Short Communication

Diversity of Methylobacterium spp. in the Rice of the Vietnamese Mekong Delta

KHOA LAI1,2, NGOC THAI NGUYEN1, HIROKI MIWA1, MICHIKO YASUDA1, HIEP HUU NGUYEN4, and SHIN OKAZAKI1,3,*

1 United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Saiwaicho 3–5–8, Fuchu, Tokyo 183–8509, Japan; 2National Agro-Forestry-Fisheries Quality Assurance Department branch 4, 271—To Ngoc Van St, Linh Dong ward, Thu Duc district, Ho Chi Minh City, Vietnam; 3Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho 3–5–8, Fuchu, Tokyo 183–8509, Japan; and 4Department of Microbial Biotechnology, Biotechnology Research and Development Institute, Cantho University, II—St 3/2, Ninh Kieu ward, Cantho City, Vietnam

(Rceived August 19, 2019—Accepted November 27, 2019—Published online January 23, 2020)

The Vietnamese Mekong delta is one of the largest rice-producing areas globally. Methylobacterium spp. are persistent colonizers of the rice plant and exert beneficial effects on plant growth and health. Sixty-one Methylobacterium strains belonging to seven species were predominantly isolated from the phyllosphere of rice cultivated in six Mekong delta provinces. Inoculation tests revealed that some strains exhibited plant growth-promoting activity. Moreover, three strains possessed the novel characteristics of inducing leaf bleaching and killing rice seedlings. These results revealed the complex diversity of Methylobacterium in Mekong delta rice and that healthy and productive rice cultivation requires a proper balance of Methylobacterium.

Key words: Methylobacterium, rice, plant growth promotion, disease, Mekong delta

The plant phyllosphere harbors diverse microbial communities and bacteria constitute the dominant population (Kembel et al., 2014). The mechanisms by which bacteria colonize and survive on leaf surfaces remain largely unknown (Burch et al., 2016). Healthy epidermal plant cells constantly leak organic and inorganic molecules, such as amino acids, sugars, and various salts to their surfaces; this may facilitate the colonization of microflora by interacting with the plant phyllosphere (Knief et al., 2012; Burch et al., 2016). Furthermore, methanol released from stomata through cell expansion attracts several bacterial strains (Nemecek-Marshall et al., 1995). The genus Methylobacterium is composed of pink-pigmented facultative methylotrophs (PPFM) that belong to the α-Proteobacteria class, and use methanol as the sole carbon and energy source (Abanda-Nkpwatt et al., 2006). Methylobacterium spp. express several genes related to methylotrophy during the colonization of plants, such as rice, citrus, peanut, lettuce, tobacco, and strawberry (Lee et al., 2006; Knief et al., 2012; Rastogi et al., 2012; Mwajita et al., 2013). Several isolates of the genus Methylobacterium have been reported to enhance plant growth by producing phytohormones, such as auxin and cytokinins, fixing atmospheric nitrogen, and utilizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity to reduce stress-related ethylene production. The dominant colonization of Methylobacterium spp. facilitates healthy growth of the rice plant by protecting it from infection and colonization by other potential pathogens (Lee et al., 2006; Kwak et al., 2014; Madhaiyan et al., 2015). Vietnam is the fifth largest rice-producing country in the world (FAO 2017), and rice export plays a pivotal part in its economic development. The Mekong Delta is the major rice-producing area in Vietnam and one of the biggest globally. Thus, healthy and productive rice cultivation is of great importance. Although the composition of the bacterial community plays an important role in the healthy growth of rice plants to produce high yields of rice, microbial diversity on the rice plant phyllosphere and rhizosphere in the Mekong delta has not yet been reported. The present study was undertaken to reveal the genetic diversity of Methylobacterium spp. in the rice of the Vietnamese Mekong delta and examine their effects on rice plant growth.

To isolate Methylobacterium spp. from rice plants, we collected rice samples at the flowering stage (60–70 d after sowing) from six provinces–Can Tho, Vinh Long, Tien Giang, Ho Chi Minh, Dong Nai, and Khanh Hoa. All rice plants sampled were Oryza sativa cultivar OM5451.

The number of samples and sampling sites are shown in Fig. 1. Fresh samples (10 g) of rice roots and shoots were separately homogenized with 90 mL sterilized 0.85% NaCl solution and plated onto Murashige and Skoog (MS) medium supplemented with 0.5% (v/v) methanol and 10 µg mL−1 cycloheximide. Plates were incubated at 28°C for 7 to 8 d. Single, well-isolated, and pinkish colonies from the isolation medium were sub-cultured, purified, and maintained in glycerol stocks. Genomic DNA of the isolates was extracted using the Wizard Genomic DNA purification kit (Promega). The 16S rRNA gene was amplified by PCR using the primers 16AF (5′-AACTGAAGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The 16S rRNA gene sequence was searched for homology by BLAST (Basic Local Alignment Search Tool) at the NCBI (National Center for Biotechnology Information) website. Sequence alignment was performed using MEGA software ver 7.0 (Kumar et al., 2016). Phylogenetic trees were constructed using the neighbor-joining method.

Rice (O. sativa Nipponbare) and lettuce (Lactuca sativa L. cv. Legacy) were used to evaluate the effects of

* Corresponding author. E-mail: sokazaki@cc.tuat.ac.jp; Tel: +81–42–367–5847; Fax: +81–42–367–5847.
Methylobacterium spp. on plant growth. Rice seeds were sterilized by soaking in 70% ethanol for 1 min and 5% sodium hypochlorite with 0.02% Tween 20 for 10 min, and subsequently washed five times with sterile water. Lettuce seeds were sterilized by soaking in 1% sodium hypochlorite solution for 5 min followed by 70% ethanol for 3 min, and subsequently washed five times with sterile water. Methylobacterium isolates were grown in 5 mL CMS medium (MS medium with sucrose 30 g L\(^{-1}\), peptone 2 g L\(^{-1}\), and tryptone 2 g L\(^{-1}\)) for 4 d, after which they were washed twice, and bacterial cells were diluted to a final concentration of 10\(^4\) cfu mL\(^{-1}\) in sterile water. Surface-sterilized seeds were soaked overnight in a bacterial suspension. Control seeds were treated with sterile distilled water without the bacterial culture. Seeds (12 for rice and 5 for lettuce) were sown in a 300-mL plant box (CUL-JAR300; Iwaki) containing 30 g sterile vermiculite (Hirukon S, Hirushi Kagaku Kogyo) and watered with 25% MS medium without sucrose. Three replicates for each treatment condition were used in all plant inoculation tests. Plants were grown in a growth chamber at 25°C (8 h dark/16 h light cycle) for 14 d. Seedling growth was evaluated by measuring leaf and root lengths and the fresh weight of leaves.

Sixty-one putative Methylobacterium strains were isolated from 24 rice plants collected from paddy fields across six provinces (Fig. 1). All 61 strains were identified by 16S rRNA gene sequencing and categorized into seven species (Fig. S1). The largest cluster belonged to M. radiotolerans (29/61 isolates), followed by M. salsuginis (17/61 isolates). Of the seven species of the genus Methylobacterium isolated from rice plants in Vietnam, five (M. komagatae, M. salsuginis, M. indicum, M. radiotolerans, and M. oryzae) were isolated from rice cultivated in other parts of the world (Madhaiyan et al., 2006; Knief et al., 2012; Kwak et al., 2014). M. rhodium was previously found in water samples (Green and Bosfield, 1983), but has never been isolated from rice. Furthermore, one of the isolates, HP16.3 was phylogenetically ambiguous and located between three species, M. platani, M. aquaticum, and M. tarhaniae, based on 16S rRNA gene phylogeny.

In the present study, Methylobacterium spp. were more frequently isolated from the shoots (80%) than from the roots (20%) (Fig. 2A and Table S1). Previous studies on the isolation of Methylobacterium spp. from rice plants demonstrated that the distribution of Methylobacterium spp. was dependent on the area of colonization (Lee et al., 2006; Sun et al., 2008; Knief et al., 2012). Metagenomics and metaproteomics analyses previously revealed that α-Proteobacterium, particularly Rhizobium and Methylobacterium, were more frequently isolated from rice plants than in the rice rhizosphere (Knief et al., 2012). Similarly, Sun et al. (2008) used 16S rDNA cloning, an amplified ribosomal DNA restriction analysis, and sequence homology comparisons to demonstrate that Methylobacterium spp. are present at a low frequency in rice roots (Sun et al., 2008); our results are consistent with these findings. Interestingly, 53% of isolates found in the rice rhizosphere belonged to M. radiotolerans (Fig. 2A). The upper part of plants (the phyllosphere) is generally considered to be a harsh environment for microbial colonization due to stress from water, UV irradiation, temperature changes, and limited nutrient availability (Lee et al., 2006; Maliti et al., 2007). One possibility is that M. radiotolerans isolates in the present study exhibit high-stress tolerance, facilitating their adaptation and survival in extreme environments, such as phyllosphere habitats. The predominant factors that transfer these bacteria into plants are still being debated. Romanovskaya et al. (2001) proposed that under natural conditions, Methylobacterium strains colonize the plant leaves, being transferred to their surface by air with soil particles (Romanovskaya et al., 2001). Furthermore, previous studies reported that Methylobacterium spp. were transferred from plants seeds to leaves (Omer et al., 2004; Madhaiyan et al., 2006). The present results indicate that environmental factors, such as temperature, air, and UV light, play a pivotal role in forming the community structure of Methylobacterium inhabitants.

We assessed the relationship between geographic locations and specific Methylobacterium species. We found that 68% isolates belonged to M. radiotolerans isolated from three provinces. Ho Chi Minh, Dong Nai, and Khanh Hoa—in the northern region of the Mekong delta. However, the southern region of the Mekong delta, consisting of Tien Giang, Vinh Long, and Can Tho province, was abundant in M. salsuginis (71%) (Fig. 2B). The southern region of the Mekong delta has recently faced the threat of saltwater intrusion, which may damage agricultural activities, including rice cultivation. Since M. salsuginis was initially isolated from the South China sea and has been well characterized, we suggest that a location with a high salt concentration in the soil influences Methylobacterium spp.
distribution in the Mekong delta (Wang et al., 2007; Vu et al., 2018). Similarly, Knief et al. (2012) examined the effects of host plant, location, time, and other bacterial communities on the composition and population size of Methylobacterium. Their findings demonstrated that the location-specific factor exerted a stronger impact than the host plant-specific factor (Knief et al., 2012). Our results are consistent with these findings indicating that specific locations influence the distribution of Methylobacterium spp.

Methylobacterium spp. promote seed germination and growth in several plants (Lee et al., 2006). Inoculation tests revealed that most Methylobacterium spp. isolated from the Mekong delta did not significantly affect rice growth (data not shown). However, some isolates exerted growth-inducing effects on rice plants (Table 1). Inoculations with Methylobacterium strains HP18.2, HP19.2, DR25.3, CP40.1, and VR43.1 enhanced plant growth and increased plant biomass over those obtained using an un-inoculated control. These isolates increased the root length from 7 to 14% and leaf weight up to 8% over those of the un-inoculated control. No significant difference was observed between these strains; however, some strains, such as HP19.2 and CP40.1, showed increased root and leaf lengths or leaf weights. Lee et al. (2006) reported that the inoculation of Methylobacterium strain CBMB20 with rice increased the rate of seed germination, seedling parameters, and seedling biomass by producing phytohormones, such as trans-zeatin riboside and indole-3-acetic acid. Other Methylobacterium strains Q4 and Q5 significantly stimulated the growth of two rice cultivars, japonica cultivar CR76 and indica cultivar A301 on four parameters—root development, leaf growth, and stem and biomass productivities (Maliti et al., 2007). Isolates of Methylobacterium spp. also effectively promoted the growth of various seed plants (Abanda-Nkpwatt et al., 2006).

Methylobacterium isolates, including those that promoted the growth of rice plants, were tested on lettuce plants. As shown in Table 1, isolates promoted the growth of lettuce plants; leaf length increased from 5 to 9% and leaf weight up to 11%. Tani et al. (2012) reported that Methylobacterium strain 22A isolated from a liquid culture of the moss R. japonicum promoted the growth of the tested seed plants, including Arabidopsis, Nicotiana, and Hordeum (Tani et al., 2012). In other studies, M. extorquens strain ME4 was shown to promote the growth of plants, such as Lycopersicon, Fragaria, Sinapis, and Nicotiana, but exerted no effect on seed germination in Triticum, Daucus, maize, Hordeum, and Pisum (Maliti et al., 2007). Our results are consistent with the findings demonstrating the positive effects of Methylobacterium spp. on plant growth.

Notably, three isolates belonging to M. indicum (VP43.2, DP28.3, and DP28.4) did not enhance plant growth, but...
exerted a negative effect on rice seedlings (Fig. 3). Following a 5-day inoculation with VP43.2, DP28.3, and DP28.4 isolates, rice leaves turned white. During this period, the growth of plant seedlings was retarded and plants died within 2 weeks. *Methylobacterium* spp. are related to more than 70 plant species. However, the mechanisms by which *Methylobacterium* spp., including *M. indicum*, exert a negative effect on plant growth remain unclear (Chaudhry et al., 2016). Thus, this is the first study to suggest that *Methylobacterium* strains cause disease-like symptoms similar to plant pathogens. The “bleaching” symptom is completely different from other disease symptoms. The “cold damage” symptom has been described as a white band on the leaf blade during exposure to cold temperatures (Da Cruz et al., 2013). “Bacterial leaf blight” caused by *Xanthomonas oryzae* was previously characterized by elongated lesions near the leaf tip or margin that are several inches long, which turn white to yellow and then gray (Sharma et al., 2017). The “bleaching” symptom caused by *Methylobacterium* strains changed the color of rice plant leaves from green to yellow, and gradually to white; infected rice plants ultimately died. Further studies are needed to identify the pathogenic genes in these isolates and elucidate the physiological mechanisms underlying this novel disease in rice.

In conclusion, the present study revealed the genetic diversity of rice-associated *Methylobacterium* spp. in the Vietnamese Mekong delta. Sixty-one isolates belonging to seven species were similar to those previously isolated from Vietnamese Mekong delta. Sixty-one isolates belonging to six species were similar to those previously isolated from rice-associated *Methylobacterium* spp., including *M. indicum*, exert a negative effect on plant growth remain unclear (Chaudhry et al., 2016). Thus, this is the first study to suggest that *Methylobacterium* strains cause disease-like symptoms similar to plant pathogens. The “bleaching” symptom is completely different from other disease symptoms. The “cold damage” symptom has been described as a white band on the leaf blade during exposure to cold temperatures (Da Cruz et al., 2013). “Bacterial leaf blight” caused by *Xanthomonas oryzae* was previously characterized by elongated lesions near the leaf tip or margin that are several inches long, which turn white to yellow and then gray (Sharma et al., 2017). The “bleaching” symptom caused by *Methylobacterium* strains changed the color of rice plant leaves from green to yellow, and gradually to white; infected rice plants ultimately died. Further studies are needed to identify the pathogenic genes in these isolates and elucidate the physiological mechanisms underlying this novel disease in rice.

Acknowledgements

We thank Kimika Suzuki (Tokyo University of Agriculture and Technology, TUAT) for her technical support. This work was supported by JSPS KAKENHI [grant number 17H04618] and the Heiwa Nakajima Foundation.

References

Abanda-Nkpwatt, D., Mäsch, M., Tschiersch, J., Boettner, M., and Schwab, W. (2006) Molecular interaction between *Methylobacterium extorquens* and seedlings: Growth promotion, methanol consumption, and localization of the methanol emission site. *J Exp Bot* 57: 4025–4032.

Burch, A.Y., Do, P.T., Shodio, A., Suslow, T.V., and Lindow, S.E. (2016) High-level culturability of epiphytic bacteria and frequency of biosurfactant producers on leaves. *Appl Environ Microbiol* 82: 5997–6009.

Chaudhry, V., Baindara, P., Pal, V.K., Chawla, N., Patil, P.B., and Korpole, S. (2016) *Methylobacterium indicum* sp. nov., a facultative methylotrophic bacterium isolated from rice seed. *Syst Appl Microbiol* 39: 25–32.

Da Cruz, R.P., Sperotto, R.A., Cargnelutti, D., Adamski, J.M., de FreitasTerra, T., and Fett, J.P. (2013) Avoiding damage and achieving cold tolerance in rice plants. *Food Energy Secur* 2: 96–119.

Grein, P.N., and Bosfield, I.J. (1983) Emendation of *Methylobacterium* *extorquens*. *Int J Syst Bacteriol* 33: 875–877.

Table 1. Effects of selected *Methylobacterium* isolates on rice and lettuce growth.

| Plant parameters               | Un-inoculated | HP18.2 | HP19.2 | DR25.3 | DP26.3 | CP40.1 | VR43.1 |
|-------------------------------|---------------|--------|--------|--------|--------|--------|--------|
| Length of rice roots (cm)     | 10.4±1.2c     | 10.9±1.3c | 11.5±1.3b | 11.4±1.2b | 10.4±1.4 | 11.2±1.2c | 11.9±1.4c |
| Length of rice leaves (cm)    | 23.0±1.9b     | 23.9±2.4b | 23.6±2.2b | 23.5±2.5b | 23.3±2.2b | 24.6±1.1c | 23.0±2.5b |
| Weight of rice leaves (g)     | 0.124±0.008b  | 0.136±0.022a | 0.134±0.007b | 0.124±0.016ab | 0.126±0.010b | 0.130±0.015bce | 0.126±0.018bce |
| Length of lettuce leaves (cm) | 5.6±0.2b      | 6.1±0.3a  | 5.9±0.3a  | 5.9±0.2a  | 6.0±0.3b  | 5.8±0.3ab | 5.8±0.4ab |
| Weight of lettuce leaves (g)  | 0.115±0.011b  | 0.125±0.011a | 0.127±0.008e | 0.121±0.010b | 0.123±0.011ab | 0.117±0.005bce | 0.117±0.005bce |

Data represent means±SD (*n*=36 for rice and *n*=15 for lettuce). Means followed by different letters are significantly different at the 5% level (ANOVA with Tukey’s test).

Fig. 3. Bleaching symptom of rice seedlings. This image was acquired following a 5-day inoculation with VP43.2, DP28.3, and DP28.4 isolates. Un-inoculated seedlings were used as the control and seedlings inoculated with HP19.2 did not exhibit any bleaching symptom. Scale bar=2 cm.
Methylobacterium in Mekong Delta Rice

Kembel, S.W., O’Connor, T.K., Arnold, H.K., Hubbell, S.P., Wright, S.J., and Green, J.L. (2014) Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. Proc Natl Acad Sci U S A 111: 13715–13720.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., et al. (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 6: 1378–1390.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33: 1870–1874.

Lee, H.S., Madhaiyan, M., Kim, C.W., Choi, S.J., Chung, K.Y., and Sa, T.M. (2006) Physiological enhancement of early growth of rice seedlings (Oryza sativa L.) by production of phytohormone of N2-fixing methylotrophic isolates. Biol Fertil Soils 42: 402–408.

Madhaiyan, M., Alex, T.H.H., Ngoh, S.T., Prithiviraj, B., and Ji, L. (2015) Leaf-residing Methylobacterium species fix nitrogen and promote biomass and seed production in Jatropha curcas. Biotechnol Biofuels 8: 1–14.

Maliti, C.M., Basile, D.V., and Corpe, W.A. (2007) Effects of Methylobacterium spp. strains on rice Oryza sativa L. callus induction, plantlet regeneration, and seedlings growth in vitro. J Torrey Bot Soc 132: 355–367.

Mwajita, M.R., Murage, H., Tani, A., and Kahangi, E.M. (2013) Evaluation of rhizosphere, rhizoplane and phyllosphere bacteria and fungi isolated from rice in Kenya for plant growth promoters. SpringerPlus 2: 1–9.

Nemecek-Marshall, M., MacDonald, R.C., Franzen, J.J., Wojciechowski, C.L., and Fall, R. (1995) Methanol emission from leaves (enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development). Plant Physiol 108: 1359–1368.

Omer, Z.S., Tombolini, R., and Gerhardson, B. (2004) Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFM). FEMS Microbiol Ecol 47: 319–326.

Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L., and Leveau, J.H.J. (2012) Leaf microbiota in an agroecosystem: Spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J 6: 1812–1822.

Romanovskaya, V.A., Stolyar, S.M., Malashenko, Y.R., and Dodatko, T.N. (2001) The ways of plant colonization by Methylobacterium strains and properties of these bacteria. Microbiology 70: 221–227.

Sun, L., Qiu, F., Zhang, X., and Dai, X. (2008) Endophytic bacterial diversity in rice (Oryza sativa L.) roots estimated by 16S rDNA sequence analysis. Microb Ecol 55: 415–424.

Tani, A., Takai, Y., Suzukawa, I., Akita, M., Murase, H., and Kimbara, K. (2012) Practical application of methanol-mediated mutualistic symbiosis between Methylobacterium species and a roof greening moss, Racomitrium japonicum. PLoS One 7: 2–10.

Vu, D.T., Yamada, T., and Ishidaira, H. (2018) Assessing the impact of sea level rise due to climate change on seawater intrusion in Mekong delta, Vietnam. Water Sci Technol 77: 1632–1639.

Wang, X., Sahr, F., Xue, T., and Sun, B. (2007) Methylobacterium salsuginis sp. nov., isolated from seawater. Int J Syst Evol Microbiol 57: 1699–1703.