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Involvement of NO_3^- in Ecophysiological Regulation of Dissimilatory Nitrate/Nitrite Reduction to Ammonium (DNRA) Is Implied by Physiological Characterization of Soil DNRA Bacteria Isolated via a Colorimetric Screening Method

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ABSTRACT Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) has recently regained attention as a nitrogen retention pathway that may potentially be harnessed to alleviate nitrogen loss resulting from denitrification. Until recently, the ecophysiology of DNRA bacteria inhabiting agricultural soils has remained largely unexplored, due to the difficulty in targeted enrichment and isolation of DNRA microorganisms. In this study, >100 DNRA bacteria were isolated from NO_3^--reducing anoxic enrichment cultures established with rice paddy soils using a newly developed colorimetric screening method. Six of these isolates, each assigned to a different genus, were characterized to improve the understanding of DNRA physiology. All the isolates carried *nrfA* and/or *nirB*, and the *Bacillus* sp. strain possessed a clade II *nosZ* gene conferring the capacity for N_2O reduction. A common prominent physiological feature observed in the isolates was NO_2^- accumulation before NH_4^+ production, which was further examined with *Citrobacter* sp. strain DNRA3 (possessing *nrfA* and *nirB*) and *Enterobacter* sp. strain DNRA5 (possessing only *nirB*). Both isolates showed inhibition of NO_2^--to-NH_4^+ reduction at submillimolar NO_3^- concentrations and downregulation of *nrfA* or *nirB* transcription when NO_3^- was being reduced to NO_2^-. In batch and chemostat experiments, both isolates produced NH_4^+ from NO_3^- reduction when incubated with excess organic electron donors, while incubation with excess NO_3^- resulted in NO_2^- buildup but no substantial NH_4^+ production, presumably due to inhibitory NO_3^- concentrations. This previously overlooked link between NO_3^- repression of NO_2^--to-NH_4^+ reduction and the C-to-N ratio regulation of DNRA activity may be a key mechanism underpinning denitrification-versus-DNRA competition in soil.

IMPORTANCE Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) is an anaerobic microbial pathway that competes with denitrification for common substrates NO_3^- and NO_2^-. Unlike denitrification, which leads to nitrogen loss and N_2O emission, DNRA reduces NO_3^- and NO_2^- to NH_4^+, a reactive nitrogen compound with a higher tendency to be retained in the soil matrix. Therefore, stimulation of DNRA has often been proposed as a strategy to improve fertilizer efficiency and reduce greenhouse gas emissions. Such attempts have been hampered by lack of insights into soil DNRA bacterial ecophysiology. Here, we have developed a new screening method for isolating DNRA-catalyzing organisms from agricultural soils without apparent DNRA activity. Physiological characteristics of six DNRA isolates were closely examined, disclosing a previously overlooked link between NO_3^- repression of NO_2^--to-NH_4^+ reduction and the C-to-N ratio regulation of DNRA activity, which may be a key to understanding why DNRA activity is rarely observed at substantial levels in nitrogen-rich agricultural soils.

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Nitrogen is an essential element for plant growth. Today, the Haber-Bosch process, used primarily for the production of nitrogen fertilizers, is singled out as the largest energy-consuming industrial process, with global energy consumption summing up to 2.5% of the total energy consumed across the globe, and naturally, nitrogen is one of the largest sources of greenhouse gases (1, 2). The increased nitrogen flux in the soil and aquatic environments as a consequence of fertilizer application to agricultural soils has also led to aggravation of various nitrogen-related environmental problems, e.g., enrichment of NO_3^- in groundwater and harmful algal blooms as a symptom of eutrophication in surface water (3). Thus, mitigation of the "nitrogen dilemma" has been regarded as one of the most pressing issues for environmental sustainability (4).

Despite the environmental consequences, nitrogen is not used efficiently in agroecosystems. Nitrogen fertilizer efficiency, i.e., the proportion of applied fertilizer nitrogen that eventually ends up in crop biomass, rarely exceeds 40% (5). One of the primary nitrogen loss pathways is nitrification followed by denitrification. Both nitrification and denitrification are also the major culprits of N_2O emissions. Several different strategies have been devised to limit nitrogen loss and N_2O emissions from soil systems, including the use of nitrification inhibitors and slow-release fertilizers (6, 7). Another possible strategy recently proposed for improved soil nitrogen management is to outcompete the denitrification pathway with the nitrogen-retaining process of dissimilatory nitrate/nitrite reduction to ammonium (DNRA) (8–11). The reduction of NO_3^- to NH_4^+ via DNRA also increases the tendency of N to be retained in the soil matrix, thereby reducing NO_3^- leaching, another substantial nitrogen loss avenue in agricultural soils (12). Dissimilatory nitrate/nitrite reduction to ammonium is catalyzed by the microorganisms carrying cytochrome c_{552} nitrite reductases (encoded by *nrfA* genes) or NADH-dependent nitrite reductases (encoded by *nirB* genes), often incorrectly generalized as assimilatory nitrite reductases (13). According to the current limited knowledge, NO_2^--to-NH_4^+ reduction may serve as the electron acceptor reaction for respiration (respiratory DNRA) or the electron dump for NADH regeneration in fermentation of complex organics (fermentative DNRA) (14–16).

Denitrification and DNRA pathways compete for common substrates, NO_3^-/NO_2^-, and thus, stimulating one would repress the other (17, 18). Previous investigations suggested that DNRA is favored in environments with high organic carbon (C) content and a limiting supply of nitrogenous electron acceptors (NO_3^-/NO_2^-) (19–21). This hypothesis was further corroborated by recent laboratory experiments with microbial enrichments and axenic microbial cultures harboring both denitrification and DNRA pathways; however, conflicting observations (e.g., in experiments with *Intrasporangium calvum* and *Deltaproteobacteria*-dominated wastewater enrichments) suggest the possibility that the observed correlation between DNRA activity and the C-to-N ratio (the ratio of C in bioavailable organic compounds to N in NO_3^-/NO_2^- in this context) may be circumstantial (18, 22–24).

The potential significance of DNRA as a key reaction determining the fate of reactive nitrogen in the environment has been considered for decades, albeit lacking sufficient evidence (25–27). Observation of dominance of DNRA over denitrification, i.e., higher NH_4^+ production than N_2O-plus-N_2 production from NO_3^-/NO_2^- reduction, has been limited to several specific highly reduced marine environments (28, 29). Nevertheless, recovery of $^{15}NH_4^+$ from $^{15}NO_3^-$ reduction in both *in situ* column studies and *ex situ* soil incubation experiments supported the presence of DNRA activity in soil environments (8, 17). Furthermore, the abundance of *nrfA* genes in several sequenced soil metagenomes suggested that microbes capable of DNRA activity may be abundant in soil communities (30, 31). Few attempts have been made to isolate and examine DNRA organisms from soils, however, presumably due to the relative insignificance of the contribution of DNRA in nitrogen-rich soils, e.g., fertilized agricultural soils, where the fate of nitrogen is most relevant to the global biogeochemical cycle (8, 17, 32).

In this era dominated by molecular microbial ecology and meta-omics, the importance of culture-based studies is often overlooked; however, meta-omics data can be effectively interpreted along ecological and biogeochemical contexts only with sound understanding of microbial physiology and metabolism (33). In this aspect, that DNRA deserves further culture-based investigation for improved understanding of soil nitrogen fate in agroecosystems is beyond doubt, even with broad availability of molecular tools and bioinformatics pipelines for culture-independent analyses of DNRA-related functional genes, e.g., *nrfA* and *nirB* (30, 34). The major bottleneck in investigation of soil DNRA ecophysiology, however, has been the difficulty in efficiently securing diverse DNRA isolates from soils, where the contribution of DNRA to anoxic NO_3^-/NO_2^- turnover is, in most cases, minor (9). Due to this difficulty, investigations of DNRA ecophysiology have relied on extrapolation of findings from experiments with limited numbers of isolates, mostly acquired from nonsoil environments (16, 18, 35–37). Furthermore, many of these isolates had been aerobically isolated and cultured for decades in laboratory settings before they were recognized as being capable of DNRA (16, 18, 37–40). Thus, the use of these isolates as representatives of soil DNRA bacteria has received criticism as lacking ecological relevance to the fate of NO_3^- in anoxic agricultural soils.

To address this issue of ecological relevance in examining soil DNRA ecophysiology, a less onerous and time-consuming method for the isolation of DNRA bacteria in denitrification-dominant agricultural soils was needed. Here, a rapid, inexpensive, high-throughput screening method was developed utilizing the well-established salicylate method for NH_4^+ detection and quantification (41). Reductive transformation of NO_3^- was examined with six DNRA organisms isolated from rice paddy soils using this novel screening method. The isolates were taxonomically assigned as *Bacillus* (belonging to the *Firmicutes* phylum) and *Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Shewanella* (belonging to the *Proteobacteria* phylum) at the genus level. Their most obvious common physiological feature was NO_3^- inhibition of NO_2^--to-NH_4^+ reduction, which had also been observed previously with *nrfA*- and-*nirB*-harboring organisms *Escherichia coli* and *Bacillus vireti* (35, 42). With a series of batch and continuous culture experiments, we identified the NO_3^- repression of DNRA activity as one of the mechanisms underpinning the widely acknowledged but controversial C-to-N ratio regulation of DNRA-versus-denitrification competition.

RESULTS

Isolation of DNRA bacteria from denitrification-dominant agricultural soil. Out of 192 colonies each from lactate- and glucose-amended rice paddy soil enrichments, both with negligible NH_4^+ production from NO_3^- reduction, 126 and 12 colonies tested DNRA positive, respectively (Fig. S1 in the supplemental material). Sequencing of the 16S rRNA gene amplicons of the positive colonies (30 randomly selected colonies from the lactate-amended enrichment and all 12 colonies from the glucose-amended enrichment) identified six bacterial genera: *Aeromonas*, *Bacillus*, and *Shewanella* (lactate-amended enrichment), *Enterobacter* and *Klebsiella* (glucose-amended enrichment), and *Citrobacter* (both enrichments) (Fig. S2). The DNRA activities in six of these isolates, each randomly selected from the isolates belonging to a unique genus, were further examined.

Identification of functional genes relevant to dissimilatory nitrogen reduction. The draft genomes of the six DNRA isolates were constructed from HiSeq sequencing reads (sequencing statistics are presented in Table S2). The functional genes potentially relevant to turnover of reactive nitrogen species or regulation of nitrogen metabolism were then analyzed in these draft genomes (Fig. 1, Table S3). The isolates that originated from lactate-enriched cultures all possessed *nrfA* genes, encoding NH_4^+-forming cytochrome c_{552} nitrite reductases. The two isolates from glucose-enriched cultures lacked *nrfA* genes but possessed *nirB* genes, suggesting that NirB-type nitrite reductase was responsible for dissimilatory reduction of NO_2^- to NH_4^+ in these organisms. *Aeromonas* sp. strain DNRA1 and *Citrobacter* sp. strain DNRA3 possessed

Isolates	Denitrification and DNRA		Denitrification			DNRA		NO reduction and detoxification			Sensors and regulators			
	$NO_3^- \rightarrow NO_2^-$ reduction		$NO_2^- \rightarrow NO$ reduction		$N_2O \rightarrow N_2$ reduction	$NO_2^- \rightarrow NH_4^+$ reduction		$NO \rightarrow N_2O$ reduction			NO_3^-/NO_2^- sensor		NO_3^-/NO_2^- response regulator	
	napA	*narG*	*nirK*	*nirS*	*nosZ*	*nirB*	*nrfA*	*norB*	*norV*	*hmpA*	*narQ*	*narX*	*narL*	*narP*
***Aeromonas* sp. DNRA1**	■					■	■			■	■		■	
***Bacillus* sp. DNRA2**	■				■		■	■		■	■		■	
***Citrobacter* sp. DNRA3**	■	■				■	■		■	■	■	■	■	■
***Shewanella* sp. DNRA4**	■						■	■			■		■	
***Enterobacter* sp. DNRA5**	■	■				■			■	■	■	■	■	
***Klebsiella* sp. DNRA6**	■	■				■			■	■		■	■	

FIG 1 Functional genes identified in the draft genomes of the six DNRA bacteria that are potentially relevant to turnover of reactive nitrogen species or regulation of dissimilatory nitrogen metabolism. The genes that were recovered in the draft genome are represented as shaded boxes. Detailed information that includes the lists of the accessory genes and their closest BLAST hits is provided in Table S3 in the supplemental material.

both *nrfA* and *nirB*. All six isolates had *napA* in their genomes, and *Citrobacter* sp. DNRA3, *Enterobacter* sp. strain DNRA5, and *Klebsiella* sp. strain DNRA6 carried *narG*, indicating the genomic potential of these organisms to reduce NO_3^- to NO_2^-. Neither *nirK* nor *nirS* (both of which encode NO-forming nitrite reductases) was present in any of the isolates; however, a clade II *nosZ* gene was identified in the draft genome of *Bacillus* sp. strain DNRA2, suggesting N_2O-reducing capability. *nosD* and *nosL* genes, encoding a maturation protein and a copper chaperone, respectively, were identified in the draft genome of *Shewanella* sp. strain DNRA4, possibly as vestiges of a functional *nos* operon. *Bacillus* sp. DRNA2 and *Shewanella* sp. DNRA4 possessed *norB* genes encoding quinol-dependent nitric oxide reductases, and *norV* and/or *hmpA* genes that encode enzymes involved in detoxification of NO were recovered in all of the sequenced draft genomes.

NO_3^- reduction by the DNRA isolates. Reductive transformation of NO_3^- was observed with the axenic cultures of the six DNRA isolates with or without 10% C_2H_2 in the headspace (Fig. 2, Fig. S3 and S4). The six isolates completely reduced the initially supplemented NO_3^- to NH_4^+ via NO_2^- with lactate or glucose as the source of electrons. Lactate-coupled NO_3^- reduction in *Aeromonas* sp. DNRA1, *Bacillus* sp. DNRA2, *Citrobacter* sp. DNRA3, and *Shewanella* sp. DNRA4 resulted in near-stoichiometric production of NH_4^+ from NO_3^-. Reduction of NO_3^- to NH_4^+ was also observed in *Enterobacter* sp. DNRA5 and *Klebsiella* sp. DNRA6 grown on glucose; however, the NH_4^+ produced only amounted to 36.3 $\pm$ 1.1 (mean $\pm$ standard deviation [SD]) and 32.1 $\pm$ 0.2 μmol, respectively, which were less than half of the added NO_3^-. As the cell densities of the glucose-fed *Enterobacter* sp. DNRA5 and *Klebsiella* sp. DNRA6 reached at least 2.5-fold higher than those of the lactate-consuming isolates, the missing nitrogen was likely due to assimilation. Despite the absence of *nirK* or *nirS* genes, N_2O production was observed in all of the isolates during NO_3^- reduction when incubated with C_2H_2. The amounts of N_2O produced varied across the isolates, ranging from 0.40 $\pm$ 0.06 μmol N_2O-N (0.4% of the added NO_3^-) for *Shewanella* sp. DNRA4 to 3.5 $\pm$ 0.3 μmol N_2O-N (3.5% of the added NO_3^-) for *Citrobacter* sp. DNRA3. In all six isolates examined, the start of N_2O production corresponded with the start of NH_4^+ production, suggesting that N_2O was a by-product of NO_2^--to-NH_4^+ reduction, not NO_3^--to-NO_2^- reduction. Of the six isolates, only *Bacillus* sp. DNRA2 showed a sub-

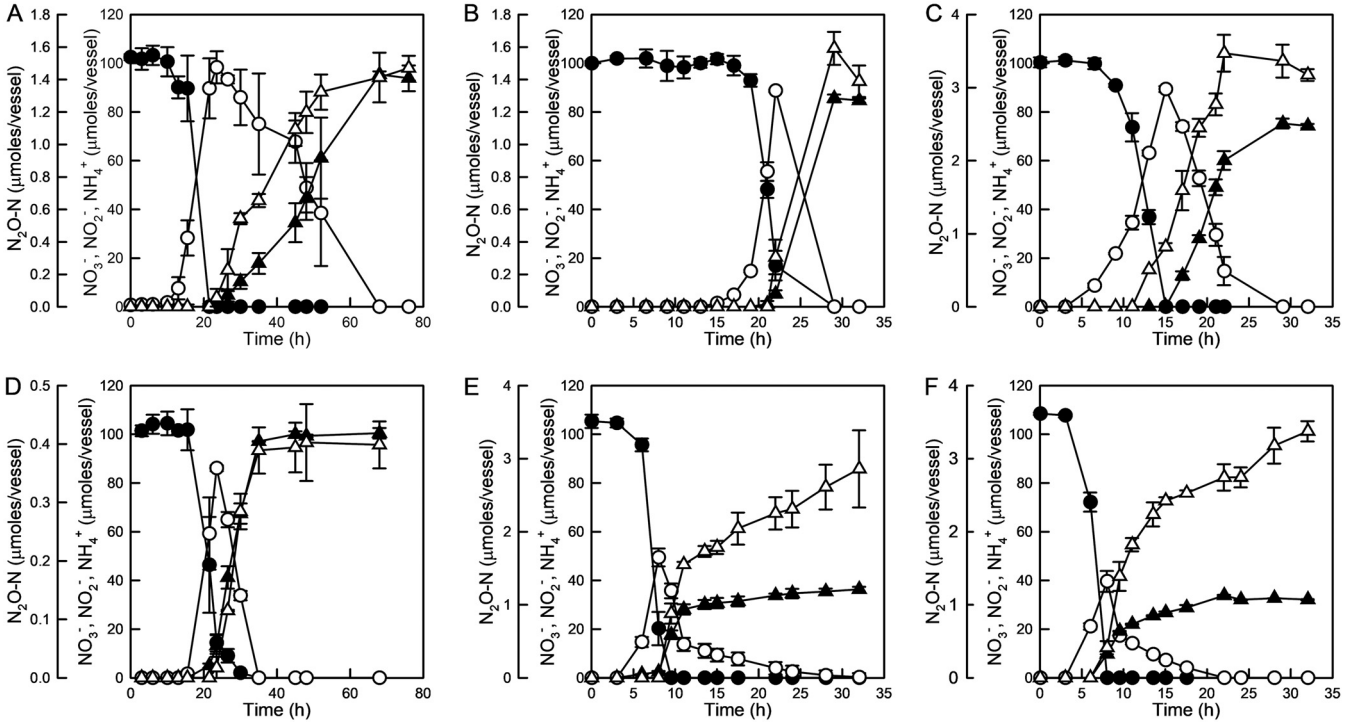

FIG 2 NO_3^- reduction monitored in 100-ml batch cultures (prepared in sealed 160-ml serum bottles with headspace consisting of 90% N_2 and 10% C_2H_2). (A) *Aeromonas* sp. DNRA1; (B) *Bacillus* sp. DNRA2; (C) *Citrobacter* sp. DNRA3; (D) *Shewanella* sp. DNRA4; (E) *Enterobacter* sp. DNRA5; (F) *Klebsiella* sp. DNRA6. The average values from biological replicates ($n = 3$) are presented, with error bars representing the standard deviations (●, NO_3^-; ○, NO_2^-; ▲, NH_4^+; △, N_2O-N).

stantially different N_2O-N time series profile when incubated without C_2H_2 (Fig. S4). The absence of N_2O accumulation suggested that N_2O consumption occurred simultaneously with DNRA in this clade II *nosZ*-harboring organism.

Accumulation of NO_2^- before reduction to NH_4^+ was consistently observed in all six isolates. Reduction of NO_2^- to NH_4^+ did not commence until >80% of NO_3^- was consumed, suggesting that NrfA- or NirB-catalyzed NO_2^--to-NH_4^+ reduction was affected by changing NO_2^- or NO_3^- concentrations. The DNRA activities of *Citrobacter* sp. DNRA3 and *Enterobacter* sp. DNRA5 were further investigated to identify whether possible causality exists between the NO_2^- or NO_3^- concentration and DNRA activity (Fig. 3). The transcription levels of *nrfA* in *Citrobacter* sp. DNRA3 and *nirB* in *Enterobacter* sp. DNRA5 were significantly higher ($P < 0.05$) after NO_3^- was depleted than before. Transcription of *nrfA* in *Citrobacter* sp. DNRA3 increased significantly ($P < 0.05$), from an *nrfA*/*recA* transcript ratio of 1.0 ± 0.6 at $t = 9$ h (0.16 ± 0.03 mM NO_3^- and 0.82 ± 0.03 mM NO_2^- remaining) to an *nrfA*/*recA* transcript ratio of 6.3 ± 0.2 at $t = 15$ h (0.33 ± 0.08 mM NO_2^- remaining). No significant change was observed with *nirB* transcription (from an *nirB*/*recA* transcript ratio of 2.04 ± 0.19 at $t = 9$ h to an *nirB*/*recA* transcript ratio of 1.61 ± 0.37 at $t = 15$ h), suggesting that NirB-type nitrite reductase was irrelevant to respiratory DNRA. Transcription of *nirB* in *Enterobacter* sp. DNRA5 followed a trend similar to that of *nrfA* in *Citrobacter* sp. DNRA3, increasing significantly from an *nirB*/*recA* transcript ratio of 1.0 ± 0.5 at $t = 6$ h (0.43 ± 0.01 mM NO_3^- and 0.37 ± 0.03 mM NO_2^- remaining) to an *nirB*/*recA* transcript ratio of 4.4 ± 0.3 at $t = 15$ h (0.31 ± 0.008 mM NO_2^- remaining) upon NO_3^- depletion ($P < 0.05$). Substrate (NO_2^-) regulation of transcription was unlikely for either *nrfA* in *Citrobacter* sp. DNRA3 or *nirB* in *Enterobacter* sp. DNRA5, as the transcription of these genes appeared unresponsive to elevated NO_2^- concentrations as long as NO_3^- was present in the medium at >0.15 mM. Thus, the NO_3^- concentration was the most probable environmental factor that affected the transcription of the genes encoding these DNRA-catalyzing nitrite reductases. The significant differences in the rates of NO_2^- reduction

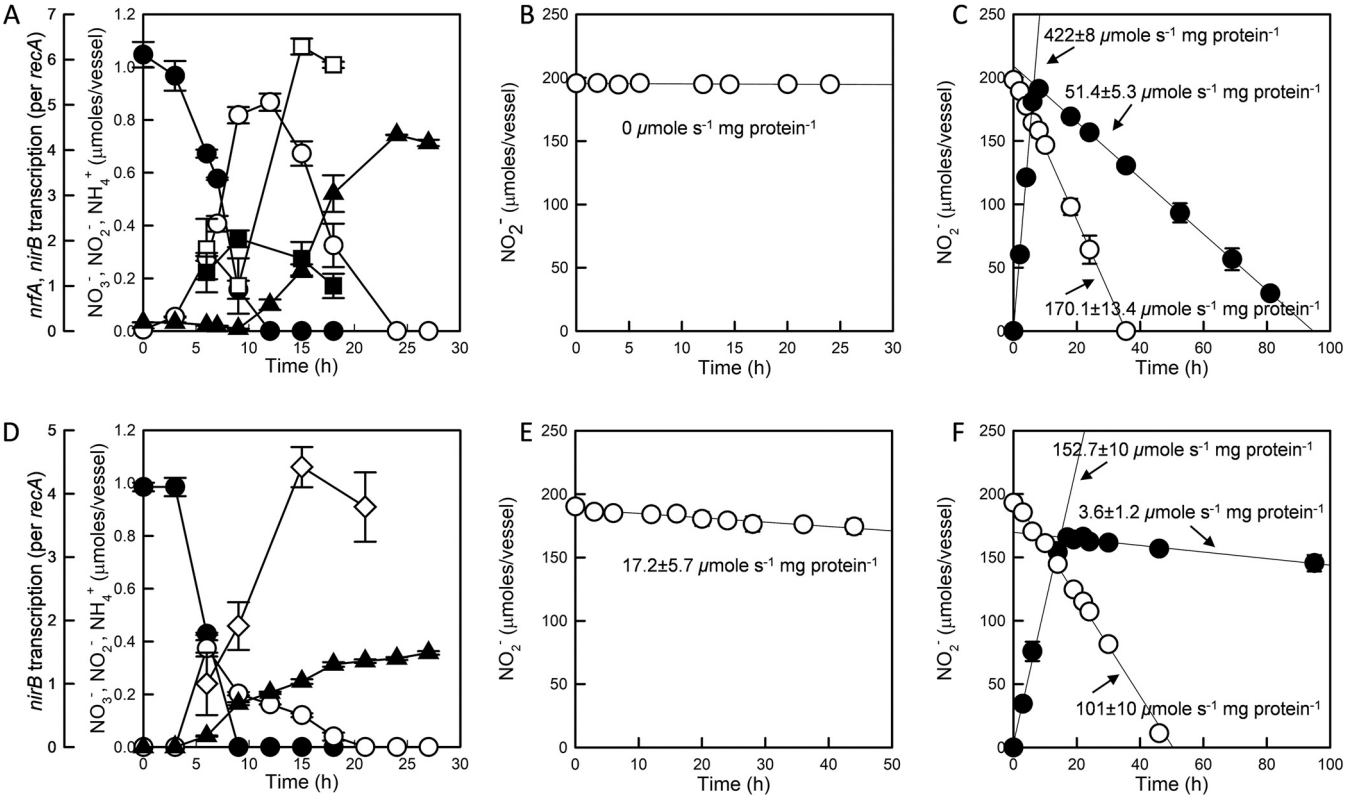

FIG 3 (A and D) Transcription of *nrfA* (□) and *nirB* (■) in *Citrobacter* sp. DNRA3 (A) and *nirB* (◇) in *Enterobacter* sp. DNRA5 (D) cells as 1 mM NO_3^- (●) was reduced to NH_4^+ (▲) via NO_2^- (○). (B, C, E, and F) Changes to the amounts of NO_2^- were monitored in the chloramphenicol-treated resting cultures of *Citrobacter* sp. DNRA3 (B and C) and *Enterobacter* sp. DNRA5 (E and F) harvested before (B and E) and after (C and F) NO_3^- depletion and resuspended in fresh medium containing 2 mM NO_2^- (○) or 2 mM NO_3^- (●). All experiments were performed in biological replicates ($n = 3$), and error bars represent the standard deviations.

measured with *Citrobacter* sp. DNRA3 or *Enterobacter* sp. DNRA5 cells harvested before and after the NO_3^- depletion and treated with chloramphenicol also supported the idea that expression of the NH_4^+-forming nitrite reductases was downregulated by the presence of NO_3^- (Fig. 3B, C, E, and F). *Citrobacter* sp. DNRA3 cells extracted before NO_3^- depletion did not exhibit significant NO_2^- reduction activity, while the cells extracted after NO_3^- depletion readily reduced NO_2^-, at a rate of 170 ± 13 μmol s^{-1} mg $protein^{-1}$. NO_2^- reduction by *Enterobacter* sp. DNRA5 cells was also ~6 times higher with the cells harvested after NO_3^- depletion (101 ± 10 μmol s^{-1} mg $protein^{-1}$) than with the cells harvested before NO_3^- depletion (17.2 ± 5.7 μmol s^{-1} mg $protein^{-1}$) ($P < 0.05$).

In the resting-cell experiments with 2 mM NO_3^- added to chloramphenicol-treated *Citrobacter* sp. DNRA3 cells harvested after NO_3^- depletion, NO_2^- accumulated up to 1.93 ± 0.04 mM at a rate of 422 ± 8 μmol s^{-1} mg $protein^{-1}$ before it was consumed at a rate of 51.4 ± 5.3 μmol s^{-1} mg $protein^{-1}$ (Fig. 3C). The negligible NO_2^- reduction activity before NO_3^- depletion suggested an additional NO_3^--mediated inhibitory mechanism in NrfA-type nitrite reductase activity apart from transcriptional regulation of the *nrfA* gene. Such repression of NO_2^- reduction activity by the presence of NO_3^- was not observed in the parallel experiment performed with *Enterobacter* sp. DNRA5 (lacking *nrfA*) and presumably utilizing NirB-type nitrite reductase (Fig. 3F).

DNRA reaction at various C-to-N ratios in batch and continuous cultivation. *Citrobacter* sp. DNRA3 and *Enterobacter* sp. DNRA5 were grown in batch and continuous cultures, each with two different C-to-N ratios, and NO_3^- reduction was monitored to investigate whether the generally perceived positive correlation between C-to-N ratio and DNRA activity may be related to the NO_3^- repression of NO_2^--to-NH_4^+ reduction (Fig. 4 and 5). When grown at the initial C-to-N ratio of 75 in batch cultures, *Citrobacter*

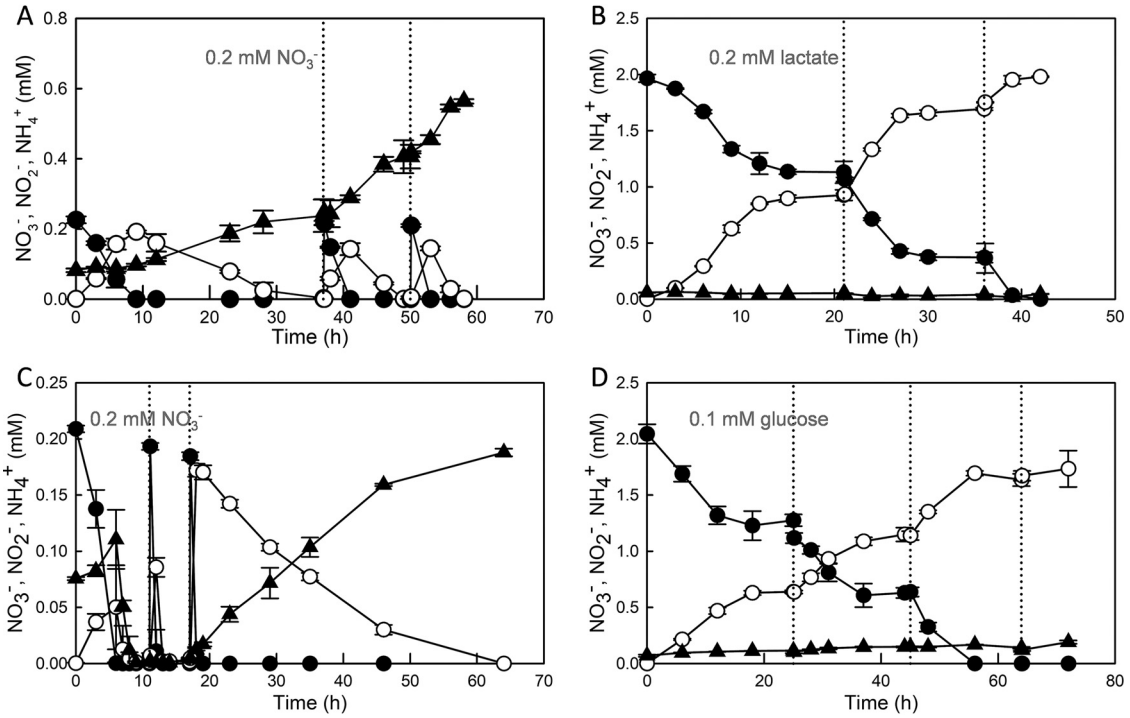

FIG 4 NO_3^- reduction observed with batch cultures of *Citrobacter* sp. DNRA3 (A and B) and *Enterobacter* sp. DNRA5 (C and D) prepared with two different initial C-to-N ratios. The high C-to-N conditions were prepared with 0.2 mM NO_3^- and 5 mM lactate (A) or 2.5 mM glucose (C), and NO_3^- was replenished to 0.2 mM upon NO_3^-/NO_2^- depletion. The low C-to-N conditions were prepared with 2.0 mM NO_3^- and 0.2 mM lactate (B) or 0.1 mM glucose (D), and the carbon sources were replenished when NO_3^-/NO_2^- reduction stopped. The dotted lines denote the time points where the limiting nutrients were replenished. The average values from biological replicates ($n = 3$) are presented, with error bars representing the standard deviations (●, NO_3^-; ○, NO_2^-; ▲, NH_4^+).

sp. DNRA3 produced NH_4^+ from NO_3^- reduction, and each addition of 20 μmol NO_3^- resulted in near-stoichiometric turnover to NH_4^+. In contrast, growth of *Citrobacter* sp. DNRA3 at the initial C-to-N ratio of 0.3 did not result in a significant increase in NH_4^+ concentration but did lead to stoichiometric NO_2^- accumulation, as NO_3^- reduction had produced 0.87 ± 0.06 mM NO_2^- when the initial reaction stopped at $t = 16$ h due to depletion of lactate. Reduction of NO_3^- to NO_2^- resumed after replenishment with 0.2 mM lactate at $t = 21$ h. Under this low C-to-N ratio incubation condition, NO_3^- was present in the culture medium throughout incubation, and the presence of NO_3^- was likely the reason for the absence of *sensu stricto* DNRA activity.

Enterobacter sp. DNRA5 incubated on glucose at a C-to-N ratio of 75 produced a significant amount of NH_4^+ only after the initially added glucose (2.19 ± 0.8 mM) was fully consumed, suggesting that substantial portions of NO_3^- and its reduction products, NO_2^- and NH_4^+, were assimilated. Upon the third addition of 0.2 mM NO_3^-, with no glucose remaining in the medium, the sequential NO_3^--to-NO_2^--to-NH_4^+ reduction was stoichiometric, suggesting that NirB-catalyzed NO_2^--to-NH_4^+ reduction was coupled to oxidation of the fermentation products. At the low C-to-N ratio, where the culture medium was replenished with 0.1 mM glucose upon a halt in NO_3^- reduction, the time series profiles of the N species concentrations were indistinguishable from those of *Citrobacter* sp. DNRA3, save for the imperfect stoichiometry between consumed NO_3^- and produced NO_2^- and the modest, albeit significant, increase in NH_4^+ concentration from 0.07 ± 0.01 mM at $t = 0$ h to 0.19 ± 0.01 mM at $t = 72$ h. The modest production of NH_4^+ was in line with the reduced but still significant NO_2^- reduction rate observed in the resting-cell cultures of *Enterobacter* sp. DNRA5 extracted before NO_3^- depletion.

A continuous culture of *Citrobacter* sp. DNRA3 that was fed with medium carrying 10 mM lactate and 2 mM NO_3^- (C-to-N ratio of 15), after attaining steady state,

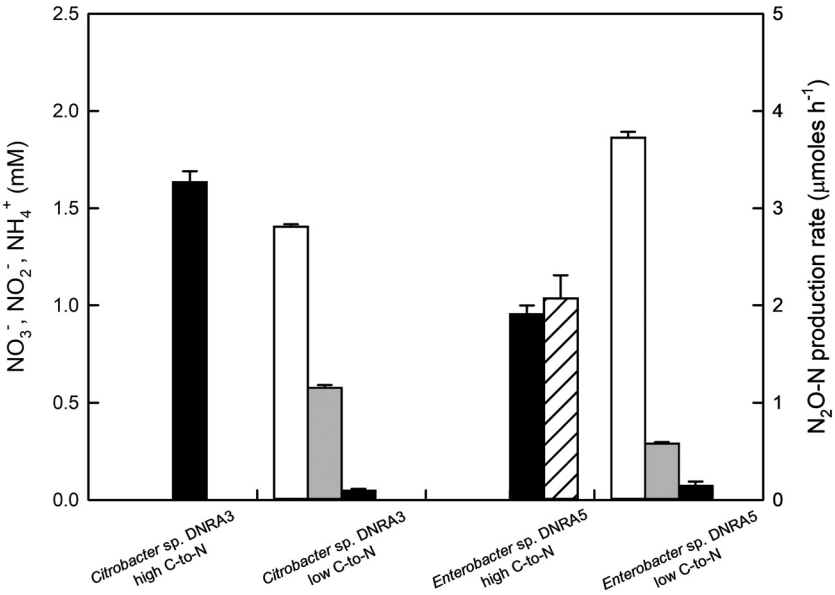

FIG 5 Steady-state concentrations of NO_3^- (white bars), NO_2^- (gray bars), and NH_4^+ (black bars) in electron acceptor limiting (high C-to-N ratio) and electron donor limiting (low C-to-N ratio) chemostat cultures of *Citrobacter* sp. DNRA3 and *Enterobacter* sp. DNRA5. The N_2O-N production rate (hatched bars) is also presented for *Enterobacter* sp. DNRA5 cultivated under the electron acceptor limiting condition, which was the only reactor culture with observed N_2O production. The average values of the three measurements taken at 6-h intervals are presented, with error bars representing the standard deviations.

contained 1.68 ± 0.09 mM NH_4^+ as the only dissolved inorganic nitrogen, indicating that NO_3^- and NO_2^- were readily reduced in the chemostat. When the reactor was fed with medium carrying 0.2 mM lactate and 2 mM NO_3^- (C-to-N ratio of 0.3), 1.41 ± 0.04 mM NO_3^- remained in the medium at steady state, due to carbon limitation. That NO_2^- was the major product of NO_3^- reduction (0.58 ± 0.04 mM at steady state) and the NH_4^+ concentration did not differ significantly from the concentration in the fresh medium ($P > 0.05$) indicated that DNRA did not proceed further beyond NO_2^-. Similarly, with *Enterobacter* sp. DNRA5, significant NH_4^+ formation was observed only in a continuous culture operated under the electron-acceptor-limiting condition, i.e., at a C-to-N ratio of 15. The steady-state NH_4^+ concentration was 0.95 ± 0.04 mM in this chemostat. In the continuous culture operated under the electron-donor-limiting condition, NO_2^- was the only dissolved nitrogen species with a significantly higher concentration than in the influent medium. Production of N_2O (2.07 ± 0.24 μmol h^{-1}) was observed only in the high C-to-N chemostat of *Enterobacter* sp. DNRA5. The absence of significant NO_2^--to-NH_4^+ reduction in the low C-to-N ratio batch and chemostat cultures, regardless of whether mediated by NrfA-type or NirB-type nitrite reductase, could be best explained as the inhibitory effect of NO_3^-.

DISCUSSION

The soil DNRA isolates newly acquired with the screening method developed in this study were assigned to six genera according to their 16S rRNA gene sequences. Several of these genera have been previously confirmed to include strains capable of carrying out DNRA (*Bacillus*, *Citrobacter*, *Enterobacter*, and *Klebsiella*). The list also included a genus generally perceived as a marine organism (*Shewanella*) and a genus without physiologically confirmed DNRA activity (*Aeromonas*) (36, 43–45). The genomic analyses of six DNRA isolates, one from each of these genera, confirmed that the possession of *nrfA* or *nirB* is necessary for a DNRA phenotype (35, 37). All four isolates utilizing lactate as the electron donor were of the *nrfA* genotype. *Enterobacter* sp. DNRA5 and *Klebsiella* sp. DNRA6 that lacked *nrfA* failed to grow on lactate under a NO_2^--reducing condition, suggesting that NrfA-type nitrite reductase is needed for respiratory NO_2^- reduction to

NH_4^+. Thus, physiological functions of NirB-catalyzed NO_2^--to-NH_4^+ reduction in these organisms may be NAD^+ regeneration for fermentation, detoxification of NO_2^-, and/or assimilatory reduction, as previously suggested (15, 37). Neither *nirS* nor *nirK* was found in any of the sequenced draft genomes; however, an *nosZ* gene was recovered in the genome of the *nrfA*-possessing *Bacillus* sp. DNRA2. Observation of NO_3^- reduction with and without C_2H_2 confirmed N_2O reduction activity in this isolate amid active DNRA, which was probably catalyzed by the NosZ encoded by this gene.

The most prominent common phenotype of the DNRA isolates was NO_2^- accumulation before NH_4^+ production, suggesting NO_3^- repression of NO_2^--to-NH_4^+ reduction. This phenotype has been consistently observed in previously studied DNRA bacteria (35, 42, 46). These previous studies attributed the NO_3^--induced repression to transcriptional regulation involving NO_3^- sensor proteins NarQ and NarX and the transcript abundances of *nrfA* in *Escherichia coli*, and transcripts of both *nrfA* and *nirB* in *B. vireti* were significantly lower when the culture was supplied with higher NO_3^- concentrations, supporting their claims. In agreement with these previous studies, *nrfA* in *Citrobacter* sp. DNRA3 and *nirB* in *Enterobacter* sp. DNRA5 exhibited at least 4.4-fold higher transcription after NO_3^- depletion than before ($P < 0.05$). Furthermore, the results from the resting-cell experiments with these isolates showed clear indications that the presence of NO_3^- at submillimolar concentrations was sufficient to inhibit activities of expressed NrfA-type nitrite reductase. However, whether the apparent inhibition was due to the redirection of electron flow analogous to what was observed with NosZ-catalyzed N_2O reduction in the presence of O_2 or to inhibition of the NrfA enzyme itself cannot be determined and is outside the scope of the current study (47). In denitrifiers, such NO_3^--mediated repression of dissimilatory NO_2^- reduction, either via transcription regulation or enzyme inhibition, has not yet been reported, and near-stoichiometric NO_2^- accumulation during NO_3^- reduction has been observed only as isolated cases (48–50). Therefore, as long as NO_3^- is present in soil matrices harboring diverse denitrifiers and DNRA-catalyzing organisms, NO_2^- produced from NO_3^- would be reduced mostly to N_2O and N_2 via denitrification, with the DNRA phenotype remaining silent.

The environmental physicochemical parameter that has been most frequently associated with DNRA activity is the C-to-N ratio. Multiple experimental evidences from culture-based experiments and field measurements have supported that DNRA is favored at high C-to-N ratios, i.e., electron acceptor limiting conditions, while denitrification is favored at low C-to-N ratios, i.e., electron donor limiting conditions (18, 20, 23). The observations from the incubation of the two isolates at the two different C-to-N ratios indicated that the NO_3^- repression of NO_2^--to-NH_4^+ reduction activity may actually be directly linked to this C-to-N ratio regulation of DNRA activity in the environment. The C-to-N ratios of soils or sediments are often inversely related to the NO_3^- contents (51). In soils with low C-to-N ratios, the NO_2^--to-NH_4^+ reduction may thus be deactivated in the DNRA-catalyzing organisms due to the high NO_3^- contents while NO_2^--to-N_2O/N_2 reduction activity remains intact in denitrifiers cohabiting the ecological niches. Even with an abundant DNRA-catalyzing population, the NO_3^- fate would still be determined by denitrification in such soils. Thus, what was previously regarded as the effect of the C-to-N ratio on the denitrification-versus-DNRA competition may be, at least in part, explained as the consequence of NO_3^- inhibition of *sensu stricto* DNRA (32).

Production of N_2O has consistently been observed in nondenitrifying organisms with DNRA phenotypes, with recovery of up to ~50% of NO_3^--N as N_2O-N (36, 37, 46, 52). Likewise, all DNRA isolates examined in this study produced N_2O during the course of NO_3^- reduction to NH_4^+ despite the absence of *nirS* or *nirK* genes in their genomes. The previously hypothesized mechanisms of N_2O production from DNRA invariably have implicated involvement of NO (37, 42, 46). Considering that *norB*, *norV*, and/or *hmp* genes were identified in the genomes of DNRA isolates, it is plausible to regard NO as the precursor of N_2O in these organisms; however, the mechanism leading to NO production from NO_3^- or NO_2^- remains unclear. The absence of N_2O production

before NO_3^- depletion in all six isolates suggested that direct N_2O production from NapA- or NarG-type nitrate reductases was unlikely. The more plausible source of NO would be NO_2^--to-NH_4^+ reduction by NrfA- and NirB-type nitrite reductases, although a reaction mechanism leading to formation of NO as a by-product has not been elucidated for either enzyme (46, 53). The results from the chemostat experiments with *Enterobacter* sp. DNRA5 further support this hypothesis, as detectable N_2O production was observed only under the high C-to-N ratio operating condition where active NO_2^--to-NH_4^+ reduction occurred.

Another noteworthy observation in this study was the absence of detectable N_2O production in the NH_4^+-producing *Citrobacter* sp. DNRA3 chemostat (under the high C-to-N ratio condition), which was contradictory to the result from batch incubation of the same DNRA isolate. This absence of N_2O production from the NO_3^--limiting chemostat was in line with the previous observations from chemostat studies of *Shewanella loihica* strain PV-4 with NO_3^-/NO_2^- as the limiting substrate, in that no detectable N_2O production was observed from the DNRA-dominant chemostat cultures (18, 54). Furthermore, in a previous study with *nrfA*-utilizing DNRA isolates of the *Citrobacter* and *Bacillus* genera, larger proportions of NO_3^- were released as N_2O when the initial C-to-N ratios were lower, i.e., the NO_3^- concentrations were higher (36). Together, these observations suggest a positive correlation between NO_3^-/NO_2^- concentrations in the surrounding environment and N_2O formation by *nrfA*-utilizing organisms, such that N_2O produced from NrfA-mediated DNRA may be negligible in environments with limiting influx of NO_3^-/NO_2^-. Also, considering that *nosZ*-possessing DNRA organisms are often found to be capable of simultaneous N_2O consumption with NO_2^--to-NH_4^+ reduction, as observed with *Bacillus* sp. DNRA2 in this study, the possibility exists that *nrfA*-type DNRA organisms may function as sinks, rather than sources, of N_2O in anoxic environments with consistent but limiting NO_3^-/NO_2^- influx.

In summary, close examination of the physiology of the DNRA organisms isolated and screened using the newly developed targeted isolation method substantially enhanced the understanding of DNRA ecophysiology. The analyses of the genomic and physiological features of lactate-oxidizing DNRA phenotypes and glucose-oxidizing DNRA phenotypes evidenced clear distinctions between NrfA-mediated respiratory DNRA and NirB-mediated fermentative DNRA. The NO_3^- inhibition of NO_2^--to-NH_4^+ reduction observed in both *nrfA*-type and *nirB*-type DNRA organisms suggested a plausible mechanistic explanation for the oft-observed C-to-N ratio effects on DNRA-versus-denitrification competition. Significant production of N_2O as a by-product of NO_2^--to-NH_4^+ reduction was also confirmed in batch cultures of all six closely examined *nrfA*- and *nirB*-type isolates; however, the observations from the chemostat incubation experiments suggested dependence of N_2O production associated with NrfA-mediated DNRA, but not NirB-mediated DNRA, on the extracellular NO_3^-/NO_2^- concentrations. The number and diversity of DNRA organisms isolated with the new isolation and screening method may appear limited to skeptics; however, the method is easily expandable in volume and is open to modifications incorporating various selective cultivation techniques that may enable the isolation of more phylogenetically and metabolically diverse DNRA organisms, which would include difficult-to-culture microorganisms. Understanding the physiology of more diverse DNRA isolates would be a sensible starting point for developing soil management techniques for enhancing the nitrogen-retaining DNRA pathway.

MATERIALS AND METHODS

Soil sampling and initial characterization. The agricultural soil used in this study was sampled from an experimental rice paddy located at the Chungnam National University (CNU) agricultural research site in Daejeon, South Korea (36°22′01.6″N, 127°21′14.3″E) in October 2018. Harvesting had been completed and there was no standing water at the time of sampling. Cover soil and plant materials were carefully removed before sampling, and approximately 1 kg of soil at 5 to 30 cm depth from the surface was collected with a stainless steel tubular soil sampler with an inner diameter of 2 cm. The sampled soils were transported to the laboratory in coolers filled with ice and stored at 4°C until use. The physico-

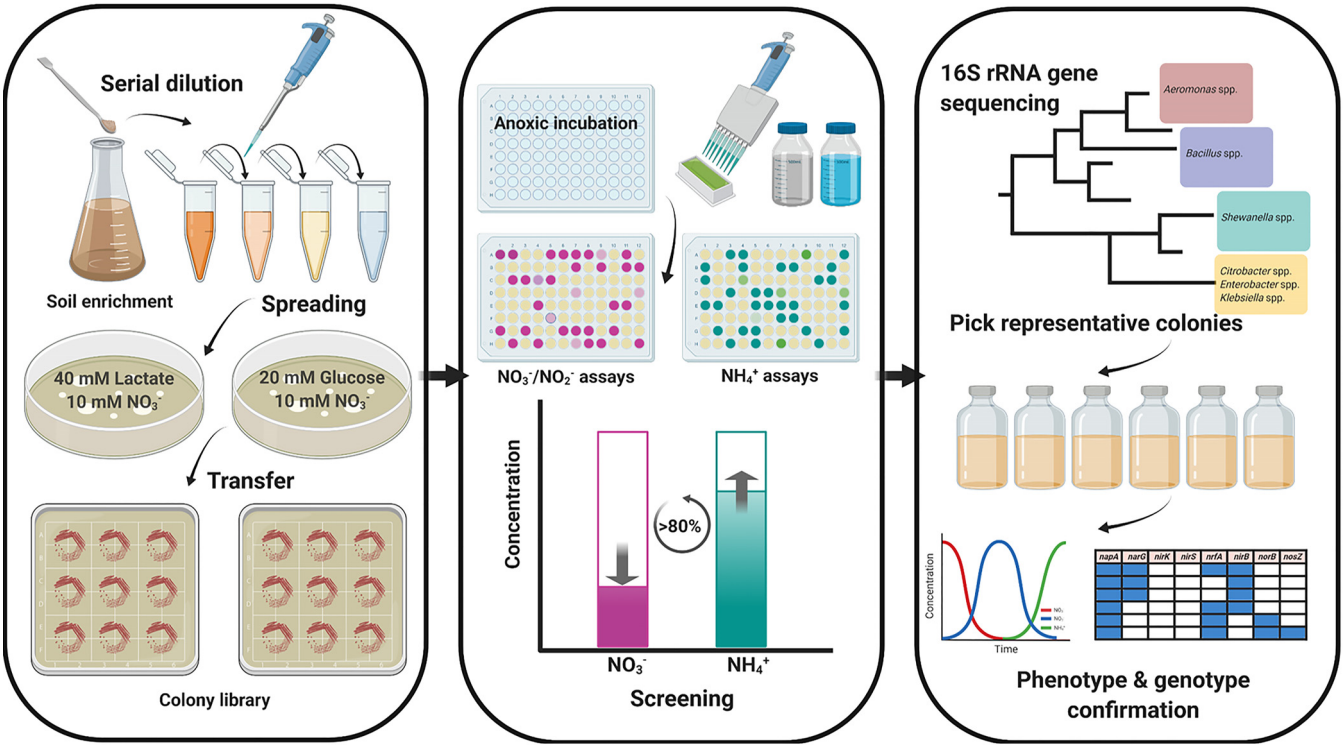

FIG 6 Schematic overview of the high-throughput screening method developed for isolation of DNRA-catalyzing organisms from agricultural soil.

chemical characteristics of this soil, including the pH, textures, and total carbon and nitrogen contents, were analyzed using standardized protocols (11).

Culture medium and growth conditions. The minimal salts medium (MSM) for enrichment, isolation, and cultivation of soil DNRA bacteria was prepared by adding, per liter of deionized distilled water, 10 mmol NaCl, 3.24 mmol Na_2HPO_4, 1.76 mmol KH_2PO_4, 0.1 mmol NH_4Cl, and 1 ml 1,000× trace element stock solution (55). For enrichment and isolation, dehydrated R2A broth (Kisanbio, Seoul, South Korea) was added to the medium as a growth supplement. To minimize the interference of NH_4^+ derived from mineralization of organic nitrogen in the ensuing DNRA-screening process, the R2A broth concentration in the medium was limited to 6.2 mg liter^{-1}. The pH of the medium was adjusted to 7.0. Pure-culture incubation and experiments were performed with 100 ml medium dispensed to 160-ml serum vials. The serum bottles were flushed with N_2 gas (≥99.999%; Special Gas, Inc., Daejeon, South Korea) for 10 min, sealed with black butyl-rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK), and crimped with aluminum crimp seals before autoclaving. The degassed, filter-sterilized, 200× vitamin stock solution was then added to the medium (55). Immediately before inoculation, sodium lactate or glucose was added as the electron donor and organic carbon source and KNO_3 as the electron acceptor. Lactate and glucose were chosen as the nonfermentable and fermentable electron donors, respectively, as both substrates had been previously reported to support DNRA reactions (18, 56, 57). Enrichment with acetate as the electron donor was also attempted but failed to yield any DNRA-positive isolates. Thus, acetate was not further considered as a potential electron donor in this study. The culture bottles were incubated with shaking at 150 rpm in the dark at room temperature (25°C). Agar plates were prepared by adding 15 g liter^{-1} Bacto agar (Becton, Dickinson, Franklin Lakes, NJ) to the liquid medium prepared with elevated concentrations of KNO_3 (12 mM) and sodium lactate or glucose (120 mM as C). The 96-well plates for DNRA screening were prepared by distributing 200-μl aliquots of the prepared culture medium to the wells in UV-sterilized 96-well clear flat-bottom microplates (Corning, Inc., Corning, NY). Agar plates and 96-well plates were prepared and incubated in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) with atmosphere consisting of 96% N_2 and 4% H_2.

High-throughput DNRA phenotype screening. A simple, novel high-throughput screening method was developed in this study for isolating DNRA-catalyzing organisms from agricultural soil enrichments without apparent DNRA activity, i.e., significant NH_4^+ production from NO_3^-/NO_2^- (Fig. 6). Anoxic soil enrichments were prepared in 250-ml Erlenmeyer flasks (Duran Group, Wertheim, Germany) in an anaerobic chamber. Rice paddy soil samples were suspended at a 1:10 (wt/vol) soil-to-medium ratio in 200 ml MSM amended with 2 mM KNO_3 and 6.67 mM sodium lactate or 3.33 mM glucose (20 mM total C concentration) and incubated for 2 weeks in the dark without shaking. The aqueous NO_3^--N, NO_2^--N, and NH_4^+-N concentrations were measured to confirm depletion of the electron acceptors and to check the extent of DNRA reaction in the enrichments. Serial dilutions of the soil enrichment cultures were spread onto agar plates, and after incubation in the anaerobic chamber, single colonies were picked to inoculate the 96-well plates loaded with fresh medium containing 1 mM NO_3^- and 3.34 mM lactate or

1.67 mM glucose. The inoculated 96-well plates were covered with an optical adhesive cover (Applied Biosystems, Foster City, CA) to prevent contamination and evaporation of the culture medium and incubated for a week.

After incubation, 100 μl of the 200-μl culture in each well was transferred to its corresponding position on a new 96-well plate. The absorbances at 600 nm, 660 nm, and 540 nm were determined using a Sunrise microplate reader (Tecan, Männedorf, Switzerland) with one of the duplicated plates to measure the possible interference of cell turbidity in the colorimetric determination of inorganic nitrogen concentrations. This plate was used to screen the wells with increased NH_4^+ concentrations indicative of DNRA activity, using salicylate-nitroprusside chemistry. To each well, 80 μl of the color reagent (containing 0.2 M sodium hydroxide, 1 M sodium salicylate, and 5.88 mM sodium nitroprusside dihydrate) and 20 μl of 5.1 mM sodium dichloroisocyanurate solution were added sequentially. The absorbance at 660 nm was measured after 30 min of incubation at 25°C, and the wells with optical density values at 660 nm (OD_{660}) that were higher than 1.6 (equivalent to 0.8 mM NH_4^+) after subtracting the OD_{660} resulting from cell turbidity were considered positive. The duplicated 96-well plate was used for sequential measurements of NO_2^--N and NO_3^--N (58). The Griess reagent was added to each well to a total volume of 200 μl, and the absorbance at 540 nm was measured after 30 min of incubation at 25°C for determination of the NO_2^--N concentration. The NO_3^--N concentration was determined after the initial Griess assay. After reducing NO_3^- to NO_2^- by adding, per well, 20 μl of 1% wt/vol vanadium(III) chloride (VCl_3; Sigma-Aldrich) prepared in 1 M HCl aqueous solution, the absorbance at 540 nm was measured to obtain the NO_3^--plus-NO_2^- concentration, from which the NO_2^- concentration was subtracted. The colonies corresponding to the wells with NH_4^+ concentrations higher than 0.8 mM and with NO_2^- and NO_3^- absent were transferred to gridded fresh agar plates and stored at 4°C until use.

Characterization of DNRA isolates. The partial 16S rRNA genes of these candidate DNRA isolates were amplified with the 27F/1492R primer set and sequenced to identify their phylogenetic affiliations. Based on these initial 16S rRNA sequencing data, one isolate per genus was randomly selected and subjected to further analyses. The DNRA activity of each isolate was confirmed by incubating the isolate with NO_3^- as the sole electron acceptor in 100 ml MSM in 160-ml serum bottle with and without 10% vol/vol C_2H_2 in the anoxic N_2 headspace. C_2H_2 inhibits N_2O reduction by NosZ, thus enabling observation of N_2O production from NO_3^- and/or NO_2^- reduction in an NosZ-harboring organism (59). The changes to the dissolved concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N, headspace concentrations of N_2O, and microbial growth (OD_{600}) were monitored until no further change was observed.

After incubation, a culture sample was collected from each of these six DNRA-positive isolates and the genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genome sequencing was performed using the HiSeq 4000 platform (Illumina, San Diego, CA) at Macrogen, Inc. (Seoul, South Korea). Quality trimming and removal of adapter sequences from raw reads was performed using Cutadapt version 2.9, and *de novo* assembly was done using SPAdes (version 3.14.0) with the minimum contig length set to 200 bp (60, 61). The quality of the draft genomes was assessed using CheckM software version 1.0.18 (62). The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used for genome annotation (63). The presence or absence of the nitrogen dissimilation functional genes was double-checked by running the *hmmsearch* command of HMMER software package version 3.1b1 with hidden Markov models (HMM), downloaded from the FunGene database (http://fungene.cme.msu.edu/), accessed on 14 October 2019 (64). This process ensured that the missing genes were not due to incompleteness of the draft genomes. The genes encoding the regulatory proteins putatively involved in nitrogen dissimilation were also searched in the annotated genome. The draft genome sequences of the six isolates were deposited to NCBI's GenBank database.

NO_3^- inhibition of NO_2^--to-NH_4^+ reduction. *Citrobacter* sp. DNRA3 carrying single copies of *nrfA* and *nirB* genes and *Enterobacter* sp. DNRA5 carrying *nirB* genes were subjected to further physiological characterization to examine whether and how NO_3^- affected NO_2^--to-NH_4^+ reduction. The resting-cell NO_3^- and NO_2^- reduction activities were examined with the cells harvested from the two distinct phases of the DNRA reaction, i.e., NO_3^--to-NO_2^- and NO_2^--to-NH_4^+ reduction. The DNRA-catalyzing isolates were grown with 5 mM NO_3^- as the electron acceptor and 40 mM lactate or 10 mM glucose as the electron donor and carbon source. The cells were harvested before and after NO_3^- depletion. Cell pellets were collected by centrifuging 200 ml culture at 10,000 $\times$ *g* for 20 min at 4°C and resuspended in 10 ml MSM. One milliliter of the cell suspension was added to a 160-ml stopper-sealed serum vial containing 100 ml of fresh MSM with N_2 headspace. Chloramphenicol (water soluble; Sigma-Aldrich) was added to a final concentration of 25 μg ml^{-1} to arrest *de novo* protein synthesis (65). These cell suspensions were then amended with 2 mM NO_3^- or NO_2^- and 6.67 mM lactate (*Citrobacter* sp. DNRA3) or 3.34 mM glucose (*Enterobacter* sp. DNRA5). The rates of change in the amounts of NO_2^- were measured and normalized to the protein mass of the resting-cell cultures.

To observe the effects of changing NO_3^- and NO_2^- concentrations on transcriptional expression of the nitrite reductase genes directly relevant to DNRA, the transcript abundances of *nrfA* and *nirB* genes in *Citrobacter* sp. DNRA3 and the *nirB* gene in *Enterobacter* sp. DNRA5 were monitored as the cells were grown with 1 mM NO_3^- and 3.34 mM lactate or 1.67 mM glucose. Collection and treatment of the samples, including extraction, purification, and reverse transcription processes, were performed using established protocols (18). Quantitative PCR (qPCR) was performed with a QuantStudio 3 real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA) using SYBR green detection chemistry, targeting *nrfA* and *nirB* in *Citrobacter* sp. DNRA3 and *nirB* in *Enterobacter* sp. DNRA5 using the primer sets listed in Table 1, as described in detail in the supplemental material.

TABLE 1 Primer sets used for PCR and qPCR

Primer	Purpose	Target gene (locus tag)	Sequence (5′ to 3′)	Amplicon size (bp)	Slope	*y* intercept	Amplification efficiency (%)	R^2	Reference or source
27F	PCR	Bacterial 16S rRNA	AGAGTTTGATCMTGGCTCAG						69
1492R			TACGGYTACCTTGTTACGACTT						
Cit_nrfA_F	qPCR	*nrfA* (HG548_21145)	ACATGCCGAAAGTGCAAAACGC	154	−3.146	34.312	102.9	0.991	This study
Cit_nrfA_R			TGAATGGCCTGTTTACGCTCGG						
Cit_nirB_F	qPCR	*nirB* (HG548_14725)	ACACCAACGACAACTTCCTGGC	166	−3.287	36.225	101.5	0.998	This study
Cit_nirB_R			AAGCCGATACGTTGAGAACCGG						
Cit_recA_F	qPCR	*recA* (HG548_20545)	GGTAAAACAACGCTGACCCTGC	186	−3.381	34.183	97.6	0.999	This study
Cit_recA_R			CAGCGCATCACAGATTTCCAGC						
Ente_nirB_F	qPCR	*nirB* (HG551_19495)	TGAAAGCGGAAACCAAAGCCG	178	−3.336	33.848	99.4	0.994	This study
Ente_nirB_R			AAGGACTTAATGCCCTCCACGC						
Ente_recA_F	qPCR	*recA* (HG551_15920)	TGGTGTGATGTTCGGTAACCCG	151	−3.255	33.021	102.8	0.996	This study
Ente_recA_R			GTTCTTCACAACCTTCACGCGG						

Batch and chemostat incubation of the DNRA-catalyzing isolates with various C-to-N ratios. Batch cultures of *Citrobacter* sp. DNRA3 and *Enterobacter* sp. DNRA5 were prepared with two different C-to-N (carbon in the organic electron donor/nitrogen in NO_3^-) ratios, and the DNRA reaction in these vessels was observed. For high C-to-N ratio incubation, the culture medium was initially prepared with 0.2 mM NO_3^- and 5 mM lactate or 2.5 mM glucose, and after each NO_3^-/NO_2^- depletion event, the culture vessels were amended with an additional batch of 0.2 mM NO_3^-. For low C-to-N ratio incubation, the initial medium contained 2 mM NO_3^- and 0.2 mM lactate or 0.1 mM glucose, and the organic electron donors were replenished upon depletion, indicated by discontinued NO_3^- reduction. The concentrations of NO_3^-, NO_2^-, and NH_4^+ were monitored throughout the incubation periods.

The chemostat cultures of the DNRA isolates were set up with a 300-ml culture in a continuously stirred 620-ml glass reactor fed fresh medium at a dilution rate of 0.05 h^{-1} (Fig. S5). The medium bottle and the reactor vessel were consistently purged with N_2 gas to maintain anoxic culture conditions during incubation. The reactor was operated with high (10 mM lactate or 5 mM glucose and 2 mM NO_3^- in the feed) and low (0.2 mM lactate or 0.1 mM glucose and 2 mM NO_3^-) C-to-N ratios. The concentrations of NO_2^-, NO_3^-, and NH_4^+ in the effluent were monitored until the reactor reached steady state, as indicated by three statistically similar NO_2^-, NO_3^-, and NH_4^+ concentrations measured at 6-h intervals. The N_2O production rate was measured after steady state was established by closing the gas inlet and outlet of the reactor and monitoring linear N_2O production.

Analytical methods. The concentrations of NH_4^+, NO_2^-, and NO_3^- were determined calorimetrically. At each sampling event, a 1-ml sample was extracted with a disposable syringe and the cell-free supernatant was subjected to spectrophotometric assays. The NH_4^+-N concentration was measured using the salicylate method and the NO_2^--N and/or NO_3^--N concentrations were determined using the Griess method (41, 66). Headspace N_2O concentrations were determined using an HP 6890 series gas chromatograph equipped with an HP-PLOT Q column and a ^{63}Ni electron capture detector (Agilent Technologies, Santa Clara, CA) (67). Helium (≥99.999%; Special Gas, Inc., Daejeon, South Korea) and 5% CH_4–95% Ar mixed gas were used as the carrier gas and the make-up gas, respectively. The injector, oven, and detector temperatures were set to 200, 85, and 250°C, respectively. Assuming equilibrium between the aqueous and gas phases, the total amounts of N_2O-N in reaction vessels were calculated using the dimensionless Henry's law constant of 1.68 at 25°C (68). The concentrations of glucose, lactate, and acetate were measured using a Prominence high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA). Protein concentrations were determined with the Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA) using a concentration series of bovine serum albumin solution as the standard.

Statistical analysis. With the exception of the chemostat experiments, all incubation experiments were performed in triplicates and the data presented as the average results of the triplicate samples along with their standard deviations. Statistical analyses were performed using R software version 3.6.3 (www.r-project.org), where the one-sample Student's *t* test was used to determine the statistical significance of temporal changes in transcript copy numbers or N species concentrations. A *P* value threshold of 0.05 was applied.

Data availability. The partial 16S rRNA gene sequences were deposited in NCBI's GenBank database (accession numbers MT426123 to MT426164). The draft genome sequences of the six confirmed DNRA isolates were deposited in NCBI's GenBank database (accession numbers JABAIU000000000, JABAIT000000000, JABAIS000000000, JABAIR000000000, JABAIQ000000000, and JABAIP000000000).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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