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Suggested role of NosZ in preventing N_2O inhibition of dissimilatory nitrite reduction to ammonium

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ABSTRACT Climate change and nutrient pollution are among the most urgent environmental issues. Enhancing the abundance and/or the activity of beneficial organisms is an attractive strategy to counteract these problems. Dissimilatory nitrate reduction to ammonium (DNRA), which theoretically improves nitrogen retention in soils, has been suggested as a microbial process that may be harnessed, especially since many DNRA-catalyzing organisms have been found to possess *nosZ* genes and the ability to respire N_2O. However, the selective advantage that may favor these *nosZ*-harboring DNRA-catalyzing organisms is not well understood. Here, the effect of N_2O on Nrf-mediated DNRA was examined in a soil isolate, *Bacillus* sp. DNRA2, possessing both *nrfA* and *nosZ* genes. The DNRA metabolism of this bacterium was observed in the presence of C_2H_2, a NosZ inhibitor, with or without N_2O, and the results were compared with C_2H_2-free controls. Cultures were also exposed to repeated oxic-anoxic transitions in the sustained presence of N_2O. The NO_2^--to-NH_4^+ reduction following oxic-to-anoxic transition was significantly delayed in NosZ-inhibited C_2H_2-amended cultures, and the inhibition was more pronounced with repeated oxic-anoxic transitions. The possibility of C_2H_2 involvement was dismissed since the cultures continuously flushed with C_2H_2/N_2 mixed gas after initial oxic incubation did not exhibit a similar delay in DNRA progression as that observed in the culture flushed with N_2O-containing gas. The findings suggest a possibility that the oft-observed *nosZ* presence in DNRA-catalyzing microorganisms secures an early transcription of their DNRA genes by scavenging N_2O, thus enhancing their capacity to compete with denitrifiers at oxic-anoxic interfaces.

IMPORTANCE Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) is a microbial energy-conserving process that reduces NO_3^- and/or NO_2^- to NH_4^+. Interestingly, DNRA-catalyzing microorganisms possessing *nrfA* genes are occasionally found harboring *nosZ* genes encoding nitrous oxide reductases, i.e., the only group of enzymes capable of removing the potent greenhouse gas N_2O. Here, through a series of physiological experiments examining DNRA metabolism in one of such microorganisms, *Bacillus* sp. DNRA2, we have discovered that N_2O may delay the transition to DNRA upon an oxic-to-anoxic transition, unless timely removed by the nitrous oxide reductases. These observations suggest a novel explanation as to why some *nrfA*-possessing microorganisms have retained *nosZ* genes: to remove N_2O that may otherwise interfere with the transition from O_2 respiration to DNRA.

KEYWORDS nitrous oxide reduction, dissimilatory nitrite reduction to ammonium, oxic-anoxic transition, *Bacillus*, transcriptional regulation

Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) is the respiratory reduction of NO_3^- and/or NO_2^- to NH_4^+ (1–3). All DNRA-catalyzing isolates examined thus far utilize organic compounds as the source of electrons, although recent culture-independent observations suggest the existence of lithotrophic DNRA in the

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environment (4, 5). As DNRA and denitrification essentially share the same electron donors and acceptors and are both anaerobic reactions activated in response to O_2 depletion, the two respiratory NO_3^-/NO_2^- pathways compete in the environment (2, 6, 7). This competition is often viewed in the context of the relative availability of organic carbon and NO_3^- /NO_2^-; as DNRA theoretically yields larger amount of energy per molecule of NO_3^- reduced, it has been hypothesized that DNRA would be competitive in reduced environments, often characterized by high C:N ratios (8–12). This redox- or C:N-ratio-controlled competition between denitrification and DNRA was demonstrated in several pure culture studies of organisms harboring both denitrification and DNRA pathways, e.g., *Shewanella loihica* PV-4, as well as in laboratory studies of complex microbial communities (5, 8, 13–15). However, environments where DNRA outcompetes denitrification, thus contributing substantially to the fate of NO_3^-, are rarely found, apart from highly reduced and/or sulfide-rich marine sediments (5, 16). If artificial stimulation of DNRA activity may be possible, either via biostimulation or bioaugmentation approaches, DNRA would have various environmental applications. Outcompeting denitrification with DNRA has been proposed as a means to improve nitrogen management of agricultural soils, as DNRA activation would reduce the amounts of nitrogen lost via denitrification and leaching (2, 17, 18). In the wastewater sector, DNRA has been suggested as a complement to the anammox process, as DNRA can reverse excessive nitrification and reduce undesired NO_3^- back to NO_2^- and NH_4^+ (19, 20). Such attractive potential applications warrant further investigation into the DNRA ecophysiology.

Previously, production of NH_4^+ from reduction of NO_3^- and NO_2^- has been verified for multiple soil isolates carrying either *nrfA* or *nirB* (3). While NirB has an assimilatory function in many organisms and thus is not exclusive to DNRA, the physiological function of the cytochrome c_{552} nitrite reductase encoded by *nrfA* is limited to the respiratory role in DNRA (3, 21, 22). Further, the NO_2^--to-NH_4^+ turnover in the microorganisms possessing *nirB* but no *nrfA* invariably required a fermentable organic substrate as the source of electrons, suggesting that NO_2^- may be used for NADH regeneration, rather than being the terminal electron acceptor for energy conservation (3, 23). For these reasons, the signature functional gene representing the DNRA pathway has long been the *nrfA* gene, and NirB-mediated NO_2^--to-NH_4^+ reduction is probably not a respiratory reaction, despite the NH_4^+ release observed with *nirB*-possessing microorganisms lacking *nrfA* (24, 25).

One of the unresolved conundrums surrounding the *nrfA* gene is its widespread co-presence with the *nosZ* gene, i.e., the gene encoding the nitrous oxide reductase, in bacterial genomes (3, 26–28). Further, several *nrfA*-possessing and DNRA-catalyzing microorganisms carrying *nosZ*, e.g., *Wolinella succinogenes*, *Anaeromyxobacter dehalogenans*, and *Bacillus vireti*, were able to reduce N_2O to N_2 (26, 27, 29). These microorganisms lack *nirS* and *nirK* and thus do not reduce NO_2^- via canonical denitrification, traditionally defined as respiratory reduction of NO_2^- where >80% of the NO_2^- is converted to N_2O and N_2 via NO (30). Release of N_2O from NrfA-mediated DNRA was demonstrated with the four *nrfA*-possessing soil isolates examined earlier (3). All four strains released 0.4–3.0% of reduced NO_3^- as N_2O, and *Bacillus* strain DNRA2, the only one of these that possessed *nosZ*, presumably consumed the N_2O that it produced, as N_2O accumulation was observed in the presence of NosZ inhibitor C_2H_2, but not in its absence (31). Energy conservation via N_2O reduction was implied in the observed cell growth in the *W. succinogens* (the *nosZ*$^+$ variant), *A. dehalogenans*, and *B. vireti* cultures fed N_2O as the sole electron acceptor together with a non-fermentable electron donor (27, 32, 33). Apparent from these observations, N_2O-reducing capability would benefit the DNRA-catalyzing organisms by enabling them to utilize the fugitive N_2O from DNRA, as well as N_2O released from other organisms in their habitat (27, 34, 35). Perhaps, as the *nosZ* genes these organisms harbor mostly belong to the clade II, which, in general, tend to exhibit higher affinities to N_2O, the possession of *nosZ* and the capability to capitalize on sub-micromolar N_2O may even be crucial for their survival in environmental niches unfavorable for DNRA in competing with denitrifiers (27, 34).

Here, using *Bacillus* sp. DNRA2 as a model organism, we focused on elucidating the ecophysiological benefits of being able to reduce N_2O, apart from utilization of N_2O for energy conservation. A series of physiological experiments were performed with *Bacillus* sp. DNRA2 growing in batch cultures, with frequent monitoring of nitrogenous gases, NO_3^-, NO_2^-, and NH_4^+ concentrations before, during, and after an oxic-to-anoxic transition. In particular, the effect of N_2O on its DNRA activity was examined. The results suggested an inhibitory effect of N_2O on DNRA activity following an oxic-anoxic transition, which was further examined by reverse-transcription qPCR targeting *nrfA* transcripts of cultures exposed to repeated oxic-anoxic spells. This study provides a previously unrecognized evolutionary explanation for possession of *nosZ* by DNRA-catalyzing microorganisms and discusses its implications for denitrification-vs-DNRA competition.

RESULTS

Effect of N_2O on DNRA in *Bacillus* sp. DNRA2 batch cultures

The batch experiments performed with *Bacillus* sp. DNRA2 with the four different gas amendments (N_2 only, N_2O/N_2, C_2H_2/N_2, or $N_2O/C_2H_2/N_2$; see Materials and Methods for details) showed that the onset of NO_2^--to-NH_4^+ reduction following O_2 consumption was delayed by the presence of N_2O (Fig. 1). The dissolved O_2 concentration decreased below

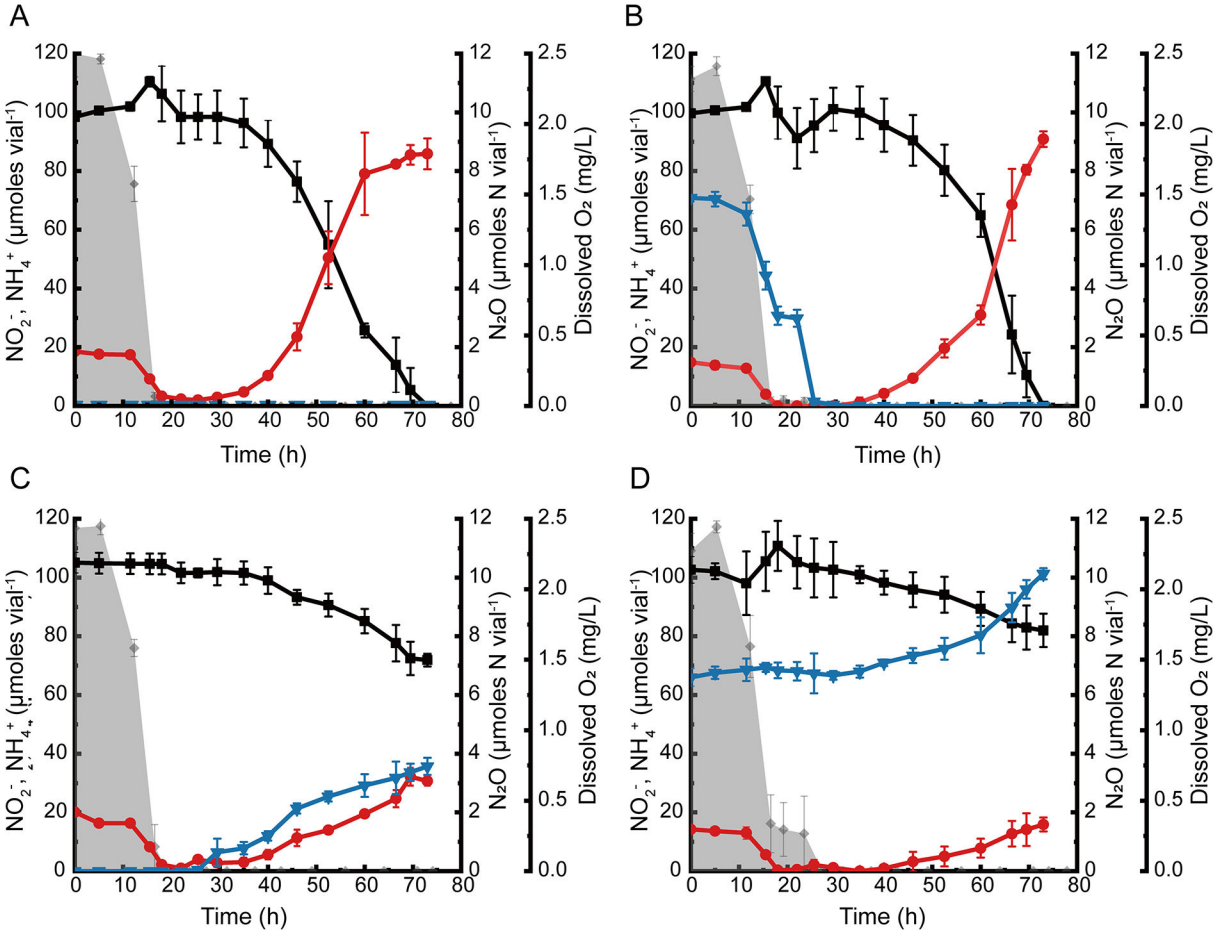

FIG 1 Incubation of 100 mL (prepared in 160 mL serum vials with the headspace consisting of 95% N_2 and 5% O_2) *Bacillus* sp. DNRA2 cultures with 1.0 mM NO_2^-. The following incubation conditions were examined: (A) control without any headspace amendment, (B) N_2O-amended condition with 3.5 µmol N_2O initially added to the culture vials, (C) C_2H_2-amended condition with 10% of the headspace replaced with C_2H_2, and (D) N_2O-and-C_2H_2-amended condition. The data points represent the average of triplicate cultures and the error bars are the standard deviations of the values obtained from triplicate cultures (■, NO_2^-; ●, NH_4^+; ▼, N_2O-N; shaded curve, dissolved oxygen).

the detection limit (0.07 mg L^{-1}) within 25 h. The NH_4^+ concentration decreased to <5 µM by 20 h in all cultures, presumably due to assimilation, as the cell concentration increased to an OD_{600} value of 0.030 ± 0.002 after O_2 depletion (the OD_{600} data are not shown, as no significant growth occurred after O_2 depletion). In the controls, 0.86 ± 0.05 mM NH_4^+ was produced from reduction of 0.98 ± 0.02 mM NO_2^- within 60 h of O_2 depletion (Fig. 1A). In the cultures amended with N_2O but not C_2H_2, N_2O was completely consumed within 10 h after O_2 depletion, before any significant consumption of NO_2^- or production of NH_4^+ occurred (Fig. 1B). When the experiment was terminated at 73 h, 1.00 ± 0.01 mM NO_2^- was reduced to 0.91 ± 0.03 mM NH_4^+, indicating that DNRA was marginally affected by the initial presence of N_2O. The inclusion of C_2H_2 to the headspace resulted in substantial delays in NO_2^- consumption and NH_4^+ production. In the cultures with the headspace initially containing C_2H_2 but no N_2O, N_2O production began at 29.5 h, and the amount of N_2O-N eventually reached 3.57 ± 0.29 µmol N_2O-N $vial^{-1}$ at 73 h, accounting for 12.0 ± 1.9% of NO_2^- that had been consumed up to this point (Fig. 1C). Reduction of NO_2^- to NH_4^+ was significantly delayed under this incubation condition, and only 3.6 ± 0.3 µmol NH_4^+ $vial^{-1}$ was detected at 73 h. In the cultures to which N_2O was added along with C_2H_2, NO_2^--to-NH_4^+ reduction was further inhibited (Fig. 1C). The amount of N_2O increased from 6.6 ± 0.3 to 10.1 ± 0.2 µmol N_2O-N $vial^{-1}$ (16.5 ± 2.2% of consumed NO_2^-). Interestingly, the anoxic cultures incubated with C_2H_2 and N_2O did not show any noticeable difference from the controls, suggesting that DNRA inhibition occurred only during the oxic-anoxic transitions (Fig. S1).

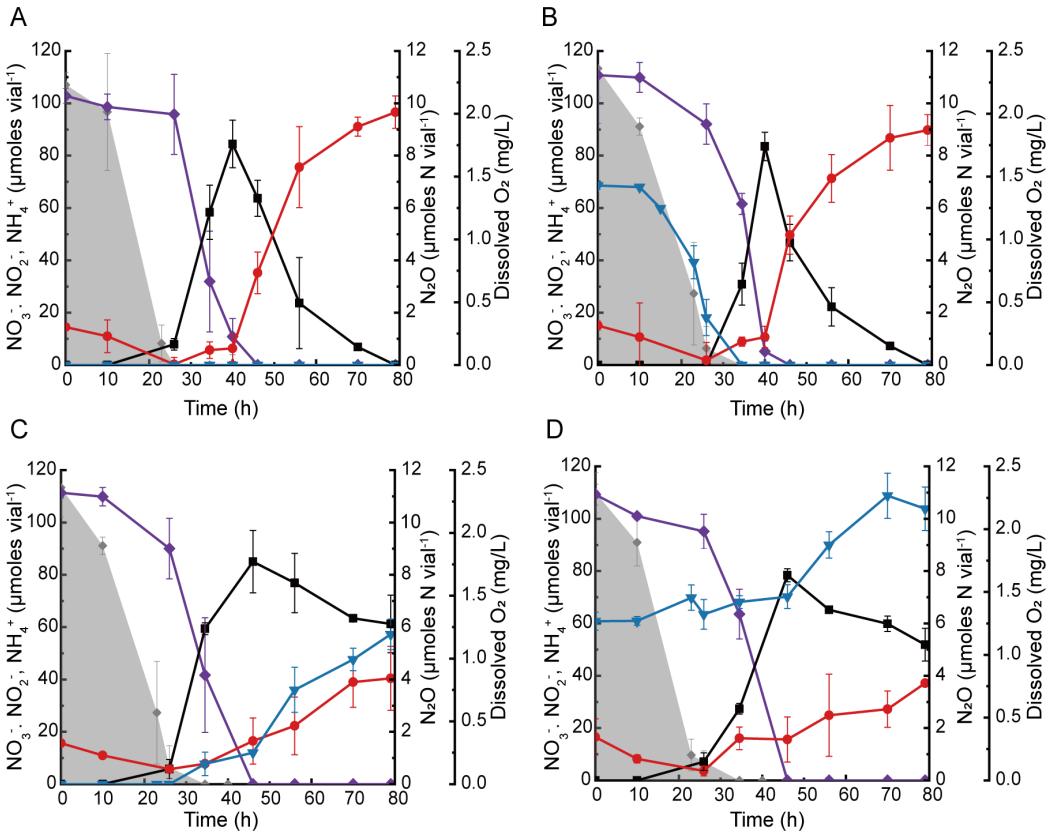

FIG 2 Incubation of 100 mL (prepared in 160 mL serum vials with the headspace consisting of 95% N_2 and 5% O_2) *Bacillus* sp. DNRA2 cultures with 1.0 mM NO_3^-. The following incubation conditions were examined: (A) control without any headspace amendment, (B) N_2O-amended condition with 3.5 µmol N_2O initially added to the culture vials, (C) C_2H_2-amended condition with 10% of the headspace replaced with C_2H_2, and (D) N_2O-and-C_2H_2-amended condition. The data points represent the average of triplicate cultures and the error bars are the standard deviations of the values obtained from triplicate cultures. (◆, NO_3^-; ■, NO_2^-; ●, NH_4^+; ▼, N_2O-N; shaded curve, dissolved oxygen).

Similar trends were observed when NO_3^- replaced NO_2^- as the electron acceptor (Fig. 2). Without C_2H_2 amendment, neither NO_3^--to-NO_2^- nor NO_2^--to-NH_4^+ reduction was noticeably affected by the initial presence of N_2O, although N_2O consumption preceded NO_3^- reduction as the culture turned anoxic (Fig. 2A and B). In the cultures amended with C_2H_2, NO_2^--to-NH_4^+ reduction that followed NO_3^--to-NO_2^- reduction was substantially slower (Fig. 2C and D). In the cultures amended with C_2H_2 but no N_2O, only 40.4 ± 12.2 of 85.0 ± 11.9 µmol NO_2^- produced from NO_3^- reduction was further reduced to NH_4^+ by the end of incubation, yielding 5.72 ± 0.58 µmol N_2O-N. The C_2H_2- and N_2O-amended cultures showed similarly slower NH_4^+ production. Only 37.2 ± 0.2 µmol NH_4^+ was produced after 79 h, while the amount of N_2O increased from 6.1 ± 0.3 to 10.4 ± 0.8 µmol N_2O-N $vial^{-1}$.

Confirmation of absence of direct C_2H_2 influence on DNRA

The possibility of C_2H_2 having contributed to the observed delays in DNRA activation following the oxic-to-anoxic transition was examined in batch reactors fed continuous gas flowthroughs (Fig. 3). In all three reactors, the initial incubation with 3:1 N_2/air mixed gas increased the cell density to OD_{600} ~0.06. Production of NH_4^+ in the reactors began after the gas source was switched to N_2, N_2/C_2H_2 mixture (9:1), or N_2/C_2H_2 mixture (13:5:2). The NH_4^+ production curves of cultures fed with N_2 were almost identical to those of cultures fed with an N_2/C_2H_2 mixture, while the reactor fed with an $N_2/C_2H_2/N_2O$ mixture showed substantially slower NO_2^--to-NH_4^+ reduction, corroborating the negative impact of N_2O on DNRA activation. These experiments were repeated with a new set of cultures, reproducing virtually indistinguishable NH_4^+ production curves (Fig. S2). These observations substantiated that the inhibition of NO_2^--to-NH_4^+ reduction observed in the C_2H_2-amended batch cultures was most likely due to an inhibitory effect of N_2O, but not C_2H_2.

N_2 and NO production during DNRA

Incubation with the closed-circuit robotized incubation system enabled the monitoring of NO and N_2 concentrations in the *Bacillus* sp. DNRA2 cultures amended with and without C_2H_2 and N_2O, showing distinct difference between the two treatments (Fig. 4). The OD_{600} values measured after O_2 depletion were 0.11 ± 0.01 and 0.091 ± 0.015 in the

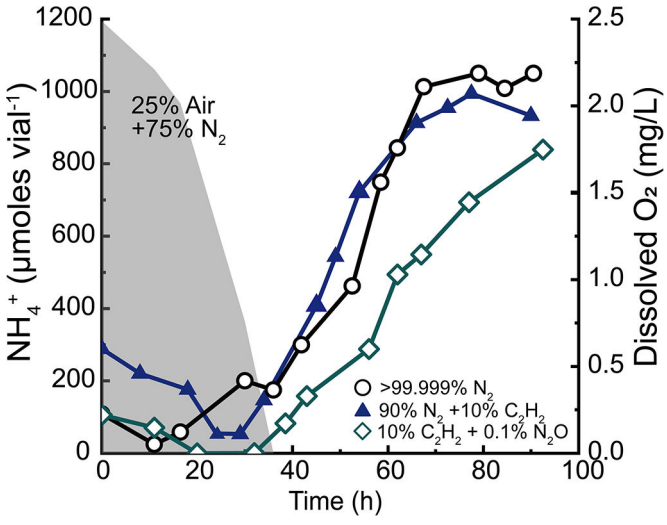

FIG 3 Production of NH_4^+ from 2 mM NO_2^- in a batch reactor containing 500 mL *Bacillus* sp. DNRA2 culture fed with continuous stream of >99.999% N_2 gas (○), 9:1 N_2/C_2H_2 mixed gas (▲), or 9:1 N_2/C_2H_2 mixed gas containing 0.1% N_2O (◇), after 30 h of aerobic incubation with 95% N_2 and 5% O_2. Dissolved oxygen concentration is presented as a shaded curve. The results from the replicate set of experiments are presented in Fig. S2.

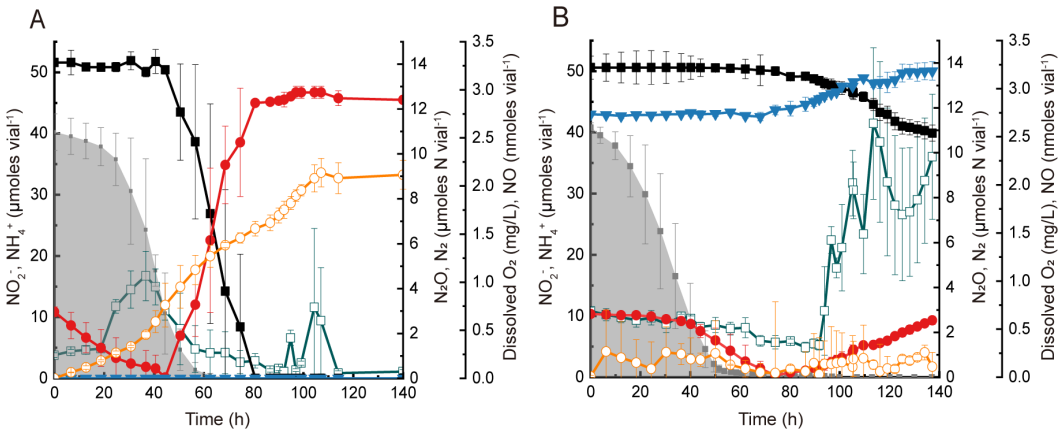

FIG 4 Respiratory kinetics of *Bacillus* sp. DNRA2 batch cultures were monitored using a robotized incubation system. About 50-mL cultures amended with 1.0 mM NO_2^- was incubated in sealed 120 mL glass vials under vigorous stirring. All vials were started with 7% O_2 in the headspace. (A) Controls without additional amendments; (B) cultures amended with 6 µmol N_2O and 10% (vol/vol) C_2H_2. The data points represent the average of triplicate cultures and the error bars represent their standard deviations (■, NO_2^-; ●, NH_4^+; ▼, N_2O-N; ○, N_2-N; □, NO; shaded curve, dissolved oxygen).

controls and C_2H_2-and-N_2O-amended cultures, respectively. The inhibitory effect of C_2H_2 and N_2O on NO_2^--to-NH_4^+ reduction was clearly reproducible. The production of 5.8 ± 1.0 µmol N_2 (10.7 ± 1.3% of reduced NO_2^-) in the controls verified that N_2O production and consumption occurred simultaneously as NO_2^- was being reduced to NH_4^+. The N_2O yield, in terms of percent of reduced NO_2^-, was significantly higher for the cultures amended with C_2H_2 and N_2O, as 17.2 ± 1.8% of reduced NO_2^- was recovered as N_2O. Notably, NO accumulated to a substantially higher level in the C_2H_2-and-N_2O-amended cultures than in the controls. While the amount of NO remained below 0.6 nmol vial^{-1} in the controls as NO_2^- was reduced to NH_4^+ (44.6–80.6 h), NO accumulated to 2.6 ± 0.8 nmol vial^{-1} from the background level (~0.5 nmol vial^{-1}) in the C_2H_2-and-N_2O-amended cultures between 91.7 and 113.6 h. Evidently, the presence of N_2O or C_2H_2 affected NO production and release in the *Bacillus* strain DNRA2 cultures undergoing DNRA.

Sustained DNRA inhibition in Nos-inhibited *Bacillus* sp. DNRA2 batch cultures subjected to oxic-anoxic alternations

Alternation of oxic and anoxic conditions via periodic replacement of the headspace gas resulted in a more pronounced N_2O impact on DNRA in *Bacillus* sp. DNRA2 cultures (Fig. 5). In the control, i.e., the batch culture without N_2O and C_2H_2, the vials contained 80.7 ± 5.0 µmol NH_4^+ after 80 h of incubation with 104.0 ± 1.9 µmol NO_2^- and 12.1 ± 1.2 µmol NH_4^+ as the initial N input. Replacement of the headspace with the oxic gas at 49.5 h immediately halted NO_2^- turnover, which was then recovered after O_2 depletion. The N_2O concentration was sustained below the detection limit throughout the incubation, suggesting that produced N_2O was immediately consumed via NosZ-catalyzed reduction. Each headspace replacement (at 43 and 80 h) resulted in an immediate decrease in NH_4^+ concentration, presumably due to assimilation. In the culture vials to which C_2H_2 and N_2O had been added, NO_2^- concentration decreased by only 17.9 ± 3.0 µmol vial^{-1}, while the amount of NH_4^+ did not increase above 17 µmol vial^{-1} at any point during incubation, clearly showing that NO_2^--to-NH_4^+ reduction was inhibited to a larger extent with the intermittent headspace replenishment. As the cultures were replenished with a gas containing a high concentration of background N_2O, no significant increase in the amount of N_2O could be observed. In all treatments, significant growth occurred only during the initial oxic incubation and immediately following the first headspace replenishment (Fig. S3).

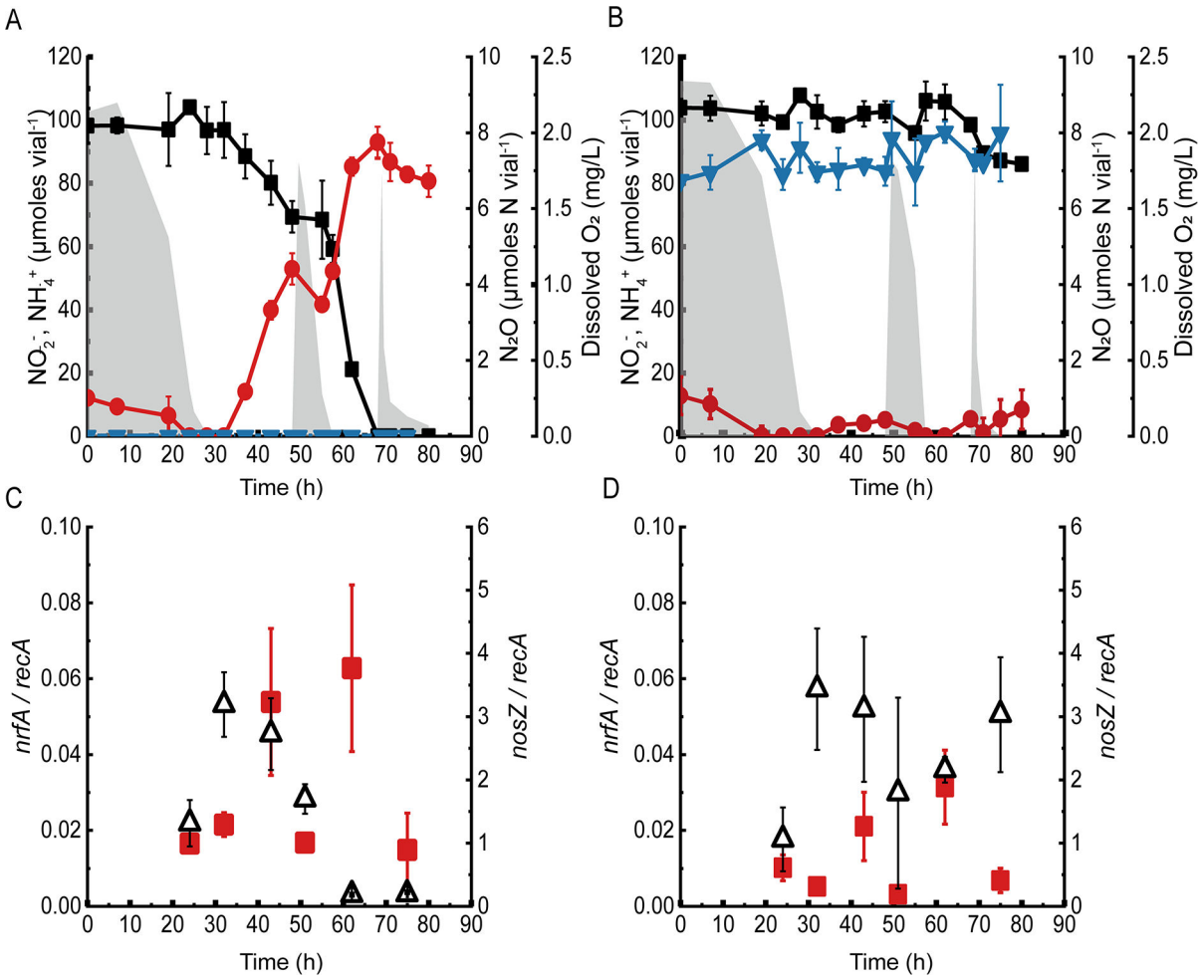

FIG 5 Incubation of *Bacillus* sp. DNRA2 cultures with headspace replenishments to simulate repeated oxic-to-anoxic transitions. The cultures initially contained 1.0 mM NO_2^-. The headspace consisted of (A) 3:1 N_2/air mixed gas or (B) 13:5:2 N_2/air/C_2H_2 mixed gas amended with 3.5 μmol N_2O (before equilibration) and was replaced with gas with the same composition at 52.5 and 70 h. The transcript copy numbers of *nrfA* (red squares) and *nosZ* (△) normalized with the copy numbers of *recA* transcripts under condition A (C) and condition B (D) were monitored with RT-qPCR. The average values obtained from biological triplicates are presented, and the error bars represent their standard deviations (black squares, NO_2^-; ●, NH_4^+; ▼, N_2O-N; shaded curve, dissolved oxygen).

The effect of N_2O on *nrfA* and *nosZ* transcription

Reverse transcription-quantitative PCR (RT-qPCR) analyses, performed with select samples from the above experiments with repeated oxic-to-anoxic transitions, showed that 15 μM dissolved N_2O was sufficient to significantly alter *nrfA* transcription in *Bacillus* strain DNRA2 (Fig. 5). With the sole exception of 75 h when NO_2^- had been depleted in the control cultures, *nrfA* transcription was significantly lower for the N_2O-and-C_2H_2-amended cultures than the control cultures (two-sample *t* test; $P < 0.05$). The fold differences between the treatments were substantial, ranging between 1.6 and 5.3. Also notable and common to both sets of cultures was that the *nrfA* transcription levels measured when O_2 was present were significantly lower than those measured during the ensuing anoxia (paired *t* -test; $P < 0.05$). For example, the *nrfA* transcription levels measured at 51 h (oxic) for the N_2O-and-C_2H_2-amended cultures and the controls ($3.2 \pm 2.6 \times 10^{-3}$ and $1.7 \pm 0.2 \times 10^{-2}$ *nrfA*/*recA*, respectively), were both significantly lower ($P < 0.05$) than those measured at 62 h (anoxic; $6.3 \pm 2.2 \times 10^{-2}$ and $3.1 \pm 1.0 \times 10^{-2}$ *nrfA*/*recA*, respectively). These RT-qPCR results clearly showed that the presence of N_2O affected *nrfA* transcription in *Bacillus* sp. DNRA2, explaining, at least partially, the inhibition of DNRA activity observed in the N_2O-and-C_2H_2-amended cultures.

The *nosZ* transcription levels were not significantly different between the control cultures and the N_2O-and-C_2H_2-amended cultures until 51 h ($P > 0.05$), but were an order of magnitude higher in the N_2O-and-C_2H_2-amended cultures beyond that time point ($P < 0.05$; Fig. 5B and D). Apparently, the presence of N_2O, i.e., the substrate of NosZ, and C_2H_2, i.e., a NosZ inhibitor, had no significant effect ($P > 0.05$) on *nosZ* transcription. The substantially decreased *nosZ* transcription levels observed toward the end of incubation in the control cultures (62 and 75 h), but not in the N_2O-and-C_2H_2-amended cultures, were probably due to depletion of the nitrogenous electron acceptors. Also notable from the RT-qPCR results was that transcription of *nosZ* was at least an order of magnitude higher than that of *nrfA* in both sets, while not being as sensitive to exposure to O_2 as that of *nrfA*.

DISCUSSION

Release of N_2O from NO_2^--to-NH_4^+ reduction has been widely observed in DNRA-catalyzing microorganisms, presumably due to the leakage of the probable intermediate NO and subsequent reduction by NO reductases (3, 26, 36–39). While most of these studies have reported N_2O yields (i.e., mole N_2O-N released per mole NO_3^- or NO_2^- consumed) below 5%, the yields varied substantially even among phylogenetically close microorganisms (3, 37). Further, the experiments with *B. vireti* showed that N_2O yields may vary depending on growth conditions and also that N_2O yields may be substantially larger (e.g., >10%) under certain incubation conditions, e.g., high NO_3^- concentration (26, 33). For long, DNRA has been perceived as a pathway that yields less N_2O than denitrification, and the DNRA-catalyzing microorganisms harboring *nosZ* have drawn particular interest as potential net consumers of environmental N_2O (2, 27, 28). However, knowing that substantial amounts of N_2O can be produced from DNRA, the widespread possession of *nosZ* by DNRA-catalyzing microorganisms may need explanations further than that merely pertaining to their N_2O-scavenging capability.

In *Bacillus* sp. DNRA2, the presence of N_2O clearly delayed NrfA-mediated NO_2^--to-NH_4^+ reduction as the culture was transitioning from aerobic to anaerobic respiration (Fig. 5C and D). The downregulated *nrfA* transcription in the presence of N_2O explained this delayed the onset of DNRA; however, the mechanism via which N_2O affects *nrfA* transcription remains unelucidated and can only be hypothesized based on the limited observations. Transcription of *nrfA* has been observed only in a surprisingly limited number of DNRA-catalyzing microorganisms, including *Escherichia coli* K-12, *W. succinogens* (the *nosZ*$^+$ variant), *S. loihica* PV-4, *B. vireti*, and *Citrobacter* sp. DNRA3 (3, 8, 13, 33, 40–43). Downregulation of *nrfA* transcription and DNRA activity in the presence of O_2 and NO_3^- has been repeatedly observed (3, 33, 40, 43). In *S. loihica* PV-4, harboring both denitrification (with NirK catalyzing NO_2^--to-NO reduction) and DNRA pathways, *nrfA* transcription was significantly affected by the electron donor- or acceptor-limitation, pH, and NO_2^-/NO_3^- ratios (8, 13, 42). The only study that reported N_2O regulation of *nrfA* transcription was that performed with *W. succinogens nosZ*$^+$ variant, where the presence of N_2O increased transcription of *nrfA*, along with those of *napA* and *nosZ* (41). Nonetheless, none of these fits into the context of the current observation.

Previous studies have repeatedly suggested the role of NO in transcription-level regulations of nitrogen cycling reactions (44–46). NO concentrations above the baseline level were observed in the N_2O-and-C_2H_2-amended culture only after the onset of DNRA; however, the departure from the control culture was evident, in that NO steadily increased with the progression of DNRA (Fig. 3). How N_2O may alter production or consumption and detoxification (presumably by NorB and HmpA, respectively) of NO remains enigmatic; however, this elevated NO level is notable as the only suggestion of N_2O impact on DNRA not directly relevant to transcriptional regulation of *nrfA*. The possibility that NO is involved in N_2O-mediated downregulation of *nrfA* transcription should not be neglected, as NO, even at nanomolar concentrations, may act as a signal initiating transcription of denitrification genes (e.g., *nirS*, *norB*, and *nosZ*) (45–48). A time-series transcriptomic analysis of *Bacillus* sp. DNRA2 cultures during and after

transition from aerobic respiration to DNRA would be an interesting follow-up study, in that it may be able to disclose the genes under influence of the N_2O presence, e.g., transcription regulators, electron transport chain enzymes, and/or even those related to vitamin B_{12} synthesis, that may help elucidate mechanistic features of the N_2O-elicited gene regulation, including possible NO involvement in the regulatory cascade (49, 50).

In DNRA-catalyzing bacteria possessing *nosZ*, N_2O reduction is often observed to occur simultaneously with DNRA (3, 29). The results from previous experiments with *W. succinogens* (the *nosZ*$^+$ variants) and *Bacillus* strain DNRA2, which comparatively examined N_2O evolution in the cultures with and without the N_2O reduction inhibitor C_2H_2, implied that NosZ-mediated N_2O reduction occurs simultaneously with DNRA in these strains (3, 29). Further, in-line N_2 monitoring verified production of N_2 from N_2O reduction during anoxic incubation of *B. vireti* and *Bacillus* sp. DNRA2, as these microorganisms reduced NO_3^-/NO_2^- to NH_4^+ (26). Utilization of fugitive N_2O from DNRA as an additional source of electron acceptors would not provide much of an energetic benefit to these organisms. Assuming a 5% N_2O yield, the additional electron-accepting capacity gained from reduction of the produced N_2O to N_2 via NosZ activity would be merely 0.22% of that gained from the dissimilatory reduction of NO_2^- to NH_4^+ (see Supplemental Material).

The current study posits a novel hypothesis that *nosZ*-possessing DNRA-catalyzing microorganisms such as *Bacillus* sp. DNRA2 may have retained *nosZ* genes, possibly acquired via horizontal gene transfers, as NosZ serves to remove N_2O, which would otherwise hamper the activation of DNRA in response to O_2-depletion (28). As NO_3^- in the environment is mostly produced from aerobic oxidation of NH_4^+, the largest anoxic pools of NO_3^- (and also NO_2^- despite at much lower concentrations) and the most vigorous NO_3^- and NO_2^- reduction activities in soils and sediments are often associated with oxic-anoxic interfaces where O_2 concentrations fluctuate, and it is likely that micro-niches in such habitats act as hotspots for N_2O accumulation from nitrification, denitrification, and/or DNRA (51–54). Any substantial delay in the transition to anaerobic respiration would be detrimental for DNRA-catalyzing microorganisms in their competition with denitrifiers (6, 8). Hasty generalization should be avoided, as simultaneous occurrence of N_2O reduction and DNRA has so far been experimentally confirmed only in *Bacillus* sp. DNRA2, *B. vireti*, and *W. succinogens*, and experimental evidence of N_2O interference with *nrfA* expression has not yet been reported for any microorganism apart from *Bacillus* sp. DNRA2. Examining whether the observed phenotype relating NosZ and NrfA functions can be further generalized would be an interesting follow-up study, which would help further understand the evolutionary implication of *nosZ* gene possession by DNRA-catalyzing microorganisms.

Bacillus sp. DNRA2, when incubated with N_2O in the presence of C_2H_2 released 12–19% of consumed NO_2-N as N_2O-N (Fig. 1D, 2D and 3B). The only other reported case with >10% conversion of NO_3^-/NO_2^- to N_2O-N in a NrfA-mediated DNRA reaction was that of *B. vireti*, which released up to 49% of reduced NO_3^- as N_2O-N when amended with 20 mM NO_3^- (26). Under the experimental conditions that resulted in the high N_2O yields, *Bacillus* sp. DNRA2 and *B. vireti* both showed a lower *nrfA* transcription level and diminished NO_2^--to-NH_4^+ reduction rates following an oxic-to-anoxic transition (33). The NrfA enzymes of the two *Bacillus* strains share a high level of amino acid sequence similarity (73% identity), suggesting a high degree of protein structure similarity. This similarity is notable, as the nitrogen dissimilation gene inventories in the two strains are substantially different. That is, *Bacillus* strain DNRA2 possesses *nap* for NO_3^- reduction and *norB* for NO reduction, while *B. vireti* possesses *nar* and *cba* encoding the menaquinol/cytochrome *c*-dependent qCuNor instead of *nap* and *norB*, respectively (3, 26). Possibly, the high yields of N_2O (or of NO, which may have been immediately reduced by nitric oxide reductases or NO detoxification enzymes) may be due to an inherent structural feature of this particular clade of NrfA. Such high N_2O yield and N_2O sensitivity of DNRA may be the rationale for the genomic observations that many of the *Bacillus* spp. harboring a *nrfA* gene in their genomes possess a *nosZ* gene (Table S1), although

verification of this rather bold hypothesis would require further experimental evidences and mechanistic explanations (55).

The N_2O effects on DNRA, as observed in *Bacillus* sp. DNRA2, may have substantial implications to the fate of nitrogen in the environment. Whether N_2O-induced delay in *nrfA* transcription and reduced DNRA activity is widely spread among DNRA-catalyzing organisms remains to be investigated, and this phenotype may possibly be limited to *Bacillus* spp. and their close relatives. Even so, *Bacillus* spp. are often an abundant group of microorganisms in agricultural soils, where the fate of NO_3^- has environmental and ecological consequences (56, 57). As DNRA-catalyzing microorganisms compete with denitrifiers for the common electron acceptors, i.e., NO_3^- and NO_2^-, any delays in NrfA activation or reduced NrfA activity would result in silencing of the DNRA phenotype (3, 6, 8). In NO_3-rich microenvironments near oxic-anoxic interfaces in soils, DNRA-catalyzing microorganisms with similar physiology as *Bacillus* sp. DNRA2 would have limited DNRA activities, if local NosZ activity lags behind production or influx of N_2O. Probably, DNRA-catalyzing *Bacillus* spp. may have retained the *nosZ* genes to increase the chance of competing against denitrifiers in such microenvironments. Supporting that NosZ was playing a crucial role in facilitating DNRA, *Bacillus* sp. DNRA2 was capable of keeping the N_2O level low and rapidly transitioning from aerobic respiration to NO_2^--to-NH_4^+ reduction when incubated without the NosZ inhibitor C_2H_2. Going one step further, these NosZ-wielding DNRA-catalyzing microorganisms may be key to collective DNRA enhancement in soils, in that they may provide relief to N_2O inhibition on DNRA activities of the surrounding microorganisms lacking *nosZ*. Whether and to what extent such hypothetical enhancement to collective nitrogen retention may occur in the soil microbiomes warrant further investigation.

MATERIALS AND METHODS

Culture medium and growth condition

The medium contained, per L, 0.58 g NaCl, 0.41 g Na_2HPO_4, 0.29 g K_2HPO_4, 5.3 mg of NH_4Cl, 6.2 mg R2A powder (Kisanbio, Seoul, South Korea), and 1 mL 1,000× trace metal solution (58). The pH was adjusted to 7.0 with 5 M HCl. Unless otherwise mentioned, batch cultures were prepared with 100 mL medium in 160 mL serum vials. For preparation of anoxic cultures, the vials were flushed with >99.9999% N_2 gas (Deokyang Co., Ulsan, South Korea) for 15 min and sealed with butyl rubber stoppers and aluminum crimps. Filter-sterilized 200× vitamin stock was added to the medium after autoclaving (59). Sodium lactate was added to a concentration of 5 mM, and KNO_3 or $NaNO_2$ was added to a concentration of 1 mM unless otherwise mentioned. For preparation of suboxic cultures, a pre-determined volume of the N_2 headspace was withdrawn and the same volume of air was injected through a 0.2-µm syringe filter (Advantec Inc., Tokyo, Japan). The medium vials were inoculated with 1 mL of *Bacillus* sp. DNRA2 preculture grown to the early stationary phase (OD_{600} ~0.03). The precultures for the suboxic cultures were grown with 5% vol/vol O_2 in the headspace as the sole electron acceptor, and those for anoxic cultures were grown with 1 mM NO_2^-. All microbial cultures were incubated in the dark at 25°C with shaking at 150 rpm, unless otherwise mentioned.

Batch observation of NO_2^-/NO_3^- reduction following oxic-to-anoxic transition

The progressions of DNRA reaction and N_2O production and consumption were observed in batch cultures of *Bacillus* sp. DNRA2 incubated with NO_2^- or NO_3^- under four different headspace compositions, to examine the possibility that N_2O may interfere or compete with DNRA reaction (3). Four sets of suboxic cultures, initially with ~5% vol/vol O_2 in the headspace, were prepared: (i) without any amendment to the culturing condition described above; (ii) with >99.999% N_2O gas (Danil Syschem Co., Seoul, South Korea) added to a targeted initial aqueous concentration of 15 µM; (iii) with 10% of

the N_2 headspace replaced with >99.99% C_2H_2 gas (Special Gas, Inc., Daejeon, South Korea) to inhibit NosZ-mediated N_2O consumption; and (iv) with both N_2O and C_2H_2 added to the aforementioned concentrations. Additionally, two sets (conditions 1 and 4) of experiments were performed with cultures incubated anoxically throughout. For measurement of the dissolved concentrations of NO_3^-, NO_2^-, and NH_4^+, 1 mL of culture sample was withdrawn, and the supernatant was collected after centrifugation and stored at −20°C. The N_2O and O_2 concentrations were measured immediately before the aqueous-phase sampling. The cultures were monitored until NO_3^- and NO_2^- were depleted in the controls (condition 1).

An additional set of batch cultivation experiments was performed to simulate repeated transitions from oxic to anoxic condition and *vice versa* that frequently occur at oxic-anoxic interfaces in soils (53). The controls (condition 1) and the cultures amended with both N_2O and C_2H_2 (condition 4) were prepared and the batch incubation experiments were performed identically to the experiments described above but with replacement of the headspace two times during the course of incubation (52 and 70.5 h), each after ensuring the absence of O_2 in both cultures. Headspace replenishing was performed by flushing the culture vials with N_2 gas for 5 min and adding, after closure of the culture vials, O_2, N_2O, and C_2H_2 back to their initial concentrations. The culture samples for RT-PCR analyses were collected at 24, 32, 43, 51.5, 62, and 75 h. The *nrfA* and *nosZ* transcripts in *Bacillus* sp. DNRA2 cultures were quantified by RT-qPCR using a previously established protocol (see Supplemental Material for a detailed method) (8).

To isolate the effect of C_2H_2 on DNRA from that of N_2O, NO_2^--to-NH_4^+ reduction by *Bacillus* sp. DNRA2 was observed in a fed-batch reactor continuously flushed with N_2 gas or 9:1 N_2:C_2H_2 mixed gas with or without 0.1% (vol/vol) N_2O (Fig. S4). A 1-L glass reactor vessel was prepared containing 490 mL medium amended with 2 mM $NaNO_2$, 10 mM lactate, and 0.2 mM NH_4Cl and inoculated with 10 mL of *Bacillus* sp. DNRA2 culture aerobically grown to OD_{600} = 0.03. The aqueous phase was stirred at 250 rpm using a magnetic bar. Initially, a synthetic gas consisting of ~95% N_2 and 5% O_2 was bubbled into the liquid phase of the reactor at 40 mL min^{-1}. After 30 h of incubation, the gas source was switched to N_2 gas or 9:1 mixture of N_2 and C_2H_2 gas with or without 0.1% N_2O. Dissolved NO_2^- and NH_4^+ concentrations were monitored until no further change was observed.

Analytical procedures

The gaseous concentration of N_2O was measured using an HP6890 series gas chromatograph equipped with an HP-PLOT/Q column and an electron capture detector (Agilent, Palo Alto, CA, USA). The injector, oven, and detector temperatures were set to 200°C, 85°C, and 250°C, respectively. The dissolved O_2 concentration was monitored using a FireStingO2 oxygen meter and fiber-optic oxygen sensor spots (Pyroscience GmbH, Aachen, Germany). The total amount of N_2O in a culture vial was calculated from the headspace concentration using the dimensionless Henry's constant of N_2O at 25°C, which was calculated to be 1.68 (60). Dissolved concentrations of NO_2^-, NO_3^-, and NH_4^+ were determined colorimetrically as previously described (61, 62). Lactate concentrations were measured using high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at the start and at the end of each incubation to confirm that the initially added amount of lactate was sufficient to deplete all added terminal electron acceptors (data presented in Table S2).

Monitoring of NO and N_2 dynamics during DNRA

Bacillus sp. DNRA2 cultures were incubated in a robotized incubation system with frequent monitoring of O_2 and relevant N-species, with particular interest in NO and N_2, which were not monitored in the other experiments described in this study. The analyses were performed as previously described with minor modifications (26, 63). Briefly, aerobic pre-cultures, raised under vigorous stirring (600 rpm) using magnetic bars

were transferred to sealed 120 mL medical flasks containing 50 mL of the R2A medium described above, to an initial OD_{600} of ~0.03. The medium was supplemented with 0.2 mM NH_4Cl and 1.0 mM $NaNO_2$. Prior to inoculation, the flasks had been made anoxic by repeated He-flushing after which 5 mL O_2 (7% in the headspace) was added with or without 0.15 mL N_2O (approx. 12 µmol N_2O-N) and 12 mL C_2H_2. The cultures were incubated at 25°C with vigorous stirring. Concentrations of the gaseous compounds were monitored automatically with a TRACE 1310 GC (Thermo Fisher Scientific, Waltham, MA, USA; O_2, CO_2, N_2O, and N_2) and a NOA 280i Sievers nitric oxide analyzer (Zysense, Weddington, NC, USA) connected to the incubation system. Aqueous samples for measurements of NO_2^- and NH_4^+ concentrations and OD_{600} were manually withdrawn. Concentrations of NO_2^- were measured as described previously (26). NH_4^+ concentration and OD_{600} were performed as described above.

Statistical analyses

All experiments, unless otherwise mentioned, were performed in triplicate. Two-sample *t* tests were used to determine the statistical significance of the pairwise comparisons between two different treatments and paired *t* test was used to determine the significance of temporal changes in the transcript copy numbers and the concentrations of the N-species. All statistical tests were performed using R software version 3.5.1 (RStudio Team 2018). The *P* values lower than the 0.05 threshold were considered significant.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental materials (mBio01540-23-S0001.pdf). Texts S1 and S2, Fig. S1 to S4, and Tables S1 to S3.

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