Inhibition of denitrification activity but not of mRNA induction in *Paracoccus denitrificans* by nitrite at a suboptimal pH

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Abstract

The influence of pH on the denitrification activity of a continuous culture of *Paracoccus denitrificans* was studied in relation to the presence of nitrite. After a transition from aerobic to anaerobic conditions at the suboptimal pH of 6.8, *P. denitrificans* was not able to build up a functional denitrification pathway. Nitrite accumulated in the medium as the predominant denitrification product. Although the nitrite reductase gene was induced properly, the enzyme could not be detected at sufficient amounts in the culture. These observations indicate that either translation was somehow inhibited, or once synthesized nitrite reductase was inactivated, possibly by the high concentrations of nitrous acid (HNO₂). Interestingly, when a *P. denitrificans* culture which was grown to steady-state under anaerobic conditions was then exposed to suboptimal pHs, cells exhibited a reduced overall denitrification activity, but neither nitrite nor any other denitrification intermediate accumulated.

Introduction

Bacterial denitrification plays an important role in the global nitrogen cycle. The complete process involves the sequential reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻), nitric oxide (NO) and nitrous oxide (N₂O), and finally to dinitrogen (N₂) (Payne, 1973). Denitrifying bacteria have been isolated from nearly every kind of environmental compartment. The ability to use oxygen as well as nitrogenous oxides as terminal electron acceptors allows them to survive under conditions where oxygen tensions tend to fluctuate. These bacteria will generally use oxygen as the preferred terminal electron acceptor under aerobic conditions (Stouthamer, 1988). However, as soon as oxygen is depleted, they will induce the electron transport chains necessary for denitrification. Denitrification is a complex respiratory process which is controlled by various environmental factors, such as pH, temperature, organic carbon source and nitrogen oxides (Błaszczuk, 1993; Firestone, 1982; Knowles, 1982; Shimizu et al., 1978).

For optimal application of denitrification, for example, in wastewater treatment plants, it is necessary that most of the NO₃⁻ will be converted to N₂, and that as little as possible NO, NO₂⁻ or N₂O will arise. For example, NO₂⁻ resp. HNO₂ (nitrous acid), the undissociated fraction of NO₂⁻, have an inhibitory effect on bacterial growth, which may disturb efficiency of the wastewater treatment process. NO₂⁻ acts by inhibiting active transport systems, oxygen uptake, and oxidative phosphorylation (Casella et al., 1986; Rowe et al., 1979). Especially due to these antimicrobial effects, NO₂⁻ is widely used as food additive (Igene et al., 1985). N₂O on its turn, is potentially harmful by enhancing the global greenhouse effect (Houghton et al., 1990; Wang et al., 1976). NO₂⁻ and N₂O will be usually formed transiently during the onset of bacterial denitrification, but disappear once the process is in stable equilibrium. However, especially the pH can determine whether NO₂⁻ and/or N₂O will accumulate to deleterious levels (Christensen & Tiedje, 1988; Kucera et al., 1986a; Thomsen et al., 1994).

In this article we describe the influence of a suboptimal external pH on the accumulation of NO₂⁻ during

the transient phase after switching from aerobic growth to denitrification, and on stability of the denitrification process in a continuous culture of *Paracoccus denitrificans*. A pH of 6.8 was chosen as a lower limit with relevance to wastewater treatment plants applying denitrification. Key factors for the denitrification process which were determined, were levels of the mRNAs for nitrate, nitrite and nitrous oxide reductases of *P. denitrificans*. Furthermore, we analyzed the formation of denitrification products (NO_2^- , NO, N_2O , N_2) and the synthesis of the *cd*₁-type nitrite reductase enzyme by using polyclonal antibodies.

Materials and methods

Organism and culture medium

Paracoccus denitrificans DSM 65 (ATCC 17741) was grown in continuous culture on a synthetic medium containing per liter 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 1.5 g KH_2PO_4 , 7.5 g KNO_3 , 2.5 g NH_4Cl , 7.4 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3.6 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.45 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 mg H_3BO_3 , 0.01 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 3.7 mg EDTA- Na_2 and 12 mg EDTA- NaFe(III) (Baumann et al., 1996). Acetate was supplied as the limiting substrate to the medium at a final concentration of 60 mM. To all cultures grown in bioreactors a 10% sterilized silicon antifoam solution was added to give a final concentration of 100 ppm (v/v).

Culture conditions for chemostat experiments

Culture conditions were, in short, a dilution rate of 0.03 h^{-1} , temperature 30°C , operating volume of the bioreactor (MBR, Wetzikon, Switzerland) 2.8 litres, and pH 6.8. Except for the pH, which previously had been maintained at 7.5, these conditions were exactly the same as described elsewhere (Baumann et al., 1996). Aerobic conditions were maintained by sparging cultures continuously with helium and oxygen to keep the dissolved oxygen concentration in the bioreactor at 90% air saturation. Anaerobic conditions were imposed by sparging with helium only. Transients were performed by switching a culture instantaneously from aerobic to anaerobic conditions.

Analytical methods

Details of the analytical procedures are given in Baumann et al. (1996). Dissolved oxygen concentration in the culture was measured *in situ* on-line. Bacterial dry weight was determined on filtered samples. Concentrations of acetate, NO_3^- and NO_2^- were analyzed in the filtrate. NO_3^- was determined by ion chromatography (Dionex Corporation, Sunnyvale USA). CO_2 , N_2O and N_2 were measured continuously by a gas-chromatograph in the off gas of the reactor. The concentration of NO was continuously measured in a chemoluminescence detector (Thermo Environmental Instrument, Franklin, MA, USA) directly coupled to the outlet of the GC. Cumulative production was determined by totalling the net production of NO_2^- , N_2O and N_2 during the first 24 h after the switch to anaerobic conditions.

RNA extraction and quantification of mRNA for denitrification enzymes

Total RNA from *P. denitrificans* was isolated from 1 ml samples taken directly from the chemostat. Cells were immediately harvested by centrifugation ($15,000 \times g$ for 30 s) and the pellet was resuspended in 0.5 ml of a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA plus 100 mM NaCl. Total RNA was extracted with acid-phenol and phenol-chloroform as described previously (Baumann et al., 1996). Similar quantities of total RNA from each RNA extraction were blotted onto positively charged nylon membranes (Qia-gen, Switzerland) and hybridized with biotin-labeled single-stranded RNA probes. Hybridization was performed as previously described (Baumann et al., 1996). Probes for the mRNAs for nitrate, nitrite and nitrous oxide reductase of *P. denitrificans* were produced *in vitro* (Baumann et al., 1996). Hybridization results were quantified by the Southern-Light chemoluminescence system (Tropix, Massachusetts, USA) and by scanning the X-ray films on a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.).

Protein isolation and immunoquantification of the nitrite reductase

P. denitrificans cells were recovered from 1 ml of cell suspension from the chemostat by centrifuging at $15,000 \times g$ for 30 s. Total proteins were isolated, separated on SDS-polyacrylamide gels and the relative cytochrome *cd*₁ amounts were determined by immuno-

Figure 1. Transition phase changes in culture parameters after an aerobic to anaerobic change of a continuous culture of *P. denitrificans* DMS 65 at pH 6.8. Time-axis indicates the time after the switch to anaerobic conditions. Symbols: dry weight of the culture (●), rate of CO₂ formation (○).

Figure 2. Transition phase changes in denitrification intermediates after an aerobic to anaerobic change of a continuous culture of *P. denitrificans* DMS 65 at pH 6.8. Time-axis indicates the time after the switch to anaerobic conditions. Symbols: nitrite concentration in culture medium (■), rates for the formation of nitric oxide (□), nitrous oxide (▲), dinitrogen (△).

logical detection on Western blots (Baumann et al., 1996).

Results and discussion

To determine the effects of a suboptimal pH on denitrification activity, we followed the induction of the denitrification pathway in a continuously growing cul-

Table 1. Cumulative production of denitrification intermediates during the first 24 hours after changing from aerobic steady state to anaerobic conditions at pH 6.8 and pH 7.5

	Cumulative production		
	NO ₂ ⁻ [mmol]	N ₂ O [mmol]	N ₂ [mmol]
pH 6.8	31.4 ^a (5.1) ^b	1.0 (0.9)	2.4 (1.7)
pH 7.5 ^c	11 (1.0)	0.2 (0.3)	21.4 (1.3)

^a Mean from three independent experiments.

^b Standard deviation.

^c Data from Baumann et al. (1996), and Baumann (unpublished).

ture of *P. denitrificans* after a switch from aerobic to anaerobic conditions. The pH of the culture medium was kept at 6.8, which is the lower limit with relevance to wastewater treatment plants applying denitrification. As upper limit usually a pH of 7.5 is considered (Metcalf & Eddy, 1979). Denitrification activity of *P. denitrificans* was analyzed by measuring the formation of all denitrification products (NO₂⁻, NO, N₂O, N₂), expression of the mRNA for nitrate, nitrite and nitrous oxide reductases and synthesis of the nitrite reductase enzyme. Furthermore, dry weight and CO₂ production were analyzed.

When a steady-state culture of *P. denitrificans* was subjected to a switch from aerobic to anaerobic growth conditions at a pH of 6.8, cells started to denitrify but were not able to reach a new steady-state with NO₃⁻ as terminal electron acceptor. We kept the culture under anaerobic conditions for 120 hours during which the bacterial biomass concentration decreased gradually (Figure 1). A slight decrease in biomass concentration during the anaerobic period was expected, since in terms of energy yield denitrification is about 30 to 40% less efficient than aerobic respiration (Stouthamer, 1988). The dry weight of our culture decreased to a much larger extent than can be explained by the lower energy gain and did not stabilize at a constant level, either. Respiration activity of *P. denitrificans* was followed by measuring CO₂ production rate (Figure 1). During a short transient period CO₂ production rate increased. At approximately seven hours it started to decline. From about 40 hours onwards it was below the detection limit indicating that the cells had dramatically reduced or even stopped their respiration activity. The only way to restore the activity of the cells that worked in our hands was to shift conditions back to aerobic (not shown). Cultures which were subjected to such a multiple aerobic to anaerobic regime (each period lasting for 24 h), in some cases were able to

develop a stabler denitrification activity at pH 6.8 after the third cycle. We think that the reason for this lays in the slow accumulation of sufficient quantities of nitrite reductase (see below).

The levels of the different denitrification intermediates indicated that the process was strikingly out of balance (Figure 2). NO_2^- and NO were the first compounds to be detected. About half an hour after oxygen in the culture was depleted *P. denitrificans* started producing NO_2^- and NO (Figure 2A). Then, from approximately three hours onwards N_2O and N_2 could be measured (Figure 2B). Independent repetitions of the experiment at pH 6.8 indicated that, although the time sequence of the production rates for the different denitrification intermediates could be slightly different, the cumulative production during the first 24 h after the change to anaerobic conditions was more or less the same (Table 1). When compared with the denitrification performance of *P. denitrificans* observed at an optimal growth pH of 7.5, the formation of denitrification intermediates at pH 6.8 was outbalanced and completely different (Table 1). Under the same growth conditions at a pH of 7.5, the cumulative N_2 production of an anaerobic steady-state culture of *P. denitrificans* was approximately 21.4 mmol, whereas only 2.4 mmol of N_2 was formed at pH 6.8. The main product of the denitrification at pH 6.8 was clearly NO_2^- . It accumulated in the culture medium at levels as high as 35 mM. Probably, the high concentrations of NO_2^- were the main cause for the considerable decline in respiratory activity and for the inhibition of cell growth. Experimental data obtained by Thomsen et al. (1994) indicate that probably the denitrification behaviour would be even more disturbed at a lower pH than 6.8. Therefore, we did not consider to repeat the same experiments at lower pH values.

It has been frequently observed that NO_2^- accumulates transiently after switching aerobically grown cultures of denitrifying bacteria to anaerobic growth conditions (Almeida et al., 1995; Calder et al., 1980; Carlson & Ingraham, 1983; Robertson & Kuenen, 1992; Shimizu et al., 1978; Thomsen et al., 1994; Waki et al., 1980). The accumulation is probably due to a retarded induction of the nitrite reductase compared to the induction of nitrate reductase (Baumann et al., 1996; Kucera et al., 1986b). We followed there the expression of the genes for the nitrate, nitrite and nitrous oxide reductase by measuring the levels of the specific mRNAs in *P. denitrificans* during the transient period (Figure 3). Unfortunately, no gene probe for the nitric oxide reductase mRNA was available to us. Therefore,

Figure 3. Transition phase changes in mRNA levels for the individual denitrification enzymes after an aerobic to anaerobic change of a steady-state aerobically growing culture of *P. denitrificans* DSM 65 at pH 6.8. mRNA levels are given as relative values compared to those under aerobic steady-state conditions (arbitrarily defined as a level of 1). Time-axis indicates the time after the switch to anaerobic conditions. Symbols: relative mRNA levels for nitrate reductase ($\circ$), for nitrite reductase ($\blacksquare$), for nitrous oxide reductase ($\square$).

Figure 4. Formation of the nitrite reductase enzyme during the transition phase of an anaerobic continuous culture of *P. denitrificans* DSM 65. Time-axis indicates the time after switch to anaerobic conditions. Formation of nitrite reductase was assayed by immunologic detection of cytochrome *cd*₁ levels on Western blots of SDS-PAGE-separated total protein extracts of culture samples at different time periods after the switch. Formation of nitrite reductase enzyme at a medium pH of 7.5 (—), at a medium pH of 6.8 ($\blacktriangle$).

its levels could not be measured here. Whereas the level of mRNAs for nitrate and nitrous oxide reductase increased as soon as oxygen in the culture was depleted, the mRNA for nitrite reductase appeared one hour later.

This pattern of sequential gene expression was similar to that observed in several other experiments performed at pH 7.5 (Baumann et al., 1996). It suggests that it was not directly the induction of the denitrification pathway which was affected at pH 6.8. Why then, did NO_2^- only transiently accumulate at pH 7.5, and did its concentrations not exceed 13 mM (Bau-

Figure 5. Effect of a pH transient on the denitrification activity of an anaerobically growing steady-state culture of *P. denitrificans*. Symbols: synthesis rate of dinitrogen ($\blacktriangle$), pH (—).

mann et al., 1996)? At pH 7.5, the cells reached a stable anaerobic steady-state with NO_3^- as electron acceptor after approximately 15 hours (Baumann et al., 1996). To determine further why *P. denitrificans* did not succeed to optimally reduce NO_2^- after an aerobic/anaerobic switch at pH 6.8, we analyzed the presence of the nitrite reductase enzyme in the cells by Western blotting using polyclonal antibodies raised against the *cd1*-type nitrite reductase (Figure 4). From two hours onwards, the level of nitrite reductase in the culture increased slightly. However, after having reached its maximum concentration at approximately five hours, the nitrite reductase concentration in the cells leveled off slowly. Compared to the induction pattern observed at pH 7.5 about 10 to 15 fold less enzyme was formed at pH 6.8 (Figure 4). This suggests that either translation of the nitrite reductase mRNA was inhibited, that the enzyme was translocated and folded improperly, or that already synthesized enzyme became inactivated and/or its conformation changed, thereby preventing immunologic detection.

We propose that the inactivation of the nitrite reductase enzyme resp. inhibition of synthesis was caused by high external NO_2^- concentrations rather than by the lower pH itself. Direct effects of nitrite have been observed before. For example, the nitrite reduction rate in *P. denitrificans* cultures decreased at NO_2^- concentrations higher than 6 mM (Shimizu et al., 1978). Probably, however, not NO_2^- itself, but its undissociated form HNO_2 is the toxic molecule (Almeida et al., 1994; Kono et al., 1994). In contrast to NO_2^- , HNO_2 -

molecules are able to diffuse rapidly and passively across the outer and inner cell membrane (Parsonage et al., 1985). HNO_2 easily dismutates to reactive intermediates, such as NO (nitric oxide) and NO^- (nitroxyl anion). These molecules are thought to react directly with heme and metal centers of proteins and forming nitrosyl-complexes (Kroneck & Zumft, 1990; Reddy et al., 1983). Estimated from the disproportionation reaction (Equation 1), the same amounts of NO_2^- will lead to approximately five times more HNO_2 at pH 6.8 than at pH 7.5.

Thus, with decreasing pH, any produced NO_2^- will become effectively more toxic to the cells (Almeida et al., 1994), which explains why the continuous culture of *P. denitrificans* growing at an external pH of 7.5 was not inhibited by high NO_2^- concentrations. At pH 6.8, the calculated concentration of HNO_2 is $17.5 \mu\text{M}$ (at 35 mM NO_2^-), which in experiments with *Pseudomonas fluorescens* caused a more than 95% inhibition of denitrification activity (Almeida et al., 1994).

Unlike NO_2^- reduction, that of NO_3^- seemed not to be affected by the high NO_2^- concentrations. This is probably due to the fact that the nitrite reductase is localized in the periplasmic space (Alefounder & Ferguson, 1980), whereas the nitrate reductase complex of *P. denitrificans* is a transmembrane protein with its active center located at the inner side of the cytoplasmic membrane (Hochstein, 1988; Stouthamer, 1988). *P. denitrificans* as a neutrophilic bacterium, is able to maintain the pH in the cytoplasm at approximately 7.5 to 8.0, irrespective of the external pH (Booth, 1985; Padan et al., 1981). This would mean that HNO_2 concentrations inside the cell would be much lower than in the periplasmic space, and that the active center of the nitrate reductase would thus be protected much better from potential damage by HNO_2 than the active center of the nitrite reductase. Similarly, it would imply that NO_2^- resp. HNO_2 would affect functioning of the NO and NO_2^- reductase, which are present at the periplasmic side (Alefounder et al., 1983; Carr & Ferguson, 1990).

To investigate if nitrite reduction would also be inhibited at a low medium pH when cells were already adapted to anaerobic conditions, we reduced the pH of the culture medium of an anaerobically growing continuous culture of *P. denitrificans* stepwise from pH 7.8

to 6.3 by 0.1 units per half-an-hour (Figure 5). Interestingly, with decreasing pH the cells showed indeed a reduced overall denitrification activity ($0.8 \text{ mM} \cdot \text{h}^{-1} \text{ NO}_3^-$ vs. $2.2 \text{ mM} \cdot \text{h}^{-1} \text{ NO}_3^-$), but no NO_2^- or any other denitrification intermediates could be detected during the time course of this experiment. This would have been expected if the denitrification course became unbalanced again. No further changes in N_2 production rate were observed within 12 h after the cultures had reached pH 6.3. Apparently, if the individual denitrification enzymes once have been formed at adequate amounts in the culture and, furthermore, exhibit the same turnover rate, NO_2^- does not accumulate and denitrification activity of *P. denitrificans* becomes much less sensitive to a suboptimal external pH.

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