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Cultivable *Methylobacterium* species diversity in rice seeds identified with whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis

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Methylobacterium **species are methylotrophic bacteria that widely inhabit plant surfaces. In addition to studies on methylotrophs as model organisms, research has also been conducted on their mechanism of plant growth promotion as well as the species–species specificity of plant–microbe interaction. We employed whole-cell matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (WC-MS) analysis, which enables the rapid and accurate identification of bacteria at the species level, to identify *Methylobacterium* isolates collected from the rice seeds of different cultivars harvested in Japan, Thailand, and Kenya. Rice seeds obtained from diverse geographical locations showed different communities of *Methylobacterium* species. We found that *M. fujisawaense*, *M. aquaticum*, *M. platani*, and *M. radiotolerans* are the most frequently isolated species, but none were isolated as common species from 18 seed samples due to the highly biased communities in some samples. These findings will contribute to the development of formulations containing selected species that promote rice growth, though it may be necessary to customize the formulations depending on the cultivars and farm conditions.**

[**Key words:** *Methylobacterium* species; Rice seeds; Whole-cell matrix-assisted laser desorption/ionization-mass spectrometry]

Methylobacterium species are members of the gram-negative *Alphaproteobacteria* class of bacteria. They are well-known facultative methylotrophs that utilize methanol and other C1 compounds. Metagenomic analysis has demonstrated that they occur as one of the major bacteria inhabiting the phyllosphere (1). Methanol is one of the major volatiles emitted from plants, with an estimated annual emission of 100 Tg (2). The predomination of *Methylobacterium* species is explained by the methanol present in the phyllosphere, the concentration of which is equivalent to 2.5–250 mM methanol added to agar medium in the case of *Arabidopsis thaliana* (3). These species produce the phytohormones auxin (4) and cytokinin (5,6), and 1-aminocyclopropane-1-carboxylate deaminase that reduces ethylene levels in plants (4,7). In addition, they show antagonistic activity toward phytopathogenic bacteria (8). Probably due to these traits, the inoculation of *Methylobacterium* onto plants results in a growth promotion effect, as reported for rice (9,10), canola (4,11), bryophytes (12,13), and other plants (14,15). This ability could be applicable for agricultural purposes.

We attempted to promote rice growth through the inoculation of *Methylobacterium* isolates. We found that the inoculated strains could not be recovered from the field-grown rice plants at the time of harvest (9), which suggests that the inoculated strains had been eliminated. Common groups of *Methylobacterium*, however, were isolated from the inoculated and non-inoculated plants, which suggest that they are specialized to rice or to the environment. To realize improved rice growth using *Methylobacterium* and to understand the mechanism of the species–species interaction, it remains necessary to accumulate more data on the interaction specificity between them.

Leaf-inhabiting *Methylobacterium* species were suggested to be seed-borne rather than from environmental sources (16). Romanovskaya et al. (8) showed that inoculation of *M. mesophilicum* into soil did not result in leaf colonization of *Zea mays*. They suggested that colonization occurs by soil particle transfer by air under natural conditions. Mizuno et al. (17,18) demonstrated that *Methylobacterium* isolates from red perilla plants grown in different sites in Japan belong solely to *M. fujisawaense* that had been isolated from the seeds, and that the seed-inoculated strain was recovered from the plant leaves grown axenically, thus supported the seed-borne hypothesis. In the case of rhizobia, *Rhizobium* strains were shown to migrate from tobacco roots to the leaf surface even when their epiphytic migration was blocked, suggesting that they migrate through plant stems (19). *Sinorhizobium* strains were shown to migrate from roots to leaves, colonizing every part of rice plants (20). In addition to these investigations on individual species, recent metagenomic analyses

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TABLE 1. Rice seeds used in this study.

Harvest year	Country	Location	Variety	Japonica/Indica	Paddy/Upland	Fertilization	Sample coding	Number of isolates	Number of *Methylobacterium* isolates	Number of non-*Methylobacterium* isolates	Remarks
2009	Japan	IPSR	Norin 18	Japonica	Paddy	Yes	JoF	14	14	0	
2009	Japan	IPSR	Norin 18	Japonica	Paddy	No	JoN	18	18	0	
2012	Japan	IPSR	Norin 18	Japonica	Paddy	Yes	No12F	20	20	0	
2012	Japan	IPSR	Norin 18	Japonica	Paddy	No	No12N	20	20	0	
2013	Japan	IPSR	Norin 18	Japonica	Paddy	Yes	No13F	40	40	0	
2013	Japan	IPSR	Norin 18	Japonica	Paddy	No	No13N	40	40	0	
2009	Japan	IPSR	Nipponbare	Japonica	Paddy	Yes	JiF	20	20	0	
2009	Japan	IPSR	Nipponbare	Japonica	Paddy	No	JiN	20	20	0	
2012	Japan	Kinki University	Nipponbare	Japonica	Paddy	Yes	R76	20	20	0	
2012	Japan	Kinki University	Nipponbare	Japonica	Paddy	Yes	R15	20	20	0	
2012	Japan	Akita Prefectural University	Nipponbare	Japonica	Paddy	Yes	AK	20	20	0	
2009	Kenya	Bunyala	Basmati 370	Indica	Paddy	No	KB	8	8	0	
2009	Kenya	Taveta	Japan 64	Indica	Paddy	No	KT	10	9	1	1 *Paenibacillus hunanensis*
2009	Kenya	Kaloleni	Matuko Nyeusi	Indica	Paddy/Upland	No	KK	15	15	0	
2009	Kenya	Mwea	Nerica 4	Japonica	Upland	Yes	KM	14	14	0	
2009	Kenya	Hola	Sindano Bahari	Japonica	Paddy	No	KH	6	4	2	2 *Paenibacillus hunanensis*
2009	Thailand	Chachoengsao province	Khao Taheng	Japonica	Paddy	No	TT	46	46	0	
2009	Thailand	Lopburi province	Khao Hom Mari	Japonica	Paddy	No	TH	42	35	7	7 *Pseudomonas oryzihabitans*
Total								393	383		

have revealed that phyllospheric communities initially mirrored airborne communities and then converged to a distinct composition in a greenhouse experiment (21). Since *Methylobacterium* species are widespread in the environment, and are found in soil, dust, air, hailstones, and rain, leaf sampling might be strongly affected by environmental factors. It is thus important to analyze the species colonizing the seeds, since endophytic microbes in seeds can be maternally passed on to plant descendants and would become the first colonizer of juvenile seedlings.

In this study, we used rice seeds of various cultivars grown in completely different environments in Japan, Kenya, and Thailand for *Methylobacterium* isolation. In this way, we examined which group can be commonly isolated, and whether rice genotype and fertilization affect the community structure. For this purpose, we used whole-cell matrix-assisted laser-desorption/ionization-mass spectrometry (WC-MS) analysis, which enables the rapid identification of unknown isolates at the species level (22). We have demonstrated the effectiveness of the method for isolates from rice leaves and barley (9). The method is only applicable to isolated bacteria, and metagenomics or specifically targeted ribosomal spacer analyses (23) are more suited for quantitative analysis of microbial community composition. As we seek to utilize the isolates for agricultural purposes, the method is convenient and rapid enough to effectively classify unidentified isolates.

MATERIALS AND METHODS

Plant materials and isolation of *Methylobacterium* species The rice seed samples used in this study are summarized in Table 1. Rice samples were used for bacteria isolation as soon as possible after harvest. Chemical fertilization at the Institute of Plant Science and Resources (IPSR), Okayama University, Akita Prefectural University, and Kinki University was applied in the amount of 5 kg each of N, P, and K per 1000 m^2. Ten rice seeds from each sample were washed extensively with sterile water. The seeds were then surface-sterilized by washing twice with 20 ml of 0.5% sodium hypochlorite and 0.005% Tween 20 for 1 min, followed by rinsing with sterile water five times. The rice seeds were husked, and the resultant brown rice was put in sterile 0.85% NaCl, and vortexed vigorously for 30 s. The resultant solution was spread onto solidified mineral medium containing 0.5% methanol (13) as the sole carbon source and 50 mg/L cycloheximide. Pink colonies that appeared after 3–7 days at 28°C were isolated and purified by streaking on plate media of the same composition.

WC-MS analysis and 16S rRNA gene sequencing All isolates were subjected to WC-MS analysis as reported previously (22). The data were analyzed with MALDI BioTyper 3.0 software (Bruker Daltonics) to construct the main spectra projection (MSP) dendrogram based on spectra similarity with the default settings (9). The type strains of *Methylobacterium* species obtained from the culture collections were included in the analysis. Representative isolates from each cluster in the MSP dendrogram were subjected to 16S rRNA gene sequencing and phylogenetic analysis as reported previously (9,22). The 16S rRNA gene sequence similarities of an isolate against those of *Methylobacterium* type strains were determined using the EzTaxon server (24). For species assignment of an isolate, pairwise sequence similarity values and nearest phylogenetic neighbors were taken into consideration.

Nucleotide accession numbers The DDBJ accession numbers for the 16S rRNA gene sequences reported in this paper are LC025976–LC026013.

RESULTS AND DISCUSSION

Isolates from Norin 18 During the 2009, 2012, and 2013 harvesting season, 147 *Methylobacterium* isolates were obtained from *Oryza sativa* cultivar Norin 18 seeds. The isolates obtained in 2009 were categorized into four species (*M. gossipiicola*, *M. fujisawaense*, *M. platani*, and *M. aquaticum*) as a result of WC-MS and 16S rRNA gene sequencing (Fig. S1). Discrimination of isolates belonging to *M. fujisawaense*, *M. oryzae*, and *M. phyllosphaerae* type strain clusters was difficult, since their 16S rRNA genes share more than 99% identity and also their WC-MS spectra have high similarity. 16S rRNA gene sequence similarity value less than