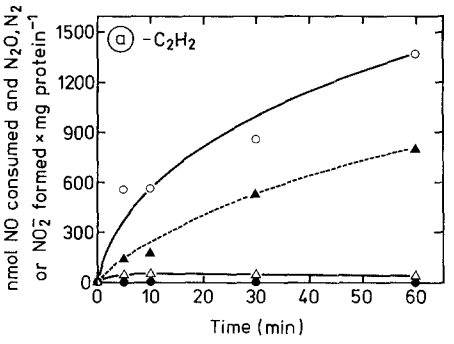
and, to some extent variable, amount of O_2 remained. It caused the immediate formation of some NO_2^- (Fig. 4). Therefore, for the present studies on NO-reductions, the assay vessels were evacuated and flushed with argon and then preincubated for several hours with the bacterial suspension in medium, however, free of electron acceptor. The consumption of the residual O_2 by bacterial respiration was monitored by gas chromatography until exhaustion prior to the start of the reaction by adding NO with a syringe.

When *P. aeruginosa* was grown anaerobically with nitrate as the sole respiratory electron acceptor for longer times (at least 3 d, diluted every day with the medium), cells of this second stage culture reduced nitrite with higher rates and had maximally formed N_2O-reductase. The only gas formed upon the addition of nitrite was N_2 (Fig. 1). These second stage cultures could also reduce NO to N_2 (94%) and N_2O (6%), and no NO_2^- was detected in the vessels within 1 h of NO addition (Fig. 5a). In the presence of 1% C_2H_2 (v/v), NO was reduced to N_2O, and N_2 formation was completely inhibited (Fig. 5b).

NO can be reduced non-enzymatically in solutions by Fe^{2+}-ascorbate (Zumft and Frunzke 1982). Controls with boiled cells, however, indicated that the formations of N_2 and N_2O from NO were of biological origin in the present study. The apparent Michaelis constant for NO was 3.3 mM. NO-reduction activity of these second stage cultures was not enhanced by preincubating with NO and was not affected by NO_3^-, NO_2^- or N_2O.

The H^+-translocations in the presence of valinomycin/K^+ were determined for NO-reductions by the oxidant pulse method (for experimental details see Danneberg et al. 1989). Garber et al. (1982) and Shapleigh and Payne (1985) reported H^+/NO ratios of 3.53 and 3.65, respectively, with *P. denitrificans*, and the present study confirms this ratio being 3.5 for the same bacterium (Table 1). H^+-translocations were almost totally diminished by the uncoupler carbonyl-cyanide-3-chlorophenyl-hydrazone (= CCCP), but was unaffected by KSCN which inhibits N_2O reductase. These experiments indicate that the energy was generated from the reduction of NO to N_2O.

In contrast, both *P. aeruginosa* and *A. brasilense* could not generate a H^+-gradient by the reduction of NO to N_2O (Table 1). The slightly negative values obtained were not statistically significant. They came from the controls which were critical in this experiment. In a typical assay with *P. aeruginosa*, oxidant pulses with the NO-containing solution gave 5.8 H^+/NO pulse, and controls with the NO-free solution (obtained by degassing the NO-containing solution after the experiment and then by assaying the same amount of the NO-free solution) gave 6.6 H^+/pulse. These H^+-translocations were driven by nitrite formation from NO and O_2. Unfortunately it was not possible to exclude O_2 entirely from the experimental system with the electrode for technical reasons (see also Garber et al. 1982; Shapleigh and Payne 1985). The results for *A. brasilense* were 6.3 H^+/NO pulse and 7.2/NO-free pulse and for *P. denitrificans*, 8.5 H^+/NO pulse and 5.0 H^+ for NO-free pulse. Clearly, there was a statistically significant difference between *P. denitrificans* and the other two bacteria. In addition, the H^+-translocation values determined for NO-pulses were not altered by adding CCCP in the case of *P. aeruginosa* and *A. brasilense* (Table 1). A higher KSCN-concentration was used than that in the preceeding study (Danneberg et al. 1989). As a result, both NO_3^- and NO_2^- reductions were partially inhibited and N_2O-reduction was totally blocked (Table 1). The experiment showed that H^+-generation from NO was not affected by KSCN in any of



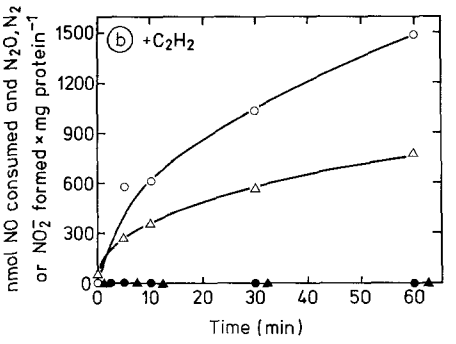


Fig. 5a, b. The utilization of NO by anaerobically grown *Pseudomonas aeruginosa*. Second stage cultures were used for this experiment which was performed with 2 ml of cells (3 mg protein/ml). The assays were started by injecting 8.9 µmol NO. For other experimental details see Materials and Methods; **a** without C_2H_2, **b** with 30% (v/v) C_2H_2 in the gas phase. ○ NO-utilization, ▲ N_2-formation, △ N_2O-formation, ● Nitrite-formation

Table 1. Stoichiometry between H^+-translocation and consumption of respiratory electron acceptors determined by the oxidant pulse method in *Pseudomonas aeruginosa*, *Azospirillum brasilense* and *Paracoccus denitrificans*

	$\frac{1}{2}O_2 \rightarrow H_2O$	$NO_3^- \rightarrow \frac{1}{2}N_2$	$NO_2^- \rightarrow \frac{1}{2}N_2$	$NO \rightarrow \frac{1}{2}N_2$	$N_2O \rightarrow N_2$
A: valinomycin treated cells					
P. aeruginosa	4.2	7.2	5.8	−0.8	2.3
A. brasilense	4.5	5.2	4.1	−0.9	2.7
A. brasilense[1]	5.6	6.6	3.8		3.1
P. denitrificans	6.5	8.7	6.6	3.5	3.5
P. denitrificans[2]	7.5	4.7	3.53	3.5	
P. denitrificans[3]			5.47	3.65	3.92
B: valinomycin treated cells + CCCP					
P. aeruginosa	0.0	0.0	0.0	0.6	0.0
A. brasilense	0.0	0.0	0.1	0.3	0.0
A. brasilense[1]	0.0	−1.3	−1.4		0.0
P. denitrificans	2.0	0.2	0.0	0.0	0.0
C: valinomycin treated cells + KSCN					
P. aeruginosa	4.2	0.2	0.4	−0.6	0
A. brasilense	4.1	0.5	0.5	−1.0	0
A. brasilense[1]	5.7	4.2	2.2		0
P. denitrificans	7.1	0	5.7	3.9	0
P. denitrificans[2]	7.8		4.5	3.6	0

The values in this table indicate the amount of H^+ translocated per molecule of electron acceptor added. Prior to all tests, the cells were preincubated with 50 μg/ml of valinomycin for 3 h. Where indicated the cuvette was supplemented with CCCP (final concentration (in μM): 15 for *P. aeruginosa*, 10 for *A. brasilense* and 30 for *P. denitrificans*. The amount of KSCN in the cuvette was (in μM): 30 for *P. aeruginosa* and *A. brasilense* and 60 for *P. denitrificans*. The 3 ml of bacterial cell suspension in the cuvette had protein contents/ml: 3.2–3.4 for *P. aeruginosa*, 2.8–4.8 for *A. brasilense* and 4.2–5.3 for *P. denitrificans*. The assays were started by adding the oxidant pulses in nmol: KNO_3 10, KNO_2 10, NO 4.2, N_2O 11, O_2 2.37. Some data from the literature are added to this table for comparison: [1] from Danneberg 1987 and Danneberg et al. 1989; [2] from Garber et al. 1982; [3] from Shapleigh and Payne (1985). Blank spaces in the table indicate that these data are not available from the literature

three bacteria. Therefore protons must have been generated by the reduction of NO to N_2O in *P. denitrificans*. The data for the H^+-translocations during O_2- and NO_3^--respirations by *A. brasilense* were slightly lower than those of the preceeding study (Danneberg et al. 1989) and also lower than those reported for O_2- and NO_3^--respirations by *P. denitrificans* (Boogerd et al. 1981). This was probably due to the fact that the experiments described above were performed with cells from batch and not from continuous cultures as previously.

Anaerobic growth experiments with NO as the sole respiratory electron acceptor were performed with second stage cultures of *P. aeruginosa* and also with *A. brasilense* and *P. denitrificans* for comparison (Table 2). Both *P. aeruginosa* and *A. brasilense* consumed almost 80% of the NO offered within 18 h and converted it quantitatively to N_2 after 4d with the transient formation of N_2O. In the presence of C_2H_2 both cultures reduced NO stoichiometrically to N_2O without the formation of N_2. The cells actively produced CO_2 regardless of the presence or absence of C_2H_2 indicating that they remained metabolically active during incubation with NO. However, only cultures which were kept C_2H_2-free could grow as indicated by the increase in protein content by about 50% for *P. aeruginosa* and about 30% for *A. brasilense* after 4d. Cells incubated with C_2H_2 rested. Both organisms could apparently meet their energy requirement for growth from the reduction of N_2O to N_2 but not from the conversion of NO to N_2O. The controls with C_2H_2 indicated that growth observed was not due to the utilization of any NO_2^- formed from NO. Nitrite reductions are known to be unaffected by C_2H_2 and should, therefore, allow growth even in the presence of C_2H_2.

In contrast, *P. denitrificans* reduced NO only to N_2O, did not evolve more CO_2 when incubated without C_2H_2 and the protein content of the culture did not increase during incubation (Table 2).

Discussion

The investigation described here showed that the end product of denitrification by *Pseudomonas aeruginosa* varied with the culture conditions. When air-grown cells were shifted to anaerobiosis, nitrate was reduced to nitrite. However, when nitrite was offered instead of nitrate, cells started to produce NO and N_2O within 15 min. Thus nitrite reductase was synthesized but blocked by nitrate, an observation that had been described for other organisms also (Payne 1973; Ji and Hollocher 1988; Körner and Zumft 1989). In these first stage cultures of *P. aeruginosa*, NO_2^--reduction proceeded beyond N_2O only to a limited extent. The new strain apparently did not synthesize N_2O-reductase immediately after transfer to anaerobiosis as *A. brasilense* did (Penteado Stephan et al. 1984). The N_2O-reductase of *P. aeruginosa* appeared to be particularly susceptible to low concentrations of O_2 and its formation required strict anaerobiosis, as already described for another strain *P. aeruginosa* (Davies et al. 1989) and for *Paracoccus halodenitrificans* (Hochstein et al. 1984). It remains to be shown whether the new strain synthesizes N_2O-reductase at low level even in air as

Table 2. Growth of *Pseudomonas aeruginosa*, *Azospirillum brasilense* and *Paracoccus denitrificans* under anaerobic conditions with NO as the sole respiratory electron acceptor

	Pseudomonas aeruginosa		*Azospirillum brasilense* Sp7		*Paracoccus denitrificans*	
	$-C_2H_2$	$+ C_2H_2$	$-C_2H_2$	$+ C_2H_2$	$-C_2H_2$	$+ C_2H_2$
1. NO content						
at the start	357	357	357	357	357	357
after 18 h	80	80	84	84	191	189
after 4 d	0	0	0	0	118	186
2. N_2O content						
after 18 h	24	126	27	124	97	105
after 4 d	0	206	0	181	106	116
3. N_2 content						
after 18 h	102	0	110	0	0	0
after 4 d	207	0	179	0	0	0
4. [N]-recovery in %	116	115	100	101	92	103
5. protein content (µg/ml)						
at the start	43.1	43.1	55.9	55.9	270	270
after 4 d	62.8	46.0	73.1	53.9	249	237
6. CO_2-content						
after 18 h	758	618	539	309	195	155
after 4 d	988	767	984	821	169	161

Growth and activity were followed under anaerobic conditions in malate medium containing 5 mM NH_4Cl in place of KNO_3. Before the assay the samples were incubated at 30°C for 4 h in a shaking water bath without any respiratory electron acceptor and the experiment was then started by injecting 257 µmol NO. The gas phase contained 2% C_2H_2 (v/v)

shown for the ZoBell strain of *P. stutzeri* (Körner and Zumft 1989). Cultures grown anaerobically for at least 3d could reduce NO_3^-, NO_2^- or N_2O to N_2. The reduction of N_2O to N_2 was accompanied by the formation of a H^+-gradient and by low but significant growth rates. Growth of *P. aeruginosa* with N_2O as the sole respiratory electron acceptor had previously been found by Bazylinski et al. (1986) but not by Carlson and Ingraham (1983).

It has been stated that NO is only an intermediate and never a terminal product of denitrification (Payne 1985) and is liberated under few and sometimes peculiar conditions (Zumft et al. 1988b). This did not apply to *P. aeruginosa*. When grown aerobically and transferred to anaerobic conditions, the new strain reduced nitrite almost quantitatively to NO at pH-values between 6.5 and 7 indicating that NO can be end product of denitrification. Such pH-dependence of NO production may have ecological implications but this has not been rigorously studied to our knowledge. Application of high nitrite concentrations also favours NO-production by *P. aeruginosa*, but such conditions hardly occur in the field.

The reduction of nitrite to nitrous oxide may have been catalyzed in total by the cytochrome cd_1 containing nitrite reductase which had also NO-reductase activity in extracts from *P. aeruginosa* (Silvestrini et al. 1979; Wharton and Weintraub 1980). It could be that nitrite reductase was modified by low pH and high nitrite concentrations to a form which easily released NO and did not convert it to N_2O (Averill and Tiedje 1982; Bessières and Henry 1984). Strikingly, isolated cytochrome oxidase (= nitrite reductase) of *P. aeruginosa* showed virtually the same substrate and pH-dependence (Silvestrini et al. 1979). In particular, NO-formation at pH values < 7.0 and N_2O-formation at pH > 7.0 were similar in intact cells of the new strain. Isolated nitrite reductase is known to catalyze the formation of N_2O from NO_2^- when associated to membranes and to produce mainly NO when solubilized. Product formation (NO or N_2O) is also dependent on the electron donor system used (NADH/PMS or ascorbate/TMPD; see Hochstein and Tomlinson 1988). All these facts suggest that NO- and N_2O-formations are both catalyzed by one and the same nitrite reductase. Such a conclusion is not in accord with recent interpretations (Silvestrini et al. 1990). It is also possible that NO_2^--reductase (catalysing the reduction of NO_2^- to NO) and NO-reductase (converting NO to N_2O) associate with each other to a tight unit in the cells. Conditions like low pH and high nitrite concentrations may loosen this functional unit and cause a net production of NO from nitrite in intact cells.

It remains to be shown whether the reduction of NO is catalysed by the nitrite reductase or by a separate NO-reductase in intact *P. aeruginosa*. The present study showed that the reduction of NO to N_2O by *P. aeruginosa* did not result in H^+-translocations and cell growth. Similarly, although *Azospirillum brasilense* grows with NO_3^-, NO_2^- or N_2O (Zimmer et al. 1984; Penteado Stephan et al. 1984) it cannot do so with NO as sole electron acceptor (this study). It is tempting to assume that NO is artificially reduced by an enzyme normally not associated with denitrification in *P. aeruginosa* and *A. brasilense*. Such an enzyme could be the heme b and c containing NO reductase recently described for *P. stutzeri* (Heiss et al. 1989). These statements are speculative but are supported by the findings that a mutant of *P. stutzeri* defective in cytochrome cd_1 of nitrite-reductase was unimpaired in NO-reduction (Zumft et al. 1988a).

The situation in *P. denitrificans* might be different. A H^+-gradient was generated from the reduction of NO to

N_2O (present study, also Garber et al. 1982; Shapleigh and Payne 1985). This implies that the properties of NO-reductase must be different in *P. denitrificans* and in *P. aeruginosa* (or *A. brasilense*). Surprisingly, the H^+-gradient generated by the reduction of NO to N_2 was not larger than that resulting from NO being reduced to N_2O. Such observations were also reported by others (Garber et al. 1982; Shapleigh and Payne 1985) without explanation. Unfortunately, *P. denitrificans* could not grow anaerobically with NO. The gas has been described as severely inhibiting N_2O-reductase and NO_3^--reductase (Frunzke and Zumft 1986; Carr et al. 1989) and may have been toxic to other metabolic reactions as indicated by the inhibition of CO_2-evolution in the present study. Thus the situation is somewhat awkward. Ideally an organism is required which forms a H^+-gradient from the reduction of NO to N_2O and grows with NO. Work with deletion mutants of such a hypothetical organism could assign the roles of NO- and NO_2^--reductases in the reduction of nitrite to nitrous oxide via nitric oxide during denitrification.

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