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Denitrification Response Patterns during the Transition to Anoxic Respiration and Posttranscriptional Effects of Suboptimal pH on Nitrogen Oxide Reductase in *Paracoccus denitrificans*▽†

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Denitrification in soil is a major source of atmospheric N_2O. Soil pH appears to exert a strong control on the N_2O/N_2 product ratio (high ratios at low pH), but the reasons for this are not well understood. To explore the possible mechanisms involved, we conducted an in-depth investigation of the regulation of denitrification in the model organism *Paracoccus denitrificans* during transition to anoxia both at pH 7 and when challenged with pHs ranging from 6 to 7.5. The kinetics of gas transformations (O_2, NO, N_2O, and N_2) were monitored using a robotic incubation system. Combined with quantification of gene transcription, this yields high-resolution data for direct response patterns to single factors. *P. denitrificans* demonstrated robustly balanced transitions from O_2 to nitric oxide-based respiration, with NO concentrations in the low nanomolar range and marginal N_2O production at an optimal pH of 7. Transcription of *nosZ* (encoding N_2O reductase) preceded that of *nirS* and *norB* (encoding nitrite and NO reductase, respectively) by 5 to 7 h, which was confirmed by observed reduction of externally supplied N_2O. Reduction of N_2O was severely inhibited by suboptimal pH. The relative transcription rates of *nosZ* versus *nirS* and *norB* were unaffected by pH, and low pH had a moderate effect on the N_2O reductase activity in cells with a denitrification proteome assembled at pH 7. We thus concluded that the inhibition occurred during protein synthesis/assembly rather than transcription. The study shed new light on the regulation of the environmentally essential N_2O reductase and the important role of pH in N_2O emission.

Denitrification is the respiratory reduction of N-oxides, which enables aerobic bacteria to sustain respiratory metabolism during anoxic spells. The process is a vital part of the global nitrogen cycle by emitting molecular nitrogen from the biosphere to the atmosphere (32). In addition, it is an important source of NO and N_2O emissions. N_2O is one of the major gases contributing to global warming (22) and was recently also recognized as the main ozone-depleting substance causing destruction of the stratospheric ozone layer (11). Complete denitrification is the reduction of nitrate to molecular nitrogen (N_2) via nitrite and the gaseous intermediates NO and N_2O. The process involves the enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR), and it is commonly anticipated that the fitness of denitrifying bacteria depends on their ability to regulate the process to avoid accumulation of toxic intermediates and to maximize energy conservation.

Denitrifying microbial communities in soils display differences in denitrification product stoichiometry (N_2O/N_2), which may explain not only the contrasting emission patterns observed from different soils, but also variations in annual N_2O/N_2 emission ratios from the same soils (10, 16). This has been taken to indicate that the composition of the denitrifying community may determine its ability to regulate the denitrification process in response to environmental conditions, thus affecting the soils' propensity to emit N_2O. Factors that are considered to be important controllers of rates and product stoichiometry of denitrification in intact soils include water content, temperature, and available NO_3^- and C substrates (28). Soil pH is another factor that appears to play a major role for N_2O emission; there is ample evidence that the N_2O/N_2 product ratio of denitrification is higher in acid than in alkaline soils (24, 25, 31). Liu et al. (unpublished data) investigated soils from three long-term liming experiments on agricultural soils and found a consistently decreasing N_2O/N_2 ratio with increasing pH within the range of 5 to 8. This pervasive pH control of the N_2O/N_2 ratio may strongly affect future emissions of N_2O from agro-ecosystems in developing countries such as China, where vast areas are gradually acidified by intensified use of nitrogen fertilizers (14).

Considering its immense implications, surprisingly few attempts have been made to unravel the mechanisms involved in the pH control of the product stoichiometry of denitrification. The traditional explanation has been that the N_2OR enzyme is more sensitive to low pH than the other reductases involved in denitrification, but there exists little evidence to confirm this. Liu et al. (unpublished data) investigated the kinetics of denitrification and transcription of functional genes in soils from long-term liming experiments and observed a consistent in-

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crease in the N_2O/N_2 product ratio with decreasing soil pH for all soils. Quantification of gene transcription demonstrated a generally lower level of expression for *nirS* (encoding nitrite reductase) and *nosZ* (encoding N_2O reductase) at pH 6 compared to pH 8, but the relative ratios of *nosZ* versus *nirS* transcripts were similar or even higher at pH 6. This strongly indicated that the higher N_2O/N_2 product ratios at pH 6 were due to a posttranscriptional effect.

Progress in understanding the role of community composition and pH control of the N_2O/N_2 emission from soils will be enhanced by further genomic and transcriptomic analyses of contrasting communities in combination with functional analyses. However, we also need complementary information from more refined studies of denitrification response patterns in isolated strains since such experiments may unravel specific mechanisms in more detail. In the present paper, we have addressed these questions by an in-depth analysis of the pH dependency of gas kinetics and gene expression of *Paracoccus denitrificans* during oxygen depletion.

The soil bacterium *P. denitrificans* is one of the paradigm species for studies of the biochemistry and regulatory biology of denitrification. It belongs to the *Alphaproteobacteria* and has an obligate respiratory metabolism involving a wide spectrum of respiratory enzymes (3). *P. denitrificans* is equipped with genes encoding three biochemically distinct oxidases (12) as well as a full set of enzymes for denitrification (NAR/periplasmic nitrate reductase [NAP], NIR, NOR, and N_2OR), which can all be coupled to the core electron transport pathway (21). The expression of genes encoding the nitrogen oxide (NO_x) reductases is regulated by factors belonging to the cyclic AMP receptor protein (CRP)/fumarate and nitrate reductase regulator (FNR) family of transcriptional regulators. The transcriptional activation of *nar*, *nir*, and *nor* in *P. denitrificans* have been shown to be mediated by such factors as a response to absence of O_2 and presence of nitrate and NO (3, 27, 30). The regulation of *nos* transcription has not been fully resolved. In *Pseudomonas aeruginosa*, NO has proven a powerful inducer of *nos* expression, probably through the FNR-like transcriptional regulator DNR (2). The substrate of N_2OR, N_2O, appears to be only a weak inducer of expression (18), and the role of O_2 in *nos* regulation is still somewhat unclear (33).

In the present study of *P. denitrificans*, we performed a series of incubations under strictly controlled conditions assessing its denitrification regulatory phenotype (DRP), the regulation and activities of the N-oxide reductases as affected by speed of oxygen depletion, concentration of N-oxides, and pH. The results demonstrated an apparently more robust DRP than that of *Agrobacterium tumefaciens* (5). Novel features of *P. denitrificans* were unraveled by the combination of transcription analyses and refined gas analyses, such as the early expression of *nosZ*, encoding N_2OR, and the detrimental effect of low pH on the assembly of this enzyme. The observations have implications for understanding the role of soil pH in controlling N_2O emissions into the atmosphere.

MATERIALS AND METHODS

Preparation of inocula and experimental setup. Three series of experiments were set up to examine (i) kinetics, (ii) pH effects, and (iii) protein assembly. All experiments were performed at 20°C with *P. denitrificans* DSM314 grown in Sistrom's medium (19), with 34 mM succinic acid as the C source and 20 mM phosphate buffer concentration unless otherwise stated.

For the kinetics experiments, 12 120-ml serum flasks, each containing a magnetic stirring bar and 50 ml Sistrom's medium supplemented with 0, 0.2, 1, or 2 mM KNO_3 or KNO_2 (see Table 2) were sealed with rubber septa and aluminum caps to ensure an airtight system. Headspace atmospheres were replaced by helium (He) in all flasks by repeated cycles of evacuation and He filling before initial oxygen levels (1 and 7%) were adjusted by adding the appropriate volumes of pure oxygen to eight of the flasks. Four flasks, one for each initial N-oxide level, were left without added oxygen and designated as 0% initial oxygen. The culture used for inoculation was grown aerobically in 50%-strength Sistrom's medium in order to prevent an excessively dense culture and with vigorous stirring to ensure full dispersal of cells. When the inoculum reached late exponential phase, $\sim 1.25 \times 10^8$ cells were injected through the septum of each of the previously prepared flasks, using a 2-ml sterile syringe.

The preparations and setup for determination of pH effects were more complex. First, 1 ml of an aerobic overnight culture, prepared from frozen stock, was added to each of five cultures destined to be used as inocula in the main experiment. The inocula were grown aerobically with vigorous stirring in 50%-strength Sistrom's medium at pHs 6.0, 6.25, 6.5, 7.0, and 7.5. The following day, all of the cultures were normalized to an optical density at 660 nm (OD_{660}) of 0.320 by dilution with sterile media at their respective pHs, and 25 ml of culture was added to flasks containing 25 ml of sterile medium at the five pH levels. Preliminary experiments had shown a high degree of reproducibility between identical treatments both between parallels within one experiment and between independent experiments. Thus, for this setup, only pH 7 and pH 6 cultures were set up in 12 parallel flasks destined for destructive sampling and gene expression analysis. These flasks were monitored with respect to oxygen consumption and accumulation of NO_x, and examples are presented in Fig. S5 in the supplemental material to demonstrate that all cultures followed the same trajectories. Both the experimental treatments and the inocula were stabilized by 100 mM phosphate buffer at their respective pH levels. However, there was a slight pH increase in all flasks during denitrification, and the average pH change at the end of incubation was 0.072 ± 0.036. All experimental cultures were supplemented with 2 mM nitrate. The cultures were allowed to grow aerobically for 1 h before the flasks were sealed by rubber septa and aluminum caps, and headspace atmosphere was replaced by He as described for the kinetics experiments. No oxygen was added after the flasks had been made anaerobic.

The protein assembly experiment was set up in order to further investigate N_2OR function and activity in response to pH. The capacity to reduce N_2O was at first studied on cultures that were pregrown aerobically so that no N_2OR enzyme would be present in the cells at the onset of denitrification. Two cultures were grown aerobically overnight at pHs 6 and 7 (100 mM phosphate buffer), and each was used to inoculate two anaerobic flasks ($\sim 10^9$ cells per flask) containing Sistrom's medium buffered at the respective pH levels and supplemented with 2 mM nitrate and 1.5 μmol pure N_2O. In addition to this, a second experiment was set up to evaluate the pH effect on N_2OR function in cultures in which the four reductases were previously assembled at pH 7. *P. denitrificans* was raised from frozen stock and grown aerobically overnight before inoculation into two parallel 120-ml flasks containing Sistrom′s medium weakly buffered (20 mM phosphate buffer) at pH 7. To ensure activation of the denitrification apparatus, oxygen was removed from the headspace and 0.2 mM nitrate was added to each of the two flasks. The cultures were allowed to reduce the available nitrate before 10 ml (approximately 4×10^9 cells) was transferred to new flasks containing Sistrom′s medium strongly buffered (100 mM phosphate buffer) at pHs 6, 6.5, 7, and 7.5. Each of the two original cultures was used as the inoculum for one full series of pH treatments (pHs 6, 6.5, 7, and 7.5). Approximately 6 μmol of pure N_2O was added to each culture, and N_2O reduction was closely monitored during the next ~40 to 50 min.

Gas measurements. After inoculation, cultures, blanks, and gas standards were placed in a thermostatic water incubator containing a serial magnetic stirrer. The cultures were continuously stirred at 850 rpm to ensure full dispersion of cells and proper gas exchange between liquid and headspace. The incubator was coupled to an autosampler connected to a Varian CP 4700 micro-GC (gas chromatograph) with a 10-m poraPLOT U and a 20-m Molsieve 5 A column (in parallel) each equipped with a thermal conductivity detector (TCD). NO measurements were performed on a chemiluminescence NO_x analyzer (model 200A; Advanced Pollution Instrumentation). This system enabled automatic real-time monitoring of the reduction of oxygen and production of CO_2, NO, N_2O, and N_2 in the headspace of active bacterial cultures. Gas measurements were made every 2 (pH experiment) or 3 (nitrate/nitrite) h or every 9 min (N_2O reduction). The instrumentation and method are described in more detail elsewhere (5, 20).

TABLE 1. List of primers used for analysis of gene expression

Purpose	Gene	Primer/probe sequence (5′→3′)[a]
Standard curve	*nirS*	Forward: GAGCCCTCGCTGGACAAC Reverse: AAGGTCGATCATATTGACCTTGC
	norB	Forward: TGTCCGAACTCCTGCCCT Reverse: TTCCAGACCATGACGAAGG
	nosZ	Forward: CAACTGGACGACTACTACGGC Reverse: GTTCAGTTCCTGATAGTCGCC
Gene expression assay	*nirS*	Forward: CTACCTGCAAAGCTTCATCACCTA Reverse: AGCAGGTAGTTCGCCATCAG Probe: FAM-TCGGCGGTCAGCTCG
	norB	Forward: GGCTGCTGGGCAAGGA Reverse: GCGATGCCGAGCTTGAC Probe: FAM-CTGGAACAGCCGAAATG
	nosZ	Forward: CAATGGCAAGCGCATCCA Reverse: GCCCTCGGTAAAGGACATGTG Probe: FAM-CAGGTCGCCGTTGTC

[a] FAM, 6-carboxyfluorescein.

Nitrite measurements and quantification of gene expression. The experiment examining pH effects included a detailed analysis of gene expression levels as well as nitrite measurements. An additional set of 13 flasks each from the pH 6 and pH 7 treatments was prepared and monitored with respect to gas kinetics alongside the main pH experiment. Samples were taken from the liquid phase of these flasks throughout the experiment—at 0, 2, 4, 6, 8, 11, 16, 21, 26, 33, 41, and 72 h—as well as from the inocula (−3 h) and before anaerobization (−1 h). Flasks were opened, and the entire contents were transferred to sterile 50-ml centrifugation tubes. Cells were pelleted by centrifugation (10 min, 4,100 × *g*) at 4°C, and 1 ml of the supernatant was stored for subsequent nitrite measurements, which were performed according to ISO 6777-1984 (17). The rest of the supernatant was decanted, and 1 ml of RNAprotect (Qiagen) was quickly added to the cell pellet. After treatment with RNAprotect, the cells were stored at −20°C pending RNA extraction. Total RNA was extracted from all samples using the RNeasy minikit (Qiagen), and residual DNA was removed by DNase I treatment (amplification-grade DNase I; Sigma), after which the concentration of RNA was measured by Nanodrop (Eppendorf). Reverse transcription (RT) was performed using the Masterscript RT-PCR system (5 Prime) and random hexamers (Invitrogen). Real-time PCR was performed on a 7900HT (Applied Biosystems) using RealMasterMix probe ROX (5 Prime) and custom gene expression assays from Applied Biosystems. Standard curves were designed using 500- to 700-bp PCR products of *nirS*, *norB*, and *nosZ* (Table 1) in known copy numbers. Primer/probe sets for *nirS*, *norB*, and *nosZ* were designed using Filebuilder from Applied Biosystems (Table 1).

The expression of *nirS*, *norB*, and *nosZ* was estimated relative to their respective standard curves (log-linear regression of threshold cycle [C_T] value against log copy number). The slopes and corresponding R^2 values of the standard curves were as follows (with two repeats): *nirS*, −4.20/−4.53 (R^2 = 0.997/0.984); *norB*, −3.49/−3.60 (R^2 = 0.998/0.991); and *nosZ*, −3.96/−3.96 (R^2 = 0.992/0.991). Real-time PCRs were set up in triplicates (subsampled from the same cDNA), and the amount of cDNA added for each sample was normalized based on Nanodrop measurements. The results were given as mRNA copies cell^{-1}, assuming that all cells contained an average of 0.1 pg total RNA (9) and that all cells were actively transcribing the denitrification genes.

RESULTS

Kinetics: influence of initial oxygen, nitrate, and nitrite concentrations. In early incubation experiments with *P. denitrificans*, we observed a high similarity between parallel flasks within the same experiments. In light of this, we decided to sacrifice replication within each experiment in order to maximize the number of treatments and rather repeat entire experiments. Comparison of repeated experiments showed good agreement, although the exact timing of oxygen depletion was somewhat variable (possibly by slight differences in the cell densities of the inocula). For this reason, the graphical presentation of the gas kinetics cannot adequately include replication. Reproducibility of the results is demonstrated, however, by calculated parameters describing oxygen concentrations at first detection of NO production (Table 2), the maximum amount of NO (Table 2), the apparent oxic and anoxic growth rates (see Tables S2 to S4 and Fig. S4 in the supplemental material), and the timing of onset of N_2 production in relation to oxygen depletion in the headspace (see Table S6 in the supplemental material). For five independent experiments with treatment with a 7% initial O_2 concentration and 2 mM nitrate at pH 7, the oxygen concentration in the liquid, [O_2], at the first appearance of detectable NO (>0.5 nM in liquid) fell within the range of 6.5 to 11 μM O_2 for all but one outlier at 1.8 μM. In the same replicates, the NO reached very similar concentrations (maximum NO concentrations were 14.8 ± 2.3 nM in liquid, equivalent to 21 ± 3 nmol NO flask^{-1}).

Figure 1 shows an example of the gas kinetics (initial O_2 concentration of 7% and initial NO_2^- concentrations of 0.2, 1, or 2 mM) in the series of batch culture experiments in which *P. denitrificans* cultures with low initial cell numbers (2.5×10^6 cells ml^{-1}) were exposed to different initial concentrations of O_2 and NO_3^- or NO_2^-. Table 2 summarizes variables for all treatments. Typically, there was a sharp increase in NO while μM concentrations of oxygen were still present. NO stabilized at an average maximum of 21.5 ± 9.7 nmol flask^{-1} (n = 45; initial nitrate/nitrite concentrations of 0 to 2 mM and initial O_2 concentration of 0 to 7%) and was maintained within this range until all nitrite was reduced. Only traces of N_2O accumulated transiently (not shown in Fig. 1); in many cases, N_2O levels did not exceed the system's detection limit (~0.5 nmol N_2O/flask). The maximum level recorded for the experiments summarized in Table 2 is 3 nmol N_2O/flask, which is less than

TABLE 2. Summary of observations through all treatments in the kinetics experiments[a]

Initial O_2 concn (vol%)[b]	Initial NA/NI concn (mM)[b]	μM O_2 at first detection of NO				NO_{max}(nmol flask^{-1})[c]				Final cell density with NA (10^6 ml^{-1})[d]
		NA		NI		NA		NI		
		Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	0	<0.1	<0.1	<0.1	0.1	22	9	7	21	13
	0.2	<0.1	<0.1	<0.1	0.2	33	22	17	16	28
	1	<0.1	<0.1	0.1	0.1	30	32	38	34	112
	2	<0.1	<0.1	0.2	0.1	39	18	41	35	212
1	0	1.1	1.5	ND[e]	<0.1	14	9	ND	7	97
	0.2	1.0	0.7	0.1	0.4	21	15	11	19	108
	1	0.8	1.1	0.9	1.4	22	14	18	38	185
	2	0.7	0.5	1.8	2.2	30	20	22	40	332
7	0	2.6	3.2	ND	ND	9	3	ND	ND	490
	0.2	9.5	6.0	5.9	8.4	13	16	17	16	490
	1	10	6.0	9.2	11	22	13	26	23	548
	2	8.3	10	9.8	12	27	20	25	23	626

[a] Experiments in which *P. denitrificans* was grown in Sistrom's medium at pH 7 with different initial concentrations of NO_3^- (NA) and NO_2^- (NI) and with different initial concentrations of O_2 in headspace. The variables shown are oxygen concentration in the liquid at first detection of NO, maximum concentration of NO measured (single-flask values for two independent experiments), and the final cell density reached for each treatment (only for a single experiment with nitrate).

[b] Basal Sistrom's medium contains approximately 17 μM NO_3^-, and the treatment with 0 O_2 contained variable traces of O_2 in the headspace (100 to 300 ppmv) (see the supplemental material for more details).

[c] NO_{max}, maximum concentration of NO.

[d] The inoculum size was 4×10^8 cells per flask for NA and 3×10^8 cells per flask for NI, equivalent to initial cell densities of 8×10^6 and 6×10^6 cells ml^{-1}, respectively.

[e] ND, not determined (no NO peak detected).

1‰ of N_2 produced at that time. Detectable N_2 production occurred 2 to 10 h after that of NO, depending on the initial oxygen level, and proceeded until all available NO_2^-- and NO_3^--N was recovered as N_2.

The final cell densities (Table 2) increased proportionally with increasing amounts of O_2 and NO_3^- electron acceptors used (average of 15×10^{13} cells mol^{-1} O_2 and 9.64×10^{13} cells mol^{-1} NO_3^-). The estimated cell yields per mol of electrons (Y_e) were thus $Y_eO_2 = 3.75 \times 10^{13}$ cells mol^{-1} electrons for O_2 and $Y_eNO_3 = 1.93 \times 10^{13}$ cells mol^{-1} electrons for NO_3^- (see the supplemental material for further details). The measured protein content per cell was 96 fg, and total C was 0.1 pg. The electron transport rates increased exponentially through the most of the oxic and anoxic phases and were used to calculate specific growth rates (μ) for growth with O_2 and NO_x as electron acceptors (for more details, see Fig. S1 and S2 and Tables S1 and S2 in the supplemental material). The average μ's were 0.19 h^{-1} (standard deviation [SD], 0.01; $n = 8$) for aerobic respiration and 0.10 h^{-1} (SD, 0.01; $n = 6$) for anoxic respiration.

pH effects. The effect of pH on oxic and anoxic growth rates was determined in separate experiments (see Tables S3 and S4 in the supplemental material). Oxic growth rates, expressed as percentages of that at pH 7, were 140, 77, 59, and 57% for pHs 7.5, 6.5, 6.25, and 6.0, respectively. Anoxic growth rates at pHs 7.5 and 6.5 were 179 and 33% of that at pH 7.

Figure 2 shows kinetics of denitrification (NO_2^-, NO, N_2O, and N_2) as affected by different pH levels in one series of cultures at pHs 6 to 7.5. Additional flasks were monitored at pH 6 and pH 7, which showed that replicate cultures followed the same trajectories (see Fig. S5 in the supplemental material). The cells used as inocula had been grown aerobically to late log phase at the respective pH levels and then were inoculated into anaerobized flasks (initial cell density of ~3×10^8 cells ml^{-1}). The onset of nitrite accumulation preceded that of NO_x gas production, and nitrite reached very high levels. At pH 7, all of the available nitrate was reduced to nitrite (2 mM) before denitrification started (Fig. 2A). The denitrification process was slower at pH 6, and accumulation of NO_2^- continued during the entire experiment, reaching 1,700 μM after 70 h. As in the previous experiments, the NO levels increased prior to the onset of N_2 production, and NO remained present within the concentration range 30 to 70 nM until all nitrate had been reduced (seen as stable N_2 plateaus for pH ≥ 6.5). The peak NO levels increased with decreasing pH, most pronounced for pH 6. The NO accumulation at pH 6 represents a minor fraction of the gases produced; the peak concentration of 80 nM in the liquid is equivalent to 112 nmol NO per flask, which is less than 3% of N_2O-N present at the same time. In the other treatments, the NO accounted for much lower percentages of the gas products.

The relative rate of N_2O reduction was severely reduced by low pH, seen as an increasing concentration of N_2O with decreasing pH (note the different scales for the two lowest pH levels in Fig. 2C). While transient for pH ≥ 6.25, N_2O accumulation at pH 6 continued throughout the incubation. The relative rate of N_2O reduction versus that of NO reduction (to N_2O) can be assessed by calculating the amount of N_2O as a percentage of N_2O plus N_2 throughout the incubation: % $N_2O = 100 \cdot N_2O/(N_2O + N_2)$. The maximum values for this percentage showed a clear pH dependency even in the higher pH range, with values of 0.08, 0.2, and 0.3% for pHs 7.5, 7.0, and 6.5, respectively. At pH 6.25, the percentage of N_2O peaked twice, at 12 h and 35 h; the percentage of N_2O for the first peak was 20%, and that for the second was 3.5%. At pH 6, the percentage of N_2O was 100% through the first 40 h and then dropped gradually to reach 40% at the end.

The expression of *nirS*, *norB*, and *nosZ* was assessed by

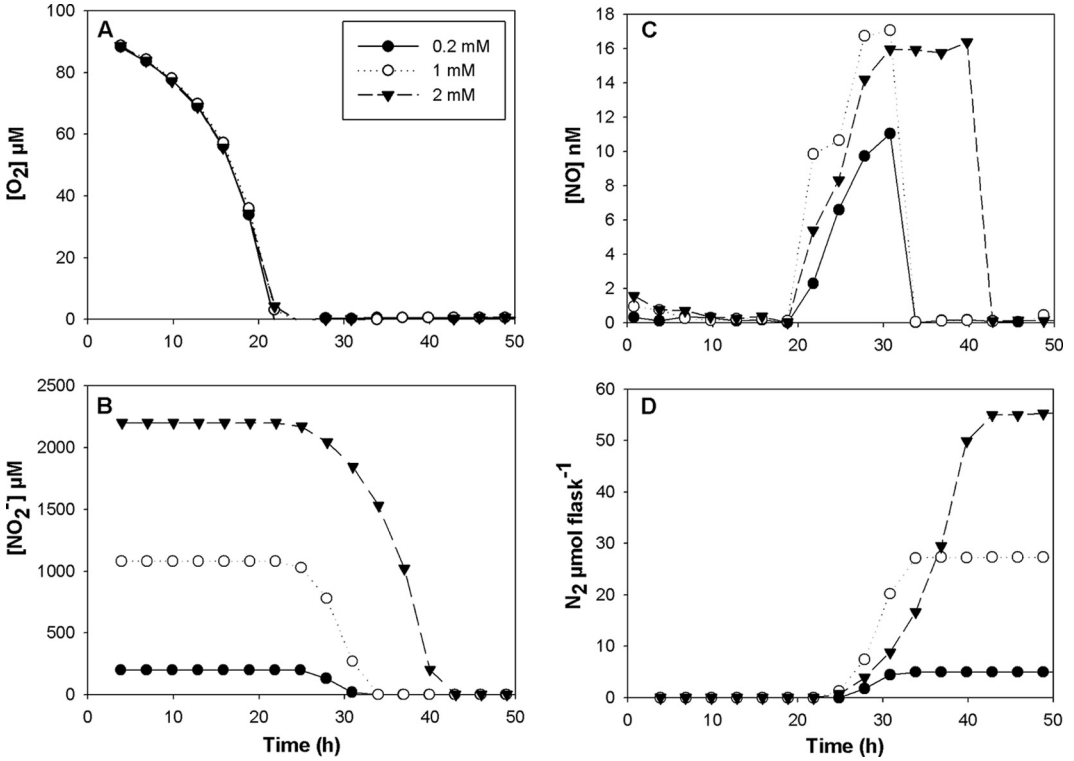

FIG. 1. Kinetics of oxygen depletion and denitrification in *P. denitrificans* when grown in sealed 120-ml flasks with 7% initial O_2 in headspace and 0.2, 1, and 2 mM nitrite in 50 ml Sistrom's medium. (A) Oxygen concentration in the liquid (μM), estimated from measured rate of O_2 depletion in headspace (20); (B) nitrite concentration (μM) calculated from accumulation of N_2 and NO; (C) NO concentration in liquid (nM) assuming equilibrium between headspace and liquid; (D) measured N_2 (μmol $flask^{-1}$) corrected for 3.4% loss for each sampling. N_2O was monitored as well, but only traces (<2 nmol $flask^{-1}$) were detectable for a brief period (not shown).

quantifying mRNA by two-step real-time RT-PCR (Fig. 3). The transcript numbers for the respective reductases, when calculated per cell, were low. For most cases, only 0.5 copy $cell^{-1}$ or less was found, and only in a couple of cases did the transcript number exceed 2.0 (assuming that all the cells present were actively transcribing the genes encoding the NO_x reductases). The timing of transcription of the three genes was hardly affected by the pH level, but the levels of gene transcription were. The maximum numbers of *norB* and *nosZ* transcripts were approximately 3 times higher at pH 7 than that at pH 6, whereas the maximum number of *nirS* transcripts was 6 times higher at pH 7 than that at pH 6. Thus, the balance in transcription of the reductase genes was affected by pH, but not in a way which could explain the near absence of N_2O reductase activity at pH 6. The timing of transcription of the individual reductase genes showed a conspicuous pattern. The transcription of *nosZ* increased sharply and apparently instantaneously upon oxygen removal, prior to significant accumulation of NO (compare with Fig. 2). In contrast, the onset of significant transcription of *nirS* and *norB* coincided with the sharp increase in NO concentration, which occurred 5 to 7 h later than the onset of *nosZ* transcription.

Protein assembly. The foregoing experiments demonstrated that pH 6 had a severe effect on the relative rate of N_2O reduction, but the quantification of gene transcripts offered no explanation for this phenomenon; the relative amount of *nosZ* transcripts was either unaffected (*nosZ*/*norB* ratio) or even increased (*nosZ*/*nirS* ratio) by low pH (pH 6 versus 7). Another conspicuous observation was that *nosZ* transcription preceded that of *nirS* and *norB* by 5 to 7 h at both pH levels. We first tested whether this early transcription of *nosZ* could result in active reduction of externally supplied N_2O prior to onset of denitrification. This was done by repeating the foregoing experiment (aerobic cultures in a medium with 2 mM NO_3^- made anoxic by He washing) but with N_2O added to the headspace at the onset of the anoxic incubation.

The results (Fig. 4) show no reduction of the externally supplied N_2O at pH 6, but in the flasks with pH 7, a rapid depletion of N_2O started approximately 5 h after anaerobization. This is 5 to 7 h before the onset of NO accumulation, and this is what would be expected based on the timing of the *nosZ* transcription versus that of *nirS* and *norB* (Fig. 3). In order to establish whether the observed effect of low pH on N_2OR was due to a direct effect of H^+ ions on the enzyme or whether instead low pH might inhibit assembly of functional enzymes, we transferred actively denitrifying cultures at pH 7 (in weakly buffered media) to new media strongly buffered at pHs 6, 6.5, 7, and 7.5. The cultures were first allowed to reduce the traces of NO_3^- (17 μM) present in the medium, and then 6 μmol of pure N_2O was injected and N_2OR activity was monitored by frequent sampling. Available N_2O was reduced until depletion within 50 min in all treatments (zero-order kinetics), and the rates estimated by linear regression are plotted in Fig. 5 against

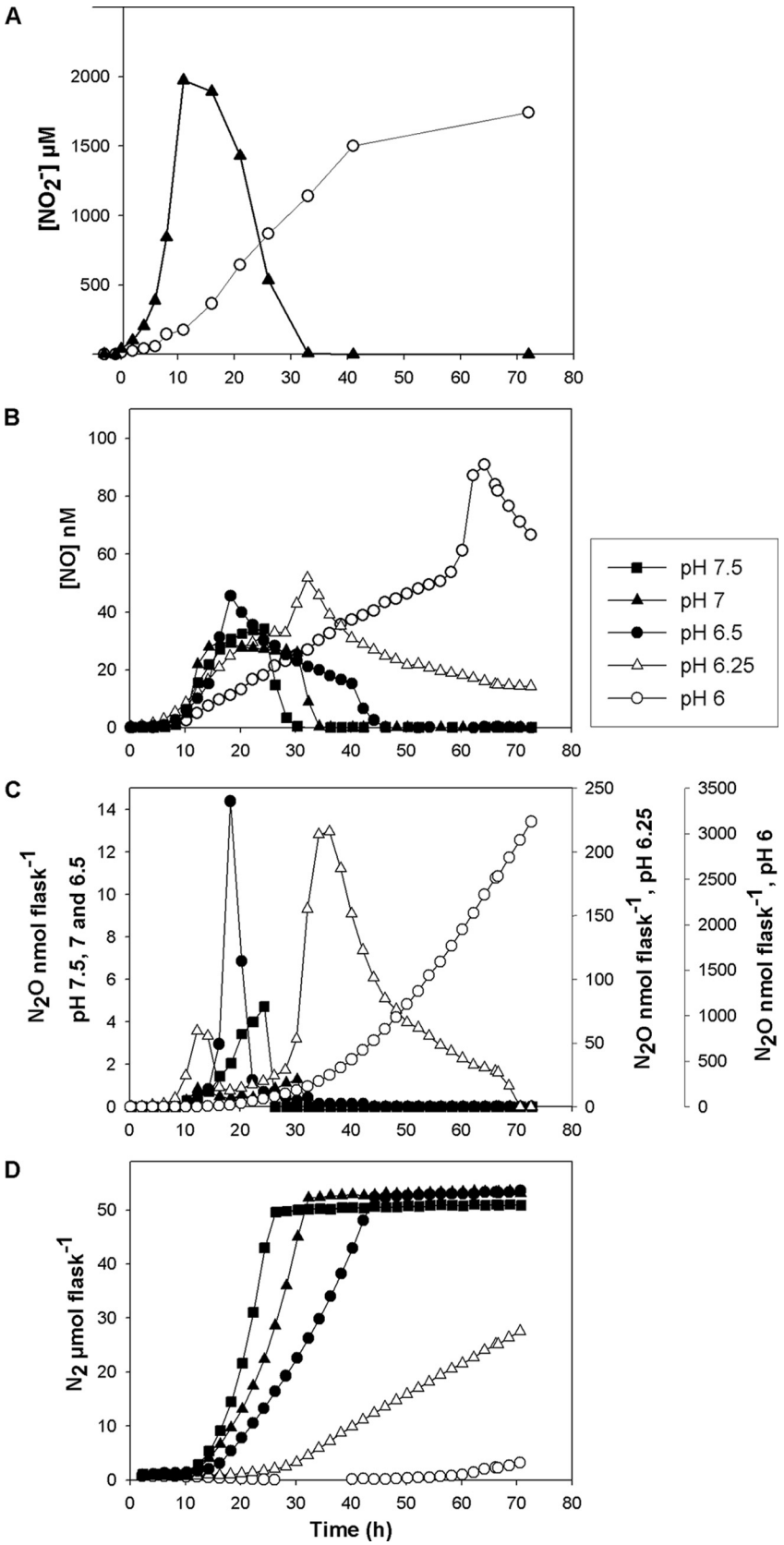

FIG. 2. Effect of pH on the reduction of N-oxides. Aerobic cultures in Sistrom's medium with 2 mM NO_3^- and pHs of 6, 6.25, 6.5, 7, and 7.5 were made anoxic by He washing (time zero). (A) Concentration of nitrite (μM; pHs of 6 and 7 only); (B) concentration of NO (nM in liquid); (C) accumulated N_2O (nmol $flask^{-1}$); (D) accumulated N_2 (μmol $flask^{-1}$).

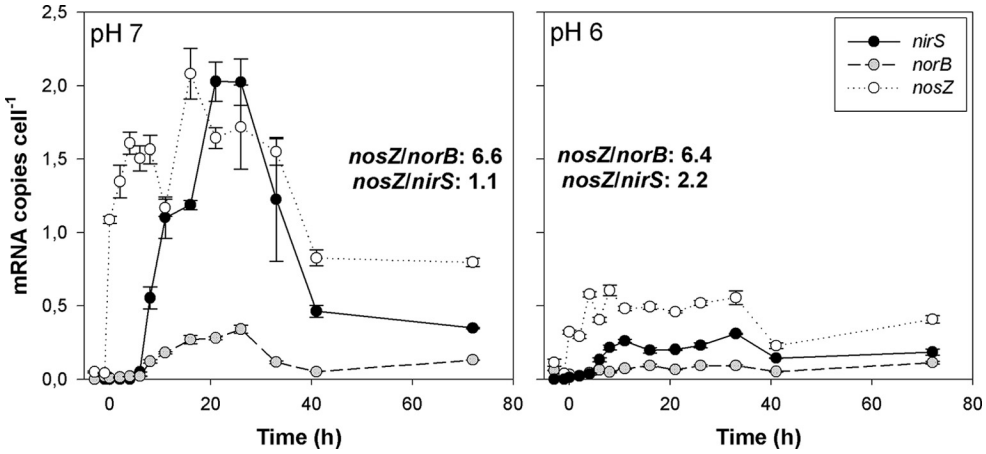

FIG. 3. Expression of the genes encoding NO_2^- reductase (*nirS*), NO reductase (*norB*), and N_2O reductase (*nosZ*) after anaerobization (by He washing at time zero) of a culture in Sistroms' medium with 2 mM NO_3^- at pH 7 (left panel) and pH 6 (right panel). The numbers of transcripts per cell (mRNA $cell^{-1}$) are given, assuming that all the cells present are transcribing. Ratios were calculated based on the four maximal expression levels for the respective genes.

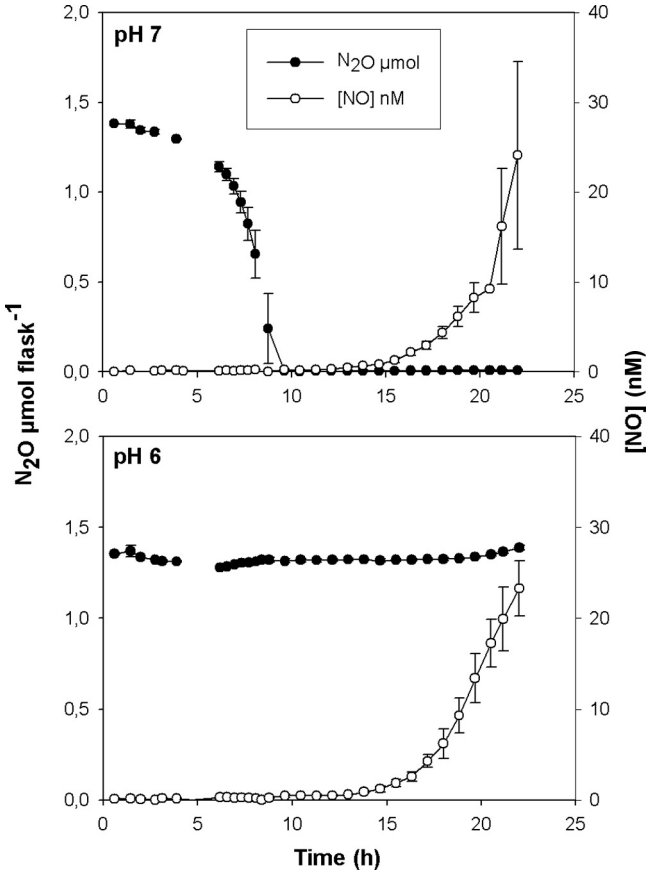

FIG. 4. Reduction of externally supplied N_2O prior to expression of *nirS*. Aerobic cultures in Sistrom's medium with 2 mM NO_3^- at pH 6 (lower panel) and pH 7 (upper panel) were made anoxic (time zero) and supplied with N_2O in the headspace. The figure shows the measured concentration of N_2O (μmol $flask^{-1}$), which should be stable if no N_2O reduction is taking place (left axis, corrected for the 3.4% dilution by each sampling), and NO accumulation (nM in liquid; right axis).

measured pH (which was somewhat higher than intended for the two lower pH levels due to the added inoculum).

The results demonstrated that functional N_2O reductase (assembled at pH 7) is quite tolerant to low pH: the activity was only marginally affected by pH within the range 6.5 to 7.5 and was only reduced by 45% (compared to the rate at pH 7) when exposed to pH 6.11. The cell density in these experiments was 4×10^9 cells per flask: thus, on a per-cell basis the N_2O reduction rates at pH 7 were 3×10^{-15} mol N_2O $cell^{-1}$ h^{-1}. In comparison, the maximum observed denitrification rate at pH 7 is 1.1×10^{-15} mol NO_3^- $cell^{-1}$ h^{-1} ($= \mu_{NO_3}/Y_{NO_3}$, where $\mu_{NO_3} = 0.104$ h^{-1} is the observed growth rate with NO_3^- as electron acceptor and $Y_{NO}3 = 9.64 \times 10^{13}$ cells mol NO_3^- is the yield per mol NO_3^- reduced to N_2), or 0.55×10^{-15} mol N_2 $cell^{-1}$ h^{-1}. Thus, the potential reduction rate of externally supplied N_2O at pH 7 (in the absence of other electron accep-

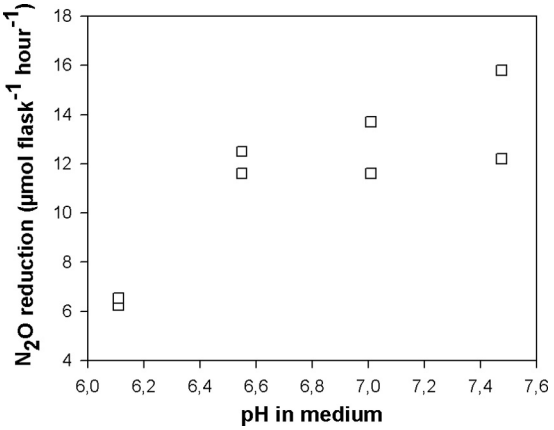

FIG. 5. Effect of pH on N_2O reductase activity in cells in which the denitrification proteome was assembled at pH 7. Actively denitrifying cultures grown in weakly buffered medium at pH 7 were transferred to strongly buffered media at different pH levels and supplied with 6 μmol pure N_2O in headspace. The figure shows the reduction rate of N_2O (μmol $flask^{-1}$ h^{-1}) in basal Sistroms' medium for single flasks, plotted against pH.

tors) was 5 to 6 times higher than the observed rate of denitrification with NO_3^- as the primary electron acceptor.

DISCUSSION

Denitrification is generally located in environments with fluctuating oxygen concentrations, such as transiently wet/flooded soils and the surface layers of sediments, since permanently anoxic environments contain little nitrate or nitrite. Thus, the primary fitness value of denitrification is to enable the organisms to sustain respiratory metabolism during anoxic spells, rather than in permanently anoxic environments. This would imply that the fitness value depends on adequate regulation to avert specific risks: organisms which do not turn off denitrification in the presence of oxygen will lose energy (21), organisms which express the denitrification proteome too late during oxygen depletion may find themselves trapped in anoxia with no available electron acceptors and thus no energy to support a later synthesis of the proteome (15), and organisms which do not manage a balanced expression of the core reductases (NIR and NOR) may become entrapped in toxic concentrations of NO (5).

We have previously demonstrated apparent shortcomings of the regulatory system for denitrification in *A. tumefaciens*. This bacterium was unable to perform a balanced expression in response to fast depletion of oxygen in combination with relatively high cell densities and millimolar concentrations of either NO_3^- or NO_2^- and accumulated NO to apparently paralyzing concentrations (5 to 10 μM NO). In the present study, *P. denitrificans* was challenged under exactly the same batch culture conditions as those for *A. tumefaciens* (Table 2 and Fig. 1), and the results demonstrated a remarkable ability to control the NO concentrations in the medium within a narrow nanomolar range under all conditions tested. While this at first glance seems to describe a shortcoming in *A. tumefaciens* compared to the robustly balanced expression of denitrification in *P. denitrificans*, it is important to keep in mind their respective natural habitats. *A. tumefaciens* is a plant pathogen which during infection must battle its hosts' defense systems. This entails NO detoxification, and thus the "sloppy" regulation of NO in the pathogen may simply reflect a high NO tolerance and even give the microbe an advantage by fending off competitors. The strict regulation of NO in the nonpathogenic organism *P. denitrificans* is most likely a necessity in an organism with a poor NO tolerance.

One surprising feature of *P. denitrificans* was the low number of mRNA copies per cell. However, the estimates were made assuming that all of the cells actively transcribed the denitrification genes. This is not necessarily the case. During transition to anoxia, there is a drastic decrease in total electron flux followed by a log-linear increase in N_2 indicative of exponential growth (see Fig. S2 in the supplemental material). We hypothesize that this phenomenon reflects that only a fraction of the population successfully switches to denitrification. Estimates based on the kinetics of electron flux suggested that as few as 2% of the cells make the transition and start denitrifying under the conditions described here. If the transcription apparatus is also subject to this limitation, the actual mRNA copy numbers per active cell may be 50 times higher than our estimates. We are currently conducting rigorous testing of this hypothesis, and the results will be published in due time.

Another conspicuous trait of *P. denitrificans* are the minute amounts of N_2O produced during transition to anoxic respiration when grown at pH $\geq$7 (N_2O/N_2 product ratio during transition, $\leq 10^{-3}$). This low relative N_2O emission from the very beginning of the anoxic respiration is attributable to the fact that expression of *nosZ* preceded that of *nirS* and *norB* by 5 to 7 h, as seen by the quantification of gene transcripts (Fig. 3) and by the early reduction of externally supplied N_2O (Fig. 4). Bauman et al. (4) similarly observed that *nosZ* transcription preceded that of *nirS* in *P. denitrificans* under somewhat different culturing conditions but did not demonstrate N_2OR activity preceding that of the other enzymes.

Signaling systems for *nosZ* activation appear to differ between bacteria and are not yet fully understood (33). Regulation of the *nos* genes is controlled by transcription factors such as Dnr, DnrD, and Nnr, belonging to the CRP/FNR family, but the details of the activation signals are not known. N_2O is considered to be only a weak inducer in most bacteria studied, while NO is thought to be a strong upregulator of the *nosZ* promoter, as well as the *nosR* and *nosD* promoters, possibly acting via one of the CRP/FNR regulators (33). O_2-sensing systems appear to be partly responsible for the activation of *nos* genes in some bacteria: for example, an FNR homologue in *P. denitrificans* (8). The combined results from gas kinetics and transcription analyses in the present study, showing the onset of *nosZ* transcription clearly preceded significant NO production (detection limit of ~0.2 ppmv, equivalent to 0.4 nM in liquid), strongly indicate that transcriptional activation of *nosZ* in *P. denitrificans* is directly regulated by oxygen. This regulatory phenotype may be an advantage in microbial communities where many of the other organisms release N_2O. By scavenging this N_2O, *P. denitrificans* may sustain its electron transport after depletion of the oxygen, thus securing the energy supply for completing the synthesis of the entire denitrification proteome.

Low pH reduced denitrification rates generally, but the reduction of N_2O to N_2 was most severely affected and was practically eliminated at pH 6 (Fig. 2). We could see three possible explanations for this, which were further investigated: (i) reduced transcription of *nosZ*; (ii) a posttranscriptional effect on the translation, assembly, or folding of the protein; or (iii) a direct pH effect on the activity of the enzyme. Transcription levels were 3 to 6 times lower at pH 6.0 than at pH 7.0 (Fig. 3), but since the *nosZ*/*norB* transcript ratios were similar at both pH levels, we ruled out the possibility of a direct pH effect at transcription level. A subsequent experiment in which N_2O was added to cultures grown at pH 6.0 and 7.0 under anaerobic conditions (Fig. 4) demonstrated that *P. denitrificans* was unable to synthesize functioning N_2OR at pH 6.0, while cells that had first been allowed to assemble the denitrification proteome at pH 7.0 did indeed reduce N_2O at pH 6.11, albeit at a 45% slower rate than at pH 7.0 (Fig. 5). This moderate inhibition of the N_2OR enzyme by low pH is in agreement with the *in vitro* studies of N_2OR from *Achromobacter cycloclastes* by Fujita et al. (13), who ascribed the effect to H^+ ions interfering with the Cu_A center of the N_2OR. Taken together, the series of experimental results strongly suggest that the deleterious effect of low pH on N_2OR occurs at a posttranscriptional

level, by interfering either with translation in the cytoplasm or with the transport and assembly/folding within the periplasm. Low external pH is unlikely to affect the translation within the cytoplasm, since *P. denitrificans* is able to maintain a pH of approximately 7.5 to 8 in the cytoplasm irrespective of external pH (7). The periplasmic pH is likely more affected by external pH. This was clearly demonstrated to be the case in *Escherichia coli* for which the cytoplasmic pH was under strict metabolic control (near pH 7), while the periplasmic pH was entirely controlled by the external pH (29). Our tentative conclusion is thus that the loss of N_2OR activity is due to unsuccessful assemblage/folding of the protein due to low pH in the periplasm.

Other functional enzymes in denitrification are located in the periplasm as well: the nitrate reductase encoded by the *nap* gene cluster and nitrite reductase are both periplasmic enzymes (6). Nitric oxide reductase is anchored in the membrane, and the large subunit resides within the membrane, but the smaller subunit containing a *c*-heme acts as a soluble periplasmic polypeptide. In fact, nitrate reductase encoded by the *nar* gene cluster is the only reductase which is likely to be unaffected by external/periplasmic pH, since it is anchored to the membrane but with the active sites located in the cytoplasm (3). Thus, N_2OR is not the only reductase whose assembly and function depend on the conditions in the periplasm. Nevertheless, it appears to be the one most seriously affected by low pH, which does not rule out misfolding as the mechanism evoked since misfolding by low pH varies among proteins (26). More research is evidently needed to identify the mechanisms involved.

Our results are in some accordance with those of Baumann et al. (4), but the interpretations are different. They suggested that the inhibitory effect of suboptimal pH was due to detrimentally high concentrations of HNO_2 derived from the accumulation of large amounts of nitrite (17 and 35 mM) during the early phase of denitrification (1, 23). Nitrite concentrations never exceeded 2 mM in our experiments, and *P. denitrificans* does not appear to be much affected by this at near-neutral pH: the oxygen consumption and aerobic growth rates were unaffected by added NO_2^- (Fig. 1), and the anoxic growth rate and cell yield per electron were also unaffected (see supplemental material). The observed reduction in anoxic growth rates at low pH, however, could tentatively be ascribed to HNO_2 toxicity, as evaluated by the model of Almeida et al. (1) (see Table S4 and a full description of the calculations in the supplemental material). In our experiments, the *nosZ* transcription clearly preceded significant accumulation of nitrite by several hours (Fig. 2. and 3.); the NO_2^- concentrations remained below 50 μM during the first 6 h in the pH 6 treatment: i.e., the HNO_2 concentration was below 0.1 μM during early transcription and assembly of N_2OR. Thus, although we cannot rule out that HNO_2 toxicity may have played a role in the experiments conducted by Bauman et al. (4), our results strongly suggest a direct effect of pH independent of any HNO_2 toxicity.

There is a growing consensus that soil pH is an important controller of N_2O emissions. The global scale of this problem is evident from recent reports on the ongoing acidification of huge areas of agricultural land in Asia (14), pinpointing the need to develop better, sustainable N_2O mitigation options. The present study provides new knowledge about the mechanisms behind denitrification phenotypic responses to environmental factors, thus contributing to such research efforts.

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