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Nitric Oxide Reductase Gene Expression and Nitrous Oxide Production in Nitrate-Grown *Pseudomonas mandelii*▽

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Pure cultures of *Pseudomonas mandelii* were incubated with or without nitrate, which acts as a substrate and an electron acceptor for denitrification. Nitric oxide reductase (*cnorB*) gene expression was measured using a quantitative reverse transcription-PCR, and nitrous oxide emissions were measured by gas chromatography. *P. mandelii* cells in either the presence or absence of nitrate demonstrated an increase in *cnorB* gene expression during the first 3 h of growth. The level of expression of *cnorB* in nitrate-amended cells remained high (average, 2.06×10^8 transcripts/μg of RNA), while in untreated cells it decreased to an average of 3.63×10^6 transcripts/μg of RNA from 4 to 6 h. Nitrous oxide accumulation in the headspace was detected at 2 h, and cumulative emissions continued to increase over a 24-h period to 101 μmol in nitrate-amended cells. *P. mandelii cnorB* gene expression was not detected under aerobic conditions. These results demonstrate that *P. mandelii cnorB* gene expression was induced 203-fold at 4 h when nitrate was present in the medium. Accumulations of N_2O indicated that the cNorB enzyme was synthesized and active.

Denitrification is the reduction of nitrate and nitrite to the gaseous compounds nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas (N_2) (29). This anaerobic process is catalyzed by nitrate, nitrite, nitric oxide, and nitrous oxide reductase enzymes, respectively (17). Incomplete biological denitrification can lead to the emission of N_2O, a potent greenhouse gas that contributes to global warming and ozone depletion (28).

Denitrifying bacteria belong to several genera, including *Pseudomonas* (8, 10, 29). Denitrification genes have been isolated from pure bacterial cultures and from environmental samples by using a range of primers (5, 6, 11, 12, 18, 21). *Pseudomonas mandelii* was first isolated from mineral water (26); however, the strain of *P. mandelii* used in this study was isolated from agricultural soil cropped for potatoes in New Brunswick, Canada, and was determined to be a dominant culturable denitrifier, accounting for 26% of the denitrifiers isolated from that soil (10).

The main factors that control denitrification in the environment include the availability of a substrate (i.e., nitrate or nitrite), oxygen limitation, the availability of carbon, and the presence of denitrifying microorganisms (23). Under oxygen-limited conditions, nitrate acts as the terminal electron acceptor in place of oxygen in a stepwise process that yields the main denitrification products N_2O and N_2 gas and generates ATP via the electron transport chain. Hence, nitrate is an excellent choice as a parameter to study the effects on denitrification gene expression (13, 23, 27).

Denitrification in pure culture has been studied previously through biochemical measurements of changes in the substrate (nitrate or nitrite) and denitrification product (N_2O) (7, 22, 24). Denitrification gene expression was also studied to understand gene regulation (1, 13) and the effect of environmental factors on expression (2–4, 15, 24, 27). However, relationships between population dynamics and denitrification activity in terms of N_2O emissions have not been studied. Recently, Philippot and Hallin (20) identified the need for additional information on the relationships among microbial diversity, density, and activity (i.e., N_2O emissions) in the environment.

The goal of our research is to understand the abundance and activity of denitrifier bacteria as a controlling factor for denitrification and N_2O emission. As a first step, this research focuses on measuring nitric oxide reductase (*cnorB*) gene expression in *P. mandelii* and N_2O emissions from denitrification with and without a nitrate substrate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. mandelii* strain PD30 was cultured in Bacto tryptic soy broth (TSB) medium (Difco; Becton, Dickinson and Company, Sparks, MD) at 30°C and maintained on tryptic soy agar plates at 4°C for short-term use and at −80°C in 15% (wt/vol) glycerol for long-term storage.

Experimental setup to measure denitrification under anaerobic conditions. *P. mandelii* PD30 was aerobically grown by inoculating a single colony into 50 ml of TSB medium in a 125-ml flask that was shaken overnight at 150 rpm in a 30°C incubator shaker (New Brunswick Co. Inc., NJ). The culture was centrifuged at 3,600 × *g* for 10 min at 25°C and resuspended in 100 ml of sterile phosphate-buffered saline (PBS). The *P. mandelii* culture was shaken aerobically for 1 h in PBS and centrifuged for 10 min, and the pellet was resuspended in 2 ml of PBS. The PBS step was performed to remove residual carbon sources and to remove traces of metabolites that may have accumulated in the TSB medium and may influence gene expression. Each set of conditions used in this study for the growth of *P. mandelii* cultures is referred to herein as a treatment. Treatments were TSB medium with nitrate and TSB medium without nitrate. Nitrate treat-

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ments were supplemented with potassium nitrate at 0.1% (wt/vol). Each treatment was set up in triplicate, and the experiment was repeated twice. The bacterial culture in PBS was used to inoculate 65 ml of sterile TSB medium to an optical density at 600 nm (OD_{600}) of 0.1 as measured by a spectrophotometer (Amersham Biosciences Ultrospec 3100 Pro; Biochrom Ltd., Cambridge, England), which corresponds to about 10^6 cells per ml of culture. The flasks were sealed using Suba-Seals, the flask atmospheres were evacuated using a vacuum pump, and the flasks were then flushed with He twice to ensure anaerobic conditions. The atmosphere was adjusted to 10% (vol/vol) acetylene–90% helium by withdrawing 10% of the headspace volume after purging and replacing it with an equivalent volume of 100% acetylene. Acetylene blocks the reduction of N_2O to N_2, allowing for the measurement of denitrification via the accumulation of N_2O in the flask.

The flasks were incubated for 24 h in a 30°C incubator with shaking at 85 rpm. Each flask was sampled at 0 h prior to substrate addition, hourly to 6 h, and at 24 h. Each sampling included removing a 3-ml aliquot of the culture to analyze gene expression and concentrations of nitrate and nitrite and a 12-ml headspace gas aliquot to analyze the concentration of N_2O in the headspace. Immediately after gas sampling, a gas mixture (10% acetylene–90% helium) was added in order to maintain the atmospheric pressure in the flask.

A 1-ml aliquot of the culture was used to monitor bacterial growth using OD and then centrifuged for 2 min, and the supernatant was stored at −20°C for nitrate and nitrite analysis. Frozen supernatant samples were analyzed for nitrate-N and nitrite-N concentrations at the Soil and Nutrient Laboratory (Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada). The remaining 2-ml culture aliquot was added to 4 ml of Bacteria Protect reagent (Qiagen Inc., Mississauga, Ontario, Canada), processed as recommended by the manufacturer, and then stored at −20°C for future RNA isolation. The 12-ml headspace gas sample was injected into evacuated Exetainers for subsequent N_2O analysis. The analysis of headspace gas for N_2O was performed by gas chromatography as described previously (11). Calculated N_2O values were corrected for dissolved N_2O in the flask and for changes in pressure and gas concentration attributed to sampling.

Experimental setup to measure denitrification under aerobic conditions. Flasks were set up as described above using an overnight *P. mandelii* culture that was centrifuged, resuspended, and shaken in PBS for 1 h. The cells were subsequently used to inoculate triplicate flasks of TSB containing 0.1% (wt/vol) potassium nitrate to an OD_{600} of 0.1. The mouths of the flasks were covered with sterile foam plugs, and the flasks were incubated aerobically at 30°C with shaking at 85 rpm. Sampling was performed at 0, 3, and 6 h for RNA extraction, followed by the quantification of *cnorB* gene expression as described above.

Total RNA extraction and genomic DNA isolation. Total RNA was isolated using the RNeasy mini kit (Qiagen Inc., Mississauga, Ontario, Canada) with a lysozyme pretreatment at 800 μg/ml, and DNase I digestion was performed using the on-column option. Genomic DNA to be used as a positive control in all subsequent quantitative reverse transcription-PCRs was isolated from *P. mandelii* cells as outlined in the DNeasy tissue kit protocol (Qiagen Inc., Mississauga, Ontario, Canada). RNA and genomic DNA were quantified using Ribogreen RNA quantitation reagent (Molecular Probes, Eugene, OR) and the Quant-iT PicoGreen double-stranded DNA assay kit (Molecular Probes, Eugene, OR), respectively.

Gene expression quantification. Gene expression quantification was performed on the Bio-Rad iCycler iQ detection system (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Previously designed primers targeting the *cnorB* region were used (11). One-step quantitative PCR was performed with 50 ng of total RNA template, 12.5 μl of 2× master mix from the Qiagen QuantiTect Sybr green reverse transcription-PCR kit, 100 nM forward primer (5′-CATGGCGC TGATAACGGG-3′) and 200 nM reverse primer (5′-CTTIACCATGCTGAAG GCG-3′), and 0.25 μl of reverse transcriptase enzyme in a final volume of 25 μl. Thermal cycling conditions were as follows: 30 min at 50°C and 13 min at 95°C, followed by 40 repeats of 15 s at 94°C, 30 s at 60°C, and 30 s at 80°C. Data collection was performed during each annealing phase. During each run, standard dilutions of digested plasmid carrying a copy of the *cnorB* gene cloned into the pGEM-T vector were included to allow for gene expression quantification. Copy numbers of plasmid standards were calculated directly from the concentration and length (in base pairs) of the extracted plasmid DNA (10). For 16S rRNA gene amplification, previously designed primers developed by López-Gutiérrez et al. (16) were adopted. One-step quantitative PCR was performed with 2.5 ng of total RNA template, 12.5 μl of 2× master mix from the Qiagen QuantiTect Sybr green reverse transcription-PCR kit, 200 nM forward primer (5′-CCTACGGGAGGCAGCAG-3′) and 200 nM reverse primer (5′-ATT CCG CGGCTGGCA-3′), and 0.25 μl of reverse transcriptase enzyme in a final volume of 25 μl. Thermal cycling conditions were as follows: 30 min at 50°C and 15 min at 95°C, followed by 35 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Data collection was performed at 72°C. For each run, an external standard curve was generated using a previously cloned copy of the 16S rRNA gene (10), and copy numbers of plasmid standards were calculated as described earlier (11). In each analysis, a negative control (no template) and a positive control (*P. mandelii* PD30 genomic DNA), as well as an RNA sample without reverse transcriptase added (to indicate genomic DNA contamination), were included. Gene expression variability within flasks, as well as among flasks, was evaluated using three flasks as described in "Experimental setup to measure denitrification under anaerobic conditions" above, and culture aliquots were obtained in triplicate from each flask at the 2-h time point. The aliquots were treated with Bacteria Protect reagent and subjected to total RNA isolation, followed by quantitative reverse transcription-PCR.

Experimental setup to measure dissolved oxygen in aerobic cultures. An experiment was devised to measure the dissolved oxygen content in a culture of aerobically grown cells to determine the amount of time required for the culture to become anaerobic. *P. mandelii* PD30 was aerobically grown in 20 ml of TSB medium and shaken overnight at 150 rpm at 30°C. The next day, the cell biomass was pelleted, the pellet was resuspended in 3 ml, and 50-μl aliquots were inoculated in triplicate into 50 ml of fresh TSB medium in 125-ml flasks. The dissolved oxygen content in the *P. mandelii* culture was measured using an oxygen meter probe (model 55 handheld dissolved oxygen and temperature system; YSI, Yellow Springs, OH) that was disinfected between measurements. *P. mandelii* cell growth in a different set of TSB flasks (triplicates) was measured to avoid potential contamination due to the use of the oxygen probe. Each flask was fitted with foam plugs and shaken at 150 rpm at 30°C. The dissolved-oxygen meter was calibrated in aerated water held at 30°C. Dissolved-oxygen and cell density (OD_{600}) measurements were taken at 0, 3, 6, 9, and 12 h.

Statistical methods. Data from both trials were pooled, and *cnorB* gene expression data and the dissolved oxygen contents, as well as nitrous oxide emission data, were tested for normality using the UNIVARIATE procedure of SAS software (version 8; SAS Institute Inc., Cary, NC) and log transformed when required. Analysis of variance was performed using the MIXED procedure of SAS software for a two-factor factorial arrangement of treatments in a completely randomized design, and the means were compared by LSMEANS ($P < 0.05$) with Tukey's adjustment. Treatment means and standard errors presented in figures were calculated from untransformed data.

RESULTS AND DISCUSSION

Our research examined the effects of the presence of nitrate on *cnorB* gene expression in pure cultures of *P. mandelii* as a first step in understanding changes in the abundance of denitrification transcripts over time and in exploring relationships between denitrifier activity and N_2O emissions. The induction of *cnorB* gene expression in *P. mandelii* occurred in both treatments between 0 and 1 h, as indicated by an increase in transcript numbers (Fig. 1). The pattern of *cnorB* gene expression was not affected by nitrate treatment for the first 3 h following the establishment of anaerobic conditions. The levels of *cnorB* gene expression measured in the treatment with nitrate were significantly higher than those in the treatment without nitrate from 4 to 24 h (Fig. 1). At 4 h, nitrate-treated *P. mandelii* cells had a 203-fold increase in *cnorB* gene expression compared with that at 0 h, whereas untreated cells had a 2-fold induction over the same time period. *cnorB* gene expression in untreated cells subsequently declined to basal levels of 1.6×10^6 gene transcripts/μg of RNA at 6 h. In contrast, *cnorB* gene expression in nitrate-treated cells was consistently measured at about 1.2×10^8 transcripts/μg of RNA, even after 6 h. A previous study using Northern blot hybridization reported that the transient expression of the *nirSTB* and *norCB* operons in *Pseudomonas stutzeri* occurred for 30 min in response to a decrease in oxygen levels and the absence of nitrate (14). These results suggest that oxygen depletion acts as an initial trigger for *cnorB* gene expression in *P. mandelii* and *P. stutzeri*. Alternatively,

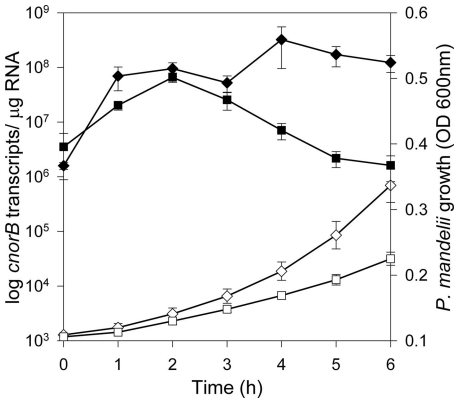

FIG. 1. *cnorB* gene expression in *P. mandelii* cells grown under anaerobic conditions in TSB medium supplemented with 0.1% potassium nitrate (◆) or in TSB medium in the absence of nitrate (■). *P. mandelii* cell growth in TSB medium with nitrate (◇) or without nitrate (□) was evaluated using OD_{600} measurements. Error bars are ±1 standard error of the mean (SEM; $n = 6$). Values were calculated from the lines of best fit described by the following linear equations: $y = -3.509x + 41.335$ ($r^2 = 0.990$) for the first experiment and $y = -3.340x + 37.516$ ($r^2 = 0.990$) for the second experiment.

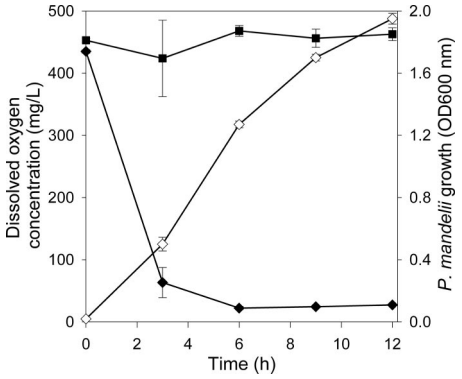

FIG. 2. Dissolved oxygen concentrations in TSB medium in the presence (◆) and absence (■) of *P. mandelii* cells. *P. mandelii* cell growth was evaluated using OD_{600} measurements (◇). Error bars are ±1 SEM ($n = 3$).

only trace amounts of nitrate or other N oxides produced from nitrate are sufficient to induce *cnorB* or *nirS* gene expression in *P. mandelii* and *P. stutzeri*. It cannot be ruled out that *P. mandelii cnorB* gene induction might have been due to a general stress response even if the 16S rRNA gene expression was constant under the same conditions (data not shown). Nonetheless, these results indicate that the regulation of the *cnorB* gene may be independent of nitrate or other N oxides as a trigger for gene expression in *P. mandelii*. Both the presence of nitrate or N oxides produced from nitrate and anaerobic conditions were required to sustain the expression of the *cnorB* gene in *P. mandelii* past 3 h of incubation. Anaerobiosis and nitrate or N oxides produced from nitrate are required for high-level denitrification gene expression in other denitrifiers, including *P. stutzeri*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* (3, 14, 15, 19).

The basal levels of about 10^6 *cnorB* gene transcripts/µg of RNA measured immediately after the commencement of anaerobic conditions in both treatments were attributed to the anaerobic state of the culture prior to inoculation and the subsequent induction of denitrification. Dissolved-oxygen measurements confirmed that the aerobic growth of *P. mandelii* cells in TSB caused a decline in dissolved oxygen concentrations from 435 to 63 mg/liter in 3 h as the cells were rapidly growing and dividing during the exponential phase of growth and a reduction to a very low concentration of 25 mg/liter for the remainder of the incubation (Fig. 2). This finding suggests that oxygen limitation may occur in aerobic preculture, which may influence the level of gene expression in subsequent denitrification experiments. Therefore, precultures should ideally be grown under the most aerobic conditions possible; however, further culture manipulation, including centrifugation, can induce denitrification genes.

Testing for aerobic denitrification at 3 and 6 h indicated an absence of *P. mandelii cnorB* gene expression even in the presence of nitrate. Similarly, the absence of induction of all four denitrification genes in *P. fluorescens* (19) and the *nar*, *nir*, and *nos* genes in *P. denitrificans* (3) under aerobic conditions was observed. In our aerobic system, oxygen levels dropped rapidly at 3 h; however, oxygen limitation commenced at about 6 h, after which we would expect the induction of the *cnorB* gene of *P. mandelii*.

Cumulative N_2O emissions in the nitrate treatment were first detected at 2 h at about 0.6 µmol, increased to 7.8 µmol after 6 h (Fig. 3), and subsequently increased to 101 µmol at 24 h (data not shown). The nitrate concentration in the medium remained nonlimiting (average of 172 mg of NO_3-N/liter over the measurement period), whereas nitrite concentrations remained low (below 8.5 mg of NO_2-N/liter) for the first 6 h of the experiment. Similarly, Carlson and Ingraham (7) showed that *P. stutzeri* has no detectable nitrite accumulation after 6 h of incubation in liquid cultures. In the present study, the absence of nitrate, no accumulation of nitrate or nitrite, and insignificant N_2O emissions were found, although *P. mandelii cnorB* gene expression was detected in the first 3 h of incubation, indicating that denitrification could not actually occur. Cumulative N_2O production levels, although detectable in the

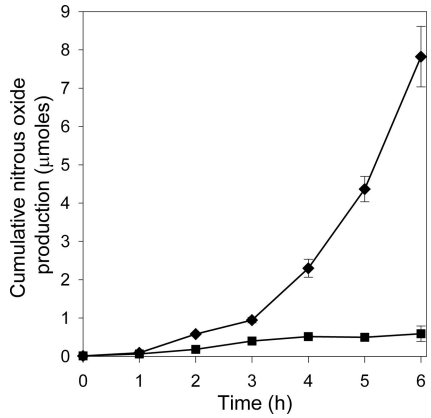

FIG. 3. Nitrous oxide production in cultures of *P. mandelii* cells grown in the presence of 0.1% potassium nitrate (◆) and in the absence of potassium nitrate (■). Error bars are ±1 SEM ($n = 6$).

nitrate-negative treatment, did not differ significantly over time and remained below 0.65 μmol over a 24-h period.

The *cnorB* gene expression may have been induced by the establishment of anaerobic conditions or, alternatively, by a stress-related response during the initiation of the experiment. It was shown previously that the expression of the 16S rRNA gene in *Staphylococcus epidermis* is more sensitive than the expression of other housekeeping genes in response to changing environments (25). To evaluate this possibility, the expression of the 16S rRNA housekeeping gene was employed to determine if it was also induced under similar conditions. The analysis of results demonstrated insignificant changes in 16S rRNA gene expression levels over time, which leads to the conclusion that *cnorB* gene induction was due to denitrification conditions present in the flasks. Accounting for the cell number and the RNA yield, as well as the content of about 10,000 ribosomes in each bacterial cell, the calculation of the number of 16S rRNA gene transcripts per microgram of RNA gave the expected result of approximately 1.7×10^{13} transcripts (9). Although the expression of the 16S rRNA gene was not induced, it is possible that other stress-related genes not tested in this research may have been induced. These results support the hypothesis that the increase in *cnorB* gene expression could be attributed to conditions that are responsible for inducing denitrification, i.e., anaerobiosis and/or a high level of nitrate.

This research was initiated with the objective of evaluating *cnorB* gene expression and N_2O activity in *P. mandelii* under defined conditions in an effort to understand the effect of nitrate on *cnorB* gene expression and N_2O emissions. In this study, limitations in oxygen concentration seemed to be the initial trigger for the expression of the *cnorB* gene, whereas the presence of nitrate or N oxides produced from nitrate allowed for the continued expression of this gene. Elevated levels of *cnorB* transcripts, combined with increasing N_2O levels, provide experimental evidence for a link between the number of gene copies and actual N_2O emissions.

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