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Effect of pH and Temperature on Denitrification Gene Expression and Activity in *Pseudomonas mandelii*[▽]

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Pseudomonas mandelii **liquid cultures were studied to determine the effect of pH and temperature on denitrification gene expression, which was quantified by quantitative reverse transcription-PCR. Denitrification was measured by the accumulation of nitrous oxide (N_2O) in the headspace in the presence of acetylene. Levels of gene expression of *nirS* and *cnorB* at pH 5 were 539-fold and 6,190-fold lower, respectively, than the levels of gene expression for cells grown at pH 6, 7, and 8 between 4 h and 8 h. Cumulative denitrification levels were 28 μmol, 63 μmol, and 22 μmol at pH 6, 7, and 8, respectively, at 8 h, whereas negligible denitrification was measured at pH 5. *P. mandelii* cells grown at 20°C and 30°C exhibited 9-fold and 94-fold increases in levels of *cnorB* expression between 0 h and 2 h, respectively, and an average 17-fold increase in levels of *nirS* gene expression. In contrast, induction of *cnorB* and *nirS* gene expression for *P. mandelii* cells grown at 10°C did not occur in the first 4 h. Levels of cumulative denitrification at 10 h were 6.6 μmol for *P. mandelii* cells grown at 10°C and 20°C and 30 μmol for cells grown at 30°C. Overall, levels of *cnorB* and *nirS* expression were relatively insensitive to pH values over the range of pH 6 to 8 but were substantially reduced at pH 5, whereas gene expression was sensitive to temperature, with induction and time to achieve maximum gene expression delayed as the temperature decreased from 30°C. Low pH and temperature negatively affected denitrification activity.**

Denitrification is a respiratory microbiological process in which nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to gaseous nitric oxide (NO), nitrous oxide (N_2O), or molecular nitrogen (N_2) under oxygen-limited conditions (33). Denitrification can result in substantial gaseous losses of N, an important plant nutrient, from agricultural fields (7, 14). N_2O depletes stratospheric ozone and contributes to global warming (28). An understanding of the environmental controls on denitrifier activity is essential for comprehending the spatial and temporal regulation of denitrification within agricultural production systems.

Denitrification is carried out by various microorganisms belonging to several genera and species of bacteria (3, 4, 27, 33). The strain of *Pseudomonas mandelii* used in this study was a dominant culturable denitrifier isolated from an agricultural field in a potato production system in New Brunswick, Canada (4).

Several environmental factors control the process of denitrification. These include oxygen availability, substrate availability (i.e., NO_2^- and NO_3^-), pH, temperature, and the abundance and species of denitrifiers. The availability of a substrate, the absence of oxygen, and the presence of active denitrifiers are the main controlling factors (24). However, pH and temperature also play a role in influencing denitrifier growth, metabolism, denitrification gene expression, and, subsequently, denitrification rate. A recent review of environmental controls on denitrifying communities and denitrification rates identified the need to link pH and temperature with denitrification gene expression as a step toward an understanding of the relationship between denitrifier community composition and function (29). Most research related to the effect of temperature and pH on denitrification has focused on denitrification rates in soils (9, 15, 16, 19, 22). Several studies have established that denitrification rates tend to decrease at low soil pH values (15, 16, 20). Parkin et al. (16) previously demonstrated a twofold decrease in denitrification rate and a threefold decrease in denitrification enzyme activity when soil pH decreased from pH 6.02 to 4.08, suggesting that a prolonged exposure to low soil pH selected a denitrifier population that was more adapted to the low-pH environment, and subsequently, N_2O reduction by the acid-tolerant population was insignificant compared to N_2O production rates. Numerous studies have demonstrated that soil pH changed the concentrations of denitrification intermediates and products (8, 12, 19). There are several studies that have investigated the effect of temperature on denitrification rates in soil. Stanford et al. (22) previously established that within a limited temperature range of 15°C to 35°C, the temperature coefficient of denitrification (Q_{10}), was about 2. This value translates to a twofold increase in denitrification for every 10°C increase in temperature in soil. Fischer and Whalen (9) also evaluated the capability of a soil microbial community to denitrify in response to temperature and calculated Q_{10} values of 1.6 and 2.8 in the temperature intervals of 7°C to 20°C and 20°C to 30°C, respectively. Holtan-Hartwig et al. (10)

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previously suggested that low temperature (0°C) exerts a challenge to denitrifying communities and affects N_2O breakdown more than the reduction of other *N*-oxides of the denitrification processes.

There is limited research on the effect of pH and temperature on denitrification gene expression in pure cultures. Most studies have focused on identifying the optimal pH for denitrification in terms of enzyme activity, denitrification intermediates and products (11, 26), and induction of mRNA (1). In pure cultures of *Pseudomonas* species, the optimum pH for denitrification, based on denitrification activity, was found to be in the range of pH 7.0 to 7.5 (11, 25). The accumulations of denitrification intermediates in pure cultures of *Paracoccus denitrificans* differed at acidic and alkaline pH values (26). The suboptimal pH of 6.8 inhibited denitrification activity in *P. denitrificans* but did not affect mRNA induction (1). There is no research that has studied the effect of temperature on denitrification gene expression.

This study determined the effect of pH and temperature on the expression of the *nirS* and *cnorB* genes and denitrification activity (i.e., N_2O emissions in the presence of acetylene) in a pure culture of *P. mandelii*. It was hypothesized that pH and temperature would differentially affect *nirS* and *cnorB* gene expression and decrease denitrification activity under suboptimal conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. mandelii* strain PD30 was cultured in 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. For long term use, *P. mandelii* cultures were stored with 15% (wt/vol) sterile glycerol at −80°C.

Experimental setup to test for pH effects. *P. mandelii* PD30 cultures were grown aerobically by inoculating a single colony into 200 ml of TSB medium in a 500-ml Erlenmeyer flask and shaking overnight at 150 rpm and 30°C (New Brunswick Co., Inc., NJ). The culture was centrifuged at 3,600 × *g* for 10 min at 25°C, resuspended in 200 ml of sterile phosphate-buffered saline (PBS), and shaken aerobically for 1 h in PBS to metabolize carbon sources and traces of metabolites that accumulated in the TSB medium which may influence gene expression. The culture was centrifuged for 10 min and resuspended in 4 ml of PBS. TSB medium was prepared, and the pHs were adjusted to pH 5.00, 6.00, 7.00, and 8.00, respectively. The media were prepared and autoclaved, and the pHs were verified to be 5.06, 6.04, 6.97 and 7.71, respectively, before inoculation.

A randomized complete block design with four treatments and three replicates, and with repeated sampling over time, was used to test for the effect of pH on denitrification gene expression and activity in *P. mandelii*. Treatments were four levels of pH (pH 5, 6, 7, and 8), and sampling was done every hour up to 8 h, followed by an additional sampling after 24 h. The experiment was conducted twice.

Flask setup and induction of denitrification conditions were performed as previously described (18). A 0-h sample for all treatments and replicates was obtained from *P. mandelii* cultures by removing a 3-ml culture aliquot from each flask to analyze gene expression and concentrations of NO_3^- and NO_2^- and by removing a 12-ml headspace gas aliquot for the N_2O concentration prior to the addition of substrate. After sampling, a sufficient volume of gas mixture (10% acetylene–90% helium) was added to maintain the flask at atmospheric pressure. Potassium nitrate was added at a final concentration of 0.1% (wt/vol) for all treatments. The flasks were incubated for 24 h in a 30°C incubator with shaking at 85 rpm.

At each sampling, optical density (OD) measurements were obtained, NO_3^- and NO_2^- analyses were performed, and samples were prepared for RNA extraction as previously described (18). The 12-ml headspace gas sampled at each time point was injected into evacuated Exetainers (Labco Limited, United Kingdom) for subsequent N_2O and carbon dioxide (CO_2) analyses. Cumulative CO_2 accumulation was used as a measure of respiration activity.

Experimental setup to test for temperature effects. A randomized complete block design with three treatments and three replicates, and with repeated sampling over time, was used to test for the effect of temperature on denitrification gene expression and activity in *P. mandelii*. Treatments consisted of three temperatures (10°C, 20°C, and 30°C), and sampling was conducted hourly to 10 h, followed by an additional sampling at 24 h. The experiment was conducted twice. Three *P. mandelii* PD30 cultures were established as described above and incubated overnight at 10°C, 20°C, or 30°C (Innova 4240 illuminated refrigerated incubator shaker; New Brunswick Scientific). The *P. mandelii* cultures were shaken aerobically for 1 h in PBS at 10°C, 20°C, or 30°C, as described above. The cells were inoculated in triplicate into TSB (pH 7.0) to an OD at 600 nm of 0.1. Flasks were sealed and evacuated, and anaerobic conditions were established as described previously (18). Potassium nitrate was added at a final concentration of 0.1% (wt/vol) for all treatments. The flasks were incubated at 10°C, 20°C, or 30°C with shaking at 85 rpm for 24 h. At each sampling, a 3-ml culture aliquot and a 12-ml headspace gas sample were withdrawn using sterile syringes. OD measurements and NO_3^-, NO_2^-, and N_2O analyses were performed as described below.

Design of *P. mandelii nirS* quantitative PCR primers. The sequence of the taxonomically well-defined *P. mandelii nirS* gene (GenBank accession number DQ518190) was aligned with similar *nirS* genes available for several *Pseudomonas* spp. including *P. lini* (accession number DQ518197), *P. migulae* (accession number DQ518195), and *Pseudomonas* spp. isolated from soil (accession numbers DQ518187, DQ518196, DQ518186, and DQ518185) and from uncultured clones (accession numbers AJ811516 and AJ811504) to find unique, conserved regions using MegAlign (Lasergene 7). *P. lini* and *P. migulae* have been isolated from soil (bulk and rhizosphere) and have been shown to be taxonomically close to *P. mandelii* (6). Primers were selected based on standard conditions for real-time quantitative PCR using PrimerSelect (Lasergene 7). The specificities of the primers were tested and verified with *P. mandelii* genomic DNA in a PCR. The product was sequenced and submitted to BLAST (NCBI). The PCR product had a 100% sequence identity to *P. mandelii nirS* (accession number DQ518190). The product was cloned the using TOPO kit (Invitrogen, Burlington, Ontario, Canada), sequenced, and verified using BLAST analysis (S. Henderson, unpublished data).

Gene expression quantification. The RNeasy minikit (Qiagen Inc., Mississauga, Ontario, Canada) was used for total RNA isolation. Modifications to the extraction protocol were described previously (18). RNA was quantified using Ribogreen RNA quantitation reagent (Molecular Probes, Eugene, OR). Gene expression quantification was performed using the Bio-Rad iCycler iQ detection system (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Previously designed primers from our research group targeting the *cnorB* region were used (5). Conditions for one-step quantitative PCR targeting the *P. mandelii cnorB* gene were the same as those described previously (5).

The level of *P. mandelii nirS* gene expression was quantified using one-step quantitative PCR with 50 ng of total RNA template, 12.5 μl of 2× master mix from the Qiagen QuantiTect SYBR green reverse transcription-PCR kit, 300 nM of forward primer (5′-ACCGCGGCCAACAACTCCAACA-3′), 500 nM of reverse primer (5′-CCGCCCTGGCCCTTGAGC-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 40 repeats of 15 s at 95°C, 30 s at 68.4°C, 30 s at 72°C, and 15 s at 83°C. Data collection was performed during the last step of each cycle at a temperature of 83°C. In a 40-cycle PCR, a no-template control was undetected.

An external standard curve was constructed using linear regression to obtain a line of best fit for the quantification of *nirS* gene copy number and transcripts. *P. mandelii nirS* primers were used to produce *nirS* PCR products that were then cloned into TOPO according to the manufacturer's instructions (Invitrogen, Burlington, Ontario, Canada). Plasmid DNA was extracted using a plasmid minikit (Qiagen Inc., Mississauga, Ontario, Canada). The plasmid was linearized by digestion with SacI (Roche, Laval, Quebec, Canada) and heat shocking to deactivate the enzymes. The linearized plasmid was quantified using Picogreen (Molecular Probes, Eugene, OR), and the size of the *nirS* insert was used to calculate the copy number. The curve was linear over a dilution range of 10^{-3} to 10^{-9} and sensitive to at least 10 copies of *nirS* per reaction.

During each run, standard dilutions of digested plasmid carrying a copy of the *nirS* gene cloned into the PCR2.1-TOPO vector were included to allow for gene quantification. A positive control consisting of *P. mandelii* genomic DNA isolated as outlined in the DNeasy tissue kit (Qiagen Inc., Mississauga, Ontario, Canada) and quantified by the Quant-iT Picogreen dsDNA assay kit (Molecular Probes, Eugene, OR) was used in each quantitative reverse transcription-PCR run.

NO_3^-, NO_2^-, N_2O, and CO_2 analyses. Frozen supernatant samples were analyzed for NO_3^--N and NO_2^--N concentrations at the Soil and Nutrient

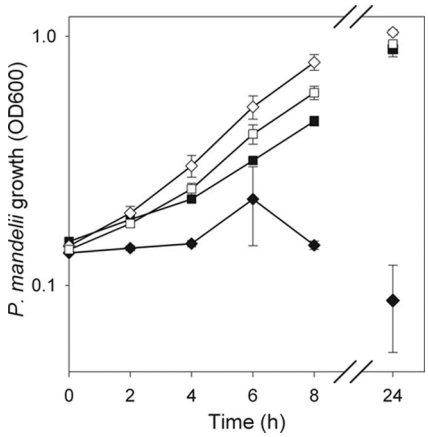

FIG. 1. *P. mandelii* growth in TSB medium at pH 5 (◆), pH 6 (■), pH 7(◇), or pH 8 (□) as measured using OD measurements obtained at 600 nm. Error bars are ±1 standard error of the mean (SEM) ($n = 6$).

Laboratory (Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada). N_2O and CO_2 analyses of headspace gas were performed by gas chromatography as previously described (4). Calculated values of cumulative denitrification (i.e., cumulative N_2O emissions in the presence of acetylene) and respiration (i.e., cumulative CO_2 emissions) were corrected for dissolved N_2O or CO_2 in the flask and for changes in pressure and gas concentrations attributed to sampling.

Statistical methods. All parameters were tested for normality using the univariate function in the SAS System for Windows (version 8; SAS Institute Inc., Cary, NC), and a log transformation was performed when required. Analysis of variance was performed using the mixed procedure of SAS. The statistical model treated duplicate experiments as blocks in order to pool data from the two experiments, and the repeated function was used to account for repeated sampling of flasks over time. Where there were significant differences between pH and temperature treatments over time, treatment means were compared by LSMEANS ($P < 0.05$) with Tukey's adjustment. Relationships among measured parameters were explored using Pearson correlations on a per-flask basis. Treatment means and standard errors presented in figures were calculated from nontransformed data.

RESULTS

Effects of pH. *P. mandelii* cultures grown at pH 5 had no significant change in cell density from 0 to 24 h and showed an average OD at 600 nm of 0.15 (Fig. 1). There was no significant increase in cell density in *P. mandelii* cultures grown at pH 6, 7, and 8, between 0 and 4 h, with the exception of pH 7, for which the cell density significantly increased between 2 and 4 h. The cell density subsequently increased rapidly after 4 h to reach averages of 0.27 for *P. mandelii* cultures grown at pH 5 and pH 6 and 0.46 for *P. mandelii* cultures grown at pH 7 and pH 8, at 6 h. At 8 h, all four treatments were significantly different from one another, with cell densities decreasing in the following order: pH 7 > pH 8 > pH 6 > pH 5. At 24 h, cell density was significantly increased compared to that at 8 h in *P. mandelii* cultures grown at pH 6 (OD of 0.89) and pH 7 and 8 (average OD of 0.99). In all four treatments, the growth medium became slightly more alkaline at 24 h, with pH values of 5.3, 6.9, 7.3, and 7.8, respectively, than at the beginning of the experiment, most likely due to the accumulation of metabolic by-products.

Gene expression levels of *nirS* and *cnorB* were differentially affected by pH treatment at different time points in *P. mandelii* cultures. At pH 5, *P. mandelii* cultures demonstrated a ninefold increase in the level of *nirS* expression at 2 h, with an average of 5.9×10^7 transcripts/μg RNA, compared with that at the start of the incubation (Fig. 2A). Similarly, at pH 5, there was a 16-fold increase in the level of *cnorB* expression at 2 h, where the number of transcripts increased from 3.6×10^4 transcripts/μg RNA to 6.0×10^5 transcripts/μg RNA (Fig. 2B). Gene expression levels for both *nirS* and *cnorB* in cultures grown at pH 5 subsequently declined at 4 h and were unchanged to the 24-h time point. In contrast, *P. mandelii* cultures grown at pH 6, 7, and 8 were not significantly different from one another for both *nirS* and *cnorB* genes and exhibited a 171-fold induction of the *nirS* gene from 0 h to 2 h, with an average of 3.6×10^9 transcripts/μg RNA at 2 h. For the *cnorB* gene, a 427-fold increase in the level of gene expression at pH

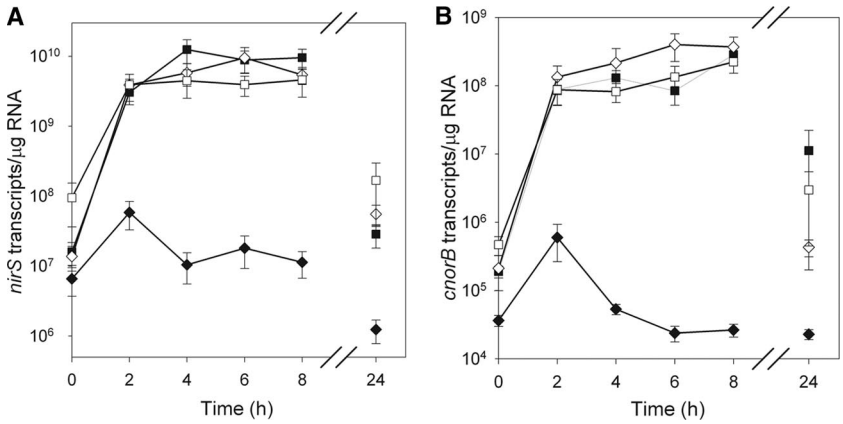

FIG. 2. *nirS* (A) and *cnorB* (B) gene expression in *P. mandelii* cultures grown in TSB medium at pH 5(◆), pH 6 (■), pH 7(◇), or pH 8 (□), supplemented with 0.1% potassium nitrate. Error bars are ±1 SEM ($n = 6$). *nirS* transcript numbers were calculated from the line of best fit described by the linear equations $y = -2.65x + 46.2$ ($r^2 = 0.99$) for the first experiment and $y = -3.01x + 47.6$ ($r^2 = 0.99$) for the second experiment. For *cnorB*, the line of best fit was described by the linear equations $y = -3.27x + 35.6$ ($r^2 = 0.98$) for the first experiment and $y = -2.77x + 34.3$ ($r^2 = 0.99$) for the second experiment.

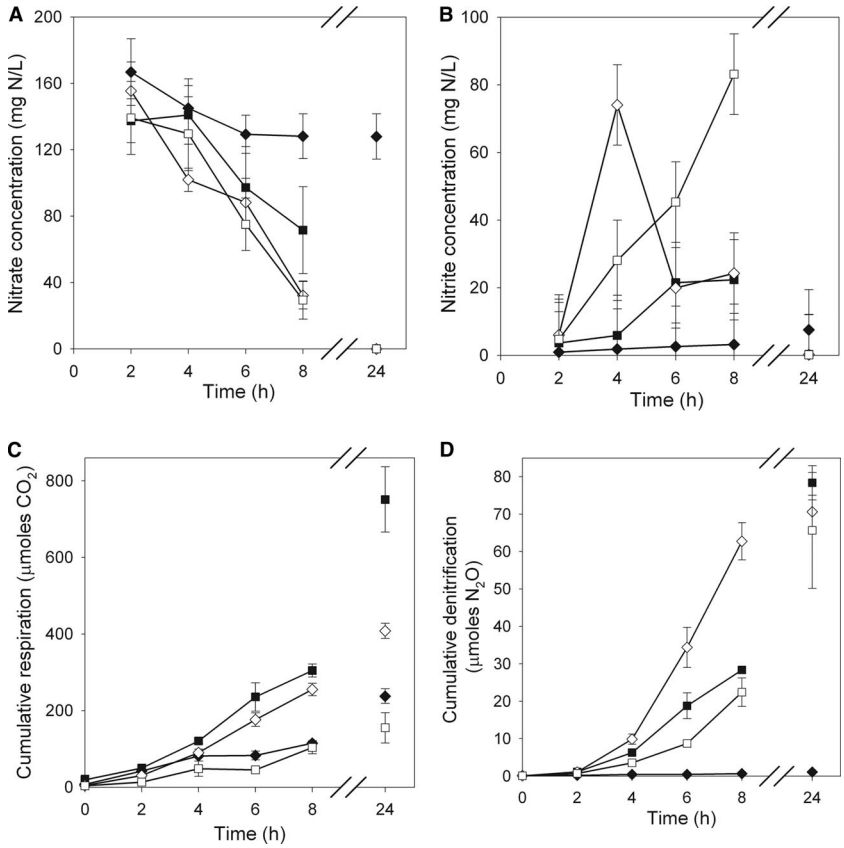

FIG. 3. NO_3^- (A) and NO_2^- (B) concentrations in liquid culture, cumulative respiration (i.e., CO_2 accumulation) (C), and cumulative denitrification (i.e., N_2O accumulation in the presence of acetylene) (D) in the headspace in TSB medium supporting the growth of denitrifying *P. mandelii* cells grown at pH 5(◆), pH 6 (■), pH 7(◇), or pH 8 (□) in the presence of NO_3^-. Error bars are ±1 SEM ($n = 6$).

6, 7, and 8 occurred from 0 to 2 h, where levels of *cnorB* transcripts increased from an average of 2.9×10^5 transcripts/μg RNA to 1.0×10^8 transcripts/μg RNA. *nirS* and *cnorB* gene transcripts in cultures grown at pH 6, 7, and 8 remained unchanged from 2 h to 8 h (averages of 2.2×10^9 transcripts/μg RNA and 1.9×10^8 transcripts/μg RNA, respectively) and then decreased to 8.4×10^7 transcripts/μg RNA and 4.9×10^6 transcripts/μg RNA, respectively, at 24 h.

NO_3^- concentrations were not significantly different among pH treatments at 2 h (Fig. 3A). Although a small amount of nitrate seemed to be reduced at pH 5, there was no significant decrease in the NO_3^- concentration between 2 and 24 h, with an average value of 139 mg NO_3^--N/liter. At 8 h, NO_3^- concentrations declined to 72 mg NO_3^--N/liter for *P. mandelii* cultures grown at pH 6 and 31 mg NO_3^--N/liter for cells grown at pH 7 and 8. By 24 h, NO_3^- had been reduced to negligible concentrations in *P. mandelii* cultures grown at pH 6, 7, and 8.

There was no significant difference in NO_2^- concentrations among time points for the pH 5 and 6 treatments, with average NO_2^- concentrations of 7.7 mg NO_2^--N/liter at pH 5 and pH 6 from 2 to 8 h (Fig. 3B). Maximum values of NO_2^- concentrations were measured at different time points for the treatments at pH 7 and 8. At 4 h, the *P. mandelii* culture grown at pH 7 had significantly higher NO_2^- concentrations (74 mg NO_2^--N/liter) than did cultures grown at pH 5, 6, and 8 (average, 12 mg NO_2^--N/liter). When cultures were grown at pH 8, NO_2^- concentrations significantly increased at 6 h, and cultures had a higher NO_2^- concentration at 8 h than with all other treatments, at 83 mg NO_2^--N/liter. Cultures grown at pH 5, 6, and 7 were not significantly different at 8 h (average, 16.6 mg NO_2^--N/liter), and by 24 h, small concentrations of NO_2^- (average, 2.0 mg NO_2^--N/liter) were measured for all four treatments.

Cumulative respiration did not increase significantly over the first 4 h of the incubation for any treatment (Fig. 3C). Significant increases in the level of cumulative respiration were measured at 6 h for pH 6 and 7 and 24 h for pH 5 and 8. At 8 h, cumulative respiration did not differ between the pH 5 and pH 8 treatments, with an average of 109 μmol, which was significantly lower than that for the pH 6 and 7 treatments, with an average of 280 μmol. The level of cumulative respiration at 24 h in *P. mandelii* cultures grown at pH 6 (751 μmol) was much greater than that at pH 7 (408 μmol). In comparison, the level of cumulative respiration at 24 h for pH 5 and 8 averaged 196 μmol.

Cumulative denitrification was differentially expressed in various pH treatments in *P. mandelii* cultures, and the response varied over time. At 8 h, the level of cumulative denitrification did not differ between the pH 6 and pH 8 treatments, with an average of 25 μmol, which was significantly lower than that of the pH 7 treatment, with an average of 63 μmol (Fig. 3D). The level of cumulative denitrification subsequently increased over

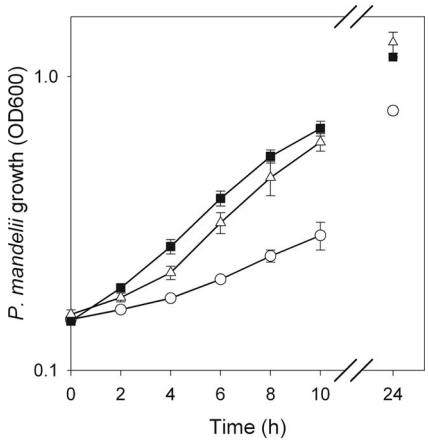

FIG. 4. *P. mandelii* growth in TSB medium at 10°C (○), 20°C (■), or 30°C (△) as measured using OD measurements obtained at 600 nm. Error bars are ±1 SEM ($n = 6$).

time for *P. mandelii* cultures grown at pH 6, 7, and 8, reaching an average value of 72 μmol at 24 h. In contrast, the pH 5 treatment demonstrated negligible cumulative denitrification over time, with an average of 0.52 μmol over the 24-h incubation (Fig. 3D).

The increase in cumulative denitrification over time (Fig. 3D) coincided with a decrease in the NO_3^- concentration (Fig. 3A), except for the pH 5 treatment, where negligible denitrification was measured. The magnitude of the NO_3^- loss was proportional to the increase in NO_2^- at 8 h, which was the time at which significant differences in both NO_3^- and NO_2^- concentrations among treatments were observed. Gene expression at 2 h, the time of maximum expression, and denitrification at 8 h, the time of significant differences in cumulative denitrification, demonstrated correlation constants of 0.47 ($P = 0.0234$) for the *nirS* gene and 0.53 ($P = 0.0092$) for the *cnorB* gene.

Effects of temperature. The density of *P. mandelii* cells grown at 10°C did not significantly increase from 0 to 10 h (average OD of 0.20) and then increased significantly at 24 h (OD of 0.77) (Fig. 4). Cell densities of *P. mandelii* cells grown at 20°C and 30°C demonstrated identical growth patterns; there was no significant change in *P. mandelii* growth from 0 h to 4 h, after which cell growth increased significantly from an average OD of 0.24 at 4 h to an OD of 0.63 at 10 h and to a final OD of 1.2 at 24 h.

A *P. mandelii* culture grown at 10°C demonstrated a small (1.5-fold) increase in the level of *nirS* expression between 0 h and 2 h, and thereafter, the level of *nirS* gene expression increased more rapidly, with 2.5×10^7 transcripts/μg RNA at 4 h and an average of 2.0×10^8 transcripts/μg RNA between 6 and 10 h (Fig. 5A). *P. mandelii* cells grown at 20°C and 30°C demonstrated a 17-fold increase in *nirS* gene expression between 0 and 2 h, reaching an average of 9.4×10^7 transcripts/μg RNA at 2 h, and maintained an average of 3.6×10^8 transcripts/μg RNA between 2 h and 10 h. The induction of *nirS* gene expression was more rapid in *P. mandelii* cells grown at 20°C and 30°C than in cells grown at 10°C; however, by 6 h, there was no significant difference among treatments (Fig. 5A). At 24 h, the 10°C treatment showed significantly higher levels of *nirS* gene expression (5.9×10^8 transcripts/μg RNA) than did the 20°C and 30°C treatments (average of 6.0×10^6 transcripts/μg RNA).

At 10°C, there was a delay in the induction of *cnorB* gene for the first 4 h (Fig. 5B). The level of *cnorB* gene expression then increased to 1.1×10^9 transcripts/μg RNA at 8 h and remained significantly unchanged from 8 h to 24 h, with an average of 1.3×10^9 transcripts/μg RNA. At 20°C, *P. mandelii* cells demonstrated a ninefold increase in the level of *cnorB* gene expression between 0 and 2 h, where the *cnorB* transcript number reached 4.6×10^7 transcripts/μg RNA at 2 h, increased to 2.6×10^9 transcripts/μg RNA at 4 h, and stayed constant until 10 h. The level of gene expression of *cnorB* subsequently declined to 3.2×10^7 transcripts/μg RNA at 24 h. *P. mandelii* cells grown at 30°C demonstrated a 94-fold increase in the level of *cnorB* gene expression between 0 and 2 h, reaching 4.6×10^8 transcripts/μg RNA at 2 h (Fig. 5B). Subsequently, there was no significant change in *cnorB* gene expression levels for the

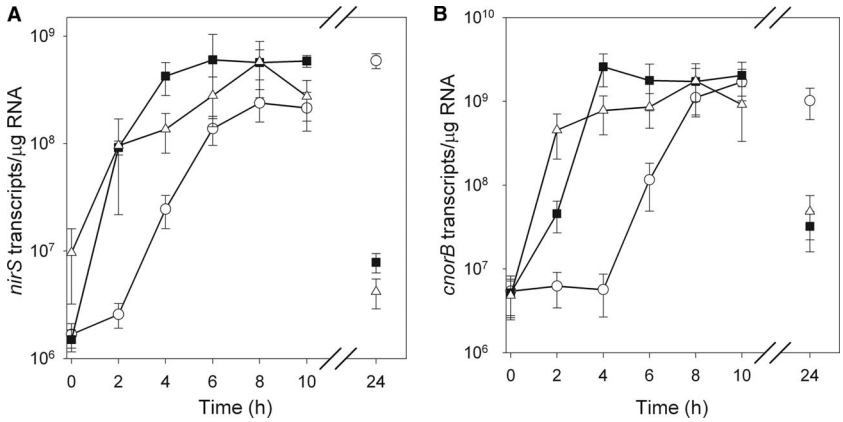

FIG. 5. *nirS* (A) and *cnorB* (B) gene expression in *P. mandelii* cultures grown in TSB medium at 10°C (○), 20°C (■), or 30°C (△), supplemented with 0.1% potassium nitrate. Error bars are ±1 SEM ($n = 6$). *nirS* transcript numbers were calculated from the line of best fit described by the linear equations $y = -2.90x + 44.1$ ($r^2 = 0.99$) for the first experiment and $y = -3.39x + 46.0$ ($r^2 = 0.99$) for the second experiment. For *cnorB*, the line of best fit was described by the linear equations $y = -4.20x + 44.4$ ($r^2 = 0.99$) for the first experiment and $y = -3.27x + 38.8$ ($r^2 = 0.99$) for the second experiment.

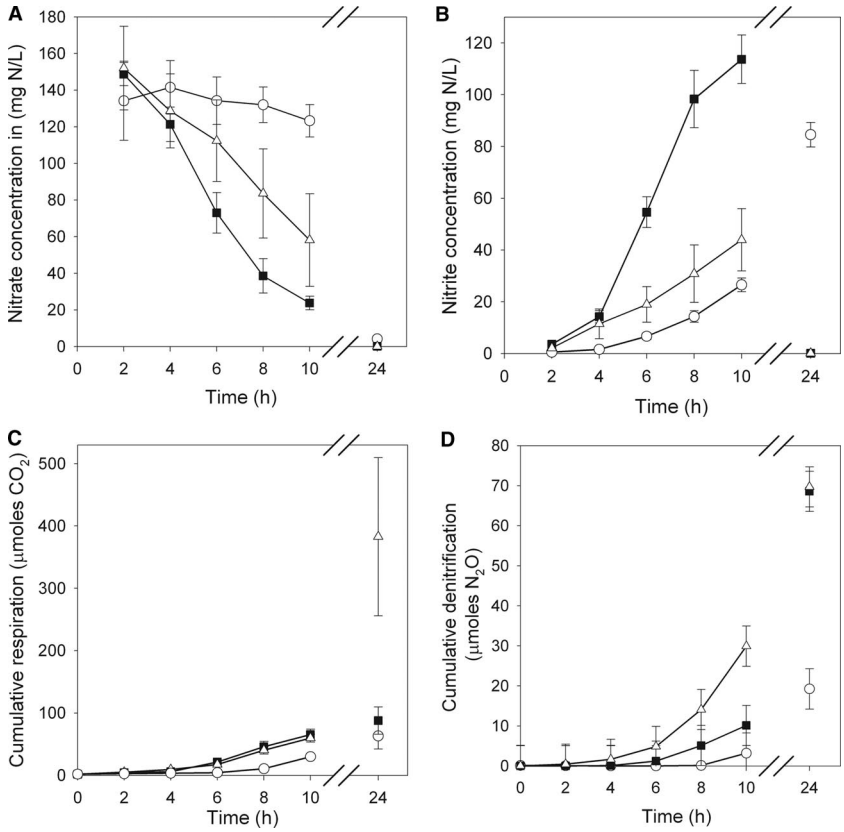

FIG. 6. NO_3^- (A) and NO_2^- (B) concentrations in liquid culture, cumulative respiration (i.e., CO_2 accumulation) (C), and cumulative denitrification (i.e., N_2O accumulation in the presence of acetylene) (D) in the headspace in TSB supporting growth of denitrifying *P. mandelii* cultures grown at 10°C (○), 20°C (■), or 30°C (△) in the presence of NO_3^-. Error bars are ±1 SEM ($n = 6$).

30°C treatment between 2 h and 10 h, with an average of 9.5×10^8 transcripts/μg RNA. The level of *cnorB* expression at 2 h for the 30°C treatment was 10-fold higher than that for the 20°C treatment and 73-fold higher than that for the 10°C treatment. Although the induction of *cnorB* gene expression was delayed in the 10°C treatment, by 8 h, there was no significant difference among all three temperature treatments, with an average of 1.5×10^9 transcripts/μg RNA. At 24 h, the level of *cnorB* expression significantly decreased compared with that at 10 h in *P. mandelii* cells grown at 20°C and 30°C, with an average of 4.0×10^7 transcripts/μg RNA, while the level of *cnorB* expression in the 10°C treatment remained similar to that of gene expression observed at 8 h, with 1.0×10^9 transcripts/μg RNA.

NO_3^- concentrations were not significantly different among treatments between 2 and 4 h (Fig. 6A). Nonlimiting NO_3^- concentrations were measured in *P. mandelii* cultures grown at 10°C for 10 h, with an average of 133 mg NO_3^--N/liter (Fig. 6A), which subsequently declined to 4.2 mg NO_3^--N/liter at 24 h. A significant decline in NO_3^- concentrations was measured at 6 and 8 h in *P. mandelii* cultures grown at 20°C and 30°C, respectively. At 10 h, NO_3^- concentrations declined to an average of 40.9 mg NO_3^--N/liter and finally to a negligible concentration of 0.1 mg NO_3^--N/liter at 24 h in *P. mandelii* cultures grown at 20°C and 30°C.

NO_2^- concentrations were not affected by temperature between 2 and 4 h for any treatment (Fig. 6B). Significant increases in NO_2^- concentrations were measured at 10, 6, and 8 h in *P. mandelii* cultures grown at 10°C, 20°C, and 30°C, respectively. At 10 h, NO_2^- concentrations increased to averages of 35.2 mg NO_2^--N/liter for *P. mandelii* cultures grown at 10°C and 30°C and 114 mg NO_2^--N/liter for *P. mandelii* cultures grown at 20°C. *P. mandelii* cultures grown at 20°C and 30°C showed essentially complete reduction of NO_2^- by 24 h (average of 0.12 mg NO_2^--N/liter), unlike *P. mandelii* culture grown at 10°C (96 mg NO_2^--N/liter). Maximum values of NO_2^- concentrations were measured at different time points among temperature treatments. The loss of NO_3^- was proportional to the increase in the NO_2^- concentration at 10 h, which was the time at which significant differences in both NO_3^- and NO_2^- concentrations among treatments were observed.

Temperature did not affect respiration between 0 and 6 h, with average emissions of 6.7 μmol, among treatments (Fig. 6C). The level of cumulative respiration was significantly higher in *P. mandelii* cells grown at 20°C and 30°C (average, 52.9 μmol) than in cells grown at 10°C (average, 20.4 μmol). At 24 h, a significant increase in the level of cumulative respiration was measured for *P. mandelii* cultures grown at 30°C only, with 383 μmol CO_2. Cultures grown at 10°C and 20°C maintained an average of 75 μmol CO_2 at 24 h. Temperature coefficients (Q_{10} values) for CO_2 of 2.2 between 10°C and 20°C and 0.9 between 20°C and 30°C at 10 h were obtained.

Temperature did not affect denitrification between 0 and 8 h, with average emissions of about 1.9 μmol among treatments (Fig. 6D). *P. mandelii* cultures grown at 10°C demonstrated no significant increase in levels of cumulative denitrification between 0 and 24 h, with average emissions of 3.3 μmol (Fig. 6D). The levels of denitrification increased significantly at 24 and 10 h in *P. mandelii* cultures grown at 20°C and 30°C, respectively. At 24 h, the level of denitrification increased to an average of 69 μmol in cells grown at 20°C and 30°C. There was no correlation between *cnorB* gene expression at 8 h, the time that it took to reach maximal denitrification gene expression for all three temperature treatments, and denitrification at 10 h, which coincided with high denitrification activity ($R = 0.11$; $P = 0.6529$), or between *nirS* gene expression at 8 h and denitrification at 10 h ($R = 0.35$; $P = 0.1612$). Temperature coefficients (Q_{10} values) for N_2O of 3.2 between 10°C and 20°C and 3.0 between 20°C and 30°C at 10 h were obtained.

DISCUSSION

In our study, levels of *nirS* and *cnorB* gene expression were not affected by pH treatment in *P. mandelii* cultures grown at pH 6, 7, and 8. A significant increase in levels of *nirS* and *cnorB* gene transcripts was observed between 0 and 2 h and was comparable to the increase in *cnorB* expression levels previously observed for *P. mandelii* cultures when NO_3^- was present in the medium (18). Very limited information is available on the effect of variable pH on denitrification gene expression in pure culture. pH 5 was the least favorable for denitrification gene expression. Although a 16-fold induction of *cnorB* and a 9-fold induction of *nirS* were observed at 2 h, negligible denitrification occurred. This observation is in agreement with data from research where an inhibition of denitrification activity was observed in *P. denitrificans* at a suboptimal pH of 6.8, which, however, conflicts with the conclusion from that same study, where no effect on mRNA induction was observed at pH 6.8 (1). The difference in the level of induction of denitrification genes observed in this study also suggests that pH had a stronger effect in negatively regulating *nirS* expression, resulting in a lower level of induction than that for *cnorB* expression.

Differences in NO_3^- utilization and NO_2^- accumulation were measured between 2 and 8 h in *P. mandelii* cultures grown at pH 6, 7, and 8. These differences may be attributed to the effect of pH on denitrification enzymes, particularly NO_3^- reductase and NO_2^- reductase (NIR). The magnitude of the NO_3^- loss was proportional to the increase in NO_2^- concentrations observed at 8 h among treatments. This observation implies that most of the NO_3^- added to the system was channeled through the denitrification process. The rate of NO_3^- utilization also varied among treatments, with a sharper decrease in NO_3^- concentrations observed at 8 h for *P. mandelii* cultures grown at pH 7 and 8 than for cells grown at pH 6. The timing of maximum NO_2^- accumulations also differed among treatments, with a higher level of NO_2^- accumulation at 8 h in cultures grown at pH 8 than at 4 h in cultures grown at pH 7. Similar to this study, NO_2^- accumulations in *P. denitrificans* cultures grown under denitrification conditions in a continuous-culture bioreactor were measured (1). Subsequently, NO_2^- accumulation was proposed to be the cause of the inactivation of the NIR enzyme in *P. denitrificans* through the formation of toxic nitrous acid (HNO_2), since the expression of the NO_2^- reductase gene was not affected by suboptimal pH. A separate study investigating the accumulation of denitrification intermediates in *Pseudomonas alcaligenes* and *Pseudomonas fluorescens* concluded that nitrite accumulation was dependent on the relative rates of nitrate and nitrite reduction (2). Nitrite accumulation may also be attributed to a lag in the synthesis of nitrite reductase (23, 30) or nitrate inhibition of NO reductase (17). In our study, it was hypothesized that enzyme activity was affected over a pH range of 6 to 8, thus causing differences in NO_3^- utilization and NO_2^- accumulation among pH treatments over time.

The rate of N_2O emissions varied during the incubation, as indicated by higher denitrification activity at 6 h and 8 h for *P. mandelii* cultures grown at pH 7 than for other pH treatments. Since gene expression was not affected at pH 6, 7, and 8, these differences are likely due to an effect of pH on nitrate reductase, NIR, or N_2O reductase activity, resulting in variable denitrification. *P. mandelii* cultures grown at pH 5 produced negligible denitrification activity compared with that for all other pH treatments. Although there have been no previous studies that investigated the effect of pH on denitrification activity in pure culture, as measured by N_2O accumulations using an acetylene block, there is evidence that the overall denitrification rate and denitrification enzyme activity were reduced by an acidic soil pH (16, 19, 21). Previous studies established that neutral-to-alkaline conditions were optimal for denitrification in cultures of *Pseudomonas* species (20, 25).

It is interesting that although negligible denitrification was observed in *P. mandelii* cultures grown at pH 5, 237 μmol CO_2 was produced at 24 h. Our NO_3 data reveal a decrease in the NO_3 concentration in cultures grown at pH 5, even though the numbers from 2 to 24 h were not statistically different. This leads to the possibility that a small amount of nitrate is consumed, and in the process, energy and CO_2 are produced. Moreover, due to the fact that CO_2 solubility increases with increasing pH, one would expect more CO_2 in the headspace of *P. mandelii* cultures grown at pH 5.

The induction of gene expression and the time to reach maximum expression were delayed as the temperature decreased from 30°C. *P. mandelii* cultures grown at 10°C demonstrated a long lag phase in growth and a subsequent delay in *nirS* and *cnorB* induction of gene expression compared with those of *P. mandelii* cells grown at 20°C and 30°C. *P. mandelii* cells grown at 30°C demonstrated a 10-fold-greater induction of *cnorB* expression at 2 h than did cells grown at 20°C; however, once maximum *cnorB* gene expression was reached at 4 h, there was no effect of temperature on *cnorB* gene expression between both treatments. The induction of *nirS* gene expression also did not differ between 20°C and 30°C treatments. Although the induction of *nirS* and *cnorB* gene expression was delayed in the 10°C treatment, by 8 h, all three temperature treatments led to the expression of maximum *nirS* and *cnorB* transcripts, thus indicating that temperature did not inhibit the transcription of denitrification genes. Temperature did, however, have a differential effect on denitrification gene expression, as is evident by differences in levels and timings of *nirS* and *cnorB* gene induction among temperature treatments. This

observation suggests an independent regulation of the *nirS* and *cnorB* genes in *P. mandelii*. There have been no studies to date that quantify the effect of temperature on denitrification gene expression.

NO_3^- utilization, NO_2^- accumulation, and denitrification activity differed among temperature treatments. The decrease in NO_3^- concentrations was proportional to the increase in NO_2^- concentrations in *P. mandelii* cultures grown at 10°C, 20°C, and 30°C, suggesting N flux mainly through the denitrification pathway. The timing of maximum NO_2^- accumulations also differed among treatments and was observed at 24 h in *P. mandelii* cultures grown at 10°C and at 10 h in cells grown at 20°C and 30°C. The level of denitrification was higher in cells grown at 30°C at 10 h than in *P. mandelii* cells grown at 10°C and 20°C. These observations suggest that temperature had a significant effect on enzyme activity. Low temperature resulted in slower enzyme rates of reaction and subsequently influenced the rate of growth and metabolism. Holtan-Hartwig et al. (10) also concluded that lower temperatures may exert a challenge to the denitrifying community, thus resulting in lower denitrification rates, as measured by N_2O production and reduction, at cooler temperatures (0°C). Alternatively, NirS synthesis may be negatively affected in *P. mandelii* at temperatures below 30°C, thus causing nitrite accumulations in the growth medium.

The pattern of CO_2 production in *P. mandelii* cultures grown at different pH values and temperatures was investigated. Previous studies have well established the concept of Q_{10}, defined as the change in denitrification, or respiration with a 10°C change in temperature (9, 13, 31, 32). When CO_2 accumulations were analyzed in *P. mandelii* cultures grown at 10°C, 20°C, and 30°C, Q_{10} values of 2.2 at temperatures between 10°C and 20°C and of 0.9 at temperatures between 20°C and 30°C were obtained. These values are comparable to those reported in previous studies, where Q_{10} values between 1.6 and 2.6 for CO_2 production were observed in soils treated with animal slurry (13). The patterns of CO_2 accumulation in *P. mandelii* cultures grown at pH 6 and pH 7 were significantly different from those of CO_2 accumulations in cultures grown at pH 5 and pH 8 (Fig. 3C). This suggests that both pH and temperature had an effect on the respiration rate in *P. mandelii*.

In our study, *P. mandelii* cultures did not exhibit a twofold increase in denitrification, as measured by N_2O emissions, with temperature increases of 10°C, averaged over 10 h. This observation is contrary to data from previous studies that established a temperature coefficient of denitrification of approximately 2 for every 10°C temperature increase in soil (9, 22). This disparity could be due to the fact that our study was conducted with pure cultures of *P. mandelii* under completely anaerobic conditions, which differ from conditions of a soil community of denitrifiers composed of members that are denitrifying at variable rates and that exist in aerobic and anaerobic microcosms.

In conclusion, a pH value of 5 negatively impacted denitrification gene expression in the *P. mandelii* strain used; however, levels of *cnorB* and *nirS* gene expression were not affected by pH values over the range of pH 6 and 8. Gene expression was sensitive to temperature, since induction and the time to reach maximum expression were delayed as the temperature decreased from 30°C. Temperature and pH influenced NO_3^- utilization, NO_2^- accumulation, as well as cumulative denitrification, leading us to conclude that both pH and temperature had a significant effect on denitrification enzyme activity.

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