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Study of the Denitrifying Enzymatic System of *Comamonas* sp. Strain SGLY2 Under Various Aeration Conditions with a Particular View on Nitrate and Nitrite Reductases

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Abstract. This paper studies the effect of oxygen on the denitrifying enzymatic system of *Comamonas* sp. It is shown that nitrate respiration can take place in the presence of oxygen. Indeed, even if a protein synthesis inhibitor is added in the medium, immediate nitrate consumption is observed in an aerobic culture inoculated with cells that have never been subjected to nitrate. Existence of a constitutive nitrate reductase could explain this phenomenon. Moreover the nitrate and nitrite reductases are active and synthesized under aerobic conditions. The different levels of inhibition of nitrate reductase activity by respiratory inhibitors and detergent, according to the aerobic and anaerobic cultures, might suggest the existence of a double nitrate reductase enzymatic system.

Bacterial denitrification is an anaerobic respiration where nitrate is reduced to nitrogen gas with nitrite, nitric oxide, and nitrous oxide as intermediates. This reaction is realized by facultative anaerobic microorganisms, especially *Pseudomonas* sp. [39]. It is an alternative way of energy production by transfer of electrons to four de novo-synthesized terminal oxidoreductases: nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NoR), and nitrous oxide reductase (N_2OR). Synthesis and activity of these enzymes are assumed to be completely repressed by oxygen and to be stimulated by one or the other of the N-oxides [31, 34].

More recently, some authors have demonstrated that synthesis and activity of denitrifying enzymes could occur under various aeration conditions. For example, *Pseudomonas stutzeri* exhibits its higher nitrate reductase activity at 1 mg $\cdot$ L^{-1} of dissolved oxygen, but the threshold value for its nitrate reductase synthesis is 5 mg $\cdot$ L^{-1} of dissolved oxygen [15]. *Thiosphaera pantotropha* denitrifies at a rate of 800 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein at 80% of air saturation [29]. Three different types of behavior can thus be distinguished in the presence of oxygen: (i) complete inhibition of denitrifying enzymes with *Paracoccus denitrificans* [14] or *Pseudomonas aeruginosa* [12]; (ii) existence of a tolerance threshold towards dissolved oxygen with *Pseudomonas stutzeri*; and (iii) co-respiration with *Thiosphaera pantotropha*, *Alcaligenes faecalis* [28, 30], *Pseudomonas nautica* [6] and *Pseudomonas sp.* [36].

Berks and associates [4, 5] have characterized the aerobic denitrifying pathway of *Thiosphaera pantotropha*, even though Thomsen and colleagues [37] have assumed that aerobic denitrification with this strain is a utopic idea because of the existence of anaerobic microzones in the kind of reactor used. They have purified a periplasmic nitrate reductase that is overexpressed in a membrane-bound nitrate reductase mutant strain [3]. They have explained the simultaneous use of oxygen and nitrate by existence of this double nitrate reductase enzymatic system: the membrane-bound nitrate reductase, inhibited by oxygen, allows aerobic expression of the periplasmic one [25].

A strain named SGLY2, and identified as *Comamonas* sp., has been isolated in our laboratory from an upflow filter submitted to various aeration conditions. This strain was shown to denitrify in the presence of high oxygen levels and to be able to co-respire the two electron acceptors [22]. In the present study, we

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characterized the enzymatic system of the strain (especially nitrate and nitrite reductases) by investigating the influence of different respiratory protein synthesis inhibitors and detergent (by modifying membrane permeability) on the oxygen-nitrate respiration system. These experiments were carried out either in batch culture under various aeration conditions or in enzymatic assays on whole cells and cell lysates.

Materials and Methods

Organisms and culture conditions. Isolation and characterization of the strain SGLY2 used in this study have been described in detail elsewhere [22]. Cells were grown on a synthetic medium: phosphate buffer 0.01 M, pH 7.0; KNO_3 ($N\text{-}NO_3 = 250$ mg $\cdot L^{-1}$) or KNO_2 ($N\text{-}NO_2 = 50$ mg $\cdot L^{-1}$); ethanol ($C\text{-}C_2H_5OH = 500$ mg $\cdot L^{-1}$); $MgSO_4$ 190 mg $\cdot L^{-1}$; $(NH_4)_2SO_4$ as nitrogen source ($N\text{-}NH_4 = 58$ mg $\cdot L^{-1}$); yeast extract (Difco) 250 mg $\cdot L^{-1}$; 1 ml $\cdot L^{-1}$ of trace element solution [22]. To study the influence of inhibitors or detergent on the oxygen-denitrifying respiration system of the strain, batch cultures were performed under three aeration conditions in 120-ml penicillin flasks (Poly Labo, Montpellier), filled with the medium described above. Anaerobic conditions were obtained by bubbling cultures with oxygen-nitrogen-free argon (argon N56 Alphagas, $O_2 < 0.5$ ppm, $N_2 < 0.5$ ppm, $CO + CO_2 < 0.1$ ppm). To obtain partial aerobic conditions, we first bubbled the cultures with argon. A known quantity of pure oxygen (oxygen C Alphagas, $N_2 < 5$ ppm) was then added into the sealed bottle, with a syringe, until it reached a concentration of 20 mmol $\cdot L^{-1}$ of gas. This addition of a large quantity of oxygen pressurized the flasks. Since no oxygen was present in the liquid phase (because of sparging with argon), a part of the oxygen gas was transferred to the liquid phase. At the beginning of the experiment, this dissolved oxygen concentration corresponded to oxygen saturation (7.8 mg $\cdot L^{-1}$ at sea level at 35°C). It subsequently dropped owing to the bacterial oxygen consumption, compensated by redistribution between the gaseous and liquid phase. Aerobic cultures were grown in cotton-wool plugs flasks filled with 50 ml of the medium described above, whereas in the two other conditions the final volume was 100 ml. The smaller liquid volume in aerobic culture allowed better gas exchange.

Inhibitors and detergent tests. Two inhibitors of cytochrome oxidases (a and a_3) at two different concentrations were tested by addition to the medium of sodium azide at 0.1 mM and 10 mM, potassium cyanide at 10 and 100 μM. Diethyldithiocarbamic acid (DDC), a copper chelator, was used as an inhibitor of copper type nitrite reductase or other copper proteins of the respiratory chain (azurin) at a final concentration of 10 mM. Erythromycin and chloramphenicol were used at 200 μg $\cdot$ ml^{-1} and 150 μg $\cdot$ ml^{-1} respectively to inhibit protein synthesis. Effect of membrane perturbations on reductases activities was observed based on triton X-100 (0.02%).

Inoculum was grown aerobically in a 15-ml cotton-wool plugs Erlenmeyer flask containing yeast extract (5 g $\cdot L^{-1}$) and peptone (15 g $\cdot L^{-1}$). After overnight growth, this culture was used to inoculate two different precultures: one 500-ml Erlenmeyer flask filled with 200 ml of the medium described above except N-oxides (this culture was named ''nonadapted to N-oxides preculture'') and one Penicillin flask filled with 200 ml of the complete medium and maintained under anaerobic conditions (this culture was named ''adapted to N-oxides preculture''). When the precultures were in the logarithmic growth phase, chloramphenicol was added to stop protein synthesis. Cells were harvested, washed, and concentrated in 0.9% NaCl solution. They were then used to inoculate the different flasks (control and test flasks) in anaerobic, partial aerobic, and fully aerobic conditions to reach a concentration of 35 mg $\cdot L^{-1}$ of proteins. Flasks were shaken in a rotary shaker (200 rpm) at 35°C to maintain a homogeneous culture.

Assays of nitrate and nitrite reductase activities. Nitrate and nitrite reductase activities were assayed on whole cells harvested from three different cultures: one 400-ml aerobic culture in a 1-L Erlenmeyer flask filled with the medium described above except N-oxides; one 400-ml aerobic culture in a 1-L Erlenmeyer flask filled with the medium described above supplied with KNO_3; and one 400-ml anaerobic culture in Penicillin flasks filled with the medium supplied with KNO_3. These three cultures were inoculated (1/200) and were then grown for 16 h. After addition of chloramphenicol (150 μg $\cdot$ ml^{-1}), cells were harvested, washed twice with 0.9% cold NaCl, and suspended in the same solution.

Nitrate and nitrite reductase activities were measured according to the combined methods of Brons and Zehnder [7] and Körner and Zumft [15]. A 4-ml mixture consisting of 2 ml 0.1 M phosphate buffer, pH 7.2; 1 ml 0.1 M KNO_3 or 1.5 ml 0.1 M KNO_2; 0.4 ml 0.5 M sodium acetate and demineralized water was pipetted into 15-ml tubes. The tubes were then flushed with argon and hermetically sealed with rubber stoppers for anaerobic tests. Different inhibitors were added to the reaction mixture: sodium azide to a final concentration of 100 μM and 10 mM, and erythromycin to a final concentration of 200 μg $\cdot$ ml^{-1}. After equilibration at 35°C in a water bath, the reaction was started by injection of the equivalent of 2.4 mg of cell protein per tube. For the next hour, a sample was withdrawn every 20 min. The reaction was stopped by eliminating the cells by centrifugation at 4°C. Nitrate reductase activity was expressed as mmol of nitrate consumed per minute per milligram of proteins. Nitrite reductase activity was expressed as nmol of nitrite consumed per minute per milligram of proteins.

Nitrate reduction was also measured in cell-free extract, obtained from a sonicated aerobic cell suspension, according to the modified procedure described by Krul and Veeningen [16]. The assay mixture consisted of 2.5 ml 0.1 M phosphate buffer, pH 7.2; 1 ml 0.1 M KNO_3; 1.25 ml benzylviologen 0.2 mg $\cdot L^{-1}$; and 0.25 ml of demineralized water. The reaction vials were sparged with argon and sealed with rubber stoppers to keep the anaerobic conditions. Thereafter, 0.5 ml of a mixed solution of 10 mg $\cdot L^{-1}$ $Na_2S_2O_4$ and 10 mg $\cdot L^{-1}$ $NaHCO_3$ (vol/vol) was supplied. After 15 min of shaking in a waterbath at 35°C, the equivalent of 100 mg $\cdot L^{-1}$ of protein extract was injected. A sample was withdrawn every 10 min in a period of 30 min. The reaction was stopped by aerating the samples in order to oxidize the residual electron donor.

Analysis of biomass, medium, and gas. Nitrate and nitrite were measured by an exchange ion chromatography system with conductivity detection (DIONEX-100). Separation and elution of the anions were carried out on an IonPacAS4A Analytical Column with a carbonate-bicarbonate eluant and a sulfuric acid regenerant.

Gas composition was analyzed by gas chromatography with a Shimadzu GC-8A apparatus with argon carrier, by use of a katharometer detector. Carbon dioxide and nitrous oxide were separated on a Haye Sep Q column (80–100 mesh, 2.0 m × 1/8 inch). Oxygen and nitrogen were separated on a molecular sieve 5A (20–100 mesh, 2.0 m × 1/8 inch). Injector and detector temperature was 100°C; column temperature was 35°C. Nitric oxide was measured on a Shimadzu-14A with helium carrier, by use of a katharometer detector. The molecular sieve 5A (80–100 mesh,

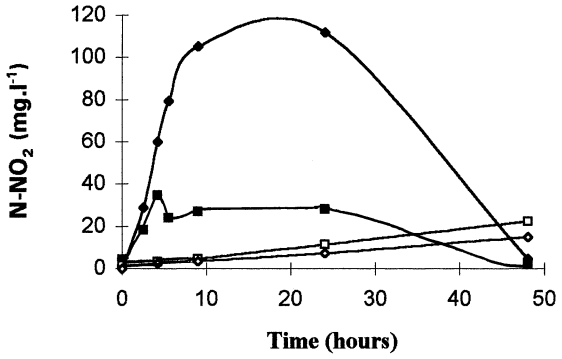

Fig. 1. Influence of chloramphenicol (150 µg · ml^{-1}) on the nitrite production during anaerobic (■, □) and partial aerobic cultures (◆, ◇) on nitrate with *Comamonas* sp. Cultures were inoculated with nonadapted preculture. Chloramphenicol was added at the beginning of the culture. ■, ◆, control cultures; □, ◇, cultures with chloramphenicol.

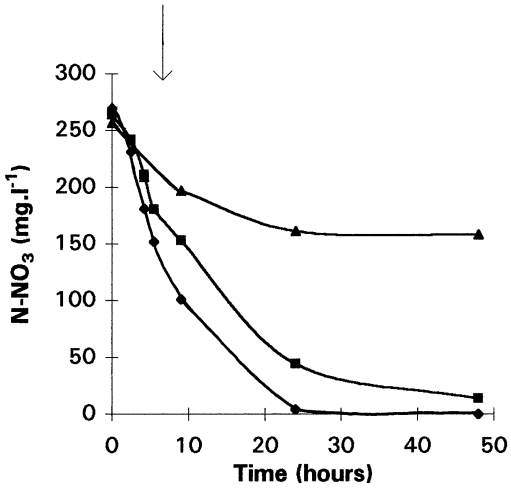

Fig. 2. Consumption of nitrate during anaerobic (■), partial aerobic (◆), and total aerobic (▲) growth conditions of *Comamonas* sp. The arrow indicates the time at which oxygen has completely disappeared in the partial aerobic culture. Cultures were inoculated with nonadapted preculture.

2.0 m × 1/8 inch) column was maintained at 220°C, injector at 150°C, and detector at 105°C.

Proteins were determined by Lowry's procedure with bovine serum albumin as standard.

Results

Existence of a constitutive nitrate reductase. Chloramphenicol (150 µg · ml^{-1}) was used to determine whether or not denitrifying enzymes were synthesized; nitrate reductase activities were measured in aerobic cultures inoculated with cells nonadapted to N-oxides. It was added during cell harvesting by centrifugation (existence of anaerobic conditions in the pellet could enhance synthesis of enzymes) and in the different batch assays. Nitrate reduction was measured by production of nitrite (Fig. 1). Addition of chloramphenicol in the anaerobic and partial aerobic cultures resulted in a sharp decrease of the nitrate reduction rate: 4 µg $N\text{-}NO_2$ · h^{-1} · mg^{-1} of protein against 85.3 µg · h^{-1} · mg^{-1} of protein in anaerobic conditions. However, an immediate production of nitrite was noticed even though the cells used to inoculate the culture were for the first time in the presence of nitrate.

Nitrate reductase activity was also measured on SGLY2 cell extracts obtained from a culture never subjected to nitrate. In this type of culture, an activity of 211 nmol NO_3^- · min^{-1} · mg^{-1} of protein was measured. In the same way, an enzymatic assay on whole cells from aerobic culture without nitrate was realized by adding erythromycin (200 µg · ml^{-1}) and chloramphenicol (150 µg · ml^{-1}) in the test tube. These protein synthesis inhibitors decreased the nitrate reductase activity: the activity, with chloramphenicol, was estimated at 49 nmol NO_3^- min^{-1} · mg^{-1} of protein, whereas 99 nmol were obtained in the control tube. This measurement of residual activity might be owing to the existence of a nitrate reductase in the aerobic culture.

Our experiments showed that a constitutive nitrate reductase is present in an aerobic culture nonadapted to N-oxides. This enzyme could be active under anaerobic as well as aerobic conditions at a small basal rate.

Synthesis and activity of nitrate and nitrite reductases under aerobic condition. The previous experiments showed that a higher nitrate reduction rate is observed when protein synthesis is not inhibited (Fig. 1). In the same way, nitrate consumption began immediately after inoculation of the medium with nonadapted cells, in both aerobic and anaerobic conditions (Fig. 2). During aerobic culture, nitrate consumption slowed down after 20 h because of a lack of carbon source. Nitrate reductase activity resulted from the activity of the constitutive nitrate reductase and of new enzyme synthesis. Thus, it seems that synthesis and activity of nitrate reductase may occur in a wide range of aeration conditions.

The same experiment was realized with nitrite as the final electron acceptor (Fig. 3). Anaerobic nitrite consumption started after a lag period of 1 day. In this case, the use of nitrite is the only way to produce energy. However, according to the literature, no nitrite reductase is synthesized in a nonadapted preculture. This lag phase corresponds to synthesis of a de novo nitrite reductase by using presumably residual energy

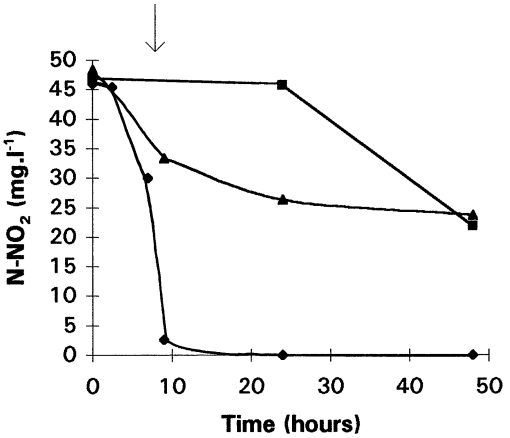

Fig. 3. Consumption of nitrite during anaerobic (■), partial aerobic (◆), and total aerobic cultures (▲) of *Comamonas* sp. The arrow indicates the time at which oxygen has completely disappeared in the partial aerobic culture. Cultures were inoculated with non-adapted preculture.

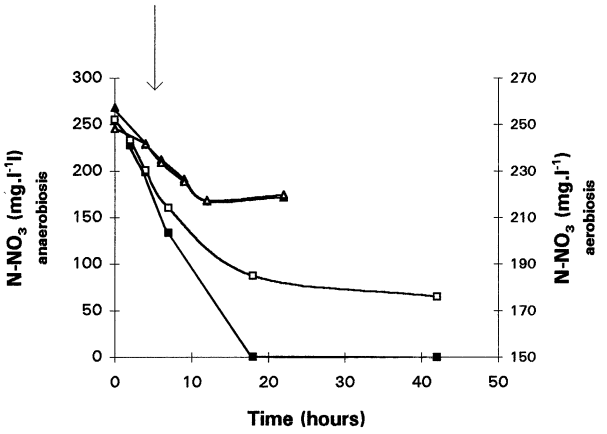

Fig. 4. Influence of triton (0.02%) on the consumption of nitrate during anaerobic (■, □) and total aerobic (▲, △) cultures of *Comamonas* sp. Cultures were inoculated with anaerobic cells. ■, ▲, control cultures; □, △; Cultures with triton; →, addition of triton to the medium.

present in the cells. In contrast, partial or fully aerobic consumption of nitrite started after a smaller lag period (4 h). This consumption was correlated with nitrous oxide and nitrogen production (data not shown). Oxygen consumption provided the energy necessary to synthesize the enzyme. Addition of chloramphenicol resulted in inhibition of nitrite consumption no matter what culture conditions were used. These experiments demonstrated that nitrite reductase, which may not be constitutive, is synthesized under aerobic conditions and that nitrite and oxygen are consumed simultaneously.

Effect of triton, sodium azide, and cyanide on nitrate reductase activity. Figure 4 shows the nitrate consumption during anaerobic and aerobic batch cultures of SGLY2 inoculated with adapted-to-N-oxide cells in the presence of triton. Addition of this detergent to the culture medium resulted in a gentle decrease in the anaerobic nitrate consumption (0.111 mg $N\text{-}NO_3 \cdot h^{-1} \cdot mg^{-1}$ of protein against 0.062 mg $N\text{-}NO_3 \cdot h^{-1} \cdot mg^{-1}$ of protein). It seems, however, to have no effect on aerobic nitrate reduction. The same results are obtained with the nonadapted to N-oxides preculture. The presence of triton X-100 perturbated the permeability properties of the cytoplasmic membrane. This implies that under anaerobic conditions, the measurement of nitrate reductase activity is correlated with the membrane, whereas aerobic nitrate reduction is independent of the membrane.

The effect of azide on nitrate reductase activity during batch assay followed exactly the same pattern observed with triton (Fig. 5). Whatever the aeration conditions and the preculture used, at the highest

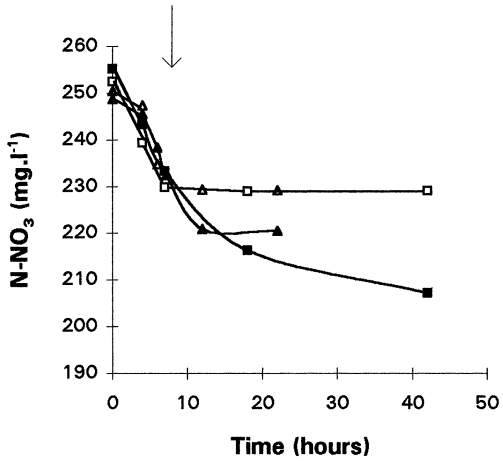

Fig. 5. Influence of sodium azide (0.1 mM) on the consumption of nitrate during anaerobic (■, □) and total aerobic (▲, △) cultures of *Comamonas* sp. Cultures were inoculated with nonadapted to N-oxides cells. ■, ▲, control cultures; □, △, cultures with azide. →, addition of azide to the medium.

concentration tested (10 mM) neither denitrification nor growth was observed. This means that the denitrifying as well as the oxygen-respiring enzymatic system was completely inhibited at this high concentration. In contrast, at 0.1 mM, the nitrate reduction rate of the anaerobic culture, inoculated with the nonadapted cells (Fig. 5), fell to 7 μg $N\text{-}NO_3 \cdot h^{-1} \cdot mg^{-1}$ of protein. This corresponds to a 97% inhibition of the denitrifying enzyme activity measured under control anaerobic conditions. During aerobic culture at 0.1 mM, no effect of azide on oxygen uptake was observed: the disappearance of oxygen in the gaseous phase was correlated with protein synthesis. The only influence

Table 1. Influence of two concentrations (0.1 and 10 mM) of sodium azide on nitrate reductase activity measured on whole cells harvested from aerobic and anaerobic precultures (see Materials and Methods for more details)

Culture conditions	Aerobic preculture			Anaerobic preculture		
Enzymatic assay	Aerobic			Aerobic		
[NaN_3] mM	0	0.1	10	0	0.1	10
Activity	99	47	4.9	155	0	0
%	100	47	4.9	100	0	0

Activity is expressed in nmol of disappeared nitrate per minute per mg of protein.

of this respiratory inhibitor on denitrifying enzymes was then noticed. Compared with the nitrate reduction rate measured under aerobic control conditions, there was a 61% inhibition of the nitrate reductase activity (Fig. 5). The same conclusions were drawn from the measurements of nitrate reductase activity in whole cells (Table 1). The presence of 0.1 mM of azide in the test tube implied a 100% fall of the nitrate reductase activity of anaerobic whole cells, whereas a 53% decrease was observed on nitrate reductase activity of aerobic whole cells. The pattern of inhibition of nitrate reductase activity was similar to the results obtained in batch assays with the highest concentration of azide. Inhibition of 95% and 100% of the nitrate reductase activities was observed in aerobic and anaerobic whole cells respectively. This difference of azide inhibition level on the nitrate reductase activity may suggest that (i) two different nitrate reductases are active according to the aeration conditions or (ii) two different electron donor pathways are used for the same nitrate reductase.

The inhibitory effect of cyanide on nitrate reduction was another aspect contributing to these two previous hypotheses. It was demonstrated that a high concentration of cyanide (100 µM) completely inhibited denitrification under anaerobiosis as well as under aerobiosis (data not shown). Figures 6a and 6b show the evolution of anions during batch culture with and without cyanide at 10 µM (added in the medium at time 0) under anaerobic (a) and partial aerobic (b) conditions. Under anaerobic conditions, nitrate consumption, with or without cyanide, followed the same pattern during 50 h. In contrast, a higher quantity of nitrite was accumulated in the presence of the respiratory inhibitor compared with the control culture. Thus, it seems that a concentration of 10 µM of cyanide has no effect on the nitrate reductase synthesized under anaerobic conditions. However, it partially inhibits the nitrite reductase or an intermediate of the respiratory

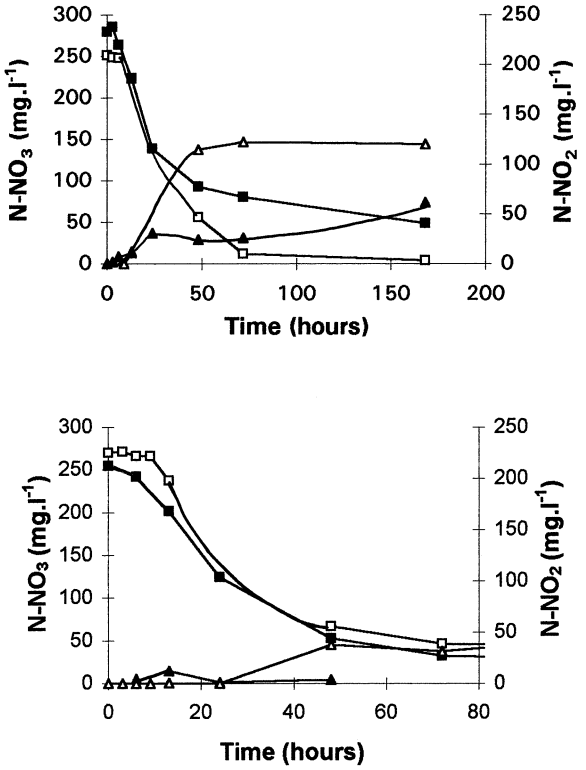

Fig. 6. Evolution of nitrate (■, □) and nitrite (▲, △) during anaerobic (A) and partial aerobic (B) cultures of *Comamonas* sp. in the presence of cyanide (□, △) at a final concentration of 10 µM. Cultures were inoculated with nonadapted to N-oxides cells.

chain between nitrate reductase and nitrite reductase. In turn, in the partial aerobic culture with cyanide, nitrates were not consumed during the period of oxygen consumption correlated with proteins synthesis (11 h). On the contrary, the concentration of nitrate sharply decreased without a lag phase in the control culture. Despite this phenomenon, after complete disappearance of oxygen in the test culture, the nitrate consumption was similar to that observed under anaerobic conditions. During fully aerobic conditions, at 10 µM, denitrification was not noticed, whereas the growth rate was similar to that obtained in the control fully aerobic culture, implying no inhibition of oxidases. This suggests that 10 µM of cyanide directly inhibits the aerobic denitrifying enzymes. Thus, cyanide influences denitrification under aerobic conditions by totally inhibiting the nitrate reduction, whereas under anaerobic conditions, no effect was observed on nitrate reduction.

Effect of triton X-100, azide, and DDC on nitrite reductase activity. Figure 4 shows that triton partially inhibited anaerobic nitrate consumption because of its effect on membrane disorganization. In the same time,

no nitrite was accumulated in the medium, whereas in the control culture the nitrite concentration increased sharply to reach a peak of 80 mg $N\text{-}NO_2 \cdot L^{-1}$. By disturbing the permeability of the cytoplasmic membrane, triton could act on the antiport system nitrate/nitrite by preventing the nitrate from joining the active site of the enzyme and by holding the nitrite in the cytoplasm. On the contrary, triton, when added in batch culture with nitrite as final electron acceptor, had no direct effect on nitrite reduction whatever the culture and preculture conditions used. This may be owing to periplasmic localization of the nitrite reductase.

Azide and DDC were chosen to show the possible existence of a copper nitrite reductase or copper intermediates as pseudoazurin in the respiratory chain: the former reacts with ferric centers, the latter links to copper centers. Nitrite reduction seems to be insensitive to respiratory inhibitors during anaerobic batch assay with nitrate as the final electron acceptor: addition of azide or DDC resulted on one hand in total inhibition of nitrate reductase activity (Fig. 5) and, on the other hand, in a complete disappearance of nitrite accumulated during the previous hours. In the same way, nitrate reductase activity measured by enzymatic assay on whole cells is expressed as the quantity of nitrate disappearing because no nitrite accumulated during the test. In the presence of the different inhibitors, batch assays with nitrite as the final electron acceptor ended up with the same conclusion: whatever the culture and preculture conditions, azide and DDC had no effect on nitrite reductase activity.

Discussion

Previous work had generally underlined that in many bacteria, synthesis and activity of denitrifying enzymes could not occur under aerobiosis. Our work leads to a modified conclusion, and the results allow us to propose a scheme to explain the nitrate-oxygen co-respiration in *Comamonas* sp. strain SGLY2. The first experiments done with the strain demonstrated its ability to use simultaneously the two electron acceptors [22]. Aeration of the culture resulted in a decrease in the nitrate reduction rate: 1.85 µmol $NO_3^- \cdot min^{-1} \cdot mg^{-1}$ of protein under anaerobic culture against 0.287 in aerated culture. These values lie close to that found with *Thiosphaera pantotropha*, in which the rate of acetate-dependent nitrate reduction is around 1.6 µmol $NO_3^- \cdot min^{-1} \cdot mg^{-1}$ of protein at dissolved oxygen concentration less than 30%, and 0.8 at 30–80% of air saturation [29]. In contrast, at a concentration of 0.25% of air saturation, no denitrification was observed with *Pseudomonas aeruginosa*, a classical oxygen-sensitive denitrifier [12]. With *Comamonas* sp., the decrease in nitrate reduction rate was correlated with a decrease in nitrogen gas production and partial nitrite, nitric oxide, and nitrous oxide accumulation. However, the presence of these denitrifying intermediates during continuous culture, at a dissolved oxygen concentration of 100% of air saturation (data not shown), implies that the four denitrifying enzymes are active and synthesized under aerobic conditions. The same conclusions are drawn, since higher aerobic nitrate–nitrite reduction rates were observed in culture without chloramphenicol compared with that obtained with the protein synthesis inhibitor (Figs. 1–3).

These aerobic nitrate and nitrite reductase activities could not be interpreted in terms of assimilation because (i) $(NH_4)_2SO_4$, used as the nitrogen source, classically inhibits nitrate assimilation, (ii) ammonium disappearance was well correlated with biomass production, and (iii) during continuous culture under oxygen-saturated conditions, nitrogen was produced, which is the direct demonstration of denitrification reaction. Van Niel and associates [38], using nitrate labeled on nitrogen, have confirmed the idea of Robertson and Kuenen [27] that a complete denitrifying system is present under aerobic conditions in *T. pantotropha*. According to the experiments done with chloramphenicol on cells never subjected to nitrate, the existence of a constitutive nitrate reductase was proposed to explain the possible aerobic denitrification in *Comamonas* sp. An opposite conclusion resulted from the same experiments made with *Paracoccus denitrificans* (NCIB 8944): nitrite, nitric oxide, nitrous oxide, and nitrogen gas were not produced during anaerobic and partial aerobic cultures with chloramphenicol. Without the protein inhibitor, anaerobic denitrification started with a long lag period of 11 h, corresponding to the synthesis of a de novo nitrate reductase (data not shown).

The close relation between the denitrifying enzymes and the electron transport pathway, the different kinds of nitrate transport, the genetic and the regulatory system of synthesis, and the activity of the enzymes are now well studied [11]. These enzymes are shown to work in vitro in the presence of oxygen [1, 3, 24]. However, in vivo, other aspects have to be considered to explain the possible aerobic denitrification. From a bioenergetic point of view, the idea of co-respiration seems illogical, because energy production is higher with oxygen than nitrate and because the main regulatory factor of denitrification is the redox potential of the respiratory chain [17]. For example, the presence of oxygen in a *P. denitrificans* culture

implies preferential diversion of electrons to oxygen owing to the modification of the redox potential of the coupler ubiquinol/ubiquinone [9, 10]. Hernandez and colleagues [13] have also shown that, using *Pseudomonas aeruginosa*, oxygen indirectly inhibits enzyme activities by oxidizing the key molecules of the antiport nitrate-nitrite system. Moreover, it is known that oxygen regulates nitrate respiration by suppressing enzyme synthesis: a FnR-like binding site, required for anaerobic gene expression in *Escherichia coli*, is present in cells of *P. denitrificans* [11, 33] and *Pseudomonas stutzeri* [8]. Bell et al. [2] have reported that *T. pantotropha* uses a periplasmic nitrate reductase while denitrifying aerobically and employs another membrane-bound reductase for anaerobic denitrification. Thus, the oxygen inhibitory effect on nitrate-nitrite antiport system is evaded. The diversion of the electron flow to the denitrifying enzymes was explained by the hypothesis of the ''bottleneck'' [26]. Using sodium azide as respiratory inhibitor, Van Niel and coworkers [38] showed that nitrogen gas production stopped immediately after the addition of 10 mM azide to the aerobic cell suspension. Conversely, 0.02 mM azide is just enough to inhibit the nitrate reductase synthesized under anaerobic conditions [23]. In the same way, the different levels of triton and azide inhibition between aerobic and anaerobic cells suggest the existence of two nitrate reductases in SGLY2: one ''aerobic'' enzyme insensitive to membrane damage caused by triton and less sensitive to azide, and one ''anaerobic'' enzyme partially inhibited by 0.02% of triton and completely inhibited at 0.1 mM of azide. Cyanide effect is another factor contributing to the hypothesis of existence of two nitrate reductases. The hypothesis of existence of two electron donor pathways for one nitrate reductase was rejected after comparison of the azide effect on nitrate and nitrite reduction under anaerobic conditions. Since azide had no effect on nitrite reductase activity, whereas it completely inhibited nitrate reductase activity, we can conclude that the nitrate reduction inhibition is a direct effect on the enzyme and not a consequence of inhibition of an intermediate of the respiratory chain. Moreover, one enzyme could not be differently inhibited by the same inhibitor. This is why existence of two nitrate reductases was proposed.

No aerobic denitrification can occur with the existence of a copper-type nitrite reductase in the denitrifying enzymatic system because of its properties to reduce oxygen to toxic peroxides. Moir and associates [21] have purified a *cdl*-type nitrite reductase from *T. pantotropha* and its presumably electron donor pseudoazurin. With *Comamonas* sp., no inhibitory effect of DDC was noticed on both anaerobic and aerobic nitrite reductase activities. On the other hand, a large amount of nitrous oxide was accumulated in the gaseous phase, perhaps owing to the blocking of a multi-copper nitrous oxide reductase. Moreover, no nitrite accumulation was observed during aerobic culture on nitrate with azide. These observations suggest the existence of a *cdl*-type nitrite reductase. According to the experiments done with and without protein inhibitor, this inducible enzyme seems to be active and synthesized under aerobic conditions. At this point, the behavior of the strain differs from that of *T. pantotropha*: using polyclonal antibodies, Moir [20] has shown that the *cdl*-type nitrite reductase was not expressed under aerobic conditions. The status of aerobic denitrifier of *T. pantotropha* is then not clear. In fact, it has been shown that there is a close relationship between *T. pantotropha* and *Paracoccus denitrificans* [19, 35]. Moreover, although a periplasmic nitrate reductase seems to be synthesized in *Paracoccus denitrificans* [32], no aerobic denitrification was noticed in this strain. Kuenen and Robertson [18], in their last experiments, observed that the aerobic denitrification rate of *T. pantotropha* is now equivalent to 5% of that found under anaerobic conditions versus 50% at the beginning of their experiments in 1989.

Physiological observations on *Comamonas* sp. strain SGLY2, using different respiratory or protein synthesis inhibitors and detergent, explain the ability of the strain to denitrify under aerobic conditions. From an ecological point of view, existence of this kind of aerobic denitrifier is interesting to explain nitrogen losses of agronomic system. It has thus to be considered in agricultural practices, especially for the mode of application of nitrogen fertilizers.

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