



# Elevated Atmospheric CO<sub>2</sub> and Nitrogen Fertilization Affect the Abundance and Community Structure of Rice Root-Associated Nitrogen-Fixing Bacteria

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Elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) results in plant growth and N limitation, yet how root-associated nitrogen-fixing bacterial communities respond to increasing atmospheric CO<sub>2</sub> and nitrogen fertilization (eN) during the growth stages of rice is unclear. Using the *nifH* gene as a molecular marker, we studied the combined effect of eCO<sub>2</sub> and eN on the diazotrophic community and abundance at two growth stages in rice (tillering, TI and heading, HI). Quantitative polymerase chain reaction (qPCR) showed that eN had no obvious effect on *nifH* abundance in rice roots under either ambient CO<sub>2</sub> (aCO<sub>2</sub>) or eCO<sub>2</sub> treatment at the TI stage; in contrast, at the HI, *nifH* copy numbers were increased under eCO<sub>2</sub> and decreased under aCO<sub>2</sub>. For rhizosphere soils, eN significantly reduced the abundance of *nifH* under both aCO<sub>2</sub> and eCO<sub>2</sub> treatment at the HI stage. Elevated CO<sub>2</sub> significantly increased the *nifH* abundance in rice roots and rhizosphere soils with nitrogen fertilization, but had no obvious effect without N addition at the HI stage. There was a significant interaction [CO<sub>2</sub> × N fertilization] effect on *nifH* abundance in root zone at the HI stage. In addition, the *nifH* copy numbers in rice roots were significantly higher at the HI stage than at the TI stage. Sequencing analysis indicated that the root-associated diazotrophic community structure tended to cluster according to the nitrogen fertilization treatment and that *Rhizobiales* were the dominant diazotrophs in all root samples at the HI stage. Additionally, nitrogen fertilization significantly increased the relative abundance of *Methylosinus* (*Methylocystaceae*) under eCO<sub>2</sub> treatment, but significantly decreased the relative abundance of *Rhizobium* (*Rhizobiaceae*) under aCO<sub>2</sub> treatment. Overall, the combined effect of eN and eCO<sub>2</sub> stimulates root-associated diazotrophic methane-oxidizing bacteria while inhibits heterotrophic diazotrophs.

**Keywords:** elevated atmospheric CO<sub>2</sub>, nitrogen fertilization, rice paddy, plant-associated nitrogen-fixing bacteria, growth stages

## INTRODUCTION

Rice is the most important staple food for half the world's population, and nearly 90% of the rice fields in the world are located in Asia (Food and Agriculture Organization of the United Nations, 2002). Rice fields act as an important carbon and nitrogen cycling interface between the atmosphere and land (Ishii et al., 2011; Tokida et al., 2011). Carbon dioxide (CO<sub>2</sub>) fixation by photosynthesis provides energy for rice plants. Atmospheric CO<sub>2</sub> concentrations have increased from approximately 270–400 ppm by human activity since preindustrial times (IPCC, 2013). Elevated concentrations of atmospheric CO<sub>2</sub> under a free-air CO<sub>2</sub> enrichment (FACE) system could increase the biomass, yield and grain quality of rice plants (Usui et al., 2014; Zhu et al., 2015). Elevated CO<sub>2</sub> (eCO<sub>2</sub>) can variously influence underground nutrient cycling, which is mainly mediated by microbial communities (García-Palacios et al., 2015). For example, CH<sub>4</sub> emissions were significantly increased by eCO<sub>2</sub> and/or temperatures in rice paddies (Tokida et al., 2011; Xie et al., 2012) due to the effects of methanogens and methanotrophs (Das and Adhya, 2012). CH<sub>4</sub> generated in soil diffuses into rice roots, transported to the shoot via aerenchyma, and finally released from micropores in the leaf sheaths (Nouchi et al., 1990). The root zone (rice root and rhizosphere soil) was previously shown to be the main area CH<sub>4</sub> oxidation in rice paddies (Bosse and Frenzel, 1997). A previous study reported that CH<sub>4</sub> oxidation and N<sub>2</sub> fixation were simultaneously activated in the root zone (root and rhizosphere soil) of wild-type rice in the low nitrogen field (Bao et al., 2014b). Further, <sup>15</sup>N-N<sub>2</sub> feeding experiments and metaproteomics analysis demonstrated that *Methylosinus* was a core diazotroph in rice roots and likely contributed to both CH<sub>4</sub> oxidation and N<sub>2</sub> fixation processes (Bao et al., 2014a; Shinoda et al., 2019). *Bradyrhizobium* and/or *Methylosinus* were frequently detected in rice roots using metagenomic analysis (Eller and Frenzel, 2001; Ikeda et al., 2014), and they were affected by the rice growth stage under eCO<sub>2</sub>, based on 16S rRNA gene analysis (Okubo et al., 2014). eCO<sub>2</sub> quantitatively changed the release of labile sugars, organic acids, and amino acids from plant roots (Bhattacharyya et al., 2013), possibly influencing the activity of rhizospheric and root-associated microbes.

In rice production, nitrogen is often a limiting factor. Supplementation with N fertilizer has increased rice grain yields over recent decades (Yang et al., 2006). However, the heavy use of N fertilizers causes various environmental impacts, such as nitrous oxide emissions, soil and water body N deposition, reactive N leaching, eutrophication and methane emissions (Ju et al., 2009). Thus, the impacts of nitrogen input on methane emissions and methane cycling bacteria, including methanotrophs and methanogens, have attracted worldwide attention (Shrestha et al., 2010; Banger et al., 2012; Bao et al., 2014a,b). N fertilization decreased the relative abundance of root-associated bacteria community, especially type II methanotrophs and *Bradyrhizobium* (Ikeda et al., 2014). Generally, N fertilizer inputs inhibit N<sub>2</sub> fixation and influence the N<sub>2</sub>-fixing bacterial community (Kumar et al., 2018), which

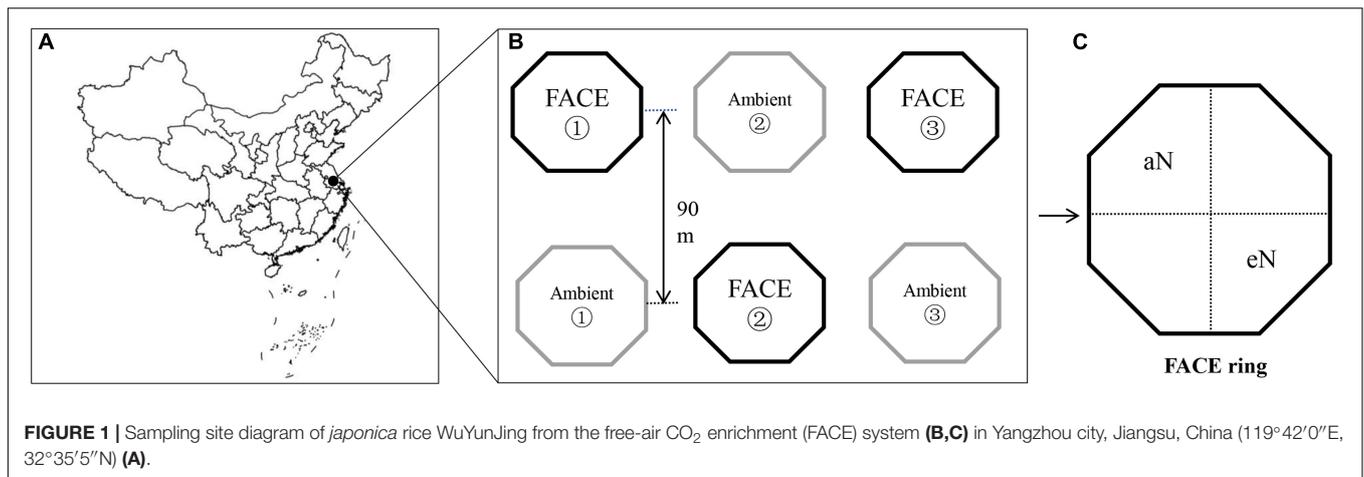
can convert atmospheric N<sub>2</sub> gas to ammonium and make it available to plants as a major nutrient (Yoneyama et al., 2019). eCO<sub>2</sub> can increase the N demand for plants, which is beneficial for N<sub>2</sub> fixation (Luo et al., 2004). A previous study showed that soil diazotrophic abundance significantly increased in two difference rice cultivars to satisfy the increased N demand under eCO<sub>2</sub> in paddy fields (Yu et al., 2018). This may be due to an increase in the organic acid content of the root system released into the soil by eCO<sub>2</sub> (Bhattacharyya et al., 2013), thus providing carbon source and energy to N<sub>2</sub>-fixing bacteria. However, plant roots associate with diverse soil-derived microbes including N<sub>2</sub>-fixing bacteria, which influences plant nutrient uptake (Berendsen et al., 2012; Shinoda et al., 2019). In addition, root-associated microorganism significantly changed between different growth stages in rice paddy field (Chaparro et al., 2014; Okubo et al., 2014). Whether the abundance and community composition of N<sub>2</sub>-fixing bacteria in the rhizosphere soils and roots of rice are affected by eCO<sub>2</sub> and nitrogen fertilization at two different stages during the rice-growing season is unclear, as is which nitrogen fixers respond to these factors.

Using qPCR and MiSeq sequencing techniques, we studied the abundance and community composition of N<sub>2</sub>-fixing bacteria in the roots of rice plants (*japonica* rice, WuYunJing) from a free-air CO<sub>2</sub> enrichment (FACE) system in Yangzhou city, Jiangsu, China. In this study, we (i) examined the combined effects of eCO<sub>2</sub> and N fertilization on the community composition of N<sub>2</sub>-fixing bacteria in the rice roots and/or rhizosphere at the tillering and heading stages and (ii) identified the N<sub>2</sub>-fixing bacteria responsive to the treatments. The results will be valuable for understanding the strategies by which root-associated N<sub>2</sub>-fixing bacterial communities form to adapt to prospective climatic changes.

## MATERIALS AND METHODS

### Study Site and Experimental Design

The experimental FACE platform was located in Zongcun Village (119°42'0"E, 32°35'5"N), Yangzhou City, Jiangsu Province (Figure 1; Yu et al., 2018). The long-term experimental platform with a rice-wheat rotation crop system started in 2004. From 2010, the rice-wheat rotation system was changed to a rice-fallow system (Yu et al., 2018). The region has a north subtropical monsoon climate with a mean annual temperature of 16°C and a mean annual precipitation of 900–1,000 mm. The experiment had a split-plot design, with CO<sub>2</sub> as the main factor and N fertilization and rice cultivar as the split-plot factors. More details about the FACE system are provided in by Zhu et al. (2016). In brief, three rectangular paddy fields were selected for their uniformity in growth and yield for use in the experiment. Within each field, a FACE plot was paired with an ambient control, and the plot centers were 90 m apart to avoid cross-contamination by CO<sub>2</sub> (Heim et al., 2009; Figure 1). The CO<sub>2</sub> levels of each FACE ring were maintained with at ambient CO<sub>2</sub> (aCO<sub>2</sub>, 400 ± 10 μmol mol<sup>-1</sup>) or eCO<sub>2</sub> (590 ± 40 μmol mol<sup>-1</sup>)



**FIGURE 1** | Sampling site diagram of *japonica* rice WuYunJing from the free-air CO<sub>2</sub> enrichment (FACE) system (B,C) in Yangzhou city, Jiangsu, China (119°42'0"E, 32°35'5"N) (A).

concentrations corresponding to the future expectations of the Intergovernmental Panel on Climate Change (IPCC, 2013). Each FACE plot was encircled with an octagonal ring (12.5 m in diameter) equipped with emission tubes surrounding the crops that injected pure CO<sub>2</sub> at approximately 50 cm above the crop canopy throughout the day. The target CO<sub>2</sub> concentration within the FACE rings was controlled by a real-time CO<sub>2</sub> monitoring system (Figure 1). The aCO<sub>2</sub> rings did not receive any supplemental CO<sub>2</sub>.

There were two N fertilization levels, no N fertilization (aN) and elevated N fertilization (eN) (Figure 1). The eN plots were fertilized with N, P, and K (in the form of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O). In the aN plots, only P and K were applied (in the form of P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O, respectively). P (90 kg ha<sup>-1</sup>) and K (90 kg ha<sup>-1</sup>) were applied as basal fertilizers to both aN and eN rice field plots before transplanting. The total nitrogen fertilization rate was 225 kg ha<sup>-1</sup>, with 40, 30, and 30% of the total amount applied to only eN rice field plots before transplanting, tillering, and heading, respectively (Zhu et al., 2016). The soil in the present study is a Shajiang-Aquic Cambisol soil and has a sandy loam texture (13.7% clay, 28.5% silt, and 57.8% sand). At the beginning of the study, the initial soil properties were analyzed and recorded as follows: soil organic carbon (17.9–18.4 g kg<sup>-1</sup> in aCO<sub>2</sub> plots and 18.5–18.6 g kg<sup>-1</sup> in eCO<sub>2</sub> plots), total N (1.6–1.7 g kg<sup>-1</sup> in aCO<sub>2</sub> plots and 2.01–2.07 g kg<sup>-1</sup> in eCO<sub>2</sub> plots), total P (0.6 g kg<sup>-1</sup> in aCO<sub>2</sub> plot and 0.7 g kg<sup>-1</sup> in eCO<sub>2</sub> plot), total K (13.8 g kg<sup>-1</sup> in aCO<sub>2</sub> plot and 14.3 g kg<sup>-1</sup> in eCO<sub>2</sub> plot), available P (8.6 mg kg<sup>-1</sup> in aCO<sub>2</sub> plot and 11.7 mg kg<sup>-1</sup> in eCO<sub>2</sub> plot), and available K (66.0 mg kg<sup>-1</sup> in aCO<sub>2</sub> plot and 75.0 mg kg<sup>-1</sup> in eCO<sub>2</sub> plot) (see Supplementary Table 1 in the Supplementary Material).

## Rice Plant and Soil Sampling

Rice plants (*japonica*, WuYunJing) with soil blocks were sampled from the aCO<sub>2</sub> rings and eCO<sub>2</sub> rings. Three plants per experimental plot were harvested on 21 July 2014 (30 d after transplanting, [DAT]), which corresponded to the TI stage (the vegetative stage of rice), and on 25 August 2014 (65 d DAT), which corresponded to the HI stage (the reproductive stage of rice). The samples were immediately transported on ice to the

laboratory. The rice roots and rhizosphere soils were sampled as described in previous studies within 1 day after field sampling (Kimura, 2004; Bao et al., 2014a). Briefly, after the rice plants with soil blocks were sampled, the soil blocks, including rice plants, were divided vertically into two equal parts for root collection. Some of the exposed roots were carefully picked from the plants using sterilized forceps and placed into a 50 mL centrifuge tube containing sterile water. The roots were also washed by centrifugation before and after sonication (90 watts for 5–10 min) and the three pellets were combined to form the rhizosphere soil samples. After the rhizosphere soil was removed, the rice root samples were transferred to new centrifuge tubes (50 mL) containing sterile water and centrifuged at 4°C for 10 min at 8,000 × g; the pellet from this centrifugation was considered the root sample. At two different growth stages, a total of 24 root and 24 rhizosphere soil samples were obtained and stored at -80°C, and molecular analysis was conducted within 6 months of sampling.

## DNA Extraction and Quantification of *nifH* Genes

Genomic DNA was extracted from root and rhizosphere soil samples using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States) according to the manufacturer's protocol. For root samples, the frozen tissues were ground into powder using a mortar and pestle under liquid nitrogen before DNA extraction. The extracted DNA was immediately stored at -20°C after measurement of the DNA concentration.

The abundance of nitrogen fixers was quantified by qPCR targeting of the *nifH* gene from 24 root and 24 rhizosphere soil samples, respectively. The reactions were performed on a CFX Connect Optical Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, United States) in volumes of 20 μL and containing 1 μL extracted DNA (approximately 50 ng), 10 μL 2 × SYBR Premix Ex Taq (Takara Biotech, Dalian, China), and 500 nM primers PolF (Poly et al., 2001) and AQER (Wartiainen et al., 2008). The parameters were those previously described in the literature (Yu et al., 2018). Briefly, the amplifications were performed with an initial denaturation step at 95°C for

30 s, followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s and extension at 72°C for 45 s. The specificity of the amplification products was confirmed by melting curve analysis, and the expected sizes (340 bp) of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide.

Standard curves were obtained using 10-fold serial dilutions of the linear *Escherichia coli*-derived vector plasmid pGEM-T Easy Vector (Promega, Madison, WI) containing a cloned *nifH* gene derived from *Bradyrhizobium diazoefficiens* USDA 110 (NZ\_CP011360). The concentration of the plasmid DNA was measured by a NanoPhotometer P-Class P-330C (IMPLEN, Munich, Germany) and used for the calculation of standard copy numbers. The  $R^2$ -values and amplification efficiencies were as follows:  $R^2 = 0.996$ – $1.000$ , efficiency =  $94.0$ – $97.0\%$ . Melting curve analysis was used to confirm the specific amplification of target genes and always showed a single peak.

## MiSeq Sequencing of *nifH* Gene Amplicons

The *nifH* gene was amplified using PCR primers PolF (TGC GAY CCS AAR GCB GAC TC) and AQER (GAC GAT GTA GAT YTC CTG) (Poly et al., 2001; Warttinen et al., 2008), which was used to detect  $N_2$ -fixing bacteria including *Alphaproteobacteria*, *Betaproteobacteria*, *Gamaproteobacteria*, and *Firmicutes* in paddy field (Yu et al., 2018). A unique 12-bp barcode was added to each sample at the 5'-end of the reverse primer. The 20 mL reaction mixtures included 4.0  $\mu$ L 5  $\times$  FastPfu Buffer (plus  $Mg^{2+}$ ), 2.0  $\mu$ L 2.5 mM each dNTP, 0.4  $\mu$ L 5 U/ $\mu$ L TransStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China), 0.8  $\mu$ L 5  $\mu$ M each primer, 0.2  $\mu$ L 20 mg  $mL^{-1}$  bovine serum albumin (BSA; Amesco, Tampa, United States) and 10 ng template DNA. The amplification conditions were as follows: initial denaturation at 95°C for 15 min, followed by 36 cycles of 94°C for 1 min, 55°C for 1 min, elongation at 72°C for 1 min, and a final extension step of 72°C for 10 min. The amplification products were approximately 340 bp. Each sample was amplified with three technical replicates, purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor™-ST (Promega, United States) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2  $\times$  300) on an Illumina MiSeq platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). In order to determine what kind of  $N_2$ -fixing bacteria was present in a large number at the heading stage, we focused on the heading stage and examined the diversity and composition of the diazotrophic community at this stage. 12 root samples at the heading stage were used for sequencing. The raw sequences (fastq files) of the 12 rice root samples at the heading stage were deposited in the Sequence Read Archive (SRA) of NCBI under accession numbers SRR10321589–SRR10321600.

## Processing of the Sequencing Data

Raw sequences were analyzed by QIIME software (Caporaso et al., 2010). All sequence reads were trimmed and assigned to

each sample based on their barcodes, and then quality filtering was performed. Sequences less than 150 bp in length were removed. Chimera removal was carried out using the software program Mothur (Schloss et al., 2009). The nucleotide sequences of *nifH* were further converted to amino acid sequences using the FunGene Pipeline of the Ribosomal Database Project (Wang et al., 2013). The sequences encoding proteins that did not match the *nifH* protein sequence or that contained termination codons were discarded. The remaining sequences were aligned against the *nifH* gene database (Gaby and Buckley, 2014). The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 90, 94, and 99% similarity, respectively, with UCLUST (Edgar, 2010; Berthrong et al., 2014), and they were used to estimate alpha diversity indices (e.g., coverage, observed species, Shannon and Simpson indices) by Mothur (Schloss et al., 2009). Taxonomic identity of the OTU clustering at 94% similarity was carried out by mapping representative OTU sequences to reference *nifH* sequences at the BLAST algorithm-based search site within GenBank. Finally, translated amino acid sequences of each representative of top 30 OTUs were aligned by using the CLUSTAL W program (Thompson et al., 1994), and neighbor-joining phylogenetic tree was constructed by using MEGA version 5 (Tamura et al., 2011). Principal component analysis (PCA) were built using “vegan” package in R (version 3.1.2).

## Statistical Analysis

The experiment design was a split-plot factor arranged within a randomized complete block design with three replications (three rectangular paddy fields). The variance analyses were performed using linear mixed-effects model to test the effect of elevated  $CO_2$ , N fertilization, and their interactions on *nifH* copy numbers, alpha diversity index (OTU numbers, Shannon and 1/Simpson), and the relative abundance of the main OTUs at the same growth stage. In these models,  $CO_2$  was treated as the fixed-effect whole-plot factor, N fertilization as the sub-plot factor, and block as the random effect factor. These analyses were performed in R (version 3.1.2). Multiple comparison was further used to determine which groups were significantly different using Duncan test in SPSS software, version 19.0 (IBM, Armonk, NY, United States). The comparison of *nifH* copy numbers at the tillering and heading stages were carried out by paired sample *t*-test procedure in SPSS software. Beta diversity was analyzed using permutational multivariate analysis of variance (PERMANOVA) to assess group differences in R (version 3.1.2). The significance level was  $p \leq 0.05$ , while  $0.05 < p \leq 0.1$  were considered marginally significant. The data are expressed as the means  $\pm$  standard deviation.

## RESULTS

### Effect of Elevated $CO_2$ and N Fertilization on *nifH* Gene Abundance in the Root Zone at Different Growth Stages of Rice

To estimate the population sizes of  $N_2$ -fixing bacteria in the root zone at different rice growth stages, we performed qPCR

assays of roots and rhizosphere soils sampled from rice grown in the paddy field at the TI stage and at the HI stage. Firstly, we examined the effects of different growth stages on the abundance of N<sub>2</sub>-fixing bacteria. The copy numbers ( $\times 10^8$  g · dry weight) of *nifH* genes in rice roots at the HI stage were significantly higher than those at the TI stage ( $t = 10.462$ ,  $p = 0.009$ ;  $t = 18.862$ ,  $p = 0.003$ ;  $t = 15.225$ ,  $p = 0.004$ ;  $t = 31.343$ ,  $p = 0.001$ ) (Supplementary Table 2), with the average increased by ~4-fold (Figure 2A). Similarly, for rhizosphere soil samples, compared to the TI stage, the copy numbers of *nifH* genes were greater at the HI stage ( $t = 8.401$ ,  $p = 0.014$ ;  $t = 4.381$ ,  $p = 0.048$ ;  $t = 11.329$ ,  $p = 0.008$ ) (Supplementary Table 2), but only decreased under the combination of aCO<sub>2</sub> and eN ( $t = -7.684$ ,  $p = 0.017$ ) (Supplementary Table 2 and Figure 2B).

Secondly, we examined the effects of elevated CO<sub>2</sub>, N fertilization, and their interaction on *nifH* copy numbers. Significant effects of elevated CO<sub>2</sub> [ $F_{(1,2)} = 37.4$ ,  $p = 0.026$ ], N fertilization [ $F_{(1,4)} = 7.896$ ,  $p = 0.048$ ], but no significant interaction [CO<sub>2</sub>  $\times$  N fertilization] [ $F_{(1,4)} = 0.877$ ,  $p = 0.402$ ] on *nifH* abundance in rice roots at the TI stage were observed (Figure 2A). Meanwhile, significant effects of elevated CO<sub>2</sub> [ $F_{(1,2)} = 47.52$ ,  $p = 0.020$ ], N fertilization [ $F_{(1,4)} = 18.80$ ,  $p = 0.012$ ] and interaction [CO<sub>2</sub>  $\times$  N fertilization] [ $F_{(1,4)} = 57.95$ ,  $p = 0.002$ ] on *nifH* abundance in rice roots at the HI stage were observed (Figure 2A). Further analyses showed that, at the HI stage, N fertilization reduced the abundance of *nifH* gene copies 2.5-fold ( $p < 0.001$ ) under aCO<sub>2</sub> but increased the abundance of *nifH* gene copies 1.2-fold ( $p = 0.031$ ) under eCO<sub>2</sub> (Figure 2A). Elevated CO<sub>2</sub> doubled the copy numbers of *nifH* in rice root with N fertilization ( $p < 0.001$ ), but had no significant effect ( $p = 0.607$ ) without N addition at the HI stage (Figure 2A). Similarly, for rhizosphere soil samples, a significant interaction [CO<sub>2</sub>  $\times$  N fertilization] on *nifH* abundance either at the TI stage [ $F_{(1,4)} = 15.21$ ,  $p = 0.018$ ] or the HI stage [ $F_{(1,4)} = 188.7$ ,  $p < 0.001$ ] was observed, but significant effects of elevated CO<sub>2</sub> [ $F_{(1,2)} = 59.14$ ,  $p = 0.017$ ], N fertilization [ $F_{(1,4)} = 450.9$ ,  $p < 0.001$ ] on *nifH* abundance were observed only at the HI stage (Figure 2B). And N fertilization significantly reduced the abundance of the *nifH* gene under either aCO<sub>2</sub> ( $p < 0.001$ ) or eCO<sub>2</sub> ( $p = 0.001$ ) treatment at the HI stage (Figure 2B). Elevated CO<sub>2</sub> also significantly increased the copy numbers of *nifH* in rice rhizosphere soils with N fertilization ( $p < 0.001$ ), but had no obvious effect without N addition ( $p = 0.109$ ) at the HI stage (Figure 2B). As explained above, the combination of elevated CO<sub>2</sub> and N fertilization has a significant effect on *nifH* abundance in rice zone (root and rhizosphere soil) at the HI stage ( $p < 0.05$ ) (Figures 2A,B).

These results suggested that rice plants have the ability to enrich N<sub>2</sub>-fixing bacteria according to the growing season. Plants set the conditions for the proliferation of more N<sub>2</sub>-fixing bacteria at the HI stage than at the TI stage, and the combined effects of eCO<sub>2</sub> and eN stimulate the abundance of N<sub>2</sub>-fixing bacteria in roots at the HI stage.

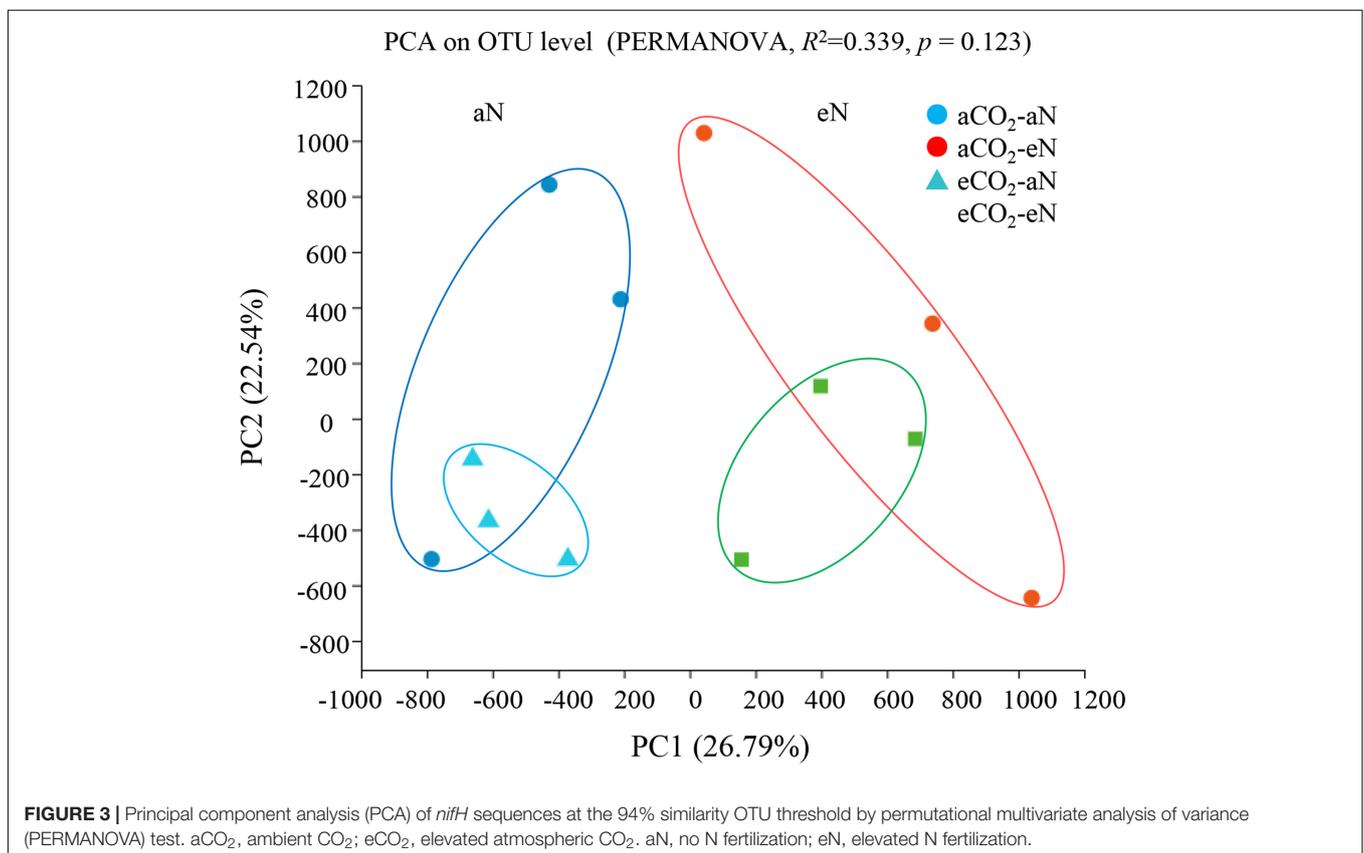
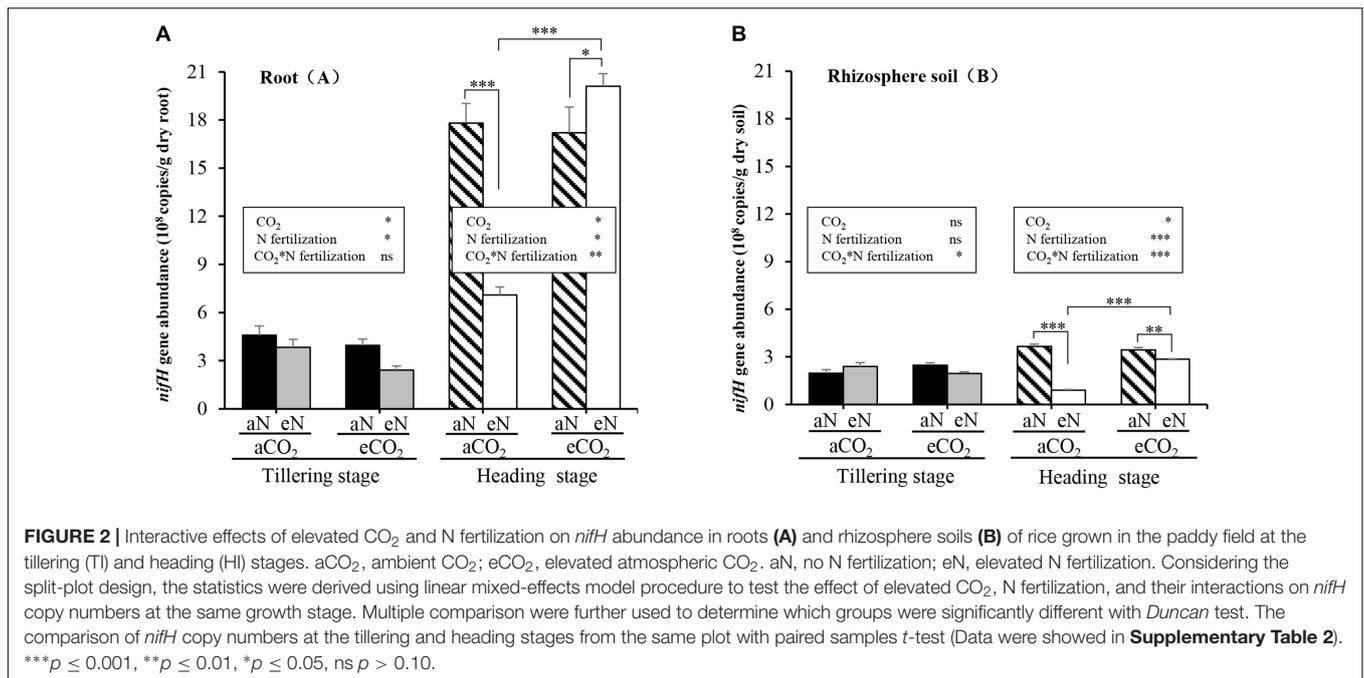
## Overview of N<sub>2</sub>-fixing Bacterial Community Structures in Rice Roots at the Heading Stage

The statistics from the high-throughput sequencing are summarized in Supplementary Table 3. *nifH* gene sequencing was performed on 12 rice roots DNA at the HI stage. After averaging each of the three parallel datasets, 47,849 high-quality reads were obtained, with 12,890, 10,998, 12,594, and 11,367 total *nifH* sequences for the aCO<sub>2</sub>-aN, aCO<sub>2</sub>-eN, eCO<sub>2</sub>-aN, and eCO<sub>2</sub>-eN plots, respectively. No significant effects of elevated CO<sub>2</sub>, N fertilization, and interaction [CO<sub>2</sub>  $\times$  N fertilization] on alpha diversity index (OTU numbers, Shannon and 1/Simpson) at the OTU cutoff values of 99, 94, and 90%, respectively, were found ( $p > 0.05$ ) (data were showed in Supplementary Table 3). At the 99% OTU level, the coverage was 97% for the four plots. The coverage (> 99%) was high for libraries from each plot when OTUs were binned at 94% similarity (Supplementary Table 3). Therefore, we constructed a phylogenetic tree and performed taxonomic classification at 94% OTU similarity in this study.

Principal component analysis (PCA) was performed using all sequence data (Figure 3) to obtain an overview of N<sub>2</sub>-fixing bacterial community shifts caused by elevated CO<sub>2</sub>, N fertilization, and their interaction. The results showed a cluster of community structures within the same N fertilization treatment, whereas no distinct separation was observed between two CO<sub>2</sub> treatments (PERMANOVA,  $p = 0.123$ ) (Figure 3).

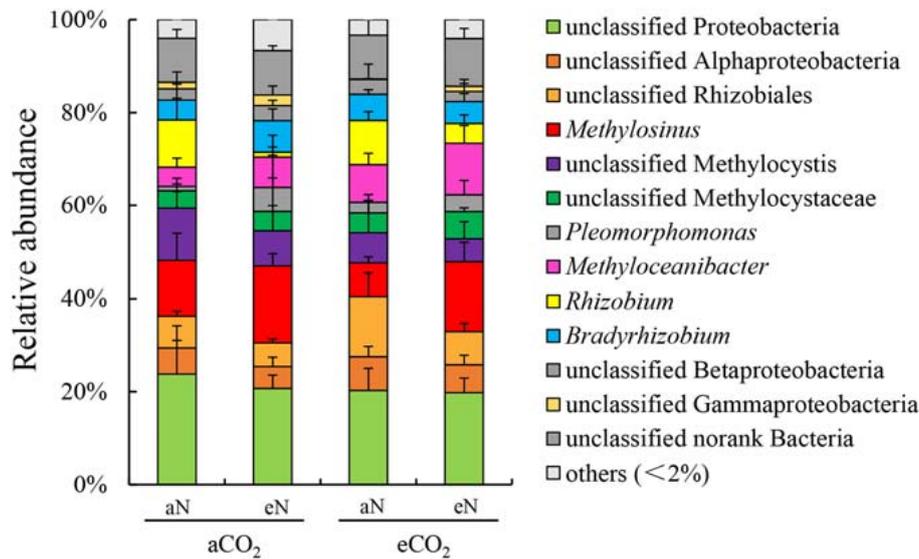
## Compositions of N<sub>2</sub>-Fixing Bacterial Communities of Rice Roots at the Heading Stage

The community compositions of root-associated N<sub>2</sub>-fixing bacteria were analyzed. Together at the order and family level, the rice root-associated N<sub>2</sub>-fixing bacterial communities were dominated by *Proteobacteria* (including unclassified *Proteobacteria*, unclassified *Alphaproteobacteria*, unclassified *Betaproteobacteria*, unclassified *Gammaproteobacteria*) (37.5–40.2%) and *Rhizobiales* (including unclassified *Rhizobiales*, *Methylocystaceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*) (45.8–49.7%) (Supplementary Figures 1A,B). Additionally, there were low abundances of unclassified bacteria (9.4–10.3%) and other families (2.9–5.0%) in all plots (Supplementary Figure 1B). N fertilization increased the relative abundance of *Methylocystaceae* (aCO<sub>2</sub>, from 16.6 to 26.0%; eCO<sub>2</sub>, from 13.8 to 24.7%) while reducing the relative abundance of *Rhizobiaceae* (aCO<sub>2</sub>, from 10.2 to 1.1%; eCO<sub>2</sub>, from 9.5 to 4.3%) under both aCO<sub>2</sub> and eCO<sub>2</sub> treatment (Supplementary Figure 1B). Combined with the community compositions at the genus level, the relative abundances of *Methylosinus* (type II methanotrophs) and *Rhizobium* (traditional N<sub>2</sub>-fixing bacteria) were changed under combined of elevated CO<sub>2</sub>, N fertilization treatment. The former showed an increasing trend, while the latter showed a decreasing trend (Figure 4).



To further explore the differences between species, we compared the total N<sub>2</sub>-fixing bacterial community at the OTU level. The top 30 OTUs were clustered into three main groups, which were affiliated with  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, and

$\gamma$ -proteobacteria (Figure 5A). The two OTUs, OTU 369 and OTU 321, were similar to *Methylosinus trichosporium* (CAD91846) (100% sequence identity) and *Rhizobium* sp. R2-708 (ALH07184) (100% sequence identity), respectively, which were affiliated



**FIGURE 4 |** Relative abundance, at the genus level, of  $N_2$ -fixing bacterial communities of rice roots at the heading stage under elevated  $CO_2$  and nitrogen fertilization treatments. a $CO_2$ , ambient  $CO_2$ ; e $CO_2$ , elevated atmospheric  $CO_2$ . aN, no N fertilization; eN, elevated N fertilization.

with  $\alpha$ -proteobacteria, Rhizobiales (Figure 5A). Linear mixed-effects model analyses showed that nitrogen fertilization had a marginally significant effect on the relative abundance of *Methylosinus trichosporium* (OTU 369) [ $F_{(1,4)} = 5.014, p = 0.089$ ], and had a significant effect *Rhizobium sp.* R2-708 (OTU 321) [ $F_{(1,4)} = 8.017, p = 0.047$ ], while no significant effects of elevated  $CO_2$  and interaction [ $CO_2 \times N$  fertilization] were observed (Figures 5B,C). In multiple comparison, N fertilization significantly increased *Methylosinus trichosporium* under e $CO_2$  treatment (the relative abundance from 7.2 to 15.0%) ( $p = 0.040$ ) (Figure 5B) and decreased *Rhizobium sp.* R2-708 (from 10.2 to 1.1%) ( $p = 0.027$ ) under a $CO_2$  treatment (Figure 5C). Under e $CO_2$  treatment, *Rhizobium sp.* R2-708 decreased with N fertilization (from 9.5 to 4.3%), but the difference was not significant ( $p = 0.156$ ) (Figure 5C).

### C1 Compound Cycle-Related Root-Associated $N_2$ -Fixing Bacteria at the Heading Stage

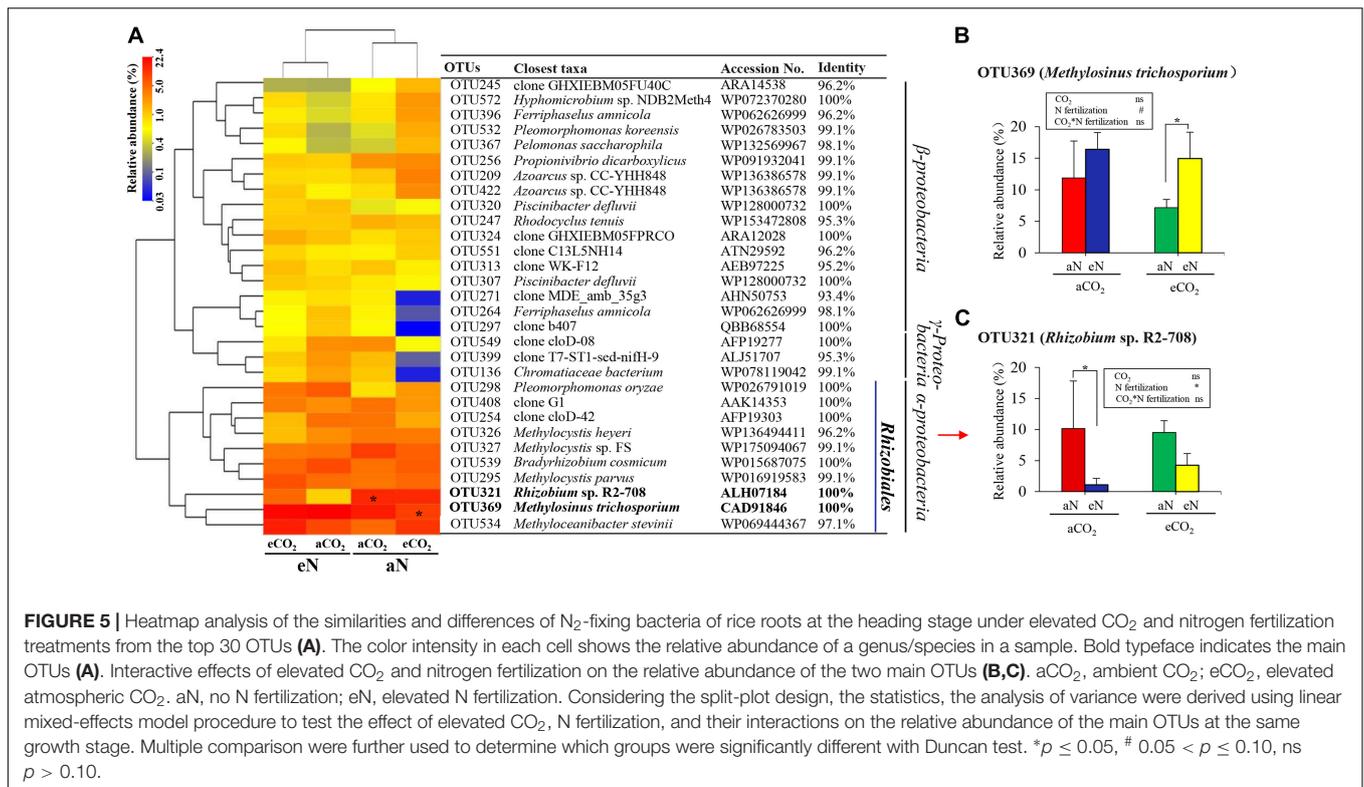
Calculating the total relative abundances at the OTU level clearly showed that  $N_2$ -fixing bacteria of the C1 compound cycle, which include mainly methane-oxidizing bacteria (*Methylosinus*, *Methylocystis*, unclassified *Methylocystis* and *Pleomorphomonas*) and methanol-oxidizing bacteria (*Methyloceanibacter*, *Rhizobium*, and *Bradyrhizobium*), were predominant in rice roots (42.64–49.03%) (Table 1).

## DISCUSSION

We demonstrated the combined effects of elevated  $CO_2$  and nitrogen fertilization on rice root-associated diazotrophic communities using qPCR and sequencing analysis. *nifH* gene

abundance significantly increased with rice growth. The combined effect of e $CO_2$  and eN stimulated the abundance of *nifH* genes in rice roots and rhizospheres at the HI stage compared at the TI stage (Figures 2A,B). Moreover, the combined effect of e $CO_2$  and eN promoted the relative abundance of root-associated methane-oxidizing bacteria while inhibiting heterotrophic diazotrophs (Figure 5). To the best of our knowledge, this study is the first to analyze the responses of the structure and gene abundance of root-associated diazotrophic communities to elevated  $CO_2$  and nitrogen fertilization treatment using the functional gene *nifH*.

The abundance of *nifH* genes is positively correlated with the rates of  $N_2$  fixation (Hsu and Buckley, 2009; Reed et al., 2011). *nifH* gene abundance in the roots and rhizospheres at the HI stage was significantly higher than that of the TI stage throughout all samples, and N fertilization decreased its abundance under a $CO_2$  treatment but increased it under e $CO_2$  treatment (Figures 2A,B). Plant growth requires more nitrogen at the HI stage than at the TI stage, and that nitrogen is acquired through the enrichment of root-associated nitrogen-fixing bacteria (Hurek and Reinhold-Hurek, 2003). Generally, nitrogen inputs inhibit  $N_2$  fixation because the nitrogenase encoded by the *nifH* gene is sensitive to nitrogen inputs (Smercina et al., 2019). The negative effect of nitrogen fertilization on  $N_2$  fixation has been well reported in legumes and other plants under ambient  $CO_2$  conditions (Reed et al., 2011; Fan et al., 2019), and these results are consistent with those of our study. In addition, a previous study carried out on the same FACE platform also showed that e $CO_2$  increased the abundance of the *nifH* gene in soil with nitrogen inputs and cultivation of the same rice cultivar (*Japonica*, WuYunJing) (Yu et al., 2018). Plant growth enhances the N demand due to the greater photosynthetic activity and plant biomass accumulation under elevated  $CO_2$  (Rogers et al., 2009). For legume plants,



**FIGURE 5 |** Heatmap analysis of the similarities and differences of  $N_2$ -fixing bacteria of rice roots at the heading stage under elevated  $CO_2$  and nitrogen fertilization treatments from the top 30 OTUs (A). The color intensity in each cell shows the relative abundance of a genus/species in a sample. Bold typeface indicates the main OTUs (A). Interactive effects of elevated  $CO_2$  and nitrogen fertilization on the relative abundance of the two main OTUs (B,C). a $CO_2$ , ambient  $CO_2$ ; e $CO_2$ , elevated atmospheric  $CO_2$ . aN, no N fertilization; eN, elevated N fertilization. Considering the split-plot design, the analysis of variance were derived using linear mixed-effects model procedure to test the effect of elevated  $CO_2$ , N fertilization, and their interactions on the relative abundance of the main OTUs at the same growth stage. Multiple comparison were further used to determine which groups were significantly different with Duncan test. \* $p \leq 0.05$ , #  $0.05 < p \leq 0.10$ , ns  $p > 0.10$ .

**TABLE 1 |** Relative abundance (%) of methylotrophs at the genus level in the total  $N_2$ -fixing bacterial communities of rice roots at the heading stage.

	Closest taxa <sup>a</sup>	Ambient $CO_2$		Elevated atmospheric $CO_2$	
		aN	eN	aN	eN
Methane oxidizing type	<i>Methylosinus trichosporium</i>	11.88 ± 5.85	16.43 ± 2.64	7.17 ± 1.29	14.94 ± 4.19
	unclassified <i>Methylocystis</i>	11.27 ± 3.56	7.58 ± 11.37	6.48 ± 6.97	4.91 ± 3.72
	<i>Methylocystis parvus</i>	3.65 ± 2.73	4.00 ± 1.16	4.21 ± 2.35	5.78 ± 0.79
	<i>Pleomorphomonas</i>	0.91 ± 0.55	5.14 ± 6.92	2.24 ± 1.67	3.59 ± 3.11
Methanol oxidizing type	<i>Methyloceanibacter stevinii</i>	4.21 ± 1.95	6.55 ± 4.70	8.12 ± 2.46	11.10 ± 3.87
	<i>Rhizobium</i>	10.15 ± 7.68	1.10 ± 1.04	9.51 ± 1.92	4.27 ± 1.87
	<i>Bradyrhizobium</i>	3.81 ± 0.13	6.25 ± 2.22	4.90 ± 1.07	4.44 ± 1.59
Total relative abundance		45.89	47.05	42.64	49.03

aN, no N fertilization; eN, elevated N fertilization.

<sup>a</sup>nifH gene sequences similarity 96–100%.

e $CO_2$  enhanced  $N_2$  fixation by increasing nodule number and biomass at later growth stages but did not reduce  $N_2$  fixation (Li et al., 2017). In addition, a  $^{15}N$  labeling experiment showed that e $CO_2$  reduced the inhibitory effect of nitrogen on the  $N_2$  fixation activity of soil cultivated with *Pisum sativum* (Butterly et al., 2016). Taken together, these results suggest that the combination of elevated  $CO_2$  and nitrogen fertilization stimulated *nifH* gene abundance in our study. Other investigations of symbiotic fixation responses to  $CO_2$  suggest that  $N_2$  fixation increases only in response to e $CO_2$  when nutrients such as P and Mo are added (van Groenigen et al., 2006), and fertilizer often contains P, Mo, and other elements. However, the mechanism of the combined effect of nitrogen fertilization and e $CO_2$  on the abundance of the diazotrophic community is complex and requires future in-depth

study. In contrast, elevated  $CO_2$  did not affect the *nifH* gene abundance of roots and rhizospheres under the treatment with no N fertilization at the HI stage in our study ( $p = 0.607$ , for roots;  $p = 0.109$ , for rhizosphere soils) (Figure 2). This result is consistent with that previously reported by van Groenigen et al. (2006), who showed using meta-analysis that elevated  $CO_2$  did not affect  $N_2$  fixation by legume plants when N fertilizer was not applied. It is likely that rice plants enrich  $N_2$ -fixing bacteria at specific growth stages, and nitrogen fertilizer mainly influences  $N_2$ -fixing bacteria not only in the root zone but also in the bulk soil of paddy rice fields under elevated  $CO_2$  treatment such as those projected for the future.

Some changes of the root-associated  $N_2$ -fixing bacterial community composition at the HI stage under the

combined effect of elevated CO<sub>2</sub> and N fertilization treatment were observed. *Methylosinus* (*Methylocystaceae*), *Methylocystis* (*Methylocystaceae*), *Rhizobium* (*Rhizobiaceae*), *Methyloceanibacter* (*Rhizobiales*), *Pleomorphomonas* (*Methylocystaceae*), *Bradyrhizobium* (*Bradyrhizobiaceae*) and other unclassified *Rhizobiales* within the order *Rhizobiales* were commonly detected as the main diazotrophs from all root samples. But eCO<sub>2</sub> increased the relative abundance of *Methylosinus trichosporium* (OTU369) under N fertilization treatment (**Figure 5B**). One Japanese FACE experiment showed that elevated CO<sub>2</sub> tended to decrease the relative abundance of *Methylocystis* and *Methylosinus* in rice roots at the ripening stage, which was observed through 16S rRNA gene sequencing; however, statistical analysis was lacking in that experiment (Okubo et al., 2014). The genera *Methylosinus* and *Methylocystis* of the family *Methylocystaceae* are methane-oxidizing bacteria that are frequently detected in rice roots and forest soils as diazotrophs (Buckley et al., 2008; Bao et al., 2014a; Shinoda et al., 2019) and in particular as the dominant group in later rice growth stages (Shrestha et al., 2010). Moreover, type II methanotrophs, including *Methylosinus*, can assimilate up to 50% of their biomass from CO<sub>2</sub> (Trotsenko and Murrell, 2008). These findings can explain the results observed in our study, i.e., the increased relative abundance of *Methylosinus trichosporium* (OTU369) under the combination of eCO<sub>2</sub> and N fertilization. Thus, the increased *nifH* gene abundance of roots under the combination of eCO<sub>2</sub> and eN might explain the increasing *nifH* copy numbers of methanotrophs (**Figure 2A**). Apart from *Methylosinus trichosporium*, N fertilization decreased the relative abundance of *Rhizobium* sp. R2-708 (OTU321) under aCO<sub>2</sub> treatment (**Figure 5C**). *Rhizobium* is a well-known N<sub>2</sub>-fixing bacterium that is frequently detected in legumes as well as in rice roots (Zahran, 1999; Bao et al., 2014a; Yoneyama et al., 2019) and other plants. Heterotrophic N<sub>2</sub>-fixing bacteria, including *Rhizobium*, are often inhibited by nitrogen inputs (Kumar et al., 2018). This result showed that compared to *Rhizobium*, root-associated diazotrophic *Methylosinus* was more stable under climate change conditions or nitrogen fertilization, even when combined. In addition, *nifH*-containing microbes are complex and high diversity, which may have a relationship with gene horizontal transfer of *nifH* (Remigi et al., 2016). Horizontal transfer of *nifH* gene is more complicated. Horizontal transfer of *nifH* gene was related to the consistency between the phylogenies of *nifH* gene (or phylogenetic tree of *nifH*, *nifD*, and *nifK*) and 16S rRNA gene (Bolhuis et al., 2010). There was no overall horizontal gene transfer of *nifH* gene across phylogeny in our study (**Figure 5A**). The main *nifH* sequences of OTUs were closely related to *Methylocystis* and *Methylosinus* of *Methylocystaceae* (type II methanotrophs), *Rhizobium* (*Rhizobiaceae*) and *Bradyrhizobiaceae*, and they closed to each other within *Rhizobiales* of *Alphaproteobacteria* (**Figure 5A**). Therefore, the result is consistent with phylogenetic analysis with 16S rRNA gene (Auman et al., 2001; Dedysh et al., 2004). But both sequences of OTU572 and OTU532 were closely related to *Hyphomicrobium* sp. NDB2 (*nifH* sequence identity, 100%) and *Pleomorphomonas koreensis* (99.1%), respectively, and were clustered into *Betaproteobacteria* (**Figure 5A**). This

result is inconsistent with a previous study showed that both *Hyphomicrobium* and *Pleomorphomonas* belong to the *Rhizobiales* of *Alphaproteobacteria* with the 16S rRNA (Hordt et al., 2020). Thus, the *nifH* of above two OTUs may be acquired through horizontal gene transfer across phylogeny.

Interestingly, most diazotrophs were related to the C1 compound cycle, which includes methane-oxidizing bacteria and methanol-utilizing bacteria, in all root samples in our study (**Table 1**). In rice paddies, methane-oxidizing bacteria are the most dominant microbes among C1 bacteria, because continuous methane production in anaerobic sediment layers or in roots provides a carbon and energy source for root-associated diazotrophic methanotrophs (Bao et al., 2014a). eCO<sub>2</sub> can significantly increase methane emissions (Tokida et al., 2011). Usually, methanotrophs are able to fix N<sub>2</sub> under N-limited conditions (Bodelier and Laanbroek, 2004; Shinoda et al., 2019). However, Zheng et al. (2019) found that N<sub>2</sub> fixation was stimulated by the substrate C/N ratio rather than by the N concentration alone. N<sub>2</sub> fixation is enhanced when substrate C is relatively plentiful and substrate N is relatively scarce (Reed et al., 2011). Thus, the availability of substrate C is critically important for N<sub>2</sub> fixation. eCO<sub>2</sub> can increase the photosynthetic C fixation capacity and biomass accumulation of rice plants (Rogers et al., 2009), which must take up large amounts of N (Shimono et al., 2009). Elevated N fertilization can help maintain a higher photosynthetic rate and ensure a higher yield under eCO<sub>2</sub> (Kimball et al., 2002). Therefore, more root-associated diazotrophic methanotrophs may be needed to provide sufficient N<sub>2</sub> fixation to meet the demand for a higher C/N ratio required for rice biomass accumulation under both eN and eCO<sub>2</sub> treatments. This may be one explanation for the N<sub>2</sub> fixation performed by root-associated diazotrophic methanotrophs at high N levels. The N<sub>2</sub>-fixing activity of diazotrophic methanotrophs under N-rich conditions needs to be studied in more detail in the future. Moreover, methanotrophic N<sub>2</sub>-fixing bacteria (*Methylosinus* and *Methylocystis*) in the roots, and other N<sub>2</sub>-fixing bacteria, such as *Bradyrhizobium*, *Rhizobium*, and *Methyloceanibacter*, which are frequently detected as methanol-oxidizing bacteria using metaproteomics or functional gene *mxoA/xoxF* sequencing analysis (Bao et al., 2014a; Macey et al., 2020), may utilize the methanol produced via the methane oxidation process to perform N<sub>2</sub> fixation. However, this relationship changes with nitrogen supplementation because the N<sub>2</sub>-fixing capacity of *Bradyrhizobium* and *Rhizobium* is inhibited by nitrogen (Kumar et al., 2018), while that of diazotrophic methanotrophs is more stable. In addition, eCO<sub>2</sub> significantly increases the total organic carbon content of rice root exudates (Bhattacharyya et al., 2013), which may influence the diversity and activity of rhizospheric and root-associated C1 bacteria. Macey et al. (2020) reported that methanol-utilizing methylootrophs were widely distributed in land plant-associated soil because they use the methanol produced as a metabolic byproduct during plant growth. Thus, C1-cycling bacteria in the root zones of both terrestrial plants and aquatic plants are important for the balance of carbon and nitrogen cycling and can increase benefits for plant growth.

## CONCLUSION

The abundance of the *nifH* gene in rice roots greatly increased at reproductive stages compared to vegetative growth stages. Elevated CO<sub>2</sub> and N fertilization significantly affect the abundance of the *nifH* gene in rice roots at two growth stages, but the interaction [CO<sub>2</sub> × N fertilization] only significantly affects *nifH* gene abundance of roots at the heading stage. Most root-associated diazotrophs (which accounted for approximately 50% of the relative abundance) were related to methanotrophs and methylotrophs at the HI stage. Of these, elevated CO<sub>2</sub> and N fertilization significantly affected the relative abundances of root-associated *Methylosinus* and *Rhizobium*. The results of this study will contribute to expanding our understanding of microorganism-related C/N dynamics in rice fields under future climate change conditions.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

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## AUTHOR CONTRIBUTIONS

ZB and CZ designed the study. JH, WC, and SZ performed the experiments. JL, MZ, YL, ZJ, RY, JZ, and ZB analyzed the data. JL and ZB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.628108/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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