Impaired Reduction of N_2O to N_2 in Acid Soils Is Due to a Posttranscriptional Interference with the Expression of *nosZ*

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ABSTRACT Accumulating empirical evidence over the last 60 years has shown that the reduction of N_2O to N_2 is impaired by low soil pH, suggesting that liming of acid soils may reduce N_2O emissions. This option has not gained much momentum in global change research, however, possibly due to limited understanding of why low pH interferes with N_2O reductase. We hypothesized that the reason is that denitrifying organisms in soils are unable to assemble functional N_2O reductase (N_2OR) at low pH, as shown to be the case for the model strain *Paracoccus denitrificans*. We tested this by experiments with bacteria extracted from soils by density gradient centrifugation. The soils were sampled from a long-term liming experiment (soil pH 4.0, 6.1, and 8.0). The cells were incubated (stirred batches, He atmosphere) at pH levels ranging from 5.7 to 7.6, while gas kinetics (NO, N_2O, and N_2) and abundances of relevant denitrification genes (*nirS*, *nirK*, and *nosZ*) and their transcripts were monitored. Cells from the most acidic soil (pH 4.0) were unable to reduce N_2O at any pH. These results warrant a closer inspection of denitrification communities of very acidic soils. Cells from the neutral soils were unable to produce functional N_2OR at pH values of ≤ 6.1, despite significant transcription of the *nosZ* gene. The N_2OR expressed successfully at pH 7.0, however, was functional over the entire pH range tested (5.7 to 7.6). These observations lend strong support to our hypothesis: low soil pH diminishes/prevents reduction of N_2O, primarily by precluding a successful assembly of functional N_2O reductase.

IMPORTANCE Impaired N_2O reduction in acid soils was first observed ~60 years ago, and the phenomenon has been rediscovered several times since then. The practical implication would be that the emissions of N_2O from cropped soils could be controlled by soil pH management, but this option has largely been ignored till now. One reason for this could be that the mechanisms involved have remained obscure. Here, we provide compelling evidence that the primary reason is that low pH interferes with the making of the enzyme N_2O reductase rather than the function of the enzyme if properly assembled. The implications are important for understanding how pH controls the kinetics of N_2O and N_2 production by denitrification. The improved understanding provides credibility for soil pH management as a way to mitigate N_2O emissions.

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Denitrification in soil is the major source of atmospheric N_2O (1, 2), which contributes to global warming and destruction of stratospheric ozone. The ultimate driver of the ongoing N_2O accumulation in the atmosphere is the input of reactive nitrogen to the biosphere through fertilization and biological nitrogen fixation in agriculture and NOx from combustion (3). This anthropogenic reactive nitrogen will sooner or later return to the atmosphere either as N_2O or N_2, depending on a plethora of factors which control the $N_2O/(N_2O + N_2)$ product ratio of denitrification within the various parts of the biosphere. This product ratio determines the atmospheric footprint of anthropogenic nitrogen, and the mechanisms controlling this ratio are a key issue where microbial ecology can possibly find solutions to a major environmental problem (4).

Wijler, Delwiche, and Nömmik (5, 6) pioneered investigations of the gaseous products of denitrification in soils and provided the first evidence for a negative effect of acidity on the rate of N_2O reduction. Since then, the phenomenon has been rediscovered several times with a variety of soils as summarized by Šimek and Cooper (7), and it now appears an indisputable fact that the $N_2O/(N_2O + N_2)$ product ratio of denitrification in soils tends to increase with increasing acidity of the soils. The implication of this would be that N_2O emission from cropped soils could be minimized by management of pH in cropped soils. However, this mitigation option has not gained much momentum, and only a few attempts have been made to rigorously test the effect of pH management on N_2O emission in agronomic field experiments as summarized by Qu et al. (8). We believe that there are two main reasons for this lack of interest: the strength of the pH control of the $N_2O/(N_2O + N_2)$ product ratio of denitrification has not been fully appreciated, and the mechanisms involved have not been understood.

Recent experiments with the model organism *Paracoccus denitrificans* (9) shed some light on the mechanisms. At pH 7, *P. denitrificans* reduced NO_3^- to N_2 with negligible amounts of N_2O emitted. At pH values of <7, transient accumulation of N_2O was

observed, which increased with decreasing pH, and at pH 6 the cultures produced only N_2O. Thus, with decreasing pH, the cells had increasing difficulty in expressing nitrous oxide reductase (N_2OR) activity. It was demonstrated that this was not due to a restriction at the transcriptional level. A series of supplementary experiments showed that N_2OR, expressed at pH 7, was fully functional at pH 6, although the specific rate was ~50% of that at pH 7. The observed pH response curve of N_2O reduction rate was in good agreement with the pH response of N_2OR *in vitro* as determined by Fujita and Dooley (10). The most plausible explanation for these observations is that low pH interferes with the assembly of the enzyme, which takes place in the periplasm (11), where pH is expected to be less controlled than in the cytoplasm (9).

We recently obtained some evidence for a similar posttranscriptional effect of low pH on the expression of N_2OR in soil bacterial communities, by measuring gene transcription and rates of N_2O reduction in soils from long-term liming experiments (12). In those experiments, where intact soils were investigated, the functional N_2OR was produced at substantially lower soil pH than the minimum for this to occur in *P. denitrificans*. This could suggest that the soils harbor bacteria with a more acid-tolerant expression of N_2OR than that of *P. denitrificans*. Direct evidence for this cannot be provided by such soil incubation experiments, however, due to the potential creation of neutral/alkaline microsites within the soil matrix. This is one reason the pH preference of soil bacteria can only be studied by extracting the cells from the soil matrix (13, 14). Another shortcoming of experiments with intact soil is that it is impossible to test whether functional N_2OR, expressed at pH 7, is functional at low pH, as shown for *P. denitrificans*.

To address these questions, we extracted cells by density gradient centrifugation from soils previously studied (12). The extracted cells were transferred to liquid medium and exposed to a range of pH levels during oxic and subsequently anoxic conditions, while the production/consumption of gases (NO, N_2O, and N_2) and the transcription of relevant functional genes (*nirS*, *nirK*, and *nosZ*) were monitored. Furthermore, we analyzed the pH robustness of functional N_2OR by first allowing the organisms to synthesize the denitrification enzymes at pH 7 and then measuring the potential for N_2O reduction at a range of pH levels (5.7 to 7.6).

RESULTS

NO, N_2O, and N_2 production. To characterize the pH-dependent kinetics of NO, N_2O, and N_2 production by denitrification, we extracted cells from the three soils (pH_s 4.0, 6.1, and 8.0), transferred them to media with 3 pH levels (pH_m 5.7, 6.1, and 7.6) in gas-tight serum vials, and replaced the air with helium (He) after a period of 28 h with oxic conditions. The entire procedure from soil to anoxic incubation is illustrated in Fig. 1. At the end of the incubation, measured pH was marginally higher (<0.2 pH units) than the initial pH. The NO, N_2O, and N_2 production during the anoxic phase is shown in Fig. 2. The pH_m had a profound effect on the rate of denitrification and the composition of its gas production. None of the communities were able to produce detectable amounts of N_2 at pH_m 5.7 and 6.1. At pH_m 7.6, the communities from the pH_s 6.1 and pH_s 8.0 soils produced transient N_2O peaks but finally converted all available NO_3^- to N_2 (50 μmol N_2 vial^{-1}). The recovery of NO_3^-–N to gaseous N was close to 100% for treatment, which reached stable plateaus of either N_2 or N_2O (2 mM NO_3^- in 50 ml = 100 μmol N).

In contrast, the community from pH_s 4.0 was unable to produce N_2 even at pH_m 7.6. This lack of N_2OR activity was not expected, and we investigated the phenomenon further in a series of experiments at several pH_m levels of around 7, using the same procedure as that for the results presented in Fig. 2. This invariably showed that practically all NO_3^- was converted to N_2O. The results of these experiments (including those presented in Fig. 2) are summarized in Fig. 3a, where the N_2O indices are plotted against pH_m. For the bacteria extracted from pH_s 4.0 soil, the indices were around 1.0 at all tested pH levels, i.e., the production of N_2 was insignificant.

Another conspicuous effect of pH_m is that the transient NO accumulation declined with increasing pH_m. This is clearly seen in Fig. 2 and further elaborated in Fig. 3b, where the NO indices are plotted against pH_m (based on the data presented in Fig. 2 and the supplemental experiments with bacteria extracted from the soil with pH_s 4.0). In addition to this direct effect of pH_m on NO accumulation, the communities were different; NO indices were highest for the community from the most acidic soil.

Since the pH_s 6.1 and 8.0 soil communities showed very similar pH-dependent denitrification phenotypes, we decided to concentrate on the soil with intermediate pH (6.1) for testing our core hypothesis regarding the pH dependency of N_2O reductase synthesis. This ensured the cells used were extracted from a soil of which the pH was within the experimental pH range (pH_m 5.7 to 7.6).

Quantification of functional genes and transcripts. Standard curves for calibration of quantitative PCR were linear for all genes studied (10^2 to 10^7 copies; $r^2 > 0.99$, efficiency of ~76 to 87%). Melting curve analysis showed one distinct peak for each expected PCR product and no nonspecific peaks. The primers for *nosZ* generated a dimer peak in the melting curve analysis, but this did not influence the accuracy of the quantitative PCR since the data were collected at 82°C, where the primer dimer had been denatured and no fluorescent signals were emitted.

Figure 4 shows the copy numbers of *nirK*, *nirS*, and *nosZ* genes (a to c) and transcripts (d to f) throughout the anoxic incubation of cells from pH_s 6.1 at pH_m 5.7, 6.1, and 7.6. The copy number of all genes increased sharply during the first 20 h of anoxic incubation at pH_m 7.6. At pH_m 6.1, a similar increase was observed for the *nosZ* and *nirS* genes (although they reached lower levels than at pH_m 7.6), whereas the copy numbers of *nirK* remained practically constant. At pH_m 5.7, the copy numbers of *nosZ* and *nirS* showed a slow increase throughout the incubation. The initial copy numbers of *nirK* genes declined with pH, reflecting changes during the 28 h of oxic preincubation at the respective pH_m (see Fig. 1). Although pH_m clearly affected the growth of denitrifying bacteria, both during the 28-h oxic preincubation (resulting in different initial number of gene copies during the anoxic incubation) and during the anoxic incubation, the pH_m had no consistent effect on the ratio between the copy numbers of *nosZ* and *nir* (*nirK* + *nirS*), as shown in Fig. S1 in the supplemental material.

Transcription of the *nirS*, *nirK*, and *nosZ* genes was quantified for the pH_s 6.1 samples when incubated in media with pH_m 5.7, 6.1, and 7.6. For *nirS* and *nosZ* genes, the transcription, presented as mRNA copy numbers, increased sharply during the first 10 to 15 h and reached somewhat higher levels at pH_m 7.6 than at pH_m 6.1 (Fig. 4). The *nirK* mRNA copy numbers were 1 to 2 orders of magnitude lower than those of *nirS*.

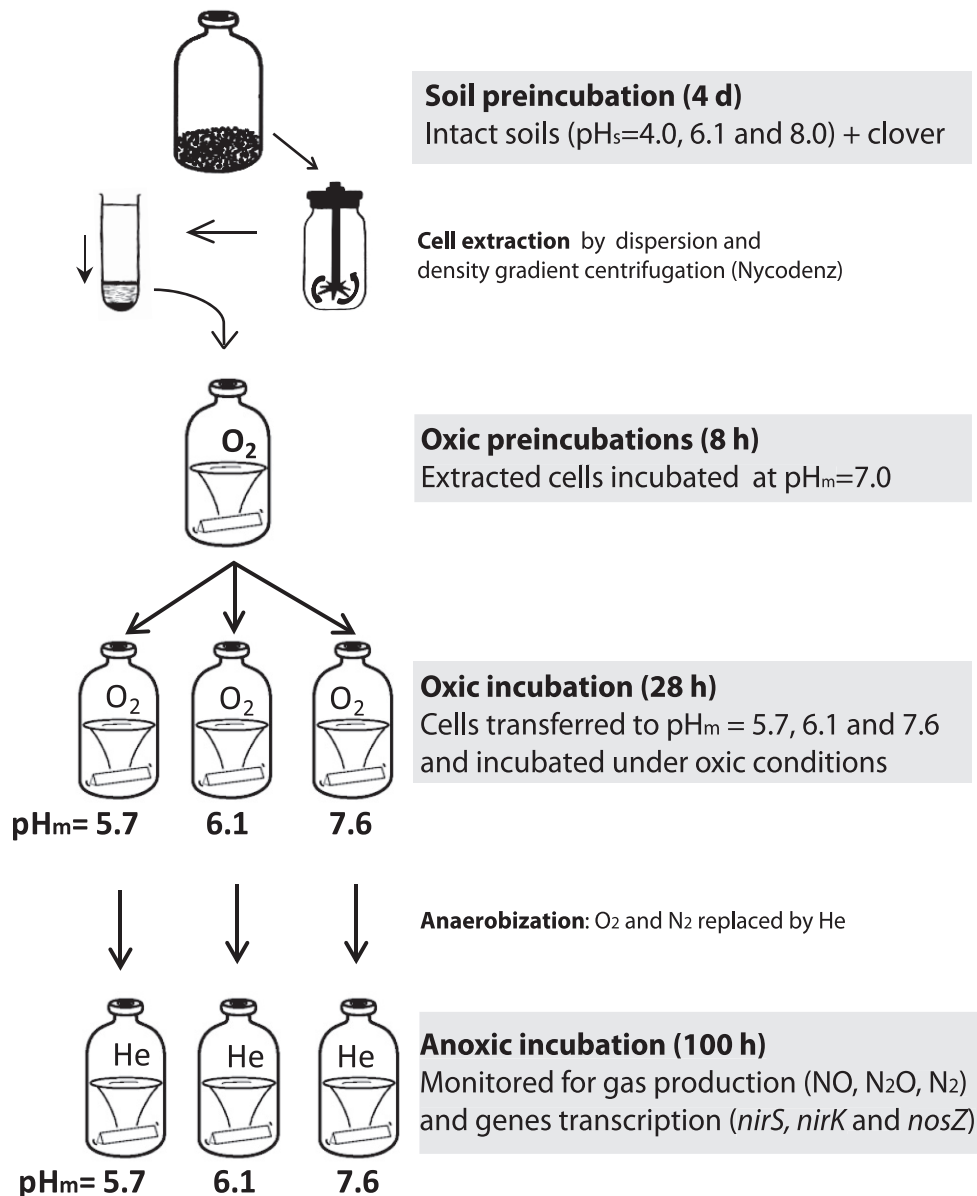To evaluate whether pH_m had any effect on the transcriptional

FIG 1 Outline of the oxic/anoxic incubations of cells extracted from soils with different pH (pH$_s$ 4.0, 6.1, and 8.0). The cells were incubated in minimal media with different pH (pH$_m$ 5.7, 6.1, and 7.6) at 15°C.

regulation of the three genes, we calculated the number of transcripts per gene (i.e., cDNA/DNA). Figure 5 shows the average values (cDNA/DNA) for the first 40 h of incubation (the ratios throughout the anoxic incubation are shown in Fig. S2 in the supplemental material). For *nosZ*, these average transcription ratios were low (0.01 to 0.02) and essentially independent of pH$_m$. Higher values were recorded for *nirS*, with a trend toward lower values at the highest pH, although this was not statistically significant ($P = 0.33$, one-way analysis of variance [ANOVA]). This trend was even more pronounced for *nirK* (and statistically significant, $P = 0.007$).

Since the gene copy numbers increased substantially during the first 20 h of anoxic incubation at pH$_m$ 6.1 and 7.6 (Fig. 4), one might suspect a major shift in the bacterial community composition due to growth of one or a few populations, representing only a small fraction of the originally extracted bacteria. We evaluated this qualitatively by running denaturing gradient gel electrophoresis (DGGE) analyses of the *nosZ* genes during the first 80 h of the pH$_m$ 7.6 incubation of cells extracted from soil with pH$_s$ 6.1. The results show a large number of bands which were essentially unaltered during the whole incubation period (see Fig. S3 in the supplemental material).

The cells extracted from the pH$_s$ 4.0 soil were unable to make functional N$_2$OR even at pH 7.0 to 7.6 (Fig. 2 and 3). To investigate if this could be due to lack of *nosZ* transcription, gene transcripts for this community were quantified during incubation in medium with pH$_m$ 7.6. The results showed that the copy numbers of *nirS* and *nosZ* transcripts were in the same range as for cells extracted from pH$_s$ 6.1 soil, but the cells were unable to reduce N$_2$O to N$_2$ (Fig. 6).
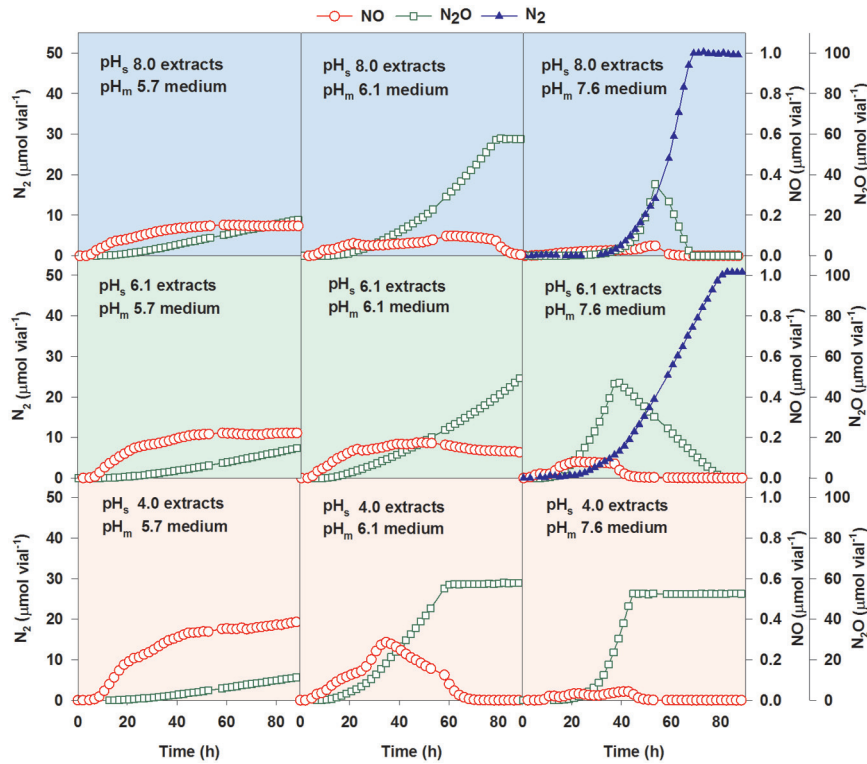
FIG 2 Kinetics of NO, N_2O, and N_2 production by bacteria from soils with different pH levels (pH_s 4.0, 6.1, and 8.0) during anoxic incubation in medium with three different pH levels (pH_m 5.7, 6.1, and 7.6). The figure shows the measured NO (red circle), N_2O (green square), and N_2 (blue triangle), all as μmol vial^{-1} (note the different scales). The production of N_2 was below the detection limit (~150 nmol vial^{-1}) for all treatments except pH_s 6.1 and pH_s 8.0 at pH_m 7.6. The pH was measured at the end of the incubation and was found to be identical to the initial pH except for a slight increase (<0.2 pH units) in the treatments with high denitrification rates.

pH sensitivity of N_2OR. The direct effect of pH on N_2OR activity and oxic respiration was tested by first producing cells with an intact denitrification proteome developed at pH 7.0 (weakly buffered medium), which were then used to inoculate vials with strongly buffered medium covering a pH range from 5.7 to 7.6 (lower pH levels could be desirable but would require another buffer system). Two sets of vials were incubated, both with a medium without NO_3^- and NO_2^- but with different treatments regarding headspace gas composition. Set 1 had 10 ml O_2 liter^{-1} in
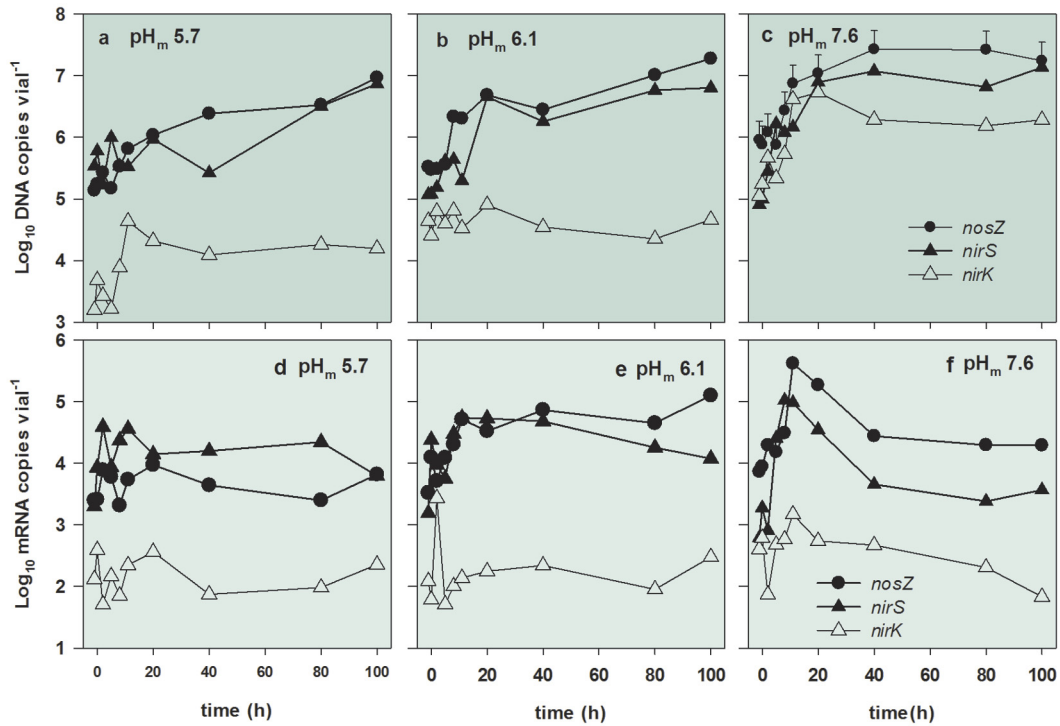
the headspace, in order to measure oxic respiration. Set 2 had anoxic (He) headspace with N_2O (~5.4 ml liter^{-1}) and nitrate-free medium, in order to measure the potential rate of N_2O reduction. The outline of the entire experiment is illustrated in Fig. S4 in the supplemental material. The results are summarized in Fig. 7, where the average rates during the first 5 h of incubation are plotted against pH_m. The oxic respiration rate (Fig. 7A) was ~1 μmol O_2 vial^{-1} h^{-1} and not much affected by pH_m. The rates of N_2O reduction (Fig. 7B) were ~1.15 μmol N_2O vial^{-1} h^{-1} at pH 7.2 and 7.6 and fell gradually with pH to reach ~0.6 μmol N_2O vial^{-1} h^{-1} at pH 5.7.

DISCUSSION

The primary denitrification experiments (Fig. 1 and 2) were designed to investigate whether any of the soils along the pH gradient from 4.0 to 8.0 harbored denitrifying bacteria that could produce functional N_2OR at lower pH than the limit observed for *P. denitrificans* (9). The results indicate that this is not the case; all three soil communities were unable to produce significant amounts of functional N_2OR at pH_m values of ≤6.1 within the time span of the incubation experiments (~100 h), suggesting that the published results from the denitrification model organism demonstrate a more general phenomenon applicable to a wide range of different bacteria. This leads to the obvious question of how organisms in intact soils with pH values of ≤6.1 were indeed able to reduce some N_2O to N_2 (albeit later/slower than in the more alkaline soils) under anoxic conditions, as demonstrated in

FIG 3 Transient accumulation of N_2O and NO by bacteria extracted from soils with different pH (pH_s 4.0, 6.1, and 8.0), depending on the pH in the medium (pH_m). Panel a shows the N_2O index plotted against pH_m (each point is the index for a single vial). The index is a measure of the relative amount of N_2O, cumulating in equation 1 in the text. Panel b shows the corresponding index for NO (see equation 2 in the text for more details).

**FIG 4** Copy numbers of the *nirS*, *nirK*, and *nosZ* genes (a to c) and the corresponding transcripts (d to f) throughout the 100 h of anoxic incubation of cells extracted from soil in which the pH (pH$_s$) was 6.1 and incubated in media of different pH (pH$_m$ 5.7, 6.1, and 7.6) (see Fig. 1). Results are shown in a separate plot for each pH level of the medium. Quantitative PCR was performed in triplicates for each sample, and average values are shown (average coefficients of variation were 32% for DNA and 40% for cDNA). Time = 0 is the time of oxygen removal.

our previous study (12). Our tentative explanation is that the successful making of N₂OR in moderately acid soils (i.e., pH of ~6.0) takes place in neutral/alkaline microsites with higher pH than the average (bulk) pH of the soil. Such microsites may be created within and around clusters of actively denitrifying cells. This has been demonstrated in biofilms (15) and utilized to differentiate between denitrifying and nondenitrifying colonies in agar (16).

We are not aware of any studies of this phenomenon in soils, but alkalinization (1 to 2 pH units) of the soil close to roots during active uptake of NO₃⁻ has been demonstrated (17). If the finding for the model organism *P. denitrificans* (9) also applies to other bacteria, a local pH value of >6.1 is needed to make the N₂OR. This would be increasingly difficult with increasing acidity of the soil, thus explaining the observation that the transient peak of N₂O increased gradually with increasing soil acidity (12). An interesting extrapolation of this is that the distribution of denitrifi-
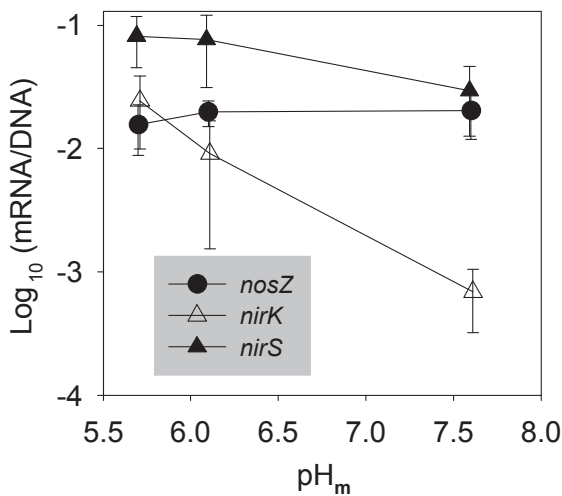
FIG 5 Average transcription ratios for *nirS* and *nosZ* genes (mRNA copies/DNA copies) during the first 40 h of anoxic incubation. The values are based on data presented in Fig. 4. Standard deviations are shown as vertical lines.
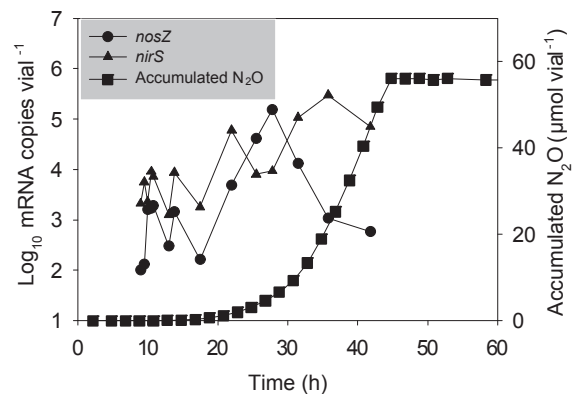
FIG 6 Transcription of *nirS* and *nosZ* during denitrification at pH$_m$ 7.6, by bacteria extracted from soil with pH$_s$ 4.0. The number of transcripts per vial is shown on the left *y* axis, and the accumulated N₂O production is shown on the right *y* axis. N₂ production was below the detection limit during the entire incubation.
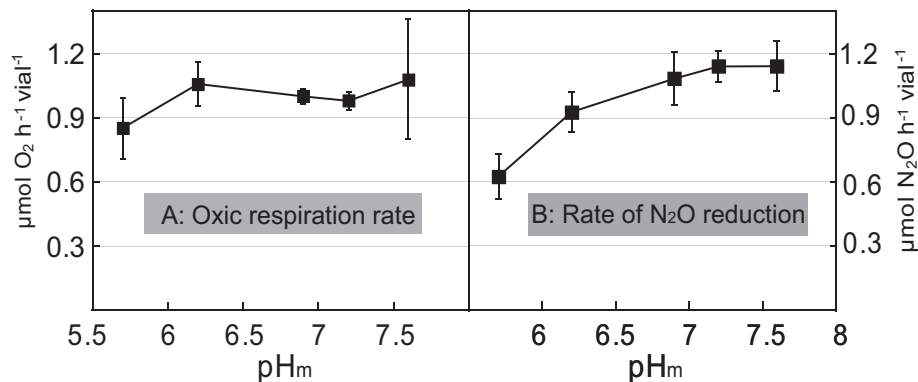
FIG 7 Effect of pH_m (pH level of the medium) on the metabolism of a denitrifying community with functional N_2O reductase was expressed at pH 7.0. The cells (extracted from soil with pH 6.1) were allowed to express denitrification at pH 7.0 and then transferred to vials with pH_m 5.7 to 7.6. (A) Rate of oxic respiration measured in vials with oxic headspace; (B) rate of N_2O reduction in vials with anoxic headspace containing N_2O (and NO_3^--free medium). All rates are averages from the first 5 h of incubation (i.e., after transfer to the different pH_m levels), and standard deviations are shown as vertical bars (3 replicate vials for panel A, n = 6 for panel B).

cation activity within the soil matrix (clumped in large colonies versus dispersed throughout the soil) could have an effect on the N_2O emission: locally neutral/high pH may be reached in micro-sites with a high density of actively denitrifying bacteria (hence production of functional N_2OR), whereas single cells within the soil matrix are unlikely to achieve this.

We were also interested in the kinetics of NO accumulation during anoxic respiration since NO is considered a strong inducer of transcription of *nir* and *nor*, coding for nitrite and nitric oxide reductase, respectively (18). NO was also recently found to induce *nosZ* transcription in *P. denitrificans* (19). The results (Fig. 2) demonstrate that the transient accumulation of NO decreased with increasing pH_m and that the community from the most acidic soil produced more NO than those from the more alkaline soils compared at the same pH_m (Fig. 3). Thus, low pH soils appear to select for organisms that produce large amounts of NO due to imbalanced denitrification. Imbalanced denitrification leading to a high net production of NO was recently demonstrated for *Agrobacterium tumefaciens* in response to changing oxygen levels (20, 21). An alternative explanation could be that low-pH soil selects for organisms that reduce all nitrate to nitrite prior to the reduction of nitrite, which has been observed for certain strains within the genus *Thauera* (22). Nitrite accumulation would enhance NO emission at moderately low pH due to chemical decomposition (23).

By quantification of gene transcription as a function of pH_m, we wanted to clarify if pH affected the transcriptional regulation of *nosZ*. The data as presented in Fig. 4 (copies vial^{-1}) could suggest that the transcription of *nosZ* is strongly repressed by low pH. However, transcription must be evaluated against the copy number of the same genes. The copy number of *nosZ* genes was clearly higher in the vials with higher pH_m, even at the onset of the anoxic incubation. This probably reflects that the organisms harboring the *nosZ* genes grow faster at high than at low pH, both during the oxic incubation and during the subsequent anoxic incubation. A stringent test is thus to calculate the ratio of mRNA copies/DNA copies of *nosZ* as shown in Fig. 5. This suggests that pH had no effect on the transcriptional regulation of the *nosZ* genes (the mRNA/DNA ratio for *nosZ* was not much affected by pH). It should be kept in mind, though, that PCR-based quantifications

of genes and transcripts are biased. The PCR primers used in this study will not capture all the genes coding for N_2OR (24). However, the mRNA/DNA ratios should be valid for the genes amplified with the primers used, since cDNA and DNA were amplified by the same set of primers. Thus, for the organisms harboring these genes, there is no evidence for a pH-dependent transcription of the *nosZ* gene. The result corroborates earlier findings (9, 12) that transcriptional regulation provides no explanation for the low N_2OR activity in acid soils.

The results for the cells extracted from soil with pH 4.0 are difficult to understand for several reasons. One would expect that if denitrifying bacteria with an acid-tolerant N_2OR expression exist, they should be selected in the most acidic soil, but the results lend no support to this notion; the bacteria extracted from the most acidic soil were unable to produce functional N_2OR even at pH values around 7.0 (Fig. 2 and 3). We hypothesized that the complete lack of N_2OR activity in the pH_s 4.0 cells could be ascribed to a "silencing" of the *nosZ* genes in this soil through mutation of the *nosZ* gene itself or another gene necessary for its expression, such as *nosR* (25). The latter was reported in a recent, comparative study of denitrification in strains within the genus *Thauera*, of which one strain was unable to reduce N_2O to N_2, apparently due to the absence of an intact *nosR* gene (22). However, the quantification of transcripts (Fig. 6) in cell extracts from the pH_s 4.0 soil in the present study lends no support to such lack of transcription, at least for the organisms whose *nosZ* genes were captured by our primers. An alternative explanation is that mutations may have occurred in other genes coding for factors involved in *nosZ* maturation or, as discussed above, that those factors do not function at low pH.

The complete lack of N_2 production by the cells from the soil with pH_s 4.0, even at pH_m values of ≥7, is in stark contrast to recent findings in our laboratory that this soil is able to express N_2OR quite effectively in response to liming (unpublished data). This may suggest a serious bias in the cell extraction by density gradient centrifugation. Nadeem et al. (26) recently investigated this by comparing the kinetics of N_2O and N_2 production by loosely attached and strongly attached cells (the latter extracted by vigorous dispersion of the pellet beneath the density gradient of the first extraction). The results demonstrated a moderate bias in

the sense that the loosely attached cells produced less N$_2$ and more N$_2$O than the strongly attached cells. This bias might be more severe for the soil with pH 4.0 than for the other soils, thus reconciling the apparently conflicting observations of the response to increased pH (intact soil responding by N$_2$O reduction, extracted cells not). This does not resolve the puzzle, however, that the cells extracted from this soil were notoriously unable to reduce N$_2$O to N$_2$ despite significant transcription of *nosZ* (Fig. 6).

In the last set of experiments, cells with an intact denitrification proteome, expressed at pH 7.0, were challenged to reduce N$_2$O at a range of pH values (Fig. 7). The results strongly support our hypothesis that the successfully assembled N$_2$OR is functional over a wide pH range and provide indirect evidence that the complete inability of the indigenous soil bacteria to produce active N$_2$OR at pH values of ≤6.1 is a posttranscriptional problem which is due to interference with the making of the enzyme. At present, we do not know why organisms are unable to make functional N$_2$OR at pH values of ≤6.1. Based on the results with *P. denitrificans*, we hypothesized that it may be due to interference with the assembly of the protein in the periplasm where the insertion of Cu^{2+} is possibly hindered by low pH (9). If so, the enzyme could be "repaired" by increasing the pH, as demonstrated for copper-deficient N$_2$OR in *Pseudomonas stutzeri* (27). In theory, this may even happen at low pH, albeit so slowly that it will not be observed within the time frame of our incubation experiments. Further investigations are needed to clarify these questions.

One would expect that acid environments select for organisms which are able to produce active N$_2$OR successfully at low pH. Our data provide compelling evidence that such organisms are not present in the soils studied. Is there any evidence for it elsewhere? Palmer and Horn (28) found high rates of N$_2$O reduction in acid (pH 4.4) peat soils, determined by anoxic incubation of soil slurries. The N$_2$O reduction was ascribed to denitrification driven by organisms with *nirS* rather than *nirK*. This is hardly evidence for acid-tolerant production of N$_2$OR, since there may exist neutral/alkaline hot spots within the soil matrix, as discussed above. Green et al. (29, 30) studied denitrifying communities in contaminated subsurface soils and found that environments with low pH (3, 4) selected for denitrifying bacteria within the genus *Rhodanobacter*. They concluded that these organisms represent an acid-tolerant group of denitrifiers, able to reduce NO$_3^-$ all the way to N$_2$. Six isolates were recently genome sequenced, confirming the presence of *nirK* and *nosZ* genes, although three of the isolates lacked the gene for nitrate reductase (31). To our knowledge, no stringent test has been conducted to evaluate the ability of *Rhodanobacter* strains to express N$_2$OR at low pH. However, the investigation by van den Heuvel et al. (32) sheds some light on this. They grew denitrifying communities from acid soils in continuous reactors at variable pH and found that low pH selected for *Rhodanobacter* strains which were evidently able to denitrify at pH as low as 4. However, although the communities were able to reduce NO$_3^-$ to N$_2$ when grown at neutral pH, they produced practically pure N$_2$O at pH values of <6.2. Thus, it may well be that *Rhodanobacter* strains are adapted to denitrify at low pH, but they appear to have the same difficulties with producing active N$_2$OR at such low pH as other bacteria.

We are aware that the primers used for *nosZ* in this study fail to amplify atypical *nosZ* genes claimed to be important in soils (33). However, this would hardly affect the validity of our conclusions regarding the lack of functional N$_2$O reductase at low pH and the

robust functioning of N$_2$O reductase (expressed at pH 7.0) at pH below 6. We are also aware that the composition of the microbial communities may have an impact on denitrification rates, the gaseous product composition, and the emissions from soils as explored in several studies, which are reviewed recently by Braker and Conrad (34). One common notion in these studies has been that high N$_2$O emission could be ascribed to the absence of organisms carrying *nosZ* (35). This appears irrelevant in our case, however, since the *nosZ* gene pool was abundant at all 3 pH$_m$ levels (12).

In conclusion, we are beginning to understand why soil acidity exerts a pervasive control of the N$_2$O/(N$_2$ + N$_2$O) product ratio of denitrification and hence on the propensity of soils to emit N$_2$O. Some empirical evidence for a direct effect of pH on N$_2$O emissions has been provided by studying the spatial distribution of N$_2$O emission and pH in forested riparian soils (36) and grassland soils (37). More rigorous testing is clearly needed to evaluate this. The immediate effect of liming acid soils may be to enhance N$_2$O emissions; however, as demonstrated by Clough et al. (38) and Baggs et al. (39), the reason for this is that liming acid soils will induce a transient enhancement of C and N mineralization and nitrification and hence also the rate of denitrification. The net outcome may thus be that liming induces a transient enhancement of N$_2$O emission despite a lowered N$_2$O/(N$_2$ + N$_2$O) product ratio of denitrification. However, the long-term effect of sustaining a high pH in agricultural soils is most likely to secure low N$_2$O emissions.

MATERIALS AND METHODS

Soil and extraction of bacteria. Peat soils (Sapric Histosol, FAO/ISRIC/ISS, containing 45% organic C, 2% organic N) were sampled from a soil liming experiment (pH$_s$ 4.0 to 8.0) established in 1978 (40), which was used previously for investigating N$_2$O production by nitrification (41) and denitrification (12). The soils were sieved (6 mm) while moist and stored moist (1.5 ml water g^{-1} soil [dry weight], i.e., ~50% air-filled porosity) at 4°C until used. The outline of the preincubation, cell extraction, and subsequent incubations is shown in Fig. 1. To raise the microbial biomass/activity prior to cell extraction, moist soil samples (20 g fresh weight = 8 g dry weight) were mixed with finely ground clover leaves (5 mg/g soil [dry weight]) and incubated for 85 h at 15°C (monitored for respiration rate and NO and N$_2$O production [see Fig. S5 in the supplemental material]). Cells were then extracted by dispersion/density gradient centrifugation as described by Bakken and Lindahl (42). The pH of soil slurries of pH 6.1 and 4.0 soils was adjusted to 7.0 prior to dispersion. The total number of cells extracted was determined by epifluorescence microscopic counts (43) in preliminary experiments (7 × 10^9 to 9 × 10^9 cells g^{-1} soil [dry weight], which is 10 to 12% of the total numbers of cells in the soils; no statistical difference between soils as tested by one-way analysis of variance [ANOVA]).

Culture conditions and general incubation procedure. The medium was the same as that used by Morley et al. (44): 3 mM (NH$_4$)$_2$SO$_4$, 5 mM glutamic acid, 1.76 mg liter^{-1} EDTA, 10 mg liter^{-1} ZnSO$_4$, 5 mg liter^{-1} FeSO$_4$, 1.5 mg liter^{-1} MnSO$_4$, 0.4 mg liter^{-1} CuSO$_4$, 0.25 mg liter^{-1} Co(NO$_3$)$_2$, 0.15 mg liter^{-1} H$_3$BO$_3$, 1 mg liter^{-1} nicotinic acid, 0.5 mg liter^{-1} thiamine, and 1 mg liter^{-1} biotin, buffered with sodium phosphate (10 mM for the oxic preculturing and 100 mM for the denitrification experiments). The concentration of NO$_3^-$ was either 1 or 2 mM (KNO$_3$), except when determining potential N$_2$O reduction, for which NO$_3^-$-free medium was used, prepared as described by Bergaust et al. (19).

All culturing was done in serum vials (120 ml) with butyl rubber septa, containing a 50-ml culture volume, vigorously stirred by Teflon-coated magnetic bars and placed in a robotized incubation system (at 15°C) which monitors the headspace concentrations of O$_2$, CO$_2$, NO, N$_2$O, and

N₂. The system is described in detail by Molstad et al. (45), who also describe the techniques to replace headspace atmosphere with He prior to inoculation.

The extracted cells (~10 ml) were transferred to vials containing 40 ml weakly buffered medium (10 mM phosphate, pH 7.0) and incubated (oxic) for 8 h. The precultured suspensions were then used to inoculate vials (5 ml vial⁻¹) containing 45 ml strongly buffered medium (100 mM phosphate, 2 mM KNO₃) at three different pH levels (pH$_m$ 5.7, 6.1, and 7.6), which were first incubated for 28 h under oxic conditions (oxic incubation [28 h], Fig. 1) while respiration rates were monitored. Measured respiration and apparent oxic growth rates (0.14 to 0.25 h⁻¹) are shown in Fig. S6 and S7 in the supplemental material. The motivation for these oxic preincubations was to secure a low level of intact denitrification enzymes in the cells prior to the anoxic incubation (preexisting denitrification enzymes would be diluted by oxic growth).

The vials were then made anoxic by evacuating and filling with pure helium (He) twice (reducing oxygen concentration to ≤250 μl liter⁻¹ = 0.4 μM in the liquid), and the incubation was continued for 100 h (anoxic incubation, Fig. 1). The incubation robot only takes 15 vials, thus it was not possible to include replicates for all 9 treatments. However, the experiment was repeated several times ($n \geq 3$), both for the community extracted from the soil with pH$_s$ 6.1 (for quantification of genes and transcripts, see below) and for the community extracted from the most acidic soil (pH$_s$ 4.0). There was generally little variation in gas kinetics results between replicates within and between experiments, as seen in Fig. 3, which condenses the results in one graph.

The NO and N₂O indices (12) were calculated in order to summarize the effect of pH$_m$ on the transient accumulation of NO and N₂O as a fraction of the total N gas produced:

$$I_{N_2O} = \int_0^T N_2O(t) / \left[\int_0^T N_2O(t) + \int_0^T NO(t) + \int_0^T N_2(t) \right] \quad (1)$$

$$I_{NO} = \int_0^T NO(t) / \left[\int_0^T N_2O(t) + \int_0^T NO(t) + \int_0^T N_2(t) \right] \quad (2)$$

where NO(t), N₂O(t), and N₂(t) are the amounts of the three gases at any time t and T is the time when all NO₃⁻ is recovered as gas (NO + N₂O + N₂).

Determination of the effect of low pH on denitrification enzymes expressed at high pH. To determine the direct effect of pH on the activity of the denitrification enzymes, cells extracted from soil with pH$_s$ 6.1 were incubated at pH$_m$ 7.0 (10 mM phosphate buffer) under anoxic conditions and allowed to deplete the 2 mM NO₃⁻ in the medium (recovered as N₂ in the headspace). This culture was then used to inoculate a series of vials containing 45 ml strongly buffered medium (100 mM phosphate buffer) with five different pH$_m$ levels ranging from 5.7 to 7.6. We prepared two sets of vials for these experiments: (i) the first set had oxic headspace (10 ml O₂ liter⁻¹) and was used to determine the oxic respiration; (ii) the second set had NO₃⁻-free medium and He atmosphere to which we added ~0.6 ml pure N₂O, resulting in ~5.4 ml N₂O liter⁻¹ in the headspace (~170 μM N₂O in the liquid). These vials were used to obtain a direct measurement of the rate of N₂O reduction.

For both sets, O₂, N₂O, and N₂ concentrations were monitored by sampling every 40 to 60 min for a period of 20 h. The measured rates (i.e., O₂ consumption in set 1; N₂O reduction in set 2) were fairly constant throughout the first 5 to 7 h of incubation, as illustrated for set 2 (see Fig. S8 in the supplemental material). Since the purpose of these experiments was to determine the direct effect of the change in pH, we report only the average rates during the first 5 h of incubation.

Extraction of nucleic acids. Gene expression was measured on cells from the pH$_s$ 6.1 soil incubated at pH$_m$ 5.6, 6.1, and 7.6 in a separate water bath, in parallel with the experiment in the incubation robot (stirring and temperature conditions were identical). Three replicate vials were sampled (entire vials) at each time point shown in Fig. 4. The cells were collected by centrifugation (10,000 × g, 10 min at 4°C) and resuspended in 1 ml of RNAprotect (Qiagen, Nordic-Norway) and quickly frozen at −20°C. The same treatment was also performed for cells form pH$_s$ 4.0 soil incubated at pH$_m$ 7.6 medium.

Total nucleic acids were isolated using a bead-beating method. To avoid RNase contamination, only certified nuclease-free tubes and diethyl pyrocarbonate (DEPC)-treated solutions (46) were used. The cell lysis, protein denaturation, and nucleic acid purification were performed by a method described in reference 47 with modifications (12, 48). The nucleic acid extracts were split into two portions, and these were treated with RNase and DNase, respectively. The extracts intended to contain only RNA were treated with an RNase-free DNase set (Qiagen, Nordic-Norway) for 30 min, but this did not remove all genomic DNA. An additional 2.5-h treatment was conducted with the DNase I kit (Sigma-Aldrich). This resulted in successful removal of all genomic DNA, as seen from the absence of PCR products from control samples that had been treated with DNase but not with reverse transcriptase. The A260/A280 ratios of both DNA and RNA were higher than 1.7 for all samples.

Reverse transcription. Reverse transcription (RT) was performed using the Superscript RT-PCR system (Invitrogen) with the method described in the manual. Two master mix solutions were prepared in separate 0.2-ml tubes. Master mix 1 contained 1 μl deoxynucleoside triphosphate (dNTP) mix (10 mM each; TaKaRa Bio Co., Japan), 2 μl random hexamer primers (50 ng/μl; Promega), total RNA, and RNase-free water up to 13 μl. Master mix 2 contained 4 μl 5× first-strand buffer, 1 μl 0.1 M dithiothreitol (DTT), 1 μl Superscript III RT enzyme (200 units/μl), 1 μl RNaseOUT recombinant RNase inhibitor (Invitrogen; 40 units/μl). Master mix 1 solution was incubated at 65°C for 5 min and put on ice for 2 min. The contents of the tube were collected by brief centrifugation before master mix 2 was added. The tube was transferred to a thermocycler (Applied Biosystems) and incubated at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. The cDNA was stored at −20°C until quantitative PCR analysis. Control samples containing the same concentration of RNA, but on which reverse transcription was not performed, were prepared for each gene (*nirS*, *nirK*, and *nosZ*) and subjected to quantitative PCR to ensure that all genomic DNA was removed by DNase.

Quantitative PCR. The genes and their transcripts were quantified with a StepOne Plus quantitative PCR system (Applied Biosystems). Gene fragments of *nirS*, *nirK*, and *nosZ* were amplified with the primer pairs cd3af, r3cd (49, 50), 1F, 5R (51), Z-F, 1622R (50, 52), respectively. A standard curve method was employed, since previous studies using the same experimental setup for batch culture incubation showed that the use of a housekeeping gene as the endogenous standard generated unstable expression levels due to the rapid changes in bacterial activity and growth (20). Construction of the standard curves, as well as the quantitative PCR program, was as described previously (12).

DGGE. DGGE was performed on extracts from pH$_s$ 6.1 soil, sampled during the incubation shown in Fig. 2. The samples from pH$_m$ 7.6 medium were taken during the anoxic incubation period at 0 h, 20 h, 40 h, 60 h, and 80 h. The *nosZ* gene was amplified with primer pair ZF and 1622R-GC (50, 52). DGGE of PCR products was performed using the method described by Muyzer et al. (53) with the use of a Dcode system (Bio-Rad Laboratories). Polyacrylamide gradient gel was made by 8% polyacrylamide with 35 to 60% denaturant (100% defined as 7 M urea and 40% formamide). The gel was run for 200 min at 200 V in 1× Tris-acetate-EDTA (TAE) buffer at a constant temperature of 60°C. The gels were stained with ethidium bromide and photographed in a GelDoc XR UV light imager (Bio-Rad).

Statistical analyses. Statistical analyses were performed using Minitab 16 (Minitab Inc., State College, PA, USA). One-way ANOVA was used to compare the cell extraction efficiencies for the different soils and for testing if the pH in the media affected the mRNA/DNA ratio for the three genes in question. Preliminary analysis of the mRNA/DNA ratio showed

that the distribution of the residuals deviated significantly from normal distribution ($P < 0.01$; tested by Kolmogorov-Smirnov's test for normality). Hence, we analyzed the log-transformed data, for which the distribution of residuals did not significantly deviate from normal distribution ($P > 0.15$). For cell extractions, log transformations prior to ANOVA were not necessary (the distribution of residuals did not deviate significantly from the normal distribution).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01383-14/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.2 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 0.5 MB.
Figure S6, DOCX file, 0.1 MB.
Figure S7, DOCX file, 0.1 MB.
Figure S8, DOCX file, 0.1 MB.

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