Diazotrophic *Anaeromyxobacter* Isolates from Soils

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ABSTRACT Biological nitrogen fixation is an essential reaction in a major pathway for supplying nitrogen to terrestrial environments. Previous culture-independent analyses based on soil DNA/RNA/protein sequencing could globally detect the nitrogenase genes/proteins of *Anaeromyxobacter* (in the class *Deltaproteobacteria*), commonly distributed in soil environments and predominant in paddy soils; this suggests the importance of *Anaeromyxobacter* in nitrogen fixation in soil environments. However, direct experimental evidence is lacking; there has been no research on the genetic background and ability of *Anaeromyxobacter* to fix nitrogen. Therefore, we verified the diazotrophy of *Anaeromyxobacter* based on both genomic and culture-dependent analyses using *Anaeromyxobacter* sp. strains PSR-1 and Red267 isolated from soils. Based on the comparison of *nif* gene clusters, strains PSR-1 and Red267 as well as strains Fw109-5, K, and diazotrophic *Geobacter* and *Pelobacter* in the class *Deltaproteobacteria* contain the minimum set of genes for nitrogenase (*nifBHDKEN*). These results imply that *Anaeromyxobacter* species have the ability to fix nitrogen. In fact, *Anaeromyxobacter* PSR-1 and Red267 exhibited N₂-dependent growth and acetylene reduction activity (ARA) *in vitro*. Transcriptional activity of the *nif* gene was also detected when both strains were cultured with N₂ gas as a sole nitrogen source, indicating that *Anaeromyxobacter* can fix and assimilate N₂ gas by nitrogenase. In addition, PSR-1- or Red267-inoculated soil showed ARA activity and the growth of the inoculated strains on the basis of RNA-based analysis, demonstrating that *Anaeromyxobacter* can fix nitrogen in the paddy soil environment. Our study provides novel insights into the pivotal environmental function, i.e., nitrogen fixation, of *Anaeromyxobacter*, which is a common soil bacterium.

IMPORTANCE *Anaeromyxobacter* is globally distributed in soil environments, especially predominant in paddy soils. Current studies based on environmental DNA/RNA analyses frequently detect gene fragments encoding nitrogenase of *Anaeromyxobacter* from various soil environments. Although the importance of *Anaeromyxobacter* as a diazotroph in nature has been suggested by culture-independent studies, there has been no solid evidence and validation from genomic and culture-based analyses that *Anaeromyxobacter* fixes nitrogen. This study demonstrates that *Anaeromyxobacter* harboring nitrogenase genes exhibits diazotrophic ability; moreover, N₂-dependent growth was demonstrated *in vitro* and in the soil environment. Our findings indicate that nitrogen fixation is important for *Anaeromyxobacter* to survive under nitrogen-deficient environments and provide a novel insight into the environmental function of *Anaeromyxobacter*, which is a common bacterium in soils.

KEYWORDS *Anaeromyxobacter*, nitrogen fixation, nitrogenase, paddy soil

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Biological nitrogen fixation is an essential step in the nitrogen cycle. Apart from anthropogenic processes such as fertilization (1), biological nitrogen fixation is the major pathway to supply nitrogen to terrestrial environments. Ammonium converted from ambient nitrogen gas via nitrogen fixation is pivotal for the nitrogen nutritional needs of plants; thus, terrestrial ecosystems and sustainable agriculture are dependent on nitrogen fixation. Since nitrogen-fixing microorganisms, i.e., diazotrophs, were first discovered in the root nodules of leguminous plants in the late 19th century (2), nitrogen fixation activity has been well demonstrated in various plant-associated and free-living prokaryotes, particularly in bacteria belonging to the *Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Cyanobacteria*, *Firmicutes*, and *Actinobacteria* (3–6). Moreover, recent genomic analyses show that a broader taxonomic range of bacteria harbors nitrogen fixation genes, suggesting that the phylogenetic diversity of diazotrophs is higher than previously believed (7).

We recently evaluated microbial communities involved in reductive nitrogen transformations, including denitrification ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$), dissimilatory nitrate reduction to ammonium (DNRA; $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$), and nitrogen fixation ($\text{N}_2 \rightarrow \text{NH}_4^+$) in paddy soils using shotgun sequencing of soil DNA and RNA, metagenomics, and metatranscriptomics (8). Nitrogen fixation genes (*nif*) and *nif* gene transcripts of *Geobacter* and *Anaeromyxobacter* (within the class *Deltaproteobacteria*) were detected much more frequently than those of other bacterial genera, suggesting that *Geobacter* and *Anaeromyxobacter* are major diazotrophs in paddy soils (8). *nif* gene fragments and Nif protein of *Anaeromyxobacter* have also been detected by culture-independent methods from other soil environments such as upland agricultural, crop rhizospheric, grassland, and polar soils (9–15), suggesting that diazotrophic *Anaeromyxobacter* occurs commonly in soil environments and has a pivotal role in nitrogen fixation. Although the reports have been limited, N_2 gas-dependent growth and nitrogen fixation activity have been demonstrated by culture-dependent methods in two species of *Geobacter*, *G. metallireducens* and *G. sulfurreducens* (16, 17). However, to date, there has been no research on the genetic and enzymatic backgrounds of *Anaeromyxobacter* nitrogenase; and the nitrogen fixation activity of *Anaeromyxobacter* has not been reported.

Anaeromyxobacter, a member of the *Myxococcales* order in the class *Deltaproteobacteria*, is a facultative anaerobe with the abilities to reduce various metals such as iron and uranium, dechlorinate aromatic compounds, and perform NO_3^- reductions to NO_2^- and ammonium via DNRA (18–20). Recent culture-dependent and -independent studies have found that *Anaeromyxobacter* is a key player driving the reduction of N_2O to N_2 in terrestrial environments (8, 21, 22). Despite possessing no nitrite reductase for catalyzing NO_2^- reduction to NO, which is an essential reaction for denitrification, *Anaeromyxobacter* can completely denitrify by itself through the chemical transformation of NO_2^- to NO via Fe^{2+} oxidation (23). Thus, *Anaeromyxobacter* carries out multiple activities to transform nitrogen, especially the reductive processes of the nitrogen cycle. Notably, DNA fragments of *Anaeromyxobacter* except for *nif* genes have also been commonly found in various soil environments such as agriculture, forest, tundra, desert, grassland, permafrost, polar, and sedimentary soils (21, 22, 24–30). Especially in paddy soils, river sediments, and some upland agricultural soils, *Anaeromyxobacter* has been reported as one of the most predominant members of the bacterial community by some culture-independent studies (8, 31–34). These observational studies highlight that *Anaeromyxobacter* is not rare but ubiquitous in soil environments and strongly suggest that *Anaeromyxobacter* would be a common and important driver of nitrogen fixation as well as other nitrogen reductive reactions in soil environments. However, as described above, the nitrogen-fixing activity of *Anaeromyxobacter* has not yet been verified at all despite its ubiquitousness in soils. Therefore, the demonstration of diazotrophic activity in *Anaeromyxobacter* is valuable for the comprehensive understanding of microbial drivers of the nitrogen cycle in soil environments. In this study, we verified the diazotrophy of *Anaeromyxobacter* based on both genomic and culture-dependent analyses using *Anaeromyxobacter* strains isolated from soils.

TABLE 1 Genomically sequenced strains of *Anaeromyxobacter*

Strain	Isolation origin	Stage of genomic sequencing (accession no.)	Size (Mb)	GC content (%)	No. of genes	<i>nif</i> genes	Reference(s)
<i>Anaeromyxobacter</i> sp. PSR-1	Arsenic-contaminated soil	Draft (BAZG000000000)	4.86	74.4	4,670	<i>nifBHDKENXA</i>	35, 36
<i>Anaeromyxobacter</i> sp. Red267	Paddy soil	Draft (BJTG000000000)	4.83	74.5	4,301	<i>nifBHDKENXAW</i>	This study
<i>A. dehalogenans</i> 2CP-1 ^T	Stream sediment	Complete (CP001359)	5.03	74.7	4,560	None	19
<i>A. dehalogenans</i> 2CP-C	Rain forest soil	Complete (CP000251)	5.01	74.9	4,522	None	19, 66
<i>Anaeromyxobacter</i> sp. Fw109-5	Nitrate- and uranium-contaminated sediment	Complete (CP000769)	5.28	73.5	4,662	<i>nifBHDKENXA</i>	37
<i>Anaeromyxobacter</i> sp. K	Terrestrial	Complete (CP001131)	5.06	74.8	4,528	<i>nifBHDKENXA</i>	— ^a

^a—, deposited in INSD database, but no valid publication.

RESULTS

Isolation and identification of *Anaeromyxobacter* derived from paddy soil. Prior to this study, genomic information from five cultured strains of *Anaeromyxobacter*, namely, PSR-1, 2CP-1^T, 2CP-C, Fw109-5, and K, isolated from sediment, contaminated soil, and forest soil has been available to the public (Table 1). Genomic information about *Anaeromyxobacter* derived from paddy soils has been lacking, although this organism is one of the predominant diazotrophs in paddy soils as suggested by our previous metatranscriptomic analysis (8). To obtain such information, we first isolated an *Anaeromyxobacter* strain from paddy soil. After 2 weeks of incubating a paddy soil slurry, the reduction of Fe³⁺ to Fe²⁺ changed the soil's color from reddish brown to gray; Fe²⁺ concentration shifted from 0.75 ± 0.03 to 498 ± 39 mg/100 g soil slurry before and after incubation, respectively. The gray soil was spread on R2Af medium and anaerobically incubated, resulting in the isolation of one anaerobic strain, designated Red267. The strain formed a red colony on R2Af, similar to that of *Anaeromyxobacter dehalogenans* 2CP-1^T (19). Comparison of a partial sequence (1,454 bp) of the 16S rRNA gene of strain Red267 to database sequences showed the closest similarity (96.56%) to *A. dehalogenans* 2CP-1^T. Based on these results, the entire genome of Red267 was sequenced and analyzed. The full 16S rRNA gene sequence of Red267 is also most similar (96.72%) to that of *A. dehalogenans* 2CP-1^T. Phylogenetic analysis based on the full-length 16S rRNA gene demonstrates that Red267 belongs to the *Anaeromyxobacter* cluster (Fig. 1A). The Red267 genome has an extremely high G+C content (74.5%), similar to those of other *Anaeromyxobacter* strains (73.5% to 74.9%) (Table 1). These results indicate that Red267 belongs to the genus *Anaeromyxobacter*.

Genetic and enzymatic backgrounds of nitrogenase in *Anaeromyxobacter*. Based on our analysis of the published and validated genomic data of six cultured *Anaeromyxobacter* strains, we found that strains PSR-1 and Red267, isolated in our previous and present studies (35, 36), respectively, as well as strains Fw109-5 and K harbor *nifBHDKENXA* (37) (Table 1). No *nif* gene was found in the complete genomes of strains 2CP-1^T and 2CP-C (Table 1). Based on an extensive survey of genomic information of the orders in the class *Deltaproteobacteria*, we found that among the order *Myxococcales*, only some representatives of *Anaeromyxobacter* harbor *nif* genes (Fig. 1A). In contrast, most strains in the order *Desulfuromonadales*, including the genera *Geobacter*, *Pelobacter*, and *Desulfuromonas*, have at least *nifHDKENB* (Fig. 1A). The *NifHDK* (comprising the essential and catalytic compartments of nitrogenase [7]) sequences in *Anaeromyxobacter* showed the closest phylogenetic relationship to those in *Geobacter*, within the order *Desulfuromonadales* (Fig. 1B).

The structure of the *nif* gene cluster of strains PSR-1 and Red267 also resembles that of other *Anaeromyxobacter* sequences; six consecutive genes (*nifHDKENX-fdxN*) are conserved in all *Anaeromyxobacter* *nif* gene clusters (Fig. 2A). The *nif* gene clusters of strains PSR-1 and Red267 are composed of genes involved in regulation (*draT* and *draG* in Red267, *fdxN*, and *nifA* [38–40]), construction of nitrogenase (*nifHDK*), and biosynthesis of FeMo cofactor (*nifBENX*). In addition, *nifBHDKENX-fdxN* shares similar features with corresponding sequences in diazotrophic *Geobacter* and *Pelobacter* (16, 17, 41).

FIG 1 Phylogenetic relationship of *Anaeromyxobacter* strains PSR-1 and Red267. Neighbor-joining phylogenies were inferred from the aligned nucleotide and amino acid sequences of 16S rRNA genes (A) and NifHDK (B), respectively, retrieved from complete genomic information (except for strains PSR-1 and Red267) listed in Table S2 in the supplemental material. ●, strains harboring a *nif* cluster comprising at least the minimum set of genes for nitrogen fixation (*nifHDKENB*). Only bootstrap values higher than 70% are shown at each branching node. Strains PSR-1 and Red267 examined in this study are highlighted by black boxes.

Moreover, the *nif* gene cluster of *Anaeromyxobacter* is more compact than that of other *Desulfuromonadales*; that is, all genes are located closer to each other in *Anaeromyxobacter* than in other genera (Fig. 2A). All *Anaeromyxobacter* strains consistently contain *nifA* and the gene encoding the GCN-related *N*-acetyltransferase, while these genes are absent in closely related species.

The catalytic site of nitrogenase is made from the NifHDK complex and comprises the gamma subunit of the iron protein and beta and alpha subunits of the molybdenum-iron protein (42). Alignment of NifH sequences showed that the P-loop/MgATP binding motif and cysteinyl ligands (Cys97 and Cys132) of the [4Fe-4S] cluster are conserved in the NifH of *Anaeromyxobacter* sp. strains PSR-1 and Red267 as well as those of other diazotrophs (Fig. 2B). Furthermore, crucial residues of the FeMo cofactor binding site in NifD (Cys275 and His442) and the P cluster in NifK (Cys70, Cys95, and Cys153) are also conserved in NifDK of all strains of *Anaeromyxobacter* (Fig. 2B).

Fixation and assimilation of N_2 . Although strains PSR-1 and Red267 are cultivable in minimum medium containing NH_4^+ with acetate and fumarate as an electron donor and acceptor, respectively (MMaf; see Materials and Methods), both strains also grew under culture conditions with N_2 as a sole nitrogen source (Fig. 3). The abundance levels of strains PSR-1 and Red267 harboring the *nif* gene cluster increased from $(5.8 \pm 0.5) \times 10^8$ and $(7.5 \pm 0.6) \times 10^8$ cells/vial to $(1.3 \pm 0.1) \times 10^{10}$ and $(1.0 \pm 0.1) \times 10^{10}$ cells/vial (mean $\pm$ standard deviation [SD]; $n = 3$), respectively, after

FIG 2 Genetic and enzymatic backgrounds of nitrogenase in *Anaeromyxobacter*. (A) Comparison of the *nif* gene cluster in *Anaeromyxobacter* and its relatives. Regions a to g (not shown) contain 13, 1, 4, 5, 3, 7, and 26 genes, respectively. Homologous genes are shown by the same colors. (B) Alignments around crucial residues of NifHDK in *Anaeromyxobacter* and other diazotrophs. Amino acid residues shaded by red indicate the MgATP binding motif (P-loop) and the [4Fe-4S] cluster ligating cysteines in NifH, FeMo cofactor ligands in NifD, and P-cluster ligands in NifK (42). Numbering of each crucial residue is derived from *Azotobacter vinelandii*, a typical diazotroph. *, amino acids conserved in sequences of all strains.

5 days of culturing. Exponential growth of strains PSR-1 and Red267 lasted for approximately 2 days before the cell abundance peaked. Neither strain grew with Ar in replacement of N_2 (data not shown). In contrast, *A. dehalogenans* 2CP-1^T harboring no *nif* gene did not grow in culture with N_2 as the sole nitrogen source; the abundance of *A. dehalogenans* 2CP-1^T remained constant at 10^8 cells/vial even after 5 days of culture (Fig. 3).

Nitrogen fixation activity and transcription of the *nif* gene. Nitrogen fixation activity was estimated based on C_2H_2 reduction activity (ARA) as described in Materials and Methods. Cultured strains PSR-1 and Red267 showed ARA $[(6.2 \pm 1.0) \times 10^{-10}]$ and $[1.5 \pm 0.1] \times 10^{-10}$ nmol C_2H_4 /h/cell, respectively) in the absence of NH_4^+ , indicating that both *nif*-containing *Anaeromyxobacter* strains fix nitrogen (Fig. 4A). Neither strain produced ethylene in the absence of acetylene (data not shown). No ARA was detected in strain 2CP-1^T, a strain harboring no *nif* gene. There have been reports that NH_4^+ inhibits the nitrogenase activity of well-known diazotrophs such as *Azospirillum* spp., *Azotobacter* spp., and *Rhizobium* spp. (43, 44) as well as diazotrophic *Geobacter* (16). The ARA levels (i.e., nitrogenase activity) of strains PSR-1 and Red267 also decreased as the NH_4^+ concentration in the medium increased, and ARA was completely inhibited by

FIG 3 Time-dependent growth of *Anaeromyxobacter* strains cultured with N_2 as a sole nitrogen source. *Anaeromyxobacter* strains with *nif* (PSR-1 [●] and Red267 [▲]) and without *nif* (2CP-1^T [■]) were grown in minimal medium without NH_4Cl containing acetate and fumarate as an electron donor and acceptor, respectively, in serum bottles with an N_2 gas atmosphere. Means $\pm$ standard deviations ($n = 3$) are shown.

more than 0.7 and 0.5 mM NH_4^+ , respectively (Fig. 4A). Transcriptional activities of *nifD* in strains PSR-1 and Red267 were significantly higher in the absence of NH_4^+ than with 1 mM NH_4^+ (Fig. 4B). Strains PSR-1 and Red267 could utilize Fe(III)-nitrilotriacetic acid (NTA) and ferrihydrite (γ -FeOOH) but not goethite (α -FeOOH) and hematite (α -Fe₂O₃) as an electron acceptor (see Table S1 in the supplemental material). The ARAs of both strains were $(1.7 \pm 0.1) \times 10^{-7}$ and $(1.2 \pm 0.3) \times 10^{-7}$ nmol C_2H_4 /h/cell when cultured with 2 mM acetate and 10 mM Fe(III)-NTA as electron donor and acceptor, respectively. Strains PSR-1 and Red267 also reduced acetylene $[(7.8 \pm 0.3) \times 10^{-9}$ and $[1.6 \pm 0.3] \times 10^{-8}$ nmol C_2H_4 /h/cell) when 10 mM ferrihydrite (γ -FeOOH) was added as an electron acceptor in replacement of 10 mM Fe(III)-NTA.

In situ nitrogen fixation activity of *Anaeromyxobacter*. After demonstrating the nitrogen-fixing abilities of strains PSR-1 and Red267 *in vitro*, we investigated whether *Anaeromyxobacter* fixes nitrogen in paddy soil environments by using a soil microcosm. We inoculated *Anaeromyxobacter* in sterilized paddy soil and incubated the soil. The number of copies of the 16S rRNA gene and ARA in the soil slurries inoculated with strain PSR-1 or strain Red267 were below the detection limit ($<10^3$ copies/g soil and

FIG 4 Inhibition effect of NH_4^+ on the nitrogen fixation by *Anaeromyxobacter*. (A) Nitrogen fixation activity of strains PSR-1 (●) and Red267 (▲) cultured with 0 to 1 mM NH_4^+ was estimated based on ARA. The actual maximum ARAs of strains PSR-1 and Red267 were $(6.2 \pm 1.0) \times 10^{-10}$ and $(1.5 \pm 0.1) \times 10^{-10}$ nmol C_2H_4 /h/cell at 0 mM NH_4^+ , respectively. (B) Transcriptional activity of *nifD* in *Anaeromyxobacter* strains cultured with or without NH_4^+ , normalized by *rpoB* transcription level. *, $P < 0.05$ by Mann-Whitney U test. Means $\pm$ standard deviations ($n = 3$) are shown.

TABLE 2 Nitrogen fixation activity of *Anaeromyxobacter* spp. in the paddy soil microcosm

Inoculum	No. of 16S rRNAs (copies/g soil)	C ₂ H ₄ (nmol/g soil/h)
Before incubation ^a	ND ^b	0
Distilled water (control)	ND	0
<i>Anaeromyxobacter</i> sp. PSR-1	$(2.38 \pm 2.14) \times 10^5$	0.24 ± 0.33
<i>Anaeromyxobacter</i> sp. Red267	$(1.39 \pm 0.56) \times 10^5$	0.58 ± 0.38

^aJust after the inoculation of each *Anaeromyxobacter* strain.^bND, not detected (under the detection limit [$<10^3$ copies/g soil]).

<0.1 nmol/vial, respectively) before the incubation, i.e., immediately after the inoculation of each strain (Table 2). After 3 days of incubation at 30°C, the number of copies of the 16S rRNA genes of strain PSR-1 and strain Red267 in the soil slurries increased to $(2.4 \pm 2.1) \times 10^5$ and $(1.4 \pm 0.6) \times 10^5$ copies/g soil, respectively, indicating that both strains grew in the soil slurries (Table 2). The reduction of acetylene was detected in slurries inoculated with strains PSR-1 and Red267 (0.24 ± 0.33 and 0.58 ± 0.38 nmol C₂H₄/g-soil/h, respectively), but not in the uninoculated slurries (Table 2).

DISCUSSION

In the present study, the diazotrophy of *Anaeromyxobacter*, which had been suggested by our previous metatranscriptomics study in paddy soils, was proven by genomic and culture-dependent analyses.

The analysis of the complete genomes of four published *Anaeromyxobacter* strains (2CP-1^T, 2CP-C, K, and Fw109-5) revealed that some *Anaeromyxobacter* strains (K and Fw109-5) possess *nif* genes. In addition to strain PSR-1, derived from arsenic-contaminated soil, we successfully isolated a new *Anaeromyxobacter* strain (Red267) from paddy soil, and the subsequent genomic analysis revealed that both strains harbor a *nif* gene cluster (Table 1). Remarkably, the 16S rRNA gene of strain PSR-1 is 99.68% and 99.74% similar to those of strains 2CP-1^T and 2CP-C, respectively, suggesting that strain PSR-1 must belong to the same species as 2CP-1^T and 2CP-C (i.e., *A. dehalogenans*). However, strain PSR-1 harbors a *nif* gene cluster, which is lacking in strains 2CP-1^T and 2CP-C. Therefore, we cannot conclude whether diazotrophs comprise a major group within the genus *Anaeromyxobacter* because of the limited genomic information and low number of isolates currently available (19, 35, 45). Moreover, even if the 16S rRNA gene of *Anaeromyxobacter* has been detected in environmental samples during polymorphism analysis of PCR amplicons of the 16S rRNA gene, it is too early to conclude about the role that *Anaeromyxobacter* plays as one of the diazotrophs in environments. Such a conclusion would require us first to expand the genomic information by isolating more *Anaeromyxobacter* strains from soil environments.

The structure of the *nif* gene cluster of *Anaeromyxobacter* is highly conserved in the genus, and it is comprised of *nifHDKENBXA*, *fdxN*, and *draT* (Fig. 2A). Although such a structure is extremely compact compared to those of the *nif* gene clusters in facultative anaerobic diazotrophs such as *Azotobacter vinelandii*, *Pseudomonas stutzeri*, *Cyanothece* sp., etc. (46), *Anaeromyxobacter* contains the minimum gene set for nitrogenase, *nifHDKENBX*, which is also conserved in diazotrophic *Geobacter* and *Pelobacter* (Fig. 2A) (16, 17, 41). In addition, the essential residues of the active site of nitrogenase have also been conserved (Fig. 2B). Therefore, it is plausible that translational products from the *nif* gene cluster in *Anaeromyxobacter* constitute active nitrogenase.

A functional *nif* gene cluster in *Anaeromyxobacter* strains PSR-1 and Red267 is suggested by N₂-dependent growth and ARA *in vitro*. These traits are not observed with strain 2CP-1^T, a strain with no *nif* genes (Fig. 3). Also, the nitrogen-fixing activity of *Anaeromyxobacter* was suppressed by NH₄⁺ (Fig. 4A), a response observed in known diazotrophs, including *Geobacter* (16, 43). Transcriptional activity of the *nif* gene was also detected when strains PSR-1 and Red267 were cultured with N₂ gas as a sole nitrogen source (Fig. 4B). These results clearly demonstrated that *Anaeromyxobacter* can fix and assimilate N₂ gas by its nitrogenase. Previous culture-dependent studies validated the physiological features of *Anaeromyxobacter*, including metal reduction,

dechlorination, N_2O reduction, and denitrification (19–21, 23), all of which are also known to play pivotal roles in soil ecosystems. This study revealed that the nitrogen fixation is also one of the physiological features of *Anaeromyxobacter*, which is a common soil bacterium, enhancing the importance of *Anaeromyxobacter* in soil ecosystems.

Ferric iron compounds are the representative electron acceptors for *Anaeromyxobacter* and a typical component in soil environments (47, 48). Strains PSR-1 and Red267 were not able to use all ferric iron compounds tested in this study as an electron acceptor; they used Fe(III)-NTA and ferrihydrite ($\gamma\text{-FeOOH}$) but not goethite ($\alpha\text{-FeOOH}$) and hematite ($\alpha\text{-Fe}_2\text{O}_3$) (see Table S1 in the supplemental material), suggesting that the nitrogen-fixing activity of *Anaeromyxobacter* in soils would be greatly affected by the forms of ferric iron compounds in each soil. In addition to ferric iron compounds, soils contain other substances that are candidates for electron acceptors, e.g., nitrate, Mn^{4+} , humic acid, etc. Therefore, it is necessary to further investigate the details regarding *Anaeromyxobacter*'s profile for availability of other electron acceptors than irons and carefully determine whether *Anaeromyxobacter* exhibits nitrogen fixation in each soil environment. To accomplish these tasks, we need to isolate more strains of *Anaeromyxobacter* and clarify their physiological characteristics, as mentioned above.

Anaeromyxobacter is known to predominate in paddy soils worldwide (8, 31, 32), and its *nif* gene transcripts and nitrogenase have been detected in previous metatranscriptomics and metaproteomics surveys of paddy soil (8, 9). The question of whether *Anaeromyxobacter* actually fixes nitrogen to survive in paddy soils was answered by a soil microcosm experiment conducted using only native paddy soil without any additives. The microcosm was inoculated with *Anaeromyxobacter* PSR-1 and Red267, and both exhibited nitrogen fixation activity (ARA). The growth of each of the inoculated strains was indicated by RNA-based analysis (Table 2), demonstrating that *Anaeromyxobacter* can fix and assimilate nitrogen in the paddy soil environment. In our previous field survey, the NH_4^+ concentration temporarily increased after fertilization; however, no or less NH_4^+ was detected both before fertilization and 4 weeks after fertilization (0 to 0.2 mmol/kg soil [49]). Acetate is present in some paddy fields at a concentration of 7.8 ± 1.9 mM throughout the year (50), a concentration at which PSR-1 and Red267 exhibited ARA *in vitro* (see Fig. S1). Moreover, they were able to use Fe(III)-NTA and ferrihydrite as an electron acceptor and showed ARA when cultured with acetate as the electron donor (Table S1). Thus, it is quite conceivable that *Anaeromyxobacter* fixes nitrogen in paddy fields where the NH_4^+ concentration is less but acetate and ferric irons adequately exist (49).

We have demonstrated that some strains of *Anaeromyxobacter* have a *nif* gene cluster in their genomes and show diazotrophic traits in the soil environment. Considering the global distribution of *Anaeromyxobacter* as described above, *Anaeromyxobacter* is likely to be involved in nitrogen fixation in various soil environments. However, it remains unclear how much they actually contribute to nitrogen fixation in nature compared to that of other diazotrophs. A PCR-based method may underestimate the amount of *Anaeromyxobacter nif* gene in soil environments because of its extremely high G+C content, as also discussed in a previous study (8). In fact, the functional gene of *Anaeromyxobacter* failed to amplify during PCR despite the improved PCR primers (51). Future studies aiming to estimate the contribution of *Anaeromyxobacter* to nitrogen fixation will find it worthwhile to summarize and reanalyze the diversity of diazotrophic microbiota using soil metagenomic and metatranscriptomic data from around the world. Moreover, stable isotope probing will be a useful addition (52, 53) to expanding culture-independent investigations.

MATERIALS AND METHODS

Soil sample. Soil was collected from paddy fields at the Niigata Agricultural Research Institute (Nagaoka, Niigata, Japan: 37°26'N, 138°52'E). This soil was also used in previous RNA-based studies (8, 48). Detailed characteristics of the soil, total carbon (12.4 g/kg), total nitrogen (0.85 g/kg), etc., were described previously (54). The soil was air dried at 25°C for >1 week and then finely crushed by a pestle and mortar. After debris and plant residues were removed by a 2-mm sieve, the prepared soil was used

for the isolation of *Anaeromyxobacter*. For *in situ* assessment of nitrogen fixation activity of *Anaeromyxobacter* as described below, the soil was used without air drying. The Fe^{2+} concentration of soil samples was measured as described previously (49).

Anaeromyxobacter strains. In addition to *Anaeromyxobacter* sp. strain PSR-1 previously isolated from arsenic-contaminated soil in Japan (35), *Anaeromyxobacter* sp. strain Red267 was newly isolated from paddy soil by the following method. The air-dried paddy soil was suspended in distilled water (soil/water, 1/1.5 [wt/vol]), and 5 ml of the resulting soil suspension was transferred to a 15-ml serum bottle. The serum bottle was autoclaved and supplemented with 0.1 g raw paddy soil and 5 μl of vitamin solution (liter^{-1} : 0.020 mg D-biotin, 0.045 mg folic acid, 0.100 mg pyridoxine-HCl, 0.050 mg thiamine-HCl, 0.050 mg nicotinic acid, 0.050 mg DL-calcium pantothenate, 0.001 mg vitamin B_{12} , 0.050 mg *p*-aminobenzoic acid, 0.050 mg DL- α -lipoic acid). Bottles were sealed with a butyl rubber stopper with an aluminum crimp. The gaseous phase of the serum bottle was exchanged with N_2/CO_2 (80:20 [vol/vol]), and then the bottle was kept at 30°C without shaking. After 2 weeks of incubation, soil slurry was spread on R2A broth “DAIGO” (Nihon Pharmaceutical, Tokyo, Japan) medium supplemented with 1.5% agar and 5 mM disodium fumarate (R2Af) (19) and incubated at 30°C for 10 days under anaerobic conditions using a sealed jar equipped with an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). After single-colony isolation, the partial sequence of the 16S rRNA gene of strain Red267 was determined and identified as described previously (55). Strains PCR-1 and Red267 were deposited to the Marine Culture Collection of China, as strains MCCC 1K04378 and MCCC 1K04211, respectively.

Both strains, PSR-1 and Red267, were subcultured using minimal medium (MM; liter^{-1} : 0.535 g NH_4Cl , 0.136 g KH_2PO_4 , 0.204 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.147 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.52 g NaHCO_3 , 1 ml trace mineral element solution [liter^{-1} : 64 mg nitrilotriacetic acid, 6.750 mg $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 0.500 mg $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$, 0.120 mg $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.500 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.500 mg ZnCl_2 , 0.125 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.050 mg H_3BO_3 , 0.120 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 5.000 mg NaCl, 0.600 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.130 mg $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$], and 1 ml vitamin solution [35]) supplemented with 2 mM acetate and 40 mM fumarate (MMaf) and anaerobically incubated in serum bottles under an N_2/CO_2 (80:20 [vol/vol]) atmosphere at 30°C without shaking. A type strain of *Anaeromyxobacter*, *A. dehalogenans* 2CP-1^T (DSM21875^T), was obtained from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen and used as a negative control in this study because it lacks *nif* gene in its genome (Table 1). Ability of *Anaeromyxobacter* to use ferric iron as an electron acceptor was tested in MM with 2 mM acetate as an electron donor based on visual change of the medium (red turbid to clear with/without black precipitation) derived from the reduction of ferric iron to ferrous iron, as described previously (56, 57).

Sequence analyses. For the draft genomic sequencing, genomic DNA was extracted from strain Red267 cells grown on R2Af using the DNeasy blood and tissue kit (Qiagen, Germany), according to the manufacturer's instructions for Gram-negative bacteria. Resulting DNA was sequenced using the Illumina HiSeq 2500 system at Beijing Novogene Bioinformatics Technology Co. Ltd. (Beijing, China), and sequence reads were assembled using SOAPdenovo version 2.04 (58). The draft genome of strain Red267 was analyzed using the DDBJ Fast Annotation and Submission Tool ver. 1.1.0 with default settings (59), with annotations added manually.

The nucleotide and amino acid sequences of the 16S rRNA gene and *nif* gene of *Anaeromyxobacter* spp. and related strains were obtained from complete and fine genomic information, which are available in the KEGG database (<https://www.genome.jp/kegg/>) (see Table S2 in the supplemental material). The draft genomic information of *Anaeromyxobacter* sp. PSR-1 (36) was retrieved from the DDBJ/ENA/GenBank database. Then, multiple alignments of nucleotide and amino acid sequences of the 16S rRNA gene and NifHDK, respectively, were generated using the ClustalW algorithm (60). Phylogenies were inferred based on the neighbor-joining and maximum likelihood methods with 1,000 replications of bootstrap tests using MEGA ver. 7.0.26 (61).

In vitro assessment of nitrogen fixation ability. After preculturing in MMaf medium for 3 days as described above, cells of *Anaeromyxobacter* spp. were rinsed with and transferred into 20 ml of nitrogen-free MMaf (i.e., MMaf without NH_4Cl) in serum bottles in which the gaseous phase had been exchanged with N_2/CO_2 gas (80:20 [vol/vol]). As a control experiment, the same system was set up using pure Ar instead of N_2/CO_2 gas. During incubation at 30°C without shaking, cell growth was monitored by direct counting of the cells using a Multisizer 3 (Beckman Coulter, CA, USA). Cells cultured with or without NH_4^+ under the same conditions were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA was extracted and purified from the cultured cells using ISOGEN with Spin Column (Nippon Gene, Tokyo, Japan) and RNA Clean and Concentrator (Zymo Research, CA, USA), respectively, according to the manufacturers' instructions and used for determining the transcriptional activity of *nif* gene by quantitative PCR (qPCR) analysis as described below.

To complement the monitoring of cell growth, nitrogen fixation activity was also measured by the traditional ARA method based on C_2H_2 reduction into C_2H_4 by nitrogenase (62). ARA experimental conditions were the same as described above, except the atmosphere in bottles was $\text{Ar}/\text{C}_2\text{H}_2$ gas (90:10 [vol/vol]). The negative control was set up using pure Ar gas in replacement of $\text{Ar}/\text{C}_2\text{H}_2$ gas. C_2H_4 production in the gaseous phase was measured by gas chromatography equipped with a fused-silica column (RT-U PLOT; Restek) as described previously (63). The inhibitory effect of NH_4^+ on nitrogen fixation activity was checked in cultures maintained at 30°C for 3 days in nitrogen-free MM added with 0 to 1 mM NH_4Cl . The effects of different concentrations of acetate on nitrogen fixation activity were evaluated based on ARA as described above on cultures incubated at 30°C for 3 days in MM supplemented with 40 mM fumarate as an electron acceptor.

In situ assessment of nitrogen fixation activity. To construct a soil microcosm, soil suspension (soil/water, 1/2.3 [wt/vol]) was placed in a serum bottle, sealed with a butyl rubber stopper and an aluminum

TABLE 3 PCR primer sets used in this study

Target gene	Direction	Sequence (5'→3')	Reference
<i>rpoB</i>	Forward	GTGGTCGATGTCGTCGAYGT	This study
	Reverse	CAACTGTTCTTCAACGCCG	This study
<i>nifD</i>	Forward	CGWCATCTTCTTCGCGAC	This study
	Reverse	TGCAGWTSACSTCCGACTTC	This study
16S rRNA	Forward (515F)	GTGCCAGCMGCCGCGGTAA	55
	Reverse (806R)	GGACTACHVGGGTWTCTAAT	55

crimp, and autoclaved. Then, the gaseous phase was exchanged with N₂/CO₂ (80:20 [vol/vol]). *Anaeromyxobacter* spp. were precultured in nitrogen-free MM supplemented with 2 mM acetate and 40 mM fumarate under N₂/CO₂ (80:20 [vol/vol]) at 30°C for 4 days. Cultured cells were collected from 20 ml of culture broth by centrifugation at 6,000 rpm, rinsed, and then resuspended in 500 μ l sterilized O₂-free water. This suspension was spiked into the soil microcosm prepared as described above. A negative control consisted of the same setup spiked with only 500 μ l of sterilized O₂-free water. After incubating at 30°C for 3 days, 2 g of soil slurry was collected, immediately frozen in liquid nitrogen, and stored at –80°C until RNA extraction. The remaining 8 g of soil slurry was subjected to ARA assays as described above. RNA was extracted from soil slurries before and after incubation as described previously (64) and used for qPCR analysis for the 16S rRNA gene of the spiked *Anaeromyxobacter* as described below.

qPCR analysis. cDNA was synthesized from prepared RNA using DNA-free RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. qPCR was performed to specifically amplify *nifD*, *rpoB*, and 16S rRNA genes of *Anaeromyxobacter* using the respective specific primer sets (Table 3) and the StepOnePlus system (Life Technologies, CA, USA) with TB Green Premix Ex Taq GC (TaKaRa Bio, Shiga, Japan). The thermal cycling for all genes was performed as follows: preheat at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 52°C for 30 s, and 72°C for 30 s.

Statistical analysis. Mann-Whitney U tests were performed using R software ver. 3.0.1 (65) to analyze data on the transcriptional activity of *nifD*.

Data availability. The partial sequence of the 16S rRNA gene and the draft genome of *Anaeromyxobacter* sp. strain Red267 have been deposited in the DDBJ/GenBank/EBI databases under accession numbers [LC488723](#) and [BJTG01000001](#) to [BJTG01000017](#), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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