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Dynamics of Denitrification Activity of *Paracoccus denitrificans* in Continuous Culture during Aerobic-Anaerobic Changes

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Induction and repression of denitrification activity were studied in a continuous culture of *Paracoccus denitrificans* during changes from aerobic to anaerobic growth conditions and vice versa. The denitrification activity of the cells was monitored by measuring the formation of denitrification products (nitrite, nitric oxide, nitrous oxide, and dinitrogen), individual mRNA levels for the nitrate, nitrite, and nitrous oxide reductases, and the concentration of the nitrite reductase enzyme with polyclonal antibodies against the cd_1-type nitrite reductase. On a change from aerobic to anaerobic respiration, the culture entered an unstable transition phase during which the denitrification pathway became induced. The onset of this phase was formed by a 15- to 45-fold increase of the mRNA levels for the individual denitrification enzymes. All mRNAs accumulated during a short period, after which their overall concentration declined to reach a stable value slightly higher than that observed under aerobic steady-state conditions. Interestingly, the first mRNAs to be formed were those for nitrate and nitrous oxide reductase. The nitrite reductase mRNA appeared significantly later, suggesting different modes of regulation for the three genes. Unlike the mRNA levels, the level of the nitrite reductase protein increased slowly during the anaerobic period, reaching a stable value about 30 h after the switch. All denitrification intermediates could be observed transiently, but when the new anaerobic steady state was reached, dinitrogen was the main product. When the anaerobic cultures were switched back to aerobic respiration, denitrification of the cells stopped at once, although sufficient nitrite reductase was still present. We could observe that the mRNA levels for the individual denitrification enzymes decreased slightly to their aerobic, uninduced levels. The nitrite reductase protein was not actively degraded during the aerobic period.

Paracoccus denitrificans is a gram-negative bacterium mainly found in soil and sewage sludge. Because of its resemblance to mitochondria (15) and its nutritional versatility (16), it has become a popular model strain for studies of electron transfer and energy conservation. Heterotrophic growth is possible both under aerobic conditions with oxygen and under anaerobic conditions with nitrate, nitrite, or nitrous oxide as the terminal electron acceptor (29).

Bacterial denitrification involves four reduction steps in which nitrate (NO_3^-) is sequentially transformed to dinitrogen (N_2) via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O). Four terminal oxidases are necessary to achieve this stepwise reduction. All four enzymes have been isolated from several bacteria and have been characterized in detail (reviewed in reference 35). Recently, the genes encoding the denitrification enzymes have been identified in different bacteria, such as *P. denitrificans*, *Pseudomonas stutzeri*, *Escherichia coli*, and *Thiosphaera pantotropha* (reviewed in reference 31).

Because of its ability to use both oxygen and nitrogenous oxides, *P. denitrificans* can survive in ecosystems with fluctuating aerobic or anaerobic conditions. When molecular oxygen is present, it is the preferred terminal electron acceptor molecule (14). As soon as oxygen is depleted, the electron transfer components required for denitrification have to be induced. In *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* the absence of oxygen alone is not sufficient to induce synthesis of the individual denitrification enzymes above a basal level. To achieve full induction, a nitrogenous oxide must be present (19, 20, 33).

Little is known about the signals and mechanisms controlling the switches between aerobic and anaerobic conditions, although recently some regulatory genes have been found and characterized (12, 24, 26, 27, 30). Even less is known about how such signalling and induction proceed over time. A very promising way to study the network of denitrification is the combined application of physiological, molecular genetic, and biochemical tools.

In this article we describe the use of gene probes for the mRNAs of the nitrate, nitrite, and nitrous oxide reductases of *P. denitrificans* to characterize the dynamics of denitrification in a continuous culture after switching growth conditions from aerobiosis to anaerobiosis and vice versa. The onset and cessation of denitrification activity in *P. denitrificans* were further investigated by monitoring the formation of denitrification products (NO_2^-, NO, N_2O, and N_2) and the synthesis of the nitrite reductase enzyme by using polyclonal antibodies raised against the cd_1-type nitrite reductase.

MATERIALS AND METHODS

Organisms and culture medium. *P. denitrificans* DSM 65 (ATCC 17741) was grown in a synthetic medium containing, per liter, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.15 g of $Na_2MoO_4 \cdot H_2O$, 1.5 g of KH_2PO_4, 7.5 g of KNO_3, 2.5 g of NH_4Cl, 7.4 mg of $CaCl_2 \cdot 2H_2O$, 1 mg of $MnSO_4 \cdot H_2O$, 3.6 mg of $ZnSO_4 \cdot 7H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 0.45 mg of $CuCl_2 \cdot 2H_2O$, 0.3 mg of H_3BO_3, 0.01 mg of $NiCl_2 \cdot 6H_2O$, 3.7 mg of EDTA-Na_2, and 12 mg of EDTA-NaFe(III). Acetate was supplied as the limiting substrate to the medium at a final concentration of

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A

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NirS consensus   (89)     I   Y   F   e   R   C   A   G   C   H   G   V
nirS consensus            ATC TAc TTC gAa CGC TGC GCC GGt TGc CAC GGc GTc
primer NIRSAC                 5'ttc gag ctc tgc gcc ggt tgc cac gg 3'

NirS consensus   (222)    l   D   T   G   Y   A   V   H   I   S   R   m
nirS consensus            cTC GAt ACC GGC TAt GCg GTG CAt ATC tcg CG. aTG
primer NIRECORV           3'g ctg tgg ccg ata cgc cac cta tag tcg g 5'
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B

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NosZ consensus   (66)     P   G   e   L   D   .   Y   Y   G   f   W   S
nosZ consensus            CCg GG. gA. CTG GA. ga. TAC TAc GGC tTC TGG ..C
primer N2OAAT                 5'caa ctg gac gtc tac tac ggc ttc tgg 3'

NosZ consensus   (160)    y   l   f   i   N   D   K   a   N   s   R   v
nosZ consensus            Tac ctg Ttc aTc AAC GAC AAG gcC AA. a.C CGC gT.
primer N2ONSI             3'g gac aag tac gta ctg tta agg tta tg 5'
```

C

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NarH consensus   (6)      Q   v   G   M   V   L   N   L   D   K   C   I
narH consensus            CAA gTC GGC ATG GTG CTg AAc CTc GAc AAg TGt ATC
primer NARNCO       5'    caa atc gcc atg gtg ctg aac ctg g 3'

NarH consensus   (173)    E   N   T   F   M   M   Y   L   P   R   L   C
narH consensus            GAA AAC ACC TTC ATG ATG TAt cTG CCG CGc CTG TGC
primer NARNSI                 3' gg aag tac tac gta gac ggc gcg ga 5'
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FIG. 1. Consensus regions and derived PCR primers for nitrite reductase (A), nitrous oxide reductase (B), and nitrate reductase (C) of *P. denitrificans* DMS 65 aligned with those of other bacteria. Conserved residues for the amino acid sequences were derived from alignments with the programs PILEUP and PRETTY. Uppercase fonts represent totally conserved residues, lowercase letters point to one aberrant residue at each specific position, and dots indicate no conservation. The position of the first amino acid residue of the aligned regions in the primary sequence of the respective *P. denitrificans* protein is indicated in parentheses. Italics indicates the restriction endonuclease sites synthesized in each primer. The sequences used for the alignments were those of the *nirS* genes of *P. denitrificans* PD1222 (9), *Pseudomonas aeruginosa* (25), and *Pseudomonas stutzeri* (17) (A); the *nosZ* genes of *Pseudomonas stutzeri* (32), *Pseudomonas aeruginosa* (36), and *P. denitrificans* NCIB8944 (13) (B); and the *narH* (5) and *narY* (6) genes of *E. coli* and the *narH* gene of *T. pantotropha* (4) (C).

60 mM. To all cultures grown in bioreactors, a 10% sterilized silicon antifoam solution was added to give a final concentration of 100 ppm (vol/vol).

E. coli DH5α (23) was used as the host strain in cloning experiments. The strain was grown in LB medium (23) supplemented with 50 μg of ampicillin per liter to maintain plasmids with the cloned *P. denitrificans* gene fragments.

Culture conditions for chemostat experiments. *P. denitrificans* was grown in continuous culture at a dilution rate of 0.03 h^{-1}. The low growth rate was chosen to mimic the long residence times of bacteria in their natural habitats, such as soil or activated sludge. The operating volume of the bioreactor (MBR, Wetzikon, Switzerland) was 2.8 liters. The temperature was maintained at 30°C, and the pH of the culture medium was kept at 7.5 by the automatic addition of 2 M NaOH or 20% (vol/vol) H_3PO_4. Aerobically grown cultures were continuously sparged with helium and oxygen to keep the dissolved oxygen concentration in the bioreactor at 90% of that of air saturation. Anaerobically grown cultures were sparged with helium only. As judged from the biomass concentration in the chemostat and the production rate of carbon dioxide, approximately 7 volume changes were required to establish a steady-state continuous culture. We started our transient experiments after a minimum of 10 reactor volume changes. Transient experiments were performed by instantaneously switching a culture which had been grown to steady state under aerobic conditions to anaerobic conditions or vice versa. Before, during, and after switching culture conditions, the cultures were sampled for denitrification products, for the concentrations of acetate and mRNAs of nitrate, nitrite, and nitrous oxide reductases, and for the presence of nitrite reductase enzyme in the cells.

Analytical methods. The dissolved oxygen concentration in the culture was measured with an oxygen electrode (Mettler-Ingold, Oberentfelden, Switzerland). The bacterial dry weight was determined by filtering 10 ml of culture through preweighed 0.2-μm-pore-size membrane filters (Nucleopore, Inc., Pleasanton, Calif.). Filters were dried overnight at 105°C.

The concentrations of acetate and NO_2^- were determined in the filtrate of cell suspensions. Cell suspensions were vacuum filtered through a 0.22-μm-pore-size filter (Millipore, Bedford, Mass.), and filtrates were stored at −20°C. Before measurements were made, the samples were slowly thawed on ice. NO_2^- was assayed as follows: 50-μl aliquots were mixed with 950 μl of 1% sulfanilic acid–0.05% *N*-naphthylene diamine-HCl dissolved in 1 M H_3PO_4. After 5 min of incubation at room temperature, the color development was complete, and the A_{540} was determined. The presence of acetate was determined enzymatically (Boehringer, Mannheim, Germany) or by ion chromatography (Dionex Corporation, Sunnyvale, Calif.).

Concentrations of N_2O and N_2 were measured on-line with a gas chromatograph (GC) (type GC-8A; Shimadzu Co., Tokyo, Japan). The effluent gas stream from the headspace of the bioreactor was passed through a 2-ml sample loop and automatically injected into the GC. The oven temperature of the GC was 80°C, and the carrier gas was helium. The GC was equipped with a thermal conductivity detector and two parallel packed columns. One column was packed with molecular sieve 5 Å 80/100 (Brechbühler AG, Schlieren, Switzerland); the other was packed with Porapak Q 80/100 (Brechbühler AG). The detection limit was 10^{-2} g of N m^{-3}.

The concentration of NO was continuously measured in a chemoluminescence detector (Thermo Environmental Instrument, Franklin, Mass.) directly coupled to the outlet of the GC. The detection limit for NO was 10^{-5} g of N m^{-3}.

The production rate (P) of the volatile denitrification products was calculated from the expression:

$$P = \frac{n \cdot F}{V}$$

where n is the concentration (in millimoles per liter) of the denitrification products in the gas phase, F is the flow rate (in liters per hour) of the effluent gas, and V is the operating volume (in liters) of the bioreactor.

Cloning of *P. denitrificans* gene fragments for denitrification enzymes. Fragments of the genes for nitrate, nitrite, nitric oxide, and nitrous oxide reductase of *P. denitrificans* had to be isolated in order to use them as gene probes in the transient experiments. Alignments were made of the known sequences of these genes from *P. denitrificans* and from other microorganisms to derive conserved regions for which we could synthesize pairs of oligonucleotides. The conserved sequences were slightly altered in order to obtain recognition sites for restriction endonucleases, which facilitated cloning afterwards (Fig. 1). The oligonucleotides were tested by PCR with total genomic DNA of *P. denitrificans*. Amplification products with the expected sizes were obtained for the nitrate, nitrite, and nitrous oxide reductase genes, but no products with the proper sizes were found for nitric oxide reductase.

The amplification products were purified by digestion with proteinase K, phenol-chloroform extraction, and ethanol pre-

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cipitation. The DNA was then dissolved and digested with the appropriate restriction enzymes. After digestion, the DNA fragments were again purified from solution and cloned into plasmid pGEM5zf$^+$ (Promega Corp., Madison, Wis.) that had been cut with the same enzymes. Ligation mixtures were transformed into *E. coli* DH5α. The DNA sequences of the inserts of selected recombinant plasmids were determined on both strands by cycle sequencing with dideoxyoligonucleotides and infrared-labelled primers for the vector (IRD41; MWG Biotech, Grabenstein, Germany) and analyzed on a model 4000 L automated sequencer (LI-COR, Inc., Lincoln, Nebr.). This analysis verified that gene fragments of nitrate, nitrite, and nitrous oxide reductase were indeed cloned. To further verify their origin, these gene fragments were hybridized to *P. denitrificans* total genomic DNA (see below).

In vitro synthesis of sense and antisense RNA for denitrification enzymes. The pGEM5zf$^+$-derived plasmids with the inserts for the three denitrification enzymes of *P. denitrificans* were used as templates to synthesize RNAs for use as probes in induction experiments. The plasmids were first linearized with appropriate restriction enzymes to obtain a transcript from the inserted DNA only. Sense and antisense RNAs were then synthesized with an in vitro translation system with either T7 or Sp6 RNA polymerases (Boehringer, Mannheim, Germany). The RNAs were labelled with biotin by incorporating biotin 16–UTP in the reaction mixture as described by the supplier (Boehringer) and purified by DNase I digestion, phenol-chloroform extraction, and ethanol precipitation.

RNA extraction. Total RNA from *P. denitrificans* was isolated from 1-ml samples taken directly from the chemostat. Cells were immediately harvested by centrifugation (15,000 rpm, 30 s), and the pellet was resuspended in 0.5 ml of TES buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl). Total RNA was then extracted with acid-phenol and phenol-chloroform as described by Aiba et al. (1). RNAs were treated with DNase I (RNase free; Boehringer), again phenol-chloroform extracted, and precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at −20°C. For dot blotting, the RNAs were centrifuged, dried, and dissolved in diethylpyrocarbonate-treated double-distilled water. The concentration of total RNA was measured at 260 nm with an RNA/DNA calculator (Gene Quant II; Pharmacia Biotech, Dübendorf, Switzerland).

Quantification of mRNA for denitrification enzymes. Similar quantities of total RNA from each RNA extraction were blotted sixfold onto positively charged nylon membranes (Qiagen, Basel, Switzerland) in a 96-well dot blot manifold, prehybridized for 1 h at 62°C in a solution of 7% sodium dodecyl sulfate (SDS), 10 g of bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.) per liter, 0.5 M sodium phosphate buffer (pH 7.2), and 1 mM EDTA (pH 8.0) and subsequently hybridized for 16 h at 62°C in the same solution with the in vitro-synthesized biotin-labelled single-stranded RNA probes. In order to detect the induction and presence of the mRNAs for nitrate, nitrite, and nitrous oxide reductase, we hybridized the antisense RNAs synthesized from the pGEM5zf$^+$-derived plasmids. As a negative control, we hybridized the sense RNAs of the same plasmids. After hybridization, the membranes were washed twice at 68°C in 0.1% SDS and 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min and then incubated for detection as described for the Southern-Light chemiluminescence system (Tropix, Bedford, Mass.). Membranes were exposed at room temperature to Hyperfilm (Amersham Life Sciences, Amersham, United Kingdom) or directly scanned for chemiluminescence in a luminometer (EG&G Berthold, Bad Wildbad, Germany). The density of the dots on the exposures was determined by scanning the X-ray films on a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.). Care was taken that no spot on the autoradiograms had a density higher than 1. Densitometrically and luminometrically determined values corresponded favorably. The densities of the spots obtained with RNA samples from the aerobic steady state, hybridized on the same filter as those from the anaerobic transition phase, were arbitrarily given a relative value of 1. In all cases, the sense RNA hybridizations revealed no signals above the background of the membrane.

Hybridization of chromosomal DNA with RNA probes. To verify the origins of the PCR-amplified gene fragments and their locations on the genome of *P. denitrificans*, we hybridized total genomic DNA with the antisense RNAs for nitrate, nitrite, and nitrous oxide reductase. Total genomic DNA was isolated according to the method of Marmur (22), digested with different restriction enzymes, and separated by electrophoresis on 0.8% agarose gels. DNA fragments were subsequently transferred to positively charged nylon membranes (Qiagen) by a standard protocol (23). Hybridization with RNA probes and visualization of the hybridization results were done as described above.

Protein isolation and immunoquantification of nitrite reductase. *P. denitrificans* cells were recovered from 1 ml of cell suspension from the chemostat by centrifugation at 15,000 rpm for 30 s. The pellet was resuspended in 50 μl of protein loading buffer (0.1 M Tris-HCl [pH 6.5], 5.2% SDS, 8.7% β-mercaptoethanol, 17.4% glycerol, 0.001% bromophenol blue), boiled for five min, and stored at −20°C. Before analysis, samples were thawed and reboiled for 2 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% gel and electroblotted onto nitrocellulose filters as described elsewhere (23). The filters were incubated overnight at 4°C with 5% skim milk powder in phosphate-buffered saline-Tween (PBS-T) buffer (8 mM Na_2HPO_4, 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.1% Tween 20 [pH 7.2]) to block nonspecific bindings. Immunological detection was carried out in PBS-T buffer. Polyclonal antibodies against cd_1-type nitrite reductase from *Pseudomonas aeruginosa* were kindly provided by Coyne et al. (8). Nitrocellulose filters were incubated at room temperature for 2 h with the antibodies (1:3,000 dilution), washed three times with PBS-T buffer, and incubated at room temperature for 2 h with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co.). Filters were washed as described above and visualized with ECL reagents (Amersham International plc, Amersham, United Kingdom). Exposed X-ray films were analyzed by densitometry.

RESULTS

Isolation of different denitrification gene fragments. In order to study the induction and cessation of the denitrification system in *P. denitrificans* after a switch from aerobiosis to anaerobiosis and vice versa, we wanted to be able to monitor the expression of specific mRNAs for denitrification enzymes. As no genes for the denitrification enzymes had been isolated from *P. denitrificans* DSM 65 at that time, we attempted to obtain some key fragments by PCR. For nitrate reductase, we aligned the sequences of *narY* and *narH* of *E. coli* (5, 6) and of *narH* from *T. pantotropha* (4). Several consensus regions were found both on the amino acid level and in the DNA sequences (Fig. 1), from which we could design oligonucleotides to be used in the PCR. From this strain we amplified a 529-bp

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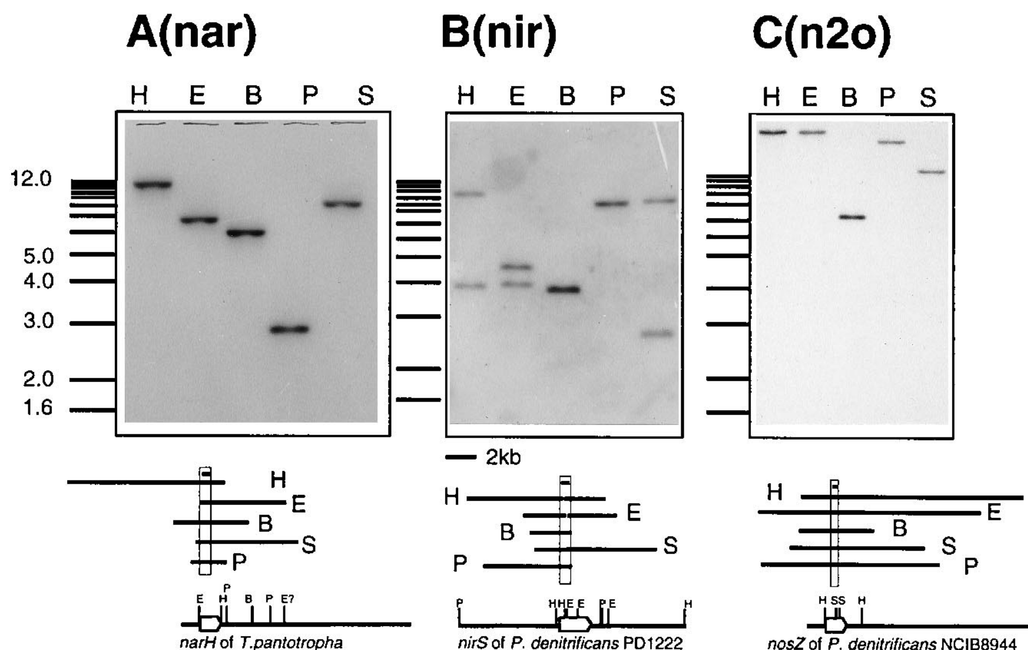

FIG. 2. Hybridizations of total genomic DNA of *P. denitrificans* DSM 65 with the PCR-derived gene fragments of *narH* (A), of *nirS* (B), and of *nosZ* (C). Size markers indicating the migration of the 1-kb fragment ladder are depicted on the left of each panel. The relative locations of the hybridizing fragments are indicated below each panel, and comparisons with the genetic and physical maps of similar regions from different bacteria are presented. The locations of the probes used are depicted with open vertical bars. (A) *narH* region of *T. pantotropha* (4); (B) *nirS* region of *P. denitrificans* PD1222 (9); (C) *nosZ* region of *P. denitrificans* NCIB8944 (13). Lanes: H, *Hin*dIII digest; E, *Eco*RI; B, *Bam*HI; P, *Pst*I; S, *Sal*I.

product, which was cloned into pGEM52f$^+$, sequenced, and found to contain a fragment of a *narH*-like gene. The DNA sequence of this fragment was most similar to a region from the *narH* gene of *T. pantotropha* (99.2% identity). This finding strongly suggests that the DNA fragment that we obtained by PCR is part of the gene for the membrane-bound nitrate reductase of *P. denitrificans*. The fragment clearly hybridized to *P. denitrificans* total genomic DNA (Fig. 2A). The restriction patterns observed were in agreement with a physical map of the *narH* gene region of *T. pantotropha* (4).

Similarly, we isolated gene fragments of a *nirS*-like gene for nitrite reductase and of a *nosZ* gene for nitrous oxide reductase (Fig. 1A and B). The *nirS* sequence that we obtained had the highest percentage identity (95.1%) to *nirS* of *P. denitrificans* Pd 1222 (9). The *nosZ* partial sequence had 90.2% identity with that of *P. denitrificans* NCIB 8944 (13). Both gene fragments clearly hybridized to the DNA of *P. denitrificans* DSM 65 (Fig. 2B and C). Interestingly, the *Hin*dIII and *Eco*RI sites were conserved in the gene fragment of *nirS* from our strain of *P. denitrificans* compared with that of *P. denitrificans* Pd 1222 (9) (data not shown). However, our hybridizations did not indicate conservation of other *Hin*dIII and *Eco*RI sites located near *nirS* (Fig. 2). We also found some differences in nearby restriction sites in the *nosZ* gene fragment compared with strain NCIB 8944 (Fig. 2). From the results, we were confident that we had obtained gene fragments for three denitrification enzymes from our strain of *P. denitrificans* that could be used as probes in the induction experiments. However, the hybridization and sequence data indicated that clear differences exist for the denitrification genes of different *P. denitrificans* strains. We did not obtain a fragment for part of *norC* for nitric oxide reductase, although consensus sequences were derived from alignment of known sequences. Therefore, the induction of *norC* was not studied here.

Response of *P. denitrificans* to a change from aerobic to anaerobic growth conditions. An aerobic steady-state culture of *P. denitrificans* was subjected to a switch from oxic to anoxic conditions. Induction of denitrification activity of the continuously growing culture was monitored by determining the formation of denitrification products; the consumption of acetate; expression of the mRNA for nitrate, nitrite, and nitrous oxide reductases and synthesis of the nitrite reductase enzyme.

The production of denitrification metabolites started about 30 min after oxygen was depleted (Fig. 3). NO_2^- was the first intermediate to be detected. Its concentration reached a maximum approximately 5 h after the switch to anaerobic conditions. Subsequently, it decreased gradually, and by 12 h it was below the detection limit. Only low levels of NO were detected. Its production rate was a 1,000 times less than the production rates of the other gaseous denitrification products. Both N_2O and N_2 were detected at 2 h after the switch. Whereas N_2O was released for only a relatively short period (Fig. 3), N_2 increased steadily until it became the dominant denitrification product, reaching a maximum production rate of 1.4 mmol $\cdot$ l^{-1} $\cdot$ h^{-1}. From approximately 6 to 12 h after switching, more N_2 was produced than during the steady state afterwards. This overproduction was due to the consumption of acetate, which accumulated during the first 4 h following the switch from aerobic to anaerobic growth conditions (Fig. 3B). After 14 h the formation of N_2 declined to a stable production rate of 1.0 mmol $\cdot$ l^{-1} $\cdot$ h^{-1}. On changing to anaerobic conditions, a slight decrease in the bacterial biomass concentration was observed (Fig. 3B).

The expression of the genes for the denitrification enzymes was monitored by measuring the levels of three specific mRNAs in the cell culture (Fig. 4). Dot blotting and hybridization with antisense RNAs for specific detection and sense RNAs for unspecific detection proved to be an easy, fast, and

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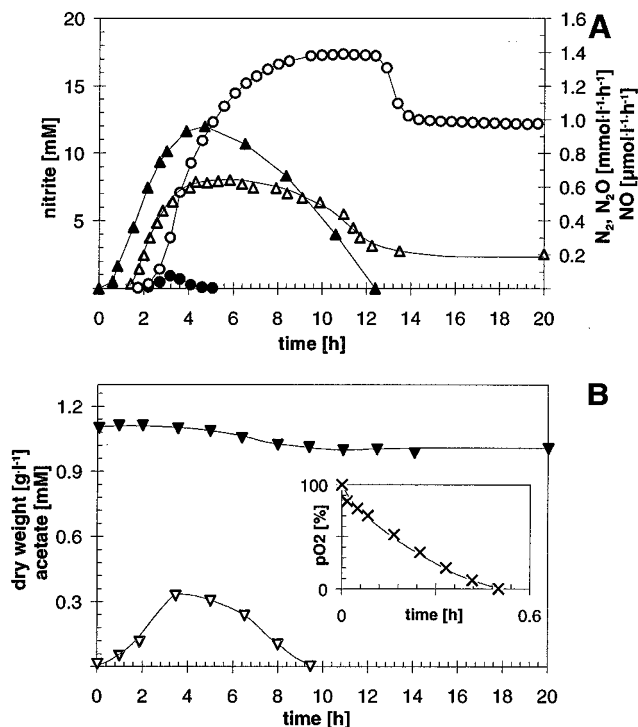

FIG. 3. Transition-phase changes in denitrification intermediates (A) and culture parameters (B) after an aerobic-to-anaerobic change of a continuous culture of *P. denitrificans* DMS 65. The time axis indicates the time after the switch to anaerobic conditions. Symbols: ▲, nitrite concentration in the culture medium; △, synthesis rate of nitric oxide; ●, synthesis rate of nitrous oxide; ○, synthesis rate of dinitrogen; ▼, dry weight of the culture; ▽, acetate concentration in the culture medium; ×, partial oxygen pressure.

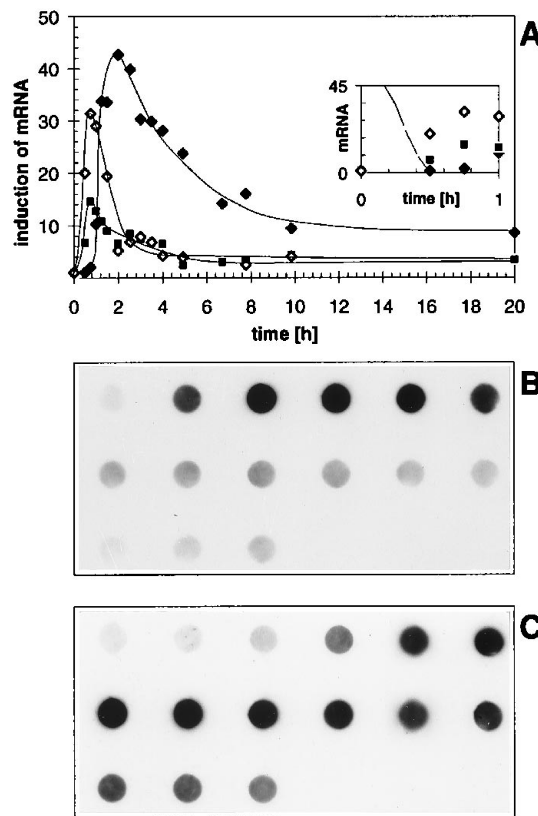

FIG. 4. Transition-phase changes in mRNA levels for individual denitrification enzymes after an aerobic-to-anaerobic change of a steady-state aerobically growing culture of *P. denitrificans* DSM 65. mRNA levels are given as relative values compared with those under aerobic steady-state conditions (arbitrarily defined as a level of 1). The time axis indicates the time after the switch to anaerobic conditions. (A) Time course of calculated relative densities of hybridization signals obtained with different antisense RNA probes. Relative mRNA levels for nitrate reductase (*narH*) (◇), nitrite reductase (*nirS*) (◆), and nitrous oxide reductase (*nosZ*) (■) are shown. Absolute densities detected from hybridization of total RNA of *P. denitrificans* transition-phase samples with the antisense RNA probe for nitrate reductase (*narH* gene fragment) (B) and for nitrite reductase (*nirS* gene fragment) (C). Each dot in panels B and C is represented in panel A, with time sampling starting at time zero onwards (from upper left to bottom right). The inset in panel A shows an enlargement of the relative mRNA levels observed during the first hour after the switch compared with the measured relative oxygen concentration in the medium (dotted line).

reliable method. It is clear, however, that we could not determine transcripts with different sizes and may have underestimated the level of specific mRNAs because of masking effects of rRNAs. We made the assumption that these masking effects were constant for all samples, allowing the determination of changes in the relative amounts of the mRNAs for denitrification. After the switch to anaerobic conditions, the genes for nitrous oxide and nitrate reductase were almost immediately induced, whereas the mRNA for nitrite reductase appeared somewhat later (Fig. 4). This delay was repeatedly observed in several independent experiments. The maximum levels of transcription of the analyzed genes increased between 15 and 45 times compared with induction levels under aerobic conditions. The *nosZ* gene was consistently induced to a level lower than those of *narH* and *nirS*. The mRNAs for both nitrous oxide and nitrate reductase appeared to decline quite rapidly to a level approximately five times that of the basal aerobic level. Interestingly, the mRNA for nitrite reductase declined more slowly.

The presence of the nitrite reductase enzyme in the cells was analyzed by Western blotting with nitrite reductase antibodies (Fig. 5). In contrast to the mRNAs where a sudden burst of synthesis was followed by a rapid decline and stabilization at a low level we observed a slow increase of the enzyme during the anaerobic period. Only after 30 h following the switch to anaerobic conditions the concentration of the nitrite reductase seemed to have come up to a stable value in the cells. The stable plateaus achieved for the production of N_2, mRNAs, and nitrite reductase indicated the end of the transient phase and a balance between the needed enzyme capacity, mRNA turnover, and protein stability under anaerobic growth conditions.

Response of *P. denitrificans* to a change from anaerobic to aerobic growth conditions. When the continuous culture had reached a new steady-state level under anaerobic conditions, we switched the cells back to aerobic respiration (Fig. 6). Except for a slight accumulation of NO_2^-, *P. denitrificans* stopped denitrification at once (Fig. 6A). As the anaerobic steady-state levels of the mRNAs for the individual denitrification enzymes were already quite low (only 5 to 10 times higher than those under aerobic conditions), only a very slight decrease in transcript abundance could be observed after the anaerobic-to-aerobic switch (Fig. 6B). Interestingly, the nitrite reductase did not appear to be actively degraded during aerobic conditions. Its overall concentration declined slowly, following the washout curve calculated for a non-renewed population of *P. denitrificans* cells which produce nitrite reductase (Fig. 6C).

Signals for induction of denitrification pathway. Aerobic steady-state cultures of *P. denitrificans* grown in a medium

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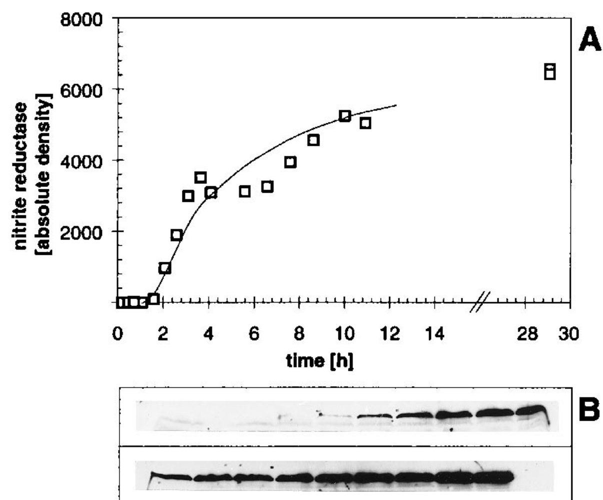

FIG. 5. Formation of nitrite reductase enzyme during transition phase of aerobic-to-anaerobic steady state in continuous culture of *P. denitrificans* DSM 65. The time axis indicates the time after the switch to anaerobic conditions. Formation of nitrite reductase was assayed by immunologic detection of cytochrome cd_1 levels on Western blots of SDS-PAGE-separated total protein extracts of culture samples at different times after the switch. (A) Graphical representation of absolute densities measured for the immunologic reaction of cd_1 protein band of each time sample; (B) time sampling starts from time zero (upper left) and continues through h 29 (lower right).

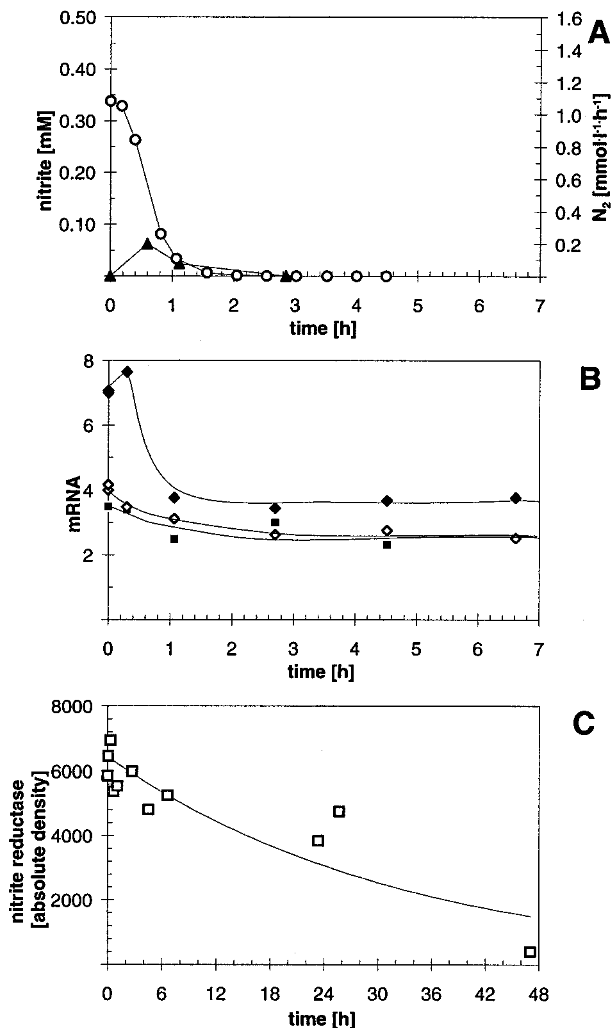

FIG. 6. Transition-phase changes in denitrification intermediates (A), relative mRNA levels for the three denitrification enzymes (B), and levels of nitrite reductase enzyme (C) after a change from anaerobic steady-state culture conditions to aerobic growth conditions of a continuous culture of *P. denitrificans* DSM 65. For other explanations, see the legends to Fig. 3 to 5. Symbols: ▲, nitrite concentration in the culture medium; ○, synthesis rate of dinitrogen; ◇, relative mRNA level for nitrate reductase (*narH*); ◆, mRNA for nitrite reductase (*nirS*); ■, mRNA for nitrous oxide reductase (*nosZ*); □, level of nitrite reductase enzyme. The line in panel C indicates the calculated washing-out curve.

containing NO_3^- exhibited a boostwise induction of the mRNAs for nitrate, nitrite, and nitrous oxide reductases on a transition from aerobic to anaerobic conditions. Apparently, the absence of oxygen played an important role in triggering the expression of the denitrification enzymes. However, in an experiment in which we switched an aerobic steady-state culture of *P. denitrificans* to anaerobic conditions without supplying it with any nitrogenous oxide (i.e., no NO_3^-, NO_2^-, or N_2O), no measurable induction of the mRNAs for *narH*, *nirS*, or *nosZ* could be observed (data not shown).

In a further attempt to determine possible effectors or conditions for the induction of the denitrification pathway, we monitored the mRNA for nitrite reductase. Since we considered NO_3^- and NO_2^- possible effectors for *nirS* induction, we pulsed 10 mM of NO_2^- to an anaerobic culture (Fig. 7) and 10 mM of NO_3^- or NO_2^- to an aerobic (data not shown) steady-state culture of *P. denitrificans*, which was pregrown on a nitrogenous oxide free medium. In all cases no additional induction of the mRNA was observed.

As mentioned previously, acetate accumulated in the medium after *P. denitrificans* was switched from aerobic to anaerobic respiration. As a consequence, acetate was no longer the growth-limiting substrate. To investigate whether this sudden availability of additional electron donors could have influenced the observed expression of the mRNA for the denitrification enzymes, we pulsed acetate (10 mM) alone and in combination with NO_2^- (10 mM) to an anaerobic steady-state culture of *P. denitrificans*. As in all other pulse experiments no additional induction of the mRNA of the nitrite reductase was observed (data not shown).

DISCUSSION

The switch from aerobic to anaerobic growth conditions necessitates the onset of the denitrification system in *P. denitrificans*. This event is the result of a network of regulated responses in the cell to new conditions. In this work we have tried to analyze this network over time, at three different levels, for a number of key elements in the denitrification: (i) expression of mRNAs for nitrate reductase, nitrite reductase, and nitrous oxide reductase; (ii) synthesis of the nitrite reductase enzyme; and (iii) formation of denitrification products. Our results show a typical sequence of transient events on induction of the denitrification activity in *P. denitrificans* before the cells enter a new steady state.

Under aerobic conditions *P. denitrificans* synthesizes only very low, basal levels of mRNAs for the denitrification enzymes. Also, on protein level the nitrite reductase enzyme was barely detectable in cultures grown aerobically. This is in contrast to *P. aeruginosa*, for example, which synthesizes nitrite reductase in the presence of oxygen, provided that NO_3^- is available (33). The denitrification pathway in *P. denitrificans* is induced under low oxygen or fully anoxic conditions, but induction requires the additional presence of a nitrogenous ox-

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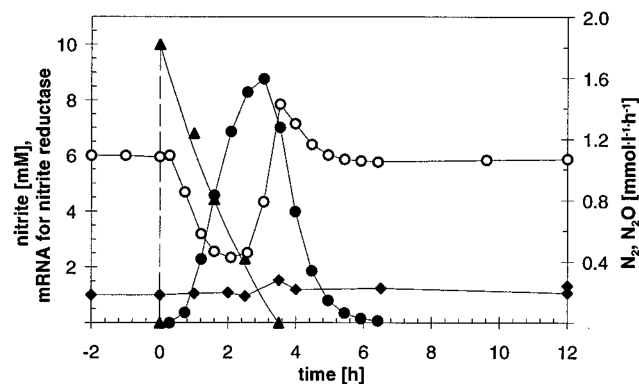

FIG. 7. Effect of adding a nitrite pulse to anaerobic steady-state culture of *P. denitrificans* on the formation of denitrification intermediates and on relative mRNA levels for nitrite reductase. For other explanations, see the legends Fig. 3 to 5. Symbols: ▲, nitrite concentration in culture medium; ●, synthesis rate of nitrous oxide; ○, synthesis rate of dinitrogen; ◆, level of nitrite reductase mRNA relative to anaerobic steady-state value.

ide, similar to that for *P. stutzeri* (19, 20). Nitrogenous oxides themselves were apparently also not enough to elicit an inducible response. For example, the addition of NO_3^- or NO_2^- to aerobically growing continuous cultures of *P. denitrificans* did not increase the basal level of the mRNAs for the individual denitrification enzymes. Secondly, we could not measure any induction of mRNA for the individual denitrification enzymes when we performed our aerobic and anaerobic transitions with a culture grown on nitrate-free medium. However, perhaps the cells were in this case not able to express detectable levels of the mRNAs, since *P. denitrificans* is unable to ferment and the cells may have quickly suffered from lack of energy when cultured without oxygen or any nitrogenous oxide.

We found that the expression of the genes for nitrate and nitrous oxide reductase occurred immediately before or directly after the oxygen concentration dropped to zero. This seems to indicate that these genes are indeed activated or derepressed when oxygen levels become low. We further found that induction of nitrate and nitrous oxide reductase mRNAs occurred simultaneously. From our Southern hybridizations we conclude that it is very unlikely that *nar* and *nos* genes are transcribed as a single polycistronic mRNA, which suggests that the same *trans*-acting regulatory protein, perhaps FnrA (31), is involved in the expression of *nar* and *nos* genes. Interestingly, we observed that the nitrite reductase gene was turned on somewhat later than *nar* and *nos* in *P. denitrificans* DSM 65, when oxygen was completely depleted from the medium, suggesting a different regulation for *nirS*. Perhaps the recently proposed Nnr protein (30) or a protein homologous to NirQ of *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* (3, 9, 31) mediate the signals for fully anoxic conditions, resulting in expression of nitrite reductase.

We found a typical time course of the overall mRNA levels during the transient period which is not uncommon for expression of mRNAs, although the stability of the transcripts can vary considerably (7, 10, 34). For instance, in contrast to the levels of *nar*, *nos*, and *nir* mRNA, which increased transiently and then decreased relatively fast, the levels of transcripts from the Pu and Pm promoters in the *Pseudomonas putida* TOL degradation pathway increased about 20-fold after induction and remained almost constant over a period of 24 h (10). The overall level of mRNA may reflect the time for two processes, one of mRNA synthesis and one of mRNA breakdown, to reach equilibrium. In addition, the mRNA synthesis rate itself may diminish because of the presence of fewer effector molecules or to a higher proportion of nonactive regulator protein. The fact that the decrease of the overall *nir* mRNA level was slower than that of *nar* or *nos* may indicate that this mRNA is more stable than the others or that regulator-effector interactions at this promoter are different.

All denitrification intermediates could be detected during the transient period, albeit at different levels. This clearly reflects the process of sequential induction of the different components of the denitrification pathway as described above. The considerably high concentrations of NO_2^- in our cultures were probably caused by the delay of expression of nitrite reductase mRNA and, consequently, a delay of synthesis of this enzyme. Retarded induction of nitrite reductase compared with the induction of nitrate reductase has been reported by others (21). The very low levels of NO measured indicate that the expression and synthesis of nitric oxide reductase were not delayed with regard to the expression and synthesis of the nitrite reductase. The end of the transition phase, and apparently a stable composition of the different denitrification enzymes, was reached when the major product of the denitrification pathway was dinitrogen.

As expected, the anaerobic steady-state cultures stopped denitrifying immediately when they were supplied with oxygen (Fig. 6). The small accumulation of NO_2^- which we observed just after the switch to aerobic conditions might have been due to the occurrence of wall growth where anaerobic conditions continued to exist for a short period in spite of the aeration of the bioreactor with oxygen. Using oxygen instead of NO_3^- is advantageous to the cells since, in terms of energy yield, NO_3^- is a less favorable terminal electron acceptor (28). The shift to oxygen conditions resulted in a slight reduction in the levels of mRNA for the denitrification proteins to the low constitutive level observed under steady-state aerobic conditions. We presume that the denitrification genes became repressed again by the presence of oxygen. Nitrite reductase enzyme was not actively degraded in the cells and was present, although declining, throughout the aerobic phase. The enzyme may even still have been active, since it was shown recently that the cd_1-type of nitrite reductase (as well as the nitric oxide and nitrous oxide reductases) can be active in the presence of molecular oxygen (11). In addition, others have shown that the nitrite reductase in *P. denitrificans* remains potentially active during the aerobic phase after a switch from anaerobic growth (18). Probably, oxygen acts indirectly by inhibiting the activity of a putative transport protein which carries NO_3^- across the cell membrane (11). The nitrate reductase, although in principle also still active under aerobic conditions (2, 14), will thus be depleted from NO_3^-; no NO_2^- will be formed, and denitrification stops. From an ecological viewpoint it makes sense that denitrifiers, such as *P. denitrificans*, do not actively degrade their denitrification enzymes, since dissolved oxygen tension is in most ecosystems continuously fluctuating. Thus, cells will benefit from keeping their set of already-synthesized denitrification enzymes required for anaerobic growth.

Up to now, very few studies of denitrification have analyzed mRNA formation, which reflects the dynamics of changing conditions more directly than does protein synthesis. This study showed that the analysis of gene networks such as denitrification on different levels simultaneously, e.g., mRNA transcription, protein synthesis, and formation of denitrification intermediates, appeared to be a good way to observe responses of such networks to changing conditions. It would be interesting to extend this approach for more of the genes involved in the different energy pathways and for other networks, to reveal

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the complexity of interacting signals, mediators, and enzyme products.

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