

Expression of Denitrification Enzymes in Response to the Dissolved Oxygen Level and Respiratory Substrate in Continuous Culture of *Pseudomonas stutzeri*

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The onset and cessation of the synthesis of denitrification enzymes of *Pseudomonas stutzeri* were investigated by using continuous culture and defined dissolved oxygen levels covering the full range of transition from air saturation to complete anaerobiosis. Expression of nitrate reductase, nitrite reductase (cytochrome *cd₁*), and N_2O reductase was controlled by discrete oxygen levels and by the nature of the nitrogenous oxide available for respiration. N_2O reductase was synthesized constitutively at a low level; for enhanced expression, oxygen concentrations were required to decrease below 5 mg of O_2 per liter. The threshold values for synthesis of nitrate reductase and cytochrome *cd₁* in the presence of nitrate were ca. 5 and ca. 2.5 mg of O_2 per liter, respectively. With nitrous oxide as the respiratory substrate, nitrite reductase was again the most sensitive to oxygen concentration; however, thresholds for all denitrification enzymes shifted to lower oxygen levels. Whereas the presence of nitrate resulted in maximum expression and nearly uniform induction of all reductases, nitrite and nitrous oxide stimulated preferably the respective enzyme catalyzing reduction. In the absence of a nitrogenous oxide, anaerobiosis did not induce enzyme synthesis to any significant degree. The accumulation of nitrite seen during both the aerobic-anaerobic and anaerobic-aerobic transition phases was caused by the differences in onset or cessation of synthesis of nitrate and nitrite reductases and an inhibitory effect of nitrate on nitrite reduction.

Denitrification represents one important system of anaerobic enzyme expression. A controversy as to whether denitrification can proceed under aerobic conditions, and to what extent (reviewed in references 28 and 32), has given way to the acceptance of denitrification in the presence of oxygen in certain species (20, 25, 26, 33). The range of oxygen levels for denitrification to occur spans 90% air saturation for *Thiosphaera pantotropha* (33) to 53% for *Alcaligenes* sp. (18) to practically completely anaerobic conditions for *Paracoccus denitrificans* (1, 13), although aerobic denitrification has also been reported for the latter organism (20). *Aquaspirillum magnetotacticum* respire oxygen below 1.8% air saturation while denitrification proceeds (3), and *T. pantotropha* is even able to simultaneously respire oxygen, denitrify, and nitrify, the latter two metabolic pathways reaching maximum activity at 25% air saturation (34).

Information extending beyond the knowledge of an oxygen dependence of the overall process is sparse; the influence of oxygen on partial reactions of the denitrification system has not yet been determined, and little is known about the regulatory interdependence of the reductases involved. Such effects are reflected by the finding that oxygen exhibits competitive behavior toward nitrate reduction (reviewed in reference 6) and in accounts of the accumulation of intermediary products. For instance, *Paracoccus halodentrificans* showed a progressive shift from dinitrogen toward nitrous oxide and nitrite as the oxygen concentration was increased (11). Nearly quantitative accumulation of nitric oxide or nitrous oxide occurred under oxygen limitation of otherwise dinitrogen-evolving denitrifiers (8). Besides oxygen, nitrate and intermediates of denitrification influence the activity of the overall process as well as the individual reductases. The presence of nitrate or

nitrite results in the induction of nitrate reductase (NaR) (4) and nitrite reductase (NiR) (38) in *Paracoccus denitrificans*. N_2O reductase (N_2OR) activity of *Pseudomonas perfectomarina* is induced by nitrate, nitrite, or nitrous oxide (30).

Considerable progress in characterizing denitrification enzymes has been made (12). Purification of the NaR (17), cytochrome *cd₁* (41), and N_2OR (5) from one organism and the production of antisera against them has now permitted analysis of individual enzyme proteins during phase shifts from aerobiosis to anaerobiosis and vice versa. Under conditions of defined oxygen levels in steady-state cultures, this approach has provided the first determinations of p_{O_2} threshold values for the synthesis of individual reductases and their modes of expression in this organism.

MATERIALS AND METHODS

Organism and culture media. *Pseudomonas stutzeri* Zo-Bell (formerly *P. perfectomarina* ATCC 14405) was grown in a synthetic medium (24) with modified concentrations of $CaCl_2 \cdot 2H_2O$ (0.1 g/liter), $MgSO_4 \cdot 7H_2O$ (0.02 g/liter), and NaCl (1 g/liter). Ammonium sulfate was added at 0.5 g/liter. Where required, the medium was supplemented with $NaNO_3$ or $NaNO_2$ at 0.5 g/liter or was sparged with N_2O . Media were adjusted to pH 6.9. Chemicals were of analytical grade and purchased from E. G. Merck AG, Darmstadt, Federal Republic of Germany.

Chemostat culture. Cells were grown in continuous culture in a glass vessel of 500-ml culture volume with stirring (500 rpm), overflow level regulation, and external temperature control at 30°C. The magnesium-limited culture medium was inoculated with 25 ml of an aerobic late-exponential-phase preculture (approximately 2×10^9 cells per ml). The latter contained no nitrogenous oxide and was itself inoculated from an overnight agar slant culture. Nutrient flow was maintained at a dilution rate of $0.12 h^{-1}$, equivalent to three

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replacement times between 24-h sampling periods, this being a minimum requirement to ensure equilibrium in the chemostat after each change in aeration (31). Stability was monitored by periodically measuring cell density as turbidity at 660 nm.

Aeration and sparging with N_2O were done by an electronic gas-blending and control system consisting of three separate mass flow meters and electromagnetically regulated, proportioning control valves for oxygen, nitrogen, and nitrous oxide (MKS Systems, Andover, Mass.). The gas mixture was filter sterilized by a PTFE filter (0.2- μ m pore size; 50 mm; Millipore Corp., Bedford, Mass.) and entered the culture medium via a fine-pored ceramic frit in the immediate vicinity of the impeller. Gases (Messer-Griesheim, Frankfurt, Federal Republic of Germany) were of purity grade 4.6 and individually filtered through 0.2- μ m stainless-steel frits.

Dissolved oxygen concentrations in the culture were measured by means of a polarographic-type electrode (WTW, Munich, Federal Republic of Germany). The electrode had an additional close-mounted stirring device that permitted a constant liquid stream over the surface of the Teflon diaphragm, thus stabilizing the liquid-film diffusion resistance (10) and preventing adhesion of gas bubbles.

Sampling technique. Aseptic sampling of the chemostat culture was done by sterile disposable syringes via a permanently mounted Luer connection port protected by a glass bell cover. Samples of 25 ml were shock cooled to 0°C, and cells were harvested by pelleting at $10,000 \times g$ in a refrigerated centrifuge. Precautionary addition of chloramphenicol and streptomycin to the sample was not found superior to rapid cooling and maintenance of the temperature below 4°C throughout the preparation procedure.

Preparation of cell extracts. Cells were washed once by centrifugation in 25 ml of 20 mM Tris hydrochloride (pH 7.2) and suspended in 1 ml of the same buffer. A crude extract was obtained by two passages through a cold 3-ml French pressure cell at 136 MPa. The homogenate was subjected to ultracentrifugation at $180,000 \times g$ for 2 h at 4°C. The supernatant was concentrated to about 100 μ l in Centricon microconcentrators (exclusion size, 10 kilodaltons). The membrane pellet was suspended in 100 μ l of Tris buffer. Both fractions were frozen at -24°C for later immunochemical analysis.

Enzyme activity assays. Harvested cells were washed once in 60 ml of phosphate buffer (pH 7.2) and suspended in the same buffer. Whole-cell activity assays for reduction of nitrate, nitrite, and nitrous oxide were done by a combination of high-performance liquid chromatography (for quantitation of nitrite) and gas chromatography (for analysis of gaseous products). Gastight, stoppered reaction vials of 8.3-ml volume contained 1.8 ml of cell suspension (at approximately 1 mg of cell protein) in 60 mM phosphate buffer (pH 7.2)—0.2 ml of lactate (0.5 M, pH 7.2). The vials were chilled on ice and then repeatedly evacuated and filled with helium on a vacuum line. After equilibration at 30°C in a shaking water bath at 120 strokes per min, reactions were started by injection of 25 μ mol of $NaNO_3$, 25 μ mol of $NaNO_2$, or 500 μ l of N_2O .

Nitrate and nitrite were determined by high-performance liquid chromatography on a model 114M isocratic system (Beckman Instruments, Inc., Fullerton, Calif.), using a column (5 by 300 mm) filled with a mixture of diatomite (Merck) and ECTEOLA cellulose (Serva, Heidelberg, Federal Republic of Germany) (9). Sample preparation consisted of pelleting of cells from the reaction mixture at $15,000 \times g$,

extraction of the supernatant with the same volume of acetonitrile, and subsequent centrifugation at $15,000 \times g$. After injection of a 50- μ l sample, the chromatogram was developed within 8 min at a flow of 0.5 ml/min and a pressure of approximately 1.8 MPa. For quantitation of nitrite in the culture medium, colorimetric determination by diazotiation with *N*-1-naphthylamino-7-sulfonic acid after coupling with sulfanilic acid was used (39).

Dinitrogen and nitrous oxide were quantitated by gas chromatography on a switched two-column system (7) consisting of activated alumina and molecular sieve columns in a modified Shimadzu 3BT chromatograph with thermal conductivity detector and a Spectra-Physics SP 4280 integrator. Protein was measured by the method of Lowry et al. (21), using bovine serum albumin (Serva) as the standard. For protein determination of whole cells, digestion with 1 N KOH preceded the assay. Membrane protein was determined by using a reagent containing 1% sodium dodecyl sulfate (22).

Immunochemical methods. Antibodies against electrophoretically homogeneous preparations of NaR, cytochrome cd_1 , and N_2OR from *P. stutzeri* were raised in New Zealand White rabbits essentially as described previously (40). Conditions for preparation of cytochrome cd_1 (16) and N_2OR (5) have been described. NaR was prepared from the membrane fraction of *P. stutzeri* by heat solubilization and subsequent chromatography on ion-exchange resin and gel filtration. Electrophoretically homogeneous material of a complex consisting of the α and β subunits was used for immunization (S. Blümle, H. Körner, and W. G. Zumft, unpublished data).

Cytochrome cd_1 and N_2OR proteins in cell extracts were determined by immunoelectrophoresis (19) as previously described (40). For electroimmunoassay of NaR, both the sample and the agarose gel contained 1% Triton X-100. Electrophoresis was performed at low field (3 V/cm) overnight. Before application, the samples were adjusted to 3 mg of protein per ml for quantitation of NaR and to 5 mg of protein per ml for quantitation of NiR and N_2OR .

RESULTS

Enzyme levels at aerobic versus anaerobic conditions. In a comparison of the concentrations of the denitrification enzymes observed at full aerobiosis (7.55 mg of O_2 per liter) with those seen under conditions of complete anaerobiosis and an intermediary stage of dissolved oxygen concentration (1.28 mg of O_2 per liter), the complementary effects of the oxygen level and the presence of nitrogenous oxides as respiratory substrates became evident (Fig. 1). Under conditions of unlimited oxygen supply, cells synthesized neither NaR nor NiR (cytochrome cd_1) but contained distinct, though small, amounts of N_2OR regardless of the presence of a nitrogenous oxide. Anaerobiosis by itself caused no appreciable expression of NaR and NiR and only a slight increase in N_2OR (Fig. 1A).

With any of the nitrogenous oxides (i.e., nitrate, nitrite, or nitrous oxide) available to the cells, oxygen depletion considerably stimulated expression of the denitrification enzymes, though to greatly different extents. The highest concentrations of NaR, NiR, and N_2OR were found with nitrate as the effector (Fig. 1B). Nitrite selectively stimulated NiR expression, whereas NaR expression remained comparatively low (Fig. 1C). In turn, nitrous oxide as the respiratory substrate did not stimulate synthesis of NiR but caused an increase in the levels of NaR and N_2OR (Fig. 1D). The response of the denitrification enzymes toward nitrite and

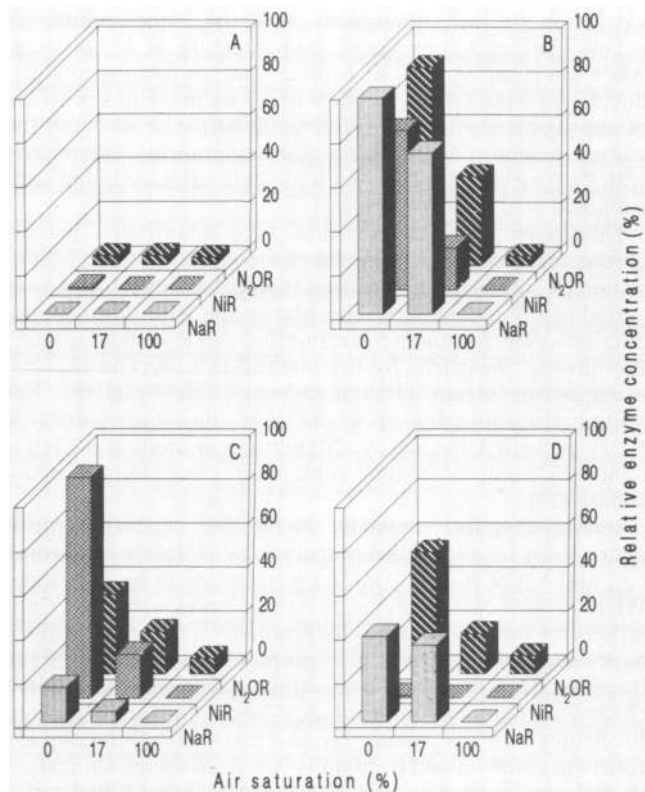


FIG. 1. Relative concentrations of denitrifying reductases of *P. stutzeri* in continuous culture at oxygen levels of 100, 17, and 0% air saturation. The maximum enzyme concentration of each reductase at any oxygen level and any nitrous oxide substrate was set at 100%. (A) Conditions without a nitrogenous oxide as respiratory substrate; (B through D) inducing effects of nitrate, nitrite, and nitrous oxide, respectively.

N_2O suggests that particularly signals controlling NiR synthesis are not, or are not fully, coupled to those of NaR and N_2OR expression.

Transition from aerobiosis to anaerobiosis. The experimental design used was chosen to yield threshold values of oxygen concentration for the onset and cessation of synthesis of the denitrification enzymes in continuous culture at gradually reduced or increased aeration. In preliminary experiments, it was determined that nutrient limitation by magnesium resulted in the most stable conditions. More commonly used limiting nutrients, such as the carbon source or phosphate, resulted in fluctuations or precipitations or, in case of N limitation, were incompatible with the questions to be answered. The minimum period between sampling was determined to be 24 h, to allow equilibrium after each small change in aeration. Removal of 25-ml samples was found not to disturb the system to any detectable degree. Each experiment included a downshift and an upshift portion in order to obtain a complete cycle of transition from oxygen saturation to anaerobiosis and back to full aerobiosis. In this way, possible lags in the response of the continuous culture system would become evident in an asymmetry of the data. The protocol of changes in aeration during the course of a complete experimental cycle is shown in Fig. 2.

Patterns of expression of NaR, NiR, and N_2OR were analyzed by immunoelectrophoresis of cell extracts (Fig. 3). In the presence of nitrate as the respiratory substrate, the maximum level of NaR coincided with fully anaerobic con-

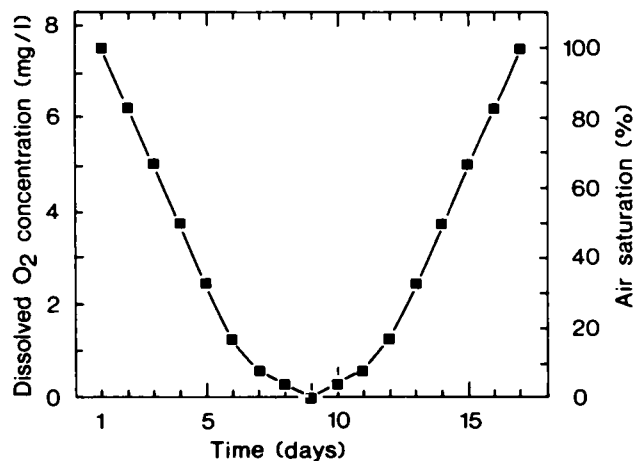


FIG. 2. Protocol of aeration changes during transition experiments from full aerobiosis to anaerobiosis to full aerobiosis.

ditions. NiR and N_2OR reached maximum expression at dissolved oxygen concentrations of about 0.6 mg of O_2 per liter. Figure 4 shows representative sets of original data for the quantitation of NaR and N_2OR by electroimmunoassay during a transition cycle. In this case, NaR showed maximum expression before full anaerobiosis was reached, whereas N_2OR (and also NiR; data not shown) displayed the pattern seen with NaR (Fig. 3). Possible reasons for the occurrence of two different patterns are discussed below.

Cultures supplied with N_2O as the respiratory substrate showed maximum concentrations for NiR and N_2OR at fully anaerobic conditions and an apparently biphasic response for NaR during the transition cycle (Fig. 5A). This pattern was also reflected in the whole-cell activity determined for the three reductases (Fig. 5B). The symmetry in both diagrams indicated that the system was stabilized at each sampling time. Slight skewing was observed only on transition from full anaerobiosis, when a lag in response to very

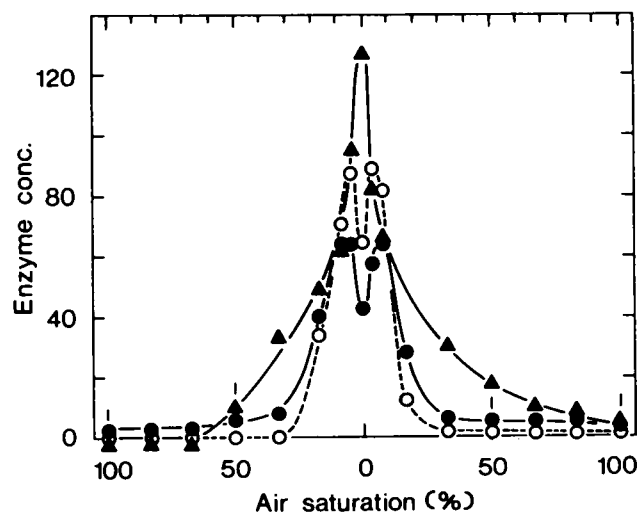


FIG. 3. Denitrification enzyme concentrations (micrograms per milligram of cell protein) in continuous culture on transition from aerobiosis (100% air saturation) via anaerobiosis to full aerobiosis. The respiratory substrate was nitrate. Symbols: \blacktriangle , NaR; \circ , NiR; \bullet , N_2OR .

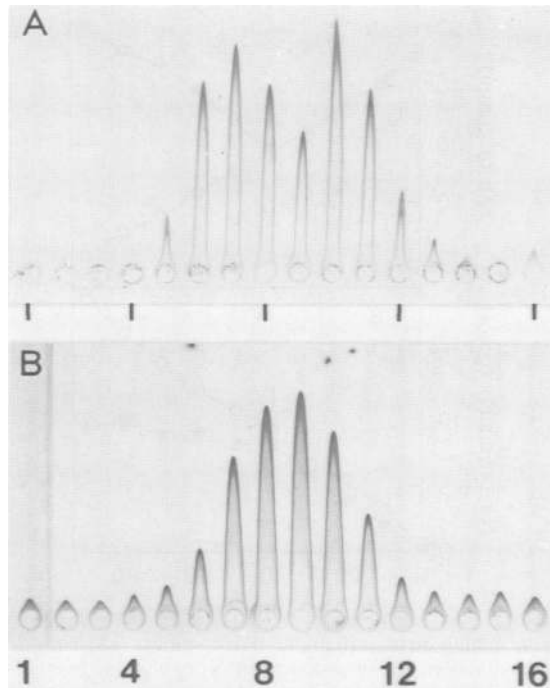


FIG. 4. Laurell immunoelectrophoresis of chemostat samples taken at various aeration levels on transition from aerobiosis (100% air saturation) to anaerobiosis to aerobiosis. Rocket height reflects relative enzyme concentration. The respiratory substrate was nitrate. (A) NaR; (B) N₂OR. Lanes 1 through 16 represent 100, 83, 67, 50, 33, 17, 8, 4, 0, 8, 17, 33, 50, 67, 83, and 100% air saturation. The rightmost sample of panel A shows some immunoreactive material caused by an accidental breakdown in aeration.

low concentrations of oxygen was discernible. At oxygen concentrations of 0.3 mg of O₂ per liter, the culture system adjusted to a different equilibrium, as evidenced by a reduced growth yield. As previously suggested for related findings (15), this adjustment reflects the lower energy yield in the complete absence of oxygen as the terminal electron acceptor and a concurrent replacement of magnesium by nitrate as the growth-limiting nutrient.

On occasion, oscillations in the concentrations and activities of all three reductases were observed; these began when oxygen levels were reduced below 0.6 mg of O₂ per liter and continued with diminishing amplitude and increasing period through the remainder of the transition cycle to full aerobiosis. Such oscillations occurred in continuous-culture systems under conditions of two or more limiting nutrient factors and are interpreted as a changeover in the nature of the limiting system component.

Critical oxygen levels for the onset or cessation of enzyme synthesis differed substantially for the three reductases and varied depending on the supplied N oxide (Table 1). N₂OR was expressed in fully aerobic cells irrespective of the N-oxide substrate at levels of approximately 5 μg/mg of soluble cell protein. The enzyme level began to markedly increase at dissolved oxygen concentrations of about 4 mg/liter with both nitrate and N₂O as respiratory substrates. NaR became detectable by immunochemical methods below O₂ levels of about 5 mg/liter with nitrate as the effector and about 4 mg/liter with nitrous oxide; NiR required the lowest levels of O₂ before becoming discernible by immunoelectrophoresis: below 2.5 mg/liter with nitrate and 0.6 mg/liter with nitrous oxide.

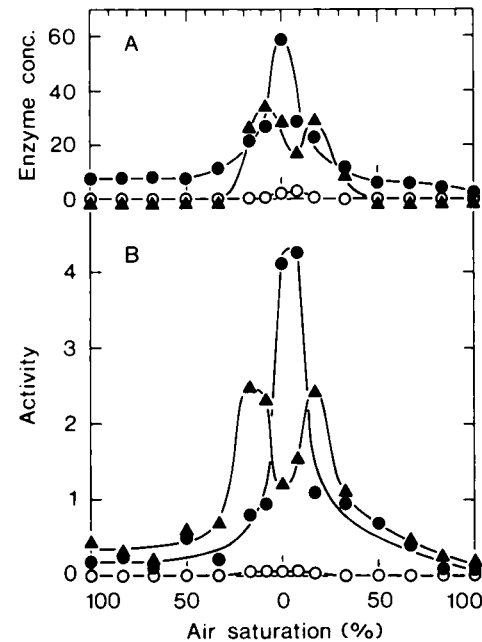


FIG. 5. Denitrification enzyme concentrations (micrograms per milligram of cell protein) (A) and activities (nanokatal per milligram of cell protein) (B) in continuous culture on transition from aerobiosis (100% air saturation) via anaerobiosis to full aerobiosis. The respiratory substrate was nitrous oxide. Symbols: ▲, NaR; ○, NiR; ●, N₂OR.

The enzymes differed only slightly in the oxygen values for half-maximum expression (Table 1), but the range of values for NiR tended to remain at lower O₂ concentrations. Data for NiR with N₂O as the respiratory substrate are less accurate because of the very low levels of enzyme observed (Fig. 5A). NiR activity was barely measurable under these conditions (Fig. 5B). N₂OR activity was high in the presence of nitrous oxide, although the absolute amount of the enzyme was lower than in cells grown on nitrate; this result confirms our previous findings on batch cultures of *P. stutzeri* (40) and indicates a peculiar mode of enzyme regulation in response to exogenous N₂O.

Accumulation of nitrite and inhibition of nitrite reduction by nitrate. At reduced levels of oxygen, nitrite accumulated in the culture medium during the critical transition phase for NaR and NiR synthesis. The effect was most pronounced at the threshold oxygen concentration for the onset or cessa-

TABLE 1. Critical dissolved oxygen concentrations for the expression of denitrification enzymes of *P. stutzeri*, determined by electroimmunoassays of crude cell extracts

Determination	Anaerobic respiration substrate	Oxygen concn (mg of O ₂ /liter) for expression of:		
		NaR	NiR	N ₂ OR
Threshold value	NO ₃	≤5.1	≤2.5	≤3.8 ^a
	N ₂ O	≤3.8	≤0.6	≤3.8 ^a
Value for half-maximal enzyme expression	NO ₃	0.8–1.6	0.7–1.2	1.0–1.6
	N ₂ O	1.6–1.8	— ^b	0.4–0.5

^a Value for start of increase from constitutive level.

^b —. No discrete value.

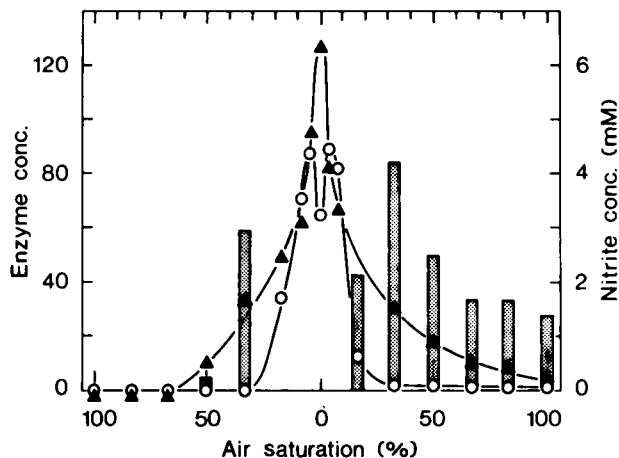


FIG. 6. Assay of nitrite concentrations (bars) in continuous culture of *P. stutzeri* on transition from aerobiosis (100% air saturation) to anaerobiosis to aerobiosis. The medium contained 0.5 g of nitrate per liter as the respiratory substrate. Curves represent concentrations (in micrograms per milligram of cell protein) of NaR (▲) and NiR (○).

tion of NiR expression but was absent when significant levels of NiR were present (Fig. 6).

Nitrate was found to inhibit nitrite reduction *in vivo*. Denitrification of nitrite by anaerobic, nitrate-grown cells ceased immediately upon addition of nitrate, evidenced by the termination of N_2 evolution from the denitrifying cells (Fig. 7); no other gaseous species was detected. This finding identified the reduction of nitrite as the sensitive step. On addition of nitrate, the level of nitrite started to increase because of the presence of NaR and lack of further reduction (Fig. 7A). This effect rendered oxygen transition studies in steady-state cultures of *P. stutzeri* with more than 6 mM nitrate as the respiratory substrate impractical, as the amount of nitrite accumulated and maintained at this level by the continuous feed of medium did not permit denitrification to proceed. The toxicity of nitrite eventually damaged the cell population and caused washout.

DISCUSSION

Previous work (reviewed in reference 27) generally ascribed the decisive effect on the synthesis of denitrification enzymes more to reduced oxygen tension than to alternative electron acceptors. Our work leads to a modified conclusion. Anaerobiosis was not sufficient for induction or appreciable stimulation of denitrification enzyme synthesis; the complementary presence of a nitrogenous oxide was a prerequisite. In turn, availability of an N oxide had to be accompanied by anaerobiosis to promote enzyme synthesis. Aerobically grown cells of *P. stutzeri* definitely contained small amounts of N_2OR , whereas NaR and NiR (cytochrome cd_1) were not detected. The observation of low N_2O reduction in aerobically grown cells of this organism (30) is in accordance with our findings.

Nitrate had the broadest effect in that it (possibly via intermediary nitrous oxides derived from its reduction) caused expression of all three reductases to maximum levels. In contrast, the other nitrogenous oxides stimulated mainly those reductases acting on the particular substrate or subsequent reductases but had lesser effects on an enzyme acting on a substrate preceding the N oxide under consider-

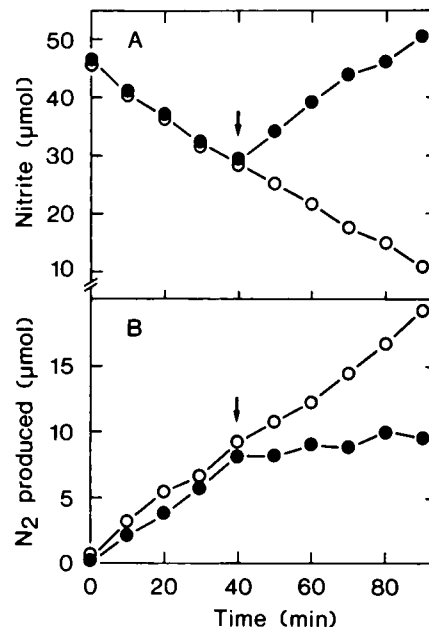


FIG. 7. Inhibition of nitrite reduction by nitrate. (A) Time course of nitrite reduction; (B) time course of N_2 evolution. Symbols: ●, sample; ○, control without nitrate addition. Assays followed the procedure described for gas chromatographic quantitation (see Materials and Methods). Reaction vials contained a suspension of anaerobically grown (respiratory substrate, nitrate), washed cells equivalent to 2 mg of cell protein; starting amount of nitrite was 25 mM; after 40 min (arrow), nitrate was added to bring the assay to 25 mM.

ation. For cells grown on nitrous oxide, this had been observed in previous bulk activity measurements (23, 30, 40). The response of N_2OR is peculiar in that exogenously provided N_2O leads to a lower level of this enzyme in *P. stutzeri* (this work; 40). For *Pseudomonas aeruginosa*, loss of N_2O reducing activity was reported when cells were grown solely on nitrous oxide (36).

Critical oxygen levels have been reported only for the onset of overall denitrification activity. This study considered enzyme expression in terms of individual reductase concentrations in reference to total cell protein. Oxygen thresholds were determined for the synthesis of NaR and NiR and for elevated synthesis from the constitutive level of N_2OR (Table 1). With oxygen present in a subsaturating concentration, the enzymes varied considerably both in the onset (or cessation) of their synthesis and in their absolute levels of expression. If N_2O instead of nitrate was present as the respiratory substrate, oxygen thresholds for enzyme synthesis were shifted to lower values.

Unexpectedly, maximum expression of the reductases did not always coincide with fully anaerobic conditions. Two different patterns were noted in the transition from aerobiosis to anaerobiosis. These showed either maximum enzyme levels at zero oxygen or maximum enzyme synthesis at levels of around 0.6 mg of O_2 per liter, with a decrease at fully anaerobic conditions. Symmetry of this behavior in a complete transition cycle supports the validity of this observation. *In vivo* activity measurements for the individual reductases paralleled this dual pattern. In every case, NaR differed in its pattern and NiR and N_2OR responded alike.

A possible explanation might be found in the fact that the shift to energy conservation exclusively by denitrification

resulted in a change in equilibrium of the continuous-culture system, as evidenced by a reduction in growth rate. Rate limiting by two factors may result in complex behavior at the switching line in the chemostat (2). Within the constraints of practical feasibility, this effect could not be alleviated by the choice of dilution rate or medium. The pattern may also reflect a stage of regulatory modulation of the three denitrification reductases, which, under stable, fully anaerobic conditions, coordinately convert nitrate to dinitrogen without accumulation of intermediates.

A further aspect contributing to the complexity of the system concerns assimilatory NaR. As the rationale for assimilatory NaR repression, ammonium was added to the culture medium. Low nitrate reduction activity, occasionally seen in washed cells from fully aerobic cultures, presumably reflects the presence of a small amount of assimilatory NaR. Immunological cross-reactivity with the antiserum raised against respiratory NaR, however, was not seen. Determination of the behavior of the assimilatory NaR under the chosen experimental protocol, and particularly of its interaction with the respiratory pathway, will require isolation of this enzyme. No bacterial assimilatory NaR, however, has been obtained in a pure state.

The range of dissolved oxygen concentrations under which denitrification activity has been reported extends to full or nearly full aerobiosis, such as for *T. pantotropha* (34), *Erythrobacter* sp. (35), or complex communities of freshwater sediment (37). Aerobic denitrification cannot take place in *P. stutzeri*, as neither NaR nor NiR is synthesized at dissolved oxygen concentrations above 5 mg of O₂ per liter. Only N₂OR was constitutively present in small amounts in fully aerobic cells.

Since the three reductases differ in the dissolved oxygen level at which synthesis commences (or exceeds the constitutive level), transient accumulation of an intermediate is likely to occur on shifting the continuous culture system from aerobic to anaerobic conditions. This effect was found for nitrite, which reached maximum concentrations at those oxygen levels at which NaR was already present in considerable amounts but synthesis of NiR had not yet begun (or had already ceased). Superimposed on this effect was the interaction of nitrate with nitrite reduction. It is known that nitrate is stoichiometrically reduced to nitrite before subsequent reactions of denitrification proceed to a gaseous product (14, 26, 27, 29). Inhibition by nitrate of nitric oxide reductase in cell extracts but not of NiR has been shown (29). Taking this into account, the instant inhibition of nitrite reduction in whole cells on addition of nitrate suggests either an indirect action on NiR or a mechanistic site of action before the enzymatic reduction step.

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