Effects of Elevated Carbon Dioxide, Elevated Temperature, and Rice Growth Stage on the Community Structure of Rice Root–Associated Bacteria

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The effects of free-air carbon dioxide enrichment (FACE) and elevated soil and water temperature (warming) on the rice root–associated bacterial community were evaluated by clone library analysis of the 16S ribosomal RNA gene. Roots were sampled at the panicle initiation and ripening stages 41 and 92 days after transplanting (DAT), respectively. The relative abundances of the methanotrophs Methylosinus and Methylocystis were increased by warming and decreased by FACE at 92 DAT, which indicated that microbial methane (CH4) oxidation in rice roots may have been influenced by global warming. The relative abundance of Burkholderia kururiensis was increased by warming at 41 DAT and by FACE or warming at 92 DAT. The abundances of methanotrophs increased during rice growth, which was likely induced by an enhancement in the emission of CH4 from the paddy fields, suggesting that CH4 is one of the predominant factors affecting the structure of the microbial community in rice roots. Marked variations in the community structure were also observed during rice growth in other genera: Bradyrhizobium, Clostridium, and an unknown genus close to Epsilonproteobacteria were abundant at 92 DAT, whereas Achromobacter was abundant at 41 DAT. These results demonstrated that the community structures of rice root-associated bacteria were markedly affected by FACE, temperature, and the rice growth stage.

Key words: carbon dioxide, methane, plant-associated bacteria, rice, warming

The atmospheric carbon dioxide concentration ([CO2]) was stable at 270 ppm for at least 1,000 years prior to the start of the industrial revolution. Since that time, [CO2] has been rising and has reached nearly 400 ppm (7). The increase in [CO2] is expected to enhance the growth and yield of C3 crops, including rice (29). Previous studies reported that increases in [CO2] quantitatively and qualitatively altered the release of labile sugars, organic acids, and amino acids from plant roots (2, 6), which may influence the activity of rhizospheric and root–associated microbes, including methanogenesis (18). Tokida et al. (32, 33) reported that the emission of CH4 from paddy fields was significantly increased by [CO2] and/or temperature elevations. CH4 generated in soil is diffused into rice roots, transported to the shoot via aerenchyma, and finally released from micropores in the leaf sheaths (25). The rhizosphere and rice roots were previously shown to be the main areas CH4 oxidation in rice paddies (4) because the oxidation of CH4 was inactive in flooded soils without oxygen derived from rice roots.

Although the responses of rice plants to elevated atmospheric [CO2] and/or temperature have been studied in detail, those of plant-associated microbes remain unknown (29, 33). The responses of plant-associated microbes to global climate changes may potentially be important because they play major roles in the flow of carbon and nitrogen as the primary utilizers of plant-derived compounds in the rhizosphere.

A technique to enrich bacterial cells obtained from plant tissues has provided a gateway to access plant-associated bacterial communities (14), and this has facilitated a deeper understanding of microbial community shifts caused by environmental factors (15, 27). In the present study, we investigated the community structure of rice root–associated bacteria in environments with elevated temperature (ET) and/or [CO2] using the bacterial cell enrichment method (14). Our results have provided an insight into carbon and nitrogen cycles in rice paddies under a changing climate.

Materials and Methods

Study site, [CO2] enrichment, and soil and water warming

The free-air carbon dioxide enrichment (FACE) and soil warming experiments were conducted in a rice paddy field at Tsukubamirai, Ibaraki, Japan (35°58′27″N, 139°59′32″E, 10 m above sea level), during the 2011 growing season. The soil there is a Fluvisol, which is typical of alluvial areas. The bulk density was 0.87 Mg m−3, and total C and N were 21.4 mg g−1 and 1.97 mg g−1, respectively. The cation exchange capacity was 202 µmolc g−1 (12). The experimental site was established in 2010, and control protocols for the FACE and warming treatments were described previously (23, 32). Briefly, four rice paddy fields were used as replicates, each with areas at ambient [CO2] (AMBI) and also at enriched [CO2] (FACE) with a target concentration of 200 µmol mol−1 above AMBI. Each treatment area was a 240-m2 octagon (“ring,” hereafter). The FACE rings used emission tubes on all eight sides at a height of approximately 30 cm above the canopy, and these released pure CO2 from wind-
ward sides to maintain a stable concentration at the center of the rings (ambient + 200 µmol mol⁻¹). The AMBI and FACE rings were separated by at least 70 m (center to center), which was previously shown to be sufficient to prevent cross-contamination by CO₂ from a FACE ring (13).

Warming treatments were also conducted by a split-plot design in each ring with two levels of soil and water temperatures: normal temperature (NT) and ET with the target of 2°C above NT. Warming was achieved using heating wires placed on the soil surface between the rows, with the water temperature continuously measured by a Pt100 thermometer (Chino Co. Ltd., Tokyo, Japan). The water and plow layer (at a depth of 10 cm) temperatures were almost uniformly elevated. The ET plot was enclosed using corrugated PVC panels to prevent an exchange of the paddy water with the surrounding area.

Rice cultivation and fertilization

Rice (Oryza sativa L. cv. Koshikihari) was sown on 25 April 2011 in seedling trays with 448 cells (Minoru Pot 448, Minoru Industrial Co., Ltd., Okayama, Japan). Three pre-germinated seeds were planted in each cell of the tray. After emergence, we raised seedlings in a puddled open field with a tunnel cloche or floating mulch for the first 2 weeks. On 25 and 26 May, seedlings at the five-leaf stage were transplanted into the rings by hand, with three seedlings per hill. Hills and rows were 15 and 30 cm apart, respectively, with a resultant density of 22.2 hills m⁻². Fertilizers were applied as a basal dressing. Nitrogen was supplied at 8 g N m⁻² (2 and 6 g N m⁻² as urea and coated urea, respectively; 4 g of LP-100 and 2 g of LP-140; JCAM-Agri Co., Ltd., Tokyo, Japan). Phosphate and potassium were applied as a compound fertilizer (Sumitomo Chemical Co., Ltd., Tokyo, Japan) containing 4.4 g P m⁻² and 8.3 g K m⁻², respectively. The method for rice cultivation and fertilization was described previously (12).

CH₄ emission measurements

The emission of CH₄ was measured weekly or biweekly between 7 June and 23 August using a closed chamber method, as described previously (16). Each chamber consisting of lower (60 cm H) and upper (60 cm H) sections was placed over 4 hills of rice plants with a basal area of 30 × 60 cm. The upper section of the chamber fit over the lower one and was supported by a water-filled groove surrounding the outer top lip of the lower section, thereby providing an airtight seal between the two sections and surrounding atmosphere. Gas samples were collected from the chamber 0, 10, and 20 min after placement of the chamber. The samples were injected into pre-evacuated 19-mL glass vials and brought back to the laboratory.

Results and Discussion

Overview of bacterial community structures

The statistics of the clone libraries are summarized in Table 1.

Table 1. DDBJ accession numbers of 16S rRNA gene sequences

| Sample   | Panicle initiation stage (41 DAT) | Ripening stage (92 DAT) |
|----------|----------------------------------|-------------------------|
| AMBI-NT  | AB836980–AB837055                 | AB837585–AB837749       |
| AMBI-ET  | AB837056–AB837234                 | AB837750–AB837919       |
| FACE-NT  | AB837235–AB837406                 | AB837920–AB838080       |
| FACE-ET  | AB837407–AB837584                 | AB838081–AB838242       |

DAT, days after transplanting; AMBI, ambient CO₂; FACE, free-air CO₂ enrichment; NT, normal soil and water temperature; ET, elevated soil and water temperature.
Table 2. Statistical characteristics of 16S rRNA gene clone libraries derived from rice roots

| Rice growth stage | Panicle initiation stage (41 DAT) | Ripening stage (92 DAT) |
|-------------------|----------------------------------|-------------------------|
|                   | AMBI NT  | ET     | AMBI NT  | ET     | AMBI NT  | ET     | AMBI NT  | ET     |
| CO2               | Temperature |
| NT               | 176  | 179  | 172  | 178  | 165  | 170  | 161  | 162  |
| FACE             |        |      |      |      |      |      |      |      |
| No. of sequences  | 28   | 22   | 27   | 28   | 52   | 45   | 48   | 32   |
| No. of OTUs (≥97% identity) | 20   | 15   | 19   | 20   | 35   | 28   | 30   | 17   |
| No. of singletons | 88.6 | 91.6 | 89.0 | 88.8 | 78.8 | 83.5 | 81.4 | 89.5 |
| Library coverage (%)a | 91.3 | 57.0 | 69.8 | 75.5 | 118.1 | 82.8 | 96.3 | 66.0 |
| Diversity indexes |      |      |      |      |      |      |      |      |
| Chao1            | 2.2 | 1.5 | 2.1 | 1.9 | 3.3 | 3.0 | 3.1 | 2.7 |
| Shannon          |      |      |      |      |      |      |      |      |

DAT, days after transplanting; AMBI, ambient CO2; FACE, free-air CO2 enrichment; NT, normal soil and water temperature; ET, elevated soil and water temperature; OTU, operational taxonomic unit.

a Coverage calculated as $C_x = 1 – (n_x/N)$, where $n_x$ is the number of singletons that are encountered only once in a library and $N$ is the total number of clones.

Fig. 1. Principal coordinate analysis of the 16S rRNA gene libraries of bacterial communities in rice roots under normal and elevated [CO2] and temperature conditions. The ordination was constructed using UniFrac distances weighted by the relative abundances. Principal component 1 (PC1) and principal component 2 (PC2) are plotted on the x- and y-axes, respectively. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. Samples were collected at the panicle initiation stage (●, 41 DAT) and ripening stage (■, 92 DAT).

Table 2. In all treatments, the number of operational taxonomic units (OTUs) and the Chao1 and Shannon diversity indexes were greater at 92 DAT than at 41 DAT. These indexes were decreased in the samples at 92 DAT due to the elevation in [CO2] or soil and water temperature. No clear trend was observed in the samples at 41 DAT.

Principal coordinate analysis was performed using all sequence data (Fig. 1) in order to obtain an overview of bacterial community shifts caused by the rice growth stage and elevation in [CO2] and temperature. Samples were clearly separated along the first principal component (PC1) axis (64.86%) according to the rice growth stage, which indicated that bacterial community structures markedly changed as the host plant grew. Two tight clusters were formed according to the temperature condition in samples at 41 DAT, suggesting that community structures were more sensitive to the temperature change than to that of [CO2] at 41 DAT. Community shifts in samples at 92 DAT were more complicated. The degree of the community shift from the control (AMBI-NT) was smaller in the simultaneous treatment (FACE-ET) than in the other treatments (FACE-NT and AMBI-ET). Furthermore, FACE-NT and AMBI-ET were clustered close to each other at 92 DAT.

Phylogenetic composition

In all treatments, the abundances of Methylosinus and Methylocystis were markedly higher at 92 DAT (5.0–15.3%) than at 41 DAT (0.0–1.1%) (Table 3). At the beginning of the rice growing period, the amount of CH4 emitted was very low (Table 4). As the season progressed, it steadily increased to approximately 14–18 mg C-CH4 m$^{-2}$ h$^{-1}$ at 48 DAT (July 12), and a high emission level of CH4 was maintained until 83 DAT (August 16). These results suggest that rice roots were exposed to a large amount of CH4 between the first (41 DAT) and second sampling (92 DAT), and this may have caused the increase observed in the relative abundances of Methylosinus and Methylocystis in the rice roots at 92 DAT. The cumulative emission of CH4 during 41–90 DAT was the highest in FACE-ET (Table 4). However, the relative abundances of Methylosinus and Methylocystis in FACE-ET were intermediate among the four treatments (Table 3), which suggested that factors other than CH4 also affected the relative abundances of methanotrophs. At 92 DAT, the relative abundances of Methylosinus and Methylocystis were increased by the elevation in temperature in both AMBI and FACE plots, but were decreased by that in [CO2]. A previous FACE experiment conducted in Japan showed that nitrogen concentrations in rice plants were decreased by elevations in [CO2] (29), which was at least partially attributed to a dilution effect due to the greater production of dry matter. Many studies have suggested the stimulatory effect of nitrogen on CH4 oxidation in rice paddies (3). Such a change in the nitrogen condition may affect the activities of rice root–associated methanotrophs, leading to decreases in the relative abundance by elevations in [CO2]. Tokida et al. (32) previously reported that the emission of CH4 from paddy fields was significantly increased by elevations in [CO2], and this effect was considered to be mainly derived from an increase...
in rhizodeposition. However, our results suggest that one reason for the increase in CH₄ emission with elevations in [CO₂] may have been a decline in the abundance of methanotrophs associated with rice roots. An enhancement in CH₄ oxidation activity in rice roots is vital for breaking the positive feedback loop of CH₄ emission that will occur with increases in atmospheric [CO₂] in the future. However, we did not observe a clear increase in the emission of CH₄ by elevations in [CO₂] under the NT condition (Table 4). Therefore, the effects of [CO₂] and temperature elevation on the oxidation activity of CH₄ in paddy fields need to be studied in more detail.

Members of the genus Bradyrhizobium are important nitrogen-fixing bacteria in rice roots (5). In all treatments, the relative abundance of Bradyrhizobium, 16S rRNA reads assigned to Bradyrhizobium were extracted from clone libraries and a phylogenetic tree was constructed with the other members of bradirhizobia (Fig. 2A). Rice root–associated bradyrhizobia were clustered into two groups that were phylogenetically close to Bradyrhizobium sp. ORS278 (bradyrhizobial cluster I) (8, 26) and Bradyrhizobium jicamae (bradyrhizobial cluster II). Bradyrhizobial cluster...
I was only observed at 92 DAT (Fig. 2B), whereas bradyrhizobial cluster II was observed at both 41 and 92 DAT (Fig. 2C). In a previous study, *Bradyrhizobium* sp. ORS278 was reported to colonize the surface and intercellular space of rice roots and also fix nitrogen (5). Our clone library analysis suggested that members of bradyrhizobial cluster I may be representative nitrogen-fixing bacteria in the rice root at the ripening stage. The relative abundance of bradyrhizobial cluster I (Fig. 2B) was strongly correlated with those of *Methylosinus* and *Methylocystis* (Pearson’s correlation coef-
ficient, r = 0.83, P = 0.011; r = 0.92, P = 0.001, respectively). One explanation for this correlation was the possible metabolic coupling by which one-carbon compounds oxidized by methanotrophs such as methanol may be partially consumed by these Bradyrhizobium species. Bradyrhizobium sp. ORS278 (bradyrhizobial cluster I) has a gene for methanol oxidation (BRAD05483–BRAD05487) on the genome, and was able to oxidize methanol (Seki et al. unpublished result).

Burkholderia was the most dominant genus in all samples, except AMBI-NT at 92 DAT, (Table 3). Members of this genus differ in terms of their effects on rice plants by exhibiting pathogenic (e.g., Burkholderia glumae) (11) or symbiotic (e.g., Burkholderia kururiensis) interactions (21). A phylogenetic tree was constructed using 16S rRNA reads assigned to rice root–associated Burkholderia in the clone libraries (Fig. 3A). Most of the sequences clustered into one group that was phylogenetically close to B. kururiensis (Figs. 3A, B). This species was reported to colonize rice roots and significantly enhance rice growth by fixing nitrogen and producing the phytohormone auxin (21). Previous rice FACE experiments showed that the nitrogen concentration of rice was decreased by elevations in [CO₂] (29), which suggested that nitrogen availability is a limiting factor in an elevated (18.5%) (Table 3). These results suggested that the effects of [CO₂] enrichment (FACE), elevated soil & water temperature (warming), and rice growth stages markedly affected the microbial communities of rice root-associated bacteria including Methylosinus sp., Methylocystis sp., Burkholderia kururiensis, Bradyrhizobium sp., Clostridium sp., and an unknown genus (OTU164) close to Epsilonproteobacteria. Most of these bacteria play important roles in the metabolism of C and N in the environment through, for example, nitrogen fixation and methane oxidation. The results of the present study will contribute to improving our understanding of microbe-mediated CN dynamics in paddy rice fields under a changing climate.

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