Physiological characterization of nitrate ammonifying bacteria isolated from rice paddy soil via a newly developed high-throughput screening method

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ABSTRACT

Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) has recently gained attention as a nitrogen retention pathway that may potentially be harnessed to alleviate nitrogen loss resulting from denitrification. Until recently, 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 microbial isolates capable of DNRA have been isolated from rice paddy soil with apparent dominance of denitrification using a novel high-throughput screening method. Six of these isolates, each assigned to a disparate genus, was examined to improve understanding of DNRA physiology. All isolates carried *nrfA* and/or *nirB*, and an isolate affiliated to *Bacillus* possessed a clade II *nosZ* gene and was capable of N_2O reduction. A common prominent physiological feature observed in all DNRA isolates was NO_2^- accumulation observed before NH_4^+ production, which was further examined with *Citrobacter* sp. DNRA3 (possessing *nrfA* and *nirB*) and *Enterobacter* sp. DNRA5 (possessing only *nirB*). In both organisms, NO_2^--to-NH_4^+ reduction was inhibited by submillimolar NO_3^-, and *nrfA* or *nirB* transcription was down-regulated when NO_3^- was being reduced to NO_2^-. Both batch and chemostat incubations of these isolates with excess organic electron donors produced NH_4^+ from reduction of NO_3^-; however, incubation with excess NO_3^- resulted in NO_2^- buildup but no substantial NH_4^+ production, presumably due to NO_3^- presence. 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-vs-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 leading to nitrogen loss and N_2O emission, DNRA reduces NO_3^- and NO_2^- to NH_4^+, a reactive nitrogen with higher tendency to be retained in 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 ecophysiology. Here, we have

developed a novel high-throughput 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 key to understanding why significant DNRA activity is rarely observed in nitrogen-rich agricultural soils.

INTRODUCTION

Nitrogen is an essential element for plant growth. Today, the Haber-Bosch process, used primarily for 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, 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 impending 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%, due to nitrogen loss via sequential nitrification and denitrification reactions (5). These microbial reactions 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 nitrogen-retaining DNRA (dissimilatory nitrate/nitrite reduction to ammonium) pathway (8-11). The DNRA pathway is the reduction of NO_3^-/NO_2^- to NH_4^+ catalyzed by the microorganisms equipped with cytochrome c_{552} nitrite reductases (encoded by *nrfA* genes) or NADH-dependent nitrite reductases (encoded by *nirB* genes) generally perceived as assimilatory nitrite reductases (12).

85 According to current limited knowledge, NO_2^--to-NH_4^+ reduction may serve as the electron acceptor
86 reaction for respiration (respiratory DNRA) or electron dump for NADH regeneration in fermentation
87 of complex organics (fermentative DNRA) (13-15).
88
89 Denitrification and DNRA pathways compete for common substrates, NO_3^-/NO_2^-, and thus,
90 stimulating one would repress the other (16, 17). Previous investigations suggested that DNRA is
91 favored in environments with high organic carbon (C) content and limiting supply of nitrogenous
92 electron acceptors (NO_3^-/NO_2^-) (18-20). This hypothesis was further corroborated by recent laboratory
93 experiments with microbial enrichments and axenic microbial cultures harboring both denitrification
94 and DNRA pathways; however, conflicting observations (e.g., in experiments with *Intrasporangium*
95 *calvum* and the *Deltaproteobacteria*-dominated wastewater enrichments) suggest the possibility that
96 the observed correlation between DNRA activity and the C-to-N ratio (the ratio of C in bioavailable
97 organic compounds to N in NO_3^-/NO_2^- in this context) may be circumstantial (17, 21-23).
98
99 Microbial population responsible for DNRA in soils can now be analyzed without culturing, using
100 recently developed molecular tools targeting *nrfA* genes or metagenomics and meta-omics techniques
101 (24, 25). Nevertheless, these culture-independent analyses need to be complemented with culture-
102 based examinations into physiology of soil DNRA isolates, especially so considering that the previous
103 observations that *nrfA* abundance may be decoupled from DNRA activity and that *nirB*-possessing
104 organisms may also contribute to the overall DNRA activity (26, 27). The difficulty in securing
105 DNRA isolates from soils with apparent dominance of denitrification has been the bottleneck in
106 culture-based investigation of DNRA physiology. The traditional approach that has been used for
107 isolation of DNRA bacteria was NO_3^--amended anoxic enrichment followed by isolation via serial
108 dilution and/or single colony picking and screening for isolates capable of NH_4^+ production from
109 anaerobic incubation with NO_3^-. This approach would require extensive menial labor for screening out
110 a few DNRA isolates from overwhelming number of denitrifiers when applied to soils with apparent
111 dominance of denitrification. In fact, in the only reported case of such targeted isolation of soil DNRA
112 organisms, only three out of 80 NO_3^--reducing isolates were revealed as capable of reducing NO_3^- to

NH_4^+ (28). Not surprisingly, the model organisms examined for DNRA ecophysiology rarely originated from soil (15, 17, 29-32). Further, most of these isolates had been aerobically isolated and cultured for decades in laboratory settings before they were recognized as being capable of DNRA. Thus, use of these isolates as representatives of soil DNRA bacteria have received criticism as lacking ecological relevance to the fate of NO_3^- in anoxic agricultural soils.

To address this ecological relevance issue in examining soil DNRA ecophysiology, a less onerous and time-consuming method for isolation of DNRA bacteria in denitrification-dominant agricultural soils was in need. Here, to address this issue,a rapid inexpensive high-throughput screening method was developed utilizing the well-established salicylate method for NH_4^+ detection and quantification (33). Reductive transformation of NO_3^- was examined with six DNRA organisms isolated from rice paddy soils using this novel screening method, affiliated to *Bacillus* (belonging to *Firmicutes* phylum), *Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Shewanella* (belonging to *Proteobacteria* phylum) genera. Their most obvious common physiological feature was NO_3^- inhibition of NO_2^--to-NH_4^+ reduction, which had been also previously observed with *nrfA*-and-*nirB*-harboring organisms *Escherichia coli* and *Bacillus vireti* (34, 35). With a series of batch and continuous culture experiments, we identified this NO_3^- repression of DNRA activity as one of the mechanisms underpinning the widely acknowledged but controversial C-to-N ratio regulation of DNRA-vs-denitrification competition.

RESULTS

Isolation of DNRA bacteria from denitrification-dominant agricultural soil. Out of 192 colonies each from the 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 (Fig. S1). Sequencing of the 16S rRNA gene amplicons of the positive colonies (30 and 12 colonies from lactate- and glucose-amended enrichments, respectively) identified six bacterial genera: *Aeromonas*, *Bacillus*, *Shewanella* (lactate-amended), *Enterobacter*, *Klebsiella* (glucose-amended), and *Citrobacter* (both) (Fig. S2). The DNRA activities in six of these isolates, each belonging to a different genus, were further examined.

Identification of functional genes relevant to dissimilatory nitrogen reduction. The draft genomes of the six DNRA isolates were constructed from the HiSeq sequencing reads (the sequencing statistics 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 and Table S3). The isolates that originated from lactate-enriched cultures all possessed *nrfA* genes encoding NH_4^+-forming cytochrome c_{552} nitrite reductases, while the two isolates from glucose-enriched cultures lacked *nrfA* gene, but possessed *nirB* genes, suggesting that NirB-type nitrite reductase was responsible for dissimilatory reduction of NO_2^- to NH_4^+ in these organisms. Only *Citrobacter* sp. DNRA3 possessed both *nrfA* and *nirB*. All six isolates have *napA* in their genomes, and *Citrobacter* sp. DNRA3, *Enterobacter* sp. DNRA5, and *Klebsiella* sp. DNRA6 carry *narG*, indicating the genomic potential of these organisms to reduce NO_3^- to NO_2^-. Neither *nirK* nor *nirS*, i.e., genes encoding 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. DNRA2, suggesting N_2O-reducing capability.

NO_3^- reduction of the candidate DNRA isolates. Reductive transformation of NO_3^- was observed with the axenic cultures of the six candidate DNRA isolates with or without 10% C_2H_2 in the headspace (Fig. 2, S3, and S4). All 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, produced NH_4^+ only amounted to 36.3±1.1 and 32.1±0.2 μmoles, respectively, which were less than half of nitrogen added as NO_3^-. As the cell density of the glucose-fed *Enterobacter* sp. DNRA5 and *Klebsiella* sp. DNRA6 reached at least 2.5-fold higher than the lactate-consuming isolates, the missing nitrogen was likely due to assimilation. Despite the absence of *nirK* or *nirS* gene, N_2O production was observed in all of the examined 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±0.06 μmoles N_2O-N of *Shewanella* sp. DNRA4 to 3.5±0.3

169 μmoles N_2O-N of *Citrobacter* sp. DNRA3. In all six isolates examined, the start of N_2O production
170 corresponded with the start of NH_4^+ production, suggesting that N_2O was the byproduct of NO_2^--to-
171 NH_4^+ reduction, not NO_3^--to-NO_2^- reduction. Of the six isolates, only *Bacillus* sp. DNRA2 showed a
172 substantially different N_2O-N time series profile when incubated without C_2H_2 (Fig. S4). The absence
173 of N_2O accumulation suggested that N_2O consumption occurred simultaneously with DNRA in this
174 clade II *nosZ*-harboring organism.
175
176 Accumulation of NO_2^- before reduction to $NH_4^+$was consistently observed in all six of these isolates.
177 Reduction of NO_2^- to NH_4^+ did not commence until >80% of NO_3^- was consumed in any of the six
178 isolates, suggesting that NrfA- or NirB-catalyzed NO_2^--to-NH_4^+ reduction was affected by changing
179 NO_2^- or NO_3^- concentrations. The DNRA reactions of *Citrobacter* sp. DNRA3 and *Enterobacter* sp.
180 DNRA5 were further investigated to identify whether possible causality exists between NO_2^- or NO_3^-
181 concentration and DNRA activity (Fig. 3A-B). Transcription levels of *nrfA* in *Citrobacter* sp. DNRA3
182 and *nirB* in *Enterobacter* sp. DNRA5 were significantly higher (p<0.05) after NO_3^- was depleted than
183 before. Transcription of *nrfA* in *Citrobacter* sp. DNRA3 increased significantly (p<0.05) from 1.0±0.6
184 *nrfA*/*recA* at t=9 h (0.16±0.03 mM NO_3^- and 0.82±0.03 mM NO_2^- remaining) to 6.3±0.2 *nrfA*/*recA* at
185 t=15 h (0.33±0.08 mM NO_2^- remaining). No significant change was observed with *nirB* transcription
186 (2.04±0.19 *nirB*/*recA* at t= 9 h and 1.61±0.37 *nirB*/*recA* at t=15 h), suggesting that NirB-type nitrite
187 reductase was irrelevant to respiratory DNRA. Transcription of *nirB* in *Enterobacter* sp. DNRA5
188 followed a similar trend with that of *nrfA* in *Citrobacter* sp. DNRA3, increasing significantly after
189 depletion of NO_3^- (p<0.05). Substrate (NO_2^-) regulation of transcription was unlikely for either *nrfA* in
190 *Citrobacter* sp. DNRA3 and *nirB* in *Enterobacter* sp. DNRA5, as the transcriptions of these genes
191 appeared unresponsive to elevated NO_2^- concentrations, as long as NO_3^- was present in the medium at
192 >0.15 mM. Thus, NO_3^- concentration was the most probable environmental factor that affected the
193 transcription of the genes encoding these DNRA-catalyzing nitrite reductases. The significant
194 differences in the rates of NO_2^- reduction measured with *Citrobacter* sp. DNRA3 or *Enterobacter* sp.
195 DNRA5 cells harvested before and after the NO_3^- depletion and treated with chloramphenicol also

196 supported that expression of the NH_4^+-forming nitrite reductases were down-regulated by the presence
197 of NO_3^- (Fig. 3C-F). *Citrobacter* sp. DNRA3 cells extracted before NO_3^- depletion did not exhibit
198 significant NO_2^- reduction activity, while the cells extracted after NO_3^- depletion readily reduced NO_2^-
199 at a rate of 170±13 μmoles s^{-1} mg $protein^{-1}$. NO_2^- reduction by *Enterobacter* sp. DNRA5 cells was
200 also ~6 times higher with the cells harvested after NO_3^- depletion (101±10 μmoles s^{-1} mg $protein^{-1}$)
201 than the cells harvested before NO_3^- depletion (17.2±5.7 μmoles s^{-1} mg $protein^{-1}$; $p<0.05$).
202
203 In the resting-cell experiments with 2 mM NO_3^- added to chloramphenicol-treated *Citrobacter* sp.
204 DNRA3 cells harvested after NO_3^- depletion, NO_2^- accumulated up to 1.93±0.04 mM at a rate of
205 422±8 μmoles s^{-1} mg $protein^{-1}$ before it was consumed at a rate of 51.4±5.3 μmoles s^{-1} mg $protein^{-1}$
206 (Fig. 3E). The negligible NO_2^- reduction activity before NO_3^- depletion suggested an additional NO_3^--
207 mediated inhibitory mechanism on NrfA-type nitrite reductase activity apart from transcriptional
208 regulation of *nrfA* gene. Such repression of NO_2^- reduction activity by NO_3^- presence was not
209 observed in the parallel experiment performed with *Enterobacter* sp. DNRA5 lacking *nrfA* and
210 presumably utilizing NirB-type nitrite reductase (Fig. 3F).
211
212 **DNRA reaction at varying C-to-N ratios: batch and continuous cultivation.** *Citrobacter* sp.
213 DNRA3 and *Enterobacter* sp. DNRA5 were grown in batch and continuous cultures, each with two
214 different C-to-N ratios, and NO_3^- reduction was monitored to investigate whether the generally
215 perceived positive correlation between C-to-N ratio and DNRA activity may be related to the NO_3^--
216 repression of NO_2^--to-NH_4^+ reduction (Fig. 4 and 5). When grown at the initial C-to-N ratio of 75 in
217 batch cultures, *Citrobacter* sp. DNRA3 produced NH_4^+ from NO_3^- reduction, and each addition of 20
218 μmoles NO_3^- resulted in near-stoichiometric turnover to NH_4^+. Contrastingly, *Citrobacter* sp. DNRA3
219 grown at the initial C-to-N ratio of 0.3 did not result in significant increase in NH_4^+ concentration, but
220 led to stoichiometric NO_2^- accumulation, as NO_3^- reduction produced 0.87±0.06 mM NO_2^- when the
221 initial reaction stopped at t=16 h due to depletion of lactate. Reduction of NO_3^- to NO_2^- resumed after
222 replenishment with 0.2 mM lactate at t=21 h. At this low C-to-N ratio incubation condition, NO_3^- was

223 present in the culture medium throughout incubation, and the presence of NO_3^- was likely the reason
224 for the absence of *sensu stricto* DNRA activity.
225
226 *Enterobacter* sp. DNRA5, incubated on glucose at the C-to-N ratio of 75 produced significant amount
227 of NH_4^+ only after initially added glucose (2.19±0.8 mM) was fully consumed, suggesting that
228 substantial portions of NO_3^- and its reduction products, NO_2^- and NH_4^+, were assimilated. Upon third
229 addition of 0.2 mM NO_3^-, with no glucose remaining in the medium, the sequential NO_3^--to-NO_2^--to-
230 NH_4^+ reduction was stoichiometric, suggesting that NirB-catalyzed NO_2^--to-NH_4^+ reduction was
231 coupled to oxidation of the fermentation products. At the low C-to-N ratio, where the culture medium
232 was replenished with 0.1 mM glucose upon halt in NO_3^- reduction, the time series profiles of the N-
233 species concentrations were indistinguishable from that of *Citrobacter* sp. DNRA3, save for the
234 imperfect stoichiometry between consumed NO_3^- and produced NO_2^- and the modest, albeit
235 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.
236 The modest production of NH_4^+ was in line with the reduced, but still significant NO_2^- reduction rate
237 observed in the resting-cell cultures of *Enterobacter* sp. DNRA5 extracted before NO_3^- depletion.
238
239 The continuous culture of *Citrobacter* sp. DNRA3 fed medium carrying 10 mM lactate and 2 mM
240 NO_3^- (C-to-N ratio of 15), after attaining steady state, contained 1.68±0.09 mM NH_4^+ as the only
241 dissolved inorganic nitrogen, indicating that NO_3^- and NO_2^- were readily reduced in the chemostat.
242 When the reactor was fed the medium carrying 0.2 mM lactate and 2 mM NO_3^- (C-to-N ratio of 0.3),
243 1.41±0.04 mM NO_3^- remained in the medium at steady state, due to carbon limitation. That NO_2^- was
244 the major product of NO_3^- reduction (0.58±0.04 mM at steady state) and NH_4^+ concentration did not
245 significantly differ from the concentration in the fresh medium ($p>0.05$) indicated DNRA did not
246 proceed further than NO_2^-. Similarly, with *Enterobacter* sp. DNRA5, significant NH_4^+ formation was
247 observed only in the continuous culture operated at the electron-acceptor-limiting condition, i.e., at C-
248 to-N ratio of 15. The steady state NH_4^+ concentration was 0.95±0.04 mM in this chemostat. In the
249 continuous culture operated at electron-donor-limiting condition, NO_2^- was the only dissolved

nitrogen species with significantly higher concentration than the influent medium. Production of N_2O (2.07±0.24 μmoles hr^{-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 at the low C-to-N-ratio batch and chemostat cultures, regardless mediated by NrfA-type or NirB-type nitrite reductase, could be best explained as the inhibitory effect of NO_3^-.

DISCUSSION

For long, DNRA has been recognized as one of the key reactions determining the fate of reactive nitrogen in the environment (36-38). Recovery of $^{15}NH_4^+$ from $^{15}NO_3^-$ reduction in both *in situ* column studies and *ex situ* soil incubation experiments supported that the DNRA pathway is a significant dissimilatory reaction pathway in soil environments; however, fertilized agricultural soils with the DNRA pathway dominating over the denitrification pathway, i.e., with higher NH_4^+ production than N_2O+N_2 production from NO_3^-/NO_2^- reduction, have rarely been reported (8, 16, 39). Thus, few attempts have been made to isolate and examine DNRA organisms from agricultural soils, and interpretations of DNRA in ecological contexts, particularly with regards to the fate of fertilizer N, have relied on extrapolation of findings from experiments with limited number of isolates, most, if not all, acquired from non-soil environments (15, 17, 28, 32, 34, 40). The high-throughput DNRA screening method developed in this study enabled easy and rapid targeted isolation by systematically combining widely-used culturing and colorimetric detection techniques. The most conspicuous benefit of the method is the ease of isolating the DNRA organisms that had been considered to be difficult to enrich from soils with apparent domination of denitrification over DNRA. Examination of the physiology of the indigenous DNRA organisms in denitrification-dominant soils, isolated using this targeted isolation technique, would be a sensible approach to investigate the reasons why NH_4^+ production via DNRA is often underrepresented in the nitrogen cycling of agricultural soils. Such knowledge would be immensely useful in developing soil management techniques for enhancing the nitrogen-retaining DNRA pathway (9, 39).

277 The newly acquired soil DNRA isolates were assigned to six genera according to their 16S rRNA
278 gene sequences. Several of these genera have been previously confirmed to include strains capable of
279 carrying out DNRA (*Bacillus*, *Citrobacter*, *Enterobacter*, and *Klebsiella*). The list also included a
280 genus that has not yet been previously isolated from soil environments (*Shewanella*) and a genus
281 without physiologically confirmed DNRA activity (*Aeromonas*) (27, 41-43). The genomic analyses of
282 these phylogenetically diverse DNRA-catalyzing isolates confirmed that the possession of *nrfA* or
283 *nirB* is necessary for DNRA phenotype (32, 34). All four isolates utilizing lactate as the electron
284 donor were *nrfA* genotype. *Enterobacter* sp. DNRA5 and *Klebsiella* sp. DNRA6 lacking *nrfA* failed to
285 grow on lactate at NO_2^--reducing condition, suggesting that NrfA-type nitrite reductase is needed for
286 respiratory NO_2^- reduction to NH_4^+. Thus, physiological functions of NirB-catalyzed NO_2^--to-NH_4^+
287 reduction in these organisms may be NAD^+ regeneration for fermentation, detoxification of NO_2^-,
288 and/or assimilatory reduction, as previously suggested (14, 32). Neither *nirS* nor *nirK* was found in
289 any of the sequenced draft genomes; however, a *nosZ* gene was recovered in the genomes of the *nrfA*-
290 possessing *Bacillus* sp. DNRA2. Incubations on NO_3^- reduction with and without C_2H_2 confirmed
291 N_2O reduction activity in this isolate amid active DNRA, which was probably catalyzed by the NosZ
292 encoded by this gene.
293
294 The most easily recognizable common phenotype of the DNRA isolates was NO_2^- accumulation
295 before NH_4^+ production suggesting NO_3^- repression of NO_2^--to-NH_4^+ reduction. This phenotype has
296 been consistently observed in previously studied DNRA bacteria (34, 35, 44). These previous studies
297 attributed the NO_3^--induced repression to transcriptional regulation involving NO_3^- sensor proteins
298 NarQ and NarX, and the transcript abundances of *nrfA* in *Escherichia coli*, and both *nrfA* and *nirB* in
299 *B. vireti* were significantly lower when supplied with higher NO_3^- concentrations. In agreement with
300 these previous studies, *nrfA* in *Citrobacter* sp. DNRA3 and *nirB* in *Enterobacter* sp. DNRA5,
301 exhibited at least 5.8-fold higher transcription after NO_3^- depletion than before ($p<0.05$). Further, the
302 results from the resting-cell experiments with these isolates showed clear indications that the presence
303 of NO_3^- at sub-millimolar concentration was sufficient to inhibit activities of expressed NrfA-type
304 nitrite reductase. Whether the apparent inhibition was due to the redirection of electron flow

analogous to what was observed with NosZ-catalyzed N_2O reduction in presence of O_2 or inhibition of NrfA enzyme itself, however, cannot be determined and is outside the scope of the current study (45). 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 (46-48). 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 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 condition, while denitrification is favored at low C-to-N ratios, i.e., electron donor limiting condition (17, 19, 22). 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^--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 ratio of soils or sediments are often inversely related to the NO_3^- contents (49). In the 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 abundant DNRA-catalyzing population, N_2O and N_2 would still be the dominant terminal product of NO_3^- reduction in such soils. Thus, what was previously regarded as the effect of the C-to-N ratio on the denification-vs-DNRA competition may be, at least in part, explained as the consequence of NO_3^- inhibition of *sensu stricto* DNRA (39).

Production of N_2O has been consistently observed in non-denitrifying organisms with DNRA phenotypes, with recovery of up to ~50% of NO_3^--N as N_2O-N (28, 32, 44, 50). Likewise, all DNRA-catalyzing 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* gene in their genomes. Hypotheses surrounding the mechanisms of

333 N_2O production from DNRA have invariably involved NO (32, 35, 44). Considering that *norB*, *norV*,
334 and/or *hmp* genes were identified in the genomes of DNRA isolates, it is plausible to regard NO as the
335 precursor of N_2O in these organisms; however, the mechanism leading to NO production from NO_3^-
336 or NO_2^- remains unclear. The absence of N_2O production before NO_3^- depletion in all six isolates
337 suggested against involvement of NapA- or NarG-type nitrate reductases. The more plausible source
338 of NO would be NO_2^--to-NH_4^+ reduction by NrfA- and NirB-type nitrite reductases, although reaction
339 mechanism leading to formation of NO as a byproduct has not been elucidated for either enzyme (44,
340 51). The results from the chemostat experiments with *Enterobacter* sp. DNRA5 further support this
341 hypothesis, as detectable N_2O production was observed only at the high C-to-N ratio operating
342 condition where active NO_2^--to-NH_4^+ reduction occurred.
343
344 Another noteworthy observation regarding N_2O production by the DNRA-catalyzing organisms was
345 the absence of detectable N_2O production in the NH_4^+-producing *Citrobacter* sp. DNRA3 chemostat
346 (high C-to-N ratio), which suggested that the NO_3^- or NO_2^- concentrations in the surrounding
347 environment may have positive correlation with N_2O formation by *nrfA*-possessing organisms. In line
348 with this observation, elevated N_2O production upon incubations with media with lower C:N ratios,
349 i.e., higher NO_3^- concentration, was previously observed in *nrfA*-utilizing isolates of *Citrobacter* and
350 *Bacillus* genera (28). Together with the finding that *Bacillus* sp. DNRA2 actively consumed N_2O
351 simultaneously with NO_2^--to-NH_4^+ reduction, this observation suggests that N_2O produced from
352 NrfA-mediated DNRA may be negligible in complex soil microbiomes.
353
354 **MATERIALS AND METHODS**
355 **Soil sampling and initial characterization.** The agricultural soil used in this study was sampled
356 from an experimental rice paddy field located at Chungnam National University (CNU) agricultural
357 research site in Daejeon, Korea (36°22'01.6"N 127°21'14.3"E) in October 2018. Harvesting had been
358 complete and there was no standing water at the time of sampling. Cover soil and plant materials were
359 carefully removed before sampling and approximately 1 kg of soil at 5 to 30 cm depth from the
360 surface was collected with a stainless tubular soil sampler with an inner diameter of 2 cm. The

361 sampled soils were transported to the laboratory in coolers filled with ice and stored at 4°C until use.
362 The physico-chemical characteristics of this soil, including the pH, textures, and total carbon and
363 nitrogen contents were analyzed using standardized protocols.
364
365 **Culture medium and growth conditions.** The minimal salts medium (MSM) for enrichment,
366 isolation, and cultivation of soil DNRA bacteria was prepared by adding, per L of deionized distilled
367 water, 10 mmoles NaCl, 3.24 mmoles Na_2HPO_4, 1.76 mmoles KH_2PO_4, 0.1 mmoles NH_4Cl, and 1 mL
368 of 1000x trace element stock solution (52). For enrichment and isolation, R2A broth (Kisanbio, Seoul,
369 Korea) was added to the medium as growth supplements. To minimize the interference of NH_4^+
370 derived from mineralization of organic nitrogen in the ensuing DNRA-screening process, R2A broth
371 concentration in the medium was limited to 6.2 mg L^{-1}. The pH of the medium was adjusted to 7.0.
372 Pure-culture incubation and experiments were performed with 100 mL medium dispensed to 160-mL
373 serum vials. The serum bottles were flushed with N_2 gas (≥99.999%, Special Gas Inc., Daejeon,
374 Korea) for 10 min and sealed with black butyl-rubber stoppers (Geo-Microbial Technologies, Inc.,
375 Ochelata, OK) and crimped with aluminum crimp seals before autoclaving. The degassed filter-
376 sterilized 200X vitamin stock solution was then added to the medium (52). Immediately before
377 inoculation, sodium lactate or glucose was added as the electron donor and organic carbon source and
378 KNO_3 as the electron acceptor. Lactate and glucose were chosen as the non-fermentable and
379 fermentable electron donors, respectively, as both substrates had been previously reported to support
380 DNRA reactions (17, 53, 54). Enrichment with acetate as the electron donor was also attempted, but
381 failed to yield any DNRA-positive isolates. Thus, acetate was not further considered as a potential
382 electron donor in this study. The culture bottles were incubated with shaking at 150 rpm in dark at
383 room temperature (25°C). Agar plates were prepared by adding 15 g L^{-1} Bacto™ Agar (Becton
384 Dickinson, Franklin Lakes, NJ) to the liquid medium prepared with elevated concentrations of KNO_3
385 (12 mM) and sodium lactate or glucose (120 mM as C). The 96-well plates for DNRA-screening were
386 prepared by distributing 200-μL aliquots of the prepared culture medium to the wells in a UV-
387 sterilized 96-well clear flat bottom microplates (Corning Inc., Corning, NY). Agar plate and 96-well

388 plate were prepared and incubated in an anaerobic chamber (Coy Laboratory Products, Inc., Grass
389 Lake, MI) with atmosphere consisting of 96% N_2 and 4% H_2.
390
391 **High-throughput DNRA phenotype screening.** A simple novel high-throughput screening method
392 was developed in this study for isolating DNRA-catalyzing organisms from agricultural soils without
393 apparent DNRA activity (Fig. 6). Anoxic soil enrichments were prepared in 250-mL Erlenmeyer
394 flasks (Duran Group, Wertheim, Germany) in the anaerobic chamber. The rice paddy soil was
395 suspended at 1:10 w/v soil-to-medium ratio in 200 mL MSM amended with 2 mM KNO_3 and 6.67
396 mM sodium lactate or 3.33 mM glucose (20 mM total C concentration) and incubated for two weeks
397 in dark without shaking. The aqueous NO_3^--N, NO_2^--N, and NH_4^+-N concentrations were measured to
398 confirm depletion of the electron acceptors and to check the extent of DNRA reaction in the
399 enrichments. Serial dilutions of the soil enrichment cultures were spread onto agar plates, and after
400 incubation in the anaerobic chamber, single colonies were picked to inoculate the 96-well plates
401 loaded with fresh medium containing 1 mM NO_3^- and 3.34 mM lactate or 1.67 mM glucose. The
402 inoculated 96-well plates were covered with an optical adhesive cover (Applied Biosystems, Foster
403 City, CA) to prevent contamination and evaporation of the culture medium and incubated for a week.
404
405 After incubation, 100 μL of the 200-μL culture in each well was transferred to its corresponding
406 position on a new 96-well plate. The absorbances at 600 nm, 660 nm, and 540 nm were determined
407 using a Sunrise microplate reader (Tecan, Männedorf, Switzerland) with one of the duplicated plates
408 to measure the possible interference of cell turbidity to the colorimetric determination of inorganic
409 nitrogen concentrations. This plate was used for screening of the wells with increased NH_4^+
410 concentrations indicative of DNRA activity, using salicylate-nitroprusside chemistry. To each well,
411 80 μL of the color reagent (containing 0.2 M sodium hydroxide, 1 M sodium salicylate and 5.88 mM
412 sodium nitroprusside dihydrate) and 20 μL of 5.1 mM sodium dichloroisocyanurate solution were
413 sequentially added. The absorbance at 660 nm was measured after 30-minute incubation at 25°C, and
414 the wells with OD_{660nm} values higher than 1.6 (equivalent to 0.8 mM NH_4^+) after subtracting OD_{660nm}

415 resulting from cell turbidity were considered as positives. The duplicated 96-well plate was used for
416 sequential measurements of NO_2^--N and NO_3^--N (55). The Griess reagent was added to each well to a
417 total volume of 200-µL and the absorbance at 540 nm was measured after 30 min of incubation at
418 25°C for determination of NO_2^--N concentration. The NO_3^--N concentration was determined after the
419 initial Griess assay. After reducing NO_3^- to NO_2^- by adding per well 20 µL of 1% w/v vanadium(III)
420 chloride (VCl_3; Sigma-Aldrich) prepared in 1 M HCl aqueous solution, the absorbance at 540 nm was
421 measured to obtain the NO_3^--plus-NO_2^- concentration, from which the NO_2^- concentration was
422 subtracted. The colonies corresponding to the wells with NH_4^+ concentrations higher than 0.8 mM and
423 absence of NO_2^- and NO_3^- were transferred to gridded fresh agar plates and stored at 4°C until use.
424
425 **Characterization of DNRA isolates.** The partial 16S rRNA genes of these candidate DNRA isolates
426 were amplified with the 27F/1492R primer set and sequenced to identify their phylogenetic
427 affiliations (GenBank accession number: MT426123-64). Based on these initial 16S rRNA
428 sequencing data, one isolate per genus was selected and subjected to further analyses. The DNRA
429 activity of each isolate was confirmed by incubating the isolate with NO_3^- as the sole electron
430 acceptor in 100 mL MSM in 160-mL serum bottle with and without 10% v/v C_2H_2 in the anoxic N_2
431 headspace. C_2H_2 inhibits N_2O reduction by NosZ, thus enabling observation of N_2O production from
432 NO_3^- and/or NO_2^- reduction in a NosZ-harboring organism (56). The changes to the dissolved
433 concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N, headspace concentrations of N_2O, and the microbial
434 growth (OD_{600nm}) were monitored until no further change was observed.
435
436 After incubation, culture sample was collected from each of these six DNRA-positive isolates and the
437 genomic DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany)
438 according to the manufacturer's protocol. Genome sequencing was performed using the HiSeq4000
439 platform (Illumina, San Diego, CA) at Macrogen Inc. (Seoul, Korea). Quality trimming and removal
440 of adapter sequences from raw reads was performed using Cutadapt v2.9 and *de novo* assembly using
441 SPAdes (v3.14.0) with the minimum contig length set to 200 bp (57, 58). The quality of the draft

genomes was assessed using the CheckM software v1.0.18 (59). The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used for genome annotation (60). The presence or absence of the nitrogen dissimilation functional genes was double-checked by running *hmmsearch* command of the HMMER software package v3.1b1 with the hidden Markov models (HMM) downloaded from the FunGene database (http://fungene.cme.msu.edu/) accessed on 10/14/2019 (61). 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 for in the annotated genome. The draft genome sequences of the six isolates were deposited to the NCBI's GenBank database (accession numbers: JABAIU000000000, JABAIT000000000, JABAIS000000000, JABAIR000000000, JABAIQ000000000, and JABAIP000000000).

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 selected to further examine whether and how NO_3^- affect 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 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 10,000Xg for 20 min at 4°C, and re-suspended 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 the final concentration of 25 μg mL^{-1} to arrest *de novo* protein synthesis (62). 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 with 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, transcript abundances of *nrfA* and *nirB* genes in

Citrobacter sp. DNRA3 and *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 (17). Quantitative polymerase chain reaction 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, as described in detail in the supplemental material.

Batch and chemostat incubation of the DNRA-catalyzing isolates with varying C-to-N ratios. Batch cultures of *Citrobacter* sp. DNRA3 and *Enterobacter* sp. DNRA5 were prepared with two different C (carbon in the organic electron donor)-to-N (nitrogen in NO_3^-) ratios, and DNRA reaction was observed in these vessels. For high-C-to-N 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 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 300-mL culture in 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 was monitored until the reactor reached steady state, as indicated by three statistically similar NO_2^-, NO_3^- and NH_4^+ concentrations measured with 6-hour 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. Upon each sampling event, 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 (33, 63). Headspace N_2O concentrations were determined using a HP 6890 series gas chromatograph equipped with a HP-PLOT Q column and a ^{63}Ni electron capture detector (Agilent Technologies, Santa Clara, CA)(64). Helium (≥99.999%, Special Gas Inc., Daejeon, Korea) and CH_4 (5%)/ Ar (95%) mixed gas were used as the carrier gas and the make-up gas, respectively. The injector, oven and detector temperature were set to 200, 85, 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 (65). 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 standards.

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

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744 **Figures**

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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*	*hmp*	*narQ*	*narX*	*narL*	*narP*
***Aeromonas* sp. DNRA1**	■					■	■			■	■		■	
***Bacillus* sp. DNRA2**	■				■		■	■		■	■		■	
***Citrobacter* sp. DNRA3**	■	■				■	■		■	■	■	■	■	■
***Shewanella* sp. DNRA4**	■						■	■			■		■	
***Enterobacter* sp. DNRA5**	■	■				■			■	■	■	■	■	
***Klebsiella* sp. DNRA6**	■	■				■			■	■		■	■	

746

747 **Fig. 1.** Functional genes identified in the draft genomes of the six DNRA bacteria that are potentially
748 relevant to turnover of reactive nitrogen species or regulation of dissilatory nitrogen metabolism. The
749 genes that were recovered in the draft genome are represented as shaded boxes.

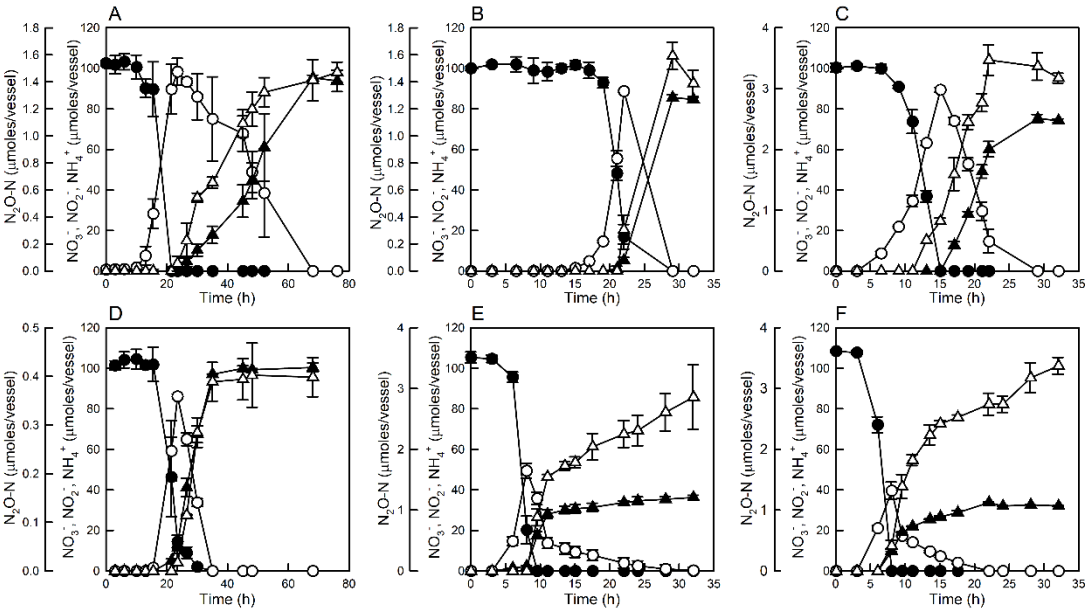


Fig. 2. NO_3^- reduction monitored in 100-mL batch cultures (prepared in sealed 160-mL serum bottles with headspace consisting of 90% N_2 and 10% C_2H_2) of (A) *Aeromonas* sp. DNRA1, (B) *Bacillus* sp. DNRA2, (C) *Citrobacter* sp. DNRA3, (D) *Shewanella* sp. DNRA4, (E) *Enterobacter* sp. DNRA5, and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

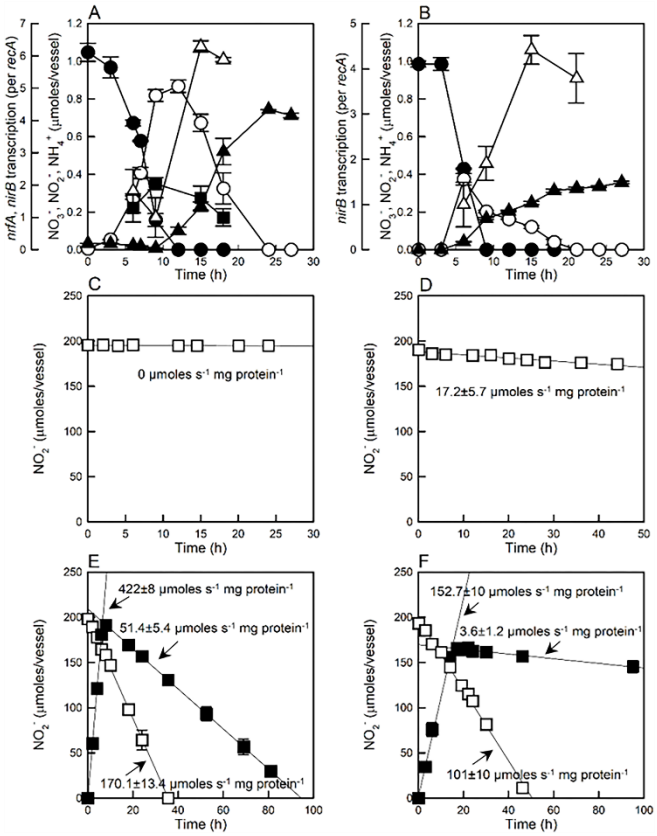


Fig. 3. Transcription of (A) *nrfA* and *nirB* in *Citrobacter* sp. DNRA2 and (B) *nirB* in *Enterobacter* sp. DNRA5 cells as 1 mM NO_3^- (●) was reduced to NH_4^+ (▲) via NO_2^- (○). Changes to the amounts of NO_2^- were monitored in the chloramphenicol-treated resting cultures of *Citrobacter* sp. DNRA2 (C, E) and *Enterobacter* sp. DNRA5 (D, F) harvested before (C, D) and after (E, 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 the error bars represent the standard deviations

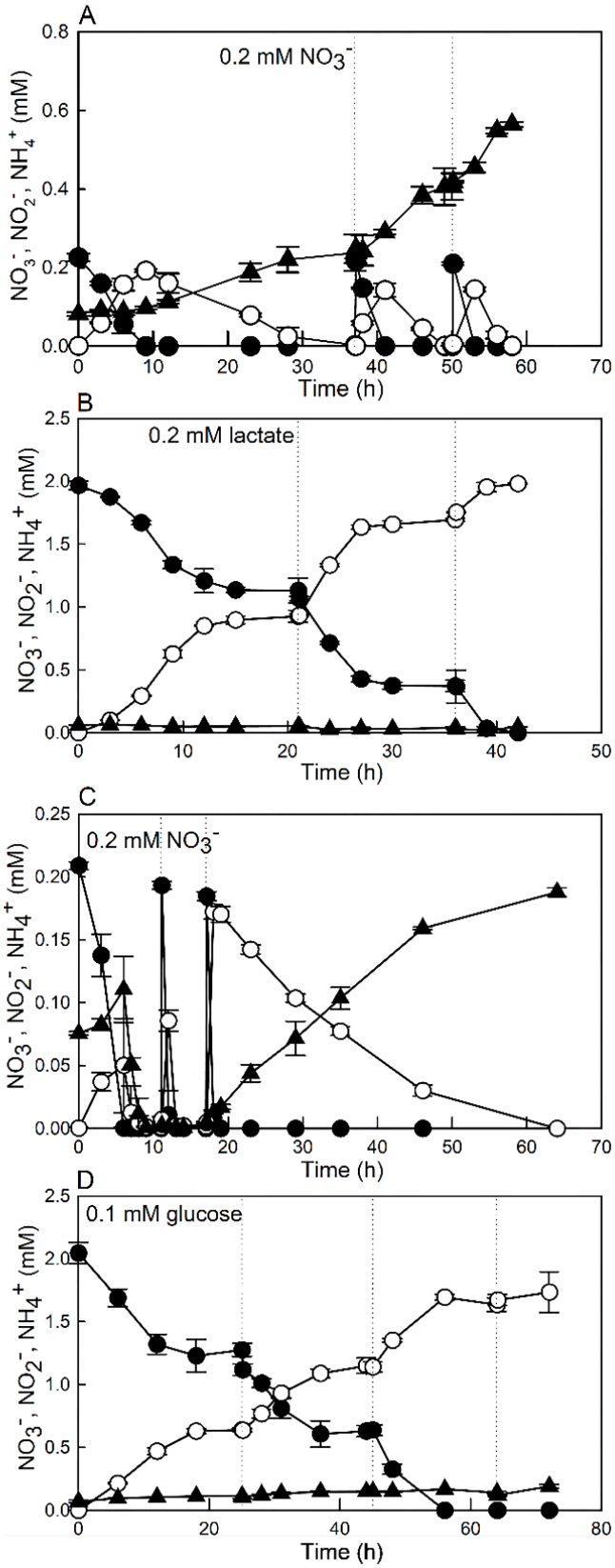
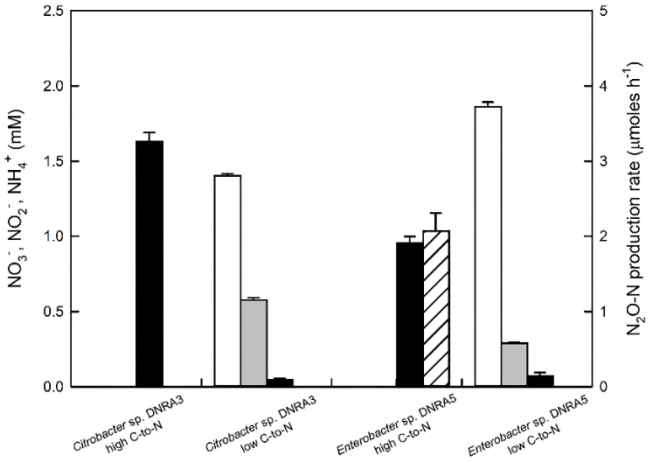


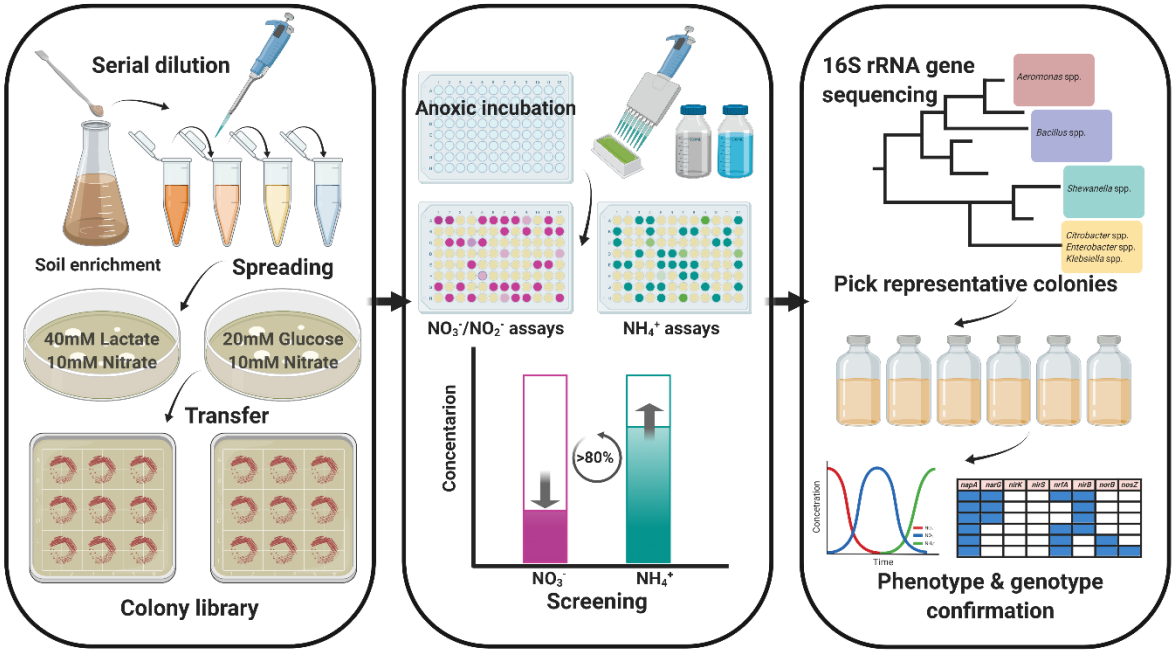
Fig. 4. NO_3^- reduction observed with batch cultures of *Citrobacter* sp. DNRA2 (A, B) and *Enterobacter* sp. DNRA5 (C, D) prepared with two different initial C-to-N ratios. The high C-to-N conditions were prepared with 0.2 mM NO_3^- and 5 mM lactate (A) or 2.5 mM glucose (C), and NO_3^-

767 was replenished to 0.2 mM upon NO_3^-/NO_2^- depletion. The low C-to-N conditions were prepared with
768 2.0 mM NO_3^- and 0.2 mM lactate (B) or 0.1 mM glucose (D) and the carbon sources were replenished
769 when NO_3^-/NO_2^- reduction stopped. The dotted lines denote the time points where the limiting
770 nutrients were replenished. The averages of biological replicates (n=3) are presented with the error
771 bars representing their standard deviations (NO_3^-: ●, NO_2^-: ○, NH_4^+: ▲, N_2O-N: △)



772

773 **Fig. 5.** Steady-state concentrations of NO_3^- (white), NO_2^- (gray), and NH_4^+ (black) in the electron
774 acceptor-limiting (high C-to-N ratio) and electron donor-limiting (low C-to-N ratio) chemostat
775 cultures of *Citrobacter* sp. DNRA2 and *Enterobacter* sp. DNRA5. N_2O-N production rate (hatched) is
776 also presented for *Enterobacter* sp. DNRA5 cultivated under the electron acceptor-limiting condition,
777 which was the only reactor culture with observed N_2O production. The average values of the three
778 measurements taken with six-hour intervals are presented with the error bars represent their standard
779 deviations



780

781 **Fig. 6.** The schematic overview of the high-throughput screening methods developed for isolation of

782 DNRA-catalyzing organisms from agricultural soil