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Distribution of Pink-pigmented Facultative Methylotrophs Isolated from the Leaves of Potato Grown in Different Regions of Japan

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Abstract

Seed tubers of potato (*Solanum tuberosum*) plants were grown in five locations in Japan: Hokkaido, Yamagata, Ibaraki, Hiroshima, and Nagasaki. One hundred colonies of pink-pigmented facultative methylotrophs (PPFMs) were isolated from potato leaves at flowering time. The 16S rRNA gene sequences of isolates were determined, and clustering analysis (≥ 99% identity) identified 11 operational taxonomic units (OTUs). Phylogenetic analysis revealed that all OTUs were grouped in genus *Methylobacterium*. The OTU M9 was distributed among all five collection sites, and other OTUs were distributed among several collection sites. These data indicated that the distribution of PPFMs in the potato phyllosphere differed according to the plant's geographical location.

Discipline: Agricultural Environment
Additional key words: 16S rRNA, *Methylobacterium*, operational taxonomic units, seed tuber

Introduction

Methylobacterium, a pink-pigmented facultative methylotroph (PPFM), is one of the most ubiquitous bacterial genera in natural environments, including soils, water, animals, and phytospheres (Chanprame et al. 1996, Lai et al. 2020, Yoshida et al. 2017, Yoshimura & Ohara 1994). Some species of *Methylobacterium* are widely known to be beneficial to plants as plant growth-promoting bacteria or as protectants from pathogens (Ardanov et al. 2012, Indiragandhi et al. 2008, Nalayuni et al. 2014, Tani et al. 2015). An understanding of the ecological traits of plant-associated microbes, such as their tissue specificity or stability in diverse environments, is essential for consistent and effective use of beneficial agents under practical agronomic circumstances. It has been reported that *Methylobacterium* mainly colonize the leaves of plants and are not dominant in roots (Chimwamurombe et al. 2016, Madhaiyan et al. 2007, Müller et al. 2016). Some reports suggest that *Methylobacterium* are transmitted to the next generation through seed in some plant species (Chimwamurombe et al. 2016, Mizuno et al. 2013). Our previous study also suggested the presence of different specific taxonomic groups of *Methylobacterium* between potato (*Solanum tuberosum*) leaves and stems (Someya et al. 2013); however, *Methylobacterium* were not dominant in roots and tubers of potato plants in soil.

In the present study, community analysis based on 16S rRNA gene sequencing was conducted on PPFMs isolated from leaves of potato plants grown in diverse locations in Japan. The results clarified the diversity and geographic heterogeneity of *Methylobacterium* in potato plants and will facilitate the application of beneficial bacteria in agricultural practice.

Materials and methods

1. Sampling of potato leaves and isolation of PPFMs

Seed tubers of potato (cultivar "Matilda") were produced at Hokkaido-Chuo Station, Center for Seeds and Seedlings, National Agriculture and Food Research

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Organization (Kitahiroshima, Hokkaido, Japan) in 2011. No PPFMs were detected from the seed tubers. Seed tubers were conserved in the seed-storage room until planting. In 2012, seed tubers were surface-sterilized and planted in five different fields: "Hokkaido" at the Memuro Research Station of the Hokkaido Agricultural Research Center, NARO (Memuro, Hokkaido, Japan); "Yamagata" at the Yamagata Integrated Agriculture Research Center (Yamagata, Yamagata, Japan); "Ibaraki" in a commercial agricultural field (Takahagi, Ibaraki, Japan); "Hiroshima" at the Western Region Agricultural Research Center, NARO (Fukuyama, Hiroshima, Japan); and "Nagasaki" at the Nagasaki Agricultural and Forestry Technical Development Center (Unzen, Nagasaki, Japan). Each field was dressed with a commercial fertilizer for basal fertilization. No pesticides were applied during the cultivation period. Plants at flowering time were sampled in May (Nagasaki), June (Hiroshima), and July (Hokkaido, Yamagata, and Ibaraki). Samples of leaves (1 g) of three plants were combined and homogenized with sterile 10 mM phosphate buffer (pH 7.2) using a mortar and pestle. An aliquot of the homogenate was spread onto ammonium mineral salts [AMS; 1 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $CaCl_2$, 4 mg of sequestrene iron complex, 0.5 g of NH_4Cl, 50 mg of Pfennig's trace element solution, and 2 mL of phosphate buffer solution (a mixture of 5% KH_2PO_4 and 5% $Na_2HPO_4 \cdot 12H_2O$) (pH 6.8)] medium agar containing 0.5% (v/v) methanol and 50 mg mL^{-1} filter-sterilized cycloheximide (Anda et al. 2011). The same sample was also spread onto R2A (Difco, Detroit, MI, USA) medium agar containing cycloheximide for counting the total culturable bacterial population. After incubating the inoculated plates at 25°C in darkness for seven days, 100 pink-pigmented colonies were randomly isolated from each sample. Single-colony isolation on AMS medium was done three times to check the purity of isolates.

2. Sequence analysis of 16S rRNA genes

Bacterial genomic DNA was prepared with a DNeasy Blood & Tissue Kit (Qiagen K.K., Tokyo, Japan). The 16S rRNA genes were amplified with Premix Taq (Takara Bio, Shiga, Japan) from genomic DNA. The primers used were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′). The thermal cycling program follows: an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Direct sequencing was conducted by the Takara Bio Dragon Genomics Center (Takara Bio, Mie, Japan) using 27F primer. Cluster analysis of sequences was done according to Someya et al. (2020). The operational taxonomic units (OTUs) in the cluster analysis were defined by 99% sequence identity. Representative sequences of OTUs were aligned using CLUSTAL X and used to build a phylogenetic tree by the neighbor-joining method (Saitou & Nei, 1987) with type strains of known species. Nucleotide sequences of the partial 16S rRNA genes of PPFMs isolated from potato leaves were deposited in the DNA Data Bank of Japan under accession numbers LC508644-LC508654.

Results and discussion

When the R2A medium agar was used for bacterial isolation, approximately 10^4-10^7 colony-forming units (CFU) of bacteria were obtained from 1 g (fresh weight) of potato leaves, for most of the samples (Fig. 1). When the AMS medium agar containing methanol was used for bacterial isolation, approximately 10^3-10^6 CFU of PPFM colonies were obtained from 1 g of potato leaves (Fig. 1). Plants and PPFM have strong relationships, with seed-transmission observed in some plant species (Chimwamurombe et al. 2016, Mizuno et al. 2013). However, we confirmed that PPFMs were not detected in the seed tubers used in this study. Therefore, it is thought that PPFM isolates from leaves of potato plants were individually colonized at each field site. Populations of PPFM were lower at Nagasaki and Hiroshima than from the locations of the other three samples (Fig. 1). In the present study, sampling was carried out at potato flowering time (May at Nagasaki, June at Hiroshima, and July at Hokkaido, Yamagata, and Ibaraki). Yoshimura & Ohara (1994) reported that populations of *Methylobacterium* in leaves of some trees increased during summer and decreased in winter. It is believed that PPFMs of potato leaves are also influenced by environmental factors, such as seasonal variances and geographical differences.

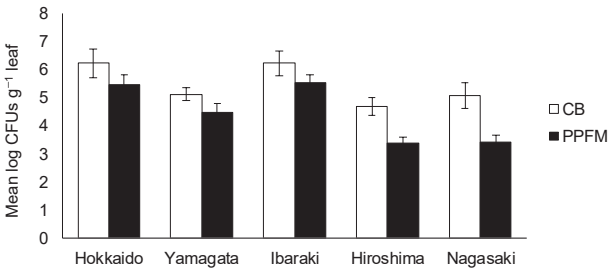

Fig. 1. Populations of culturable bacteria (CB) and pink-pigmented facultative methylotrophs (PPFM) detected from leaf samples of potato grown at five different locations in Japan: Hokkaido, Yamagata, Ibaraki, Hiroshima, and Nagasaki

A total of 500 isolates of PPFMs were obtained from potato leaves collected from the five locations. As a result of the 16S rRNA gene sequencing, 486 sequences were successfully determined. Using the RDP Classifier (https://rdp.cme.msu.edu/classifier/classifier.jsp), all of the isolates were confirmed to belong to the genus *Methylobacterium* known as PPFM. Clustering analysis (> 99% identity) revealed the presence of 11 OTUs for PPFMs in potato leaves (Table 1). Four OTUs from Hokkaido, Yamagata, and Hiroshima samples, six OTUs from the Nagasaki sample, and seven OTUs from the Ibaraki sample were detected. Among them, OTU M2 from Hokkaido, OTU M7 from Nagasaki, and OTU M10 from Ibaraki were location specific. Another eight OTUs were detected from different collection sites. Notably, OTU M9 was present in all five locations. The closest known type species of each OTU are indicated in Table 1. Detailed phylogenetic analysis indicated that the genetic diversity of potato-associated PPFMs was widespread within the entire genus *Methylobacterium* (Fig. 2).

The proportion of PPFMs comprising each OTU differed among the potato plants grown at different sites (Table 1). The OTU M9 was isolated from all collection sites; however, it was dominant in Hokkaido but not Nagasaki isolates. The OTU M5 was dominant in Nagasaki but was not isolated in the Hokkaido sample. The PPFMs of most OTUs were isolated from different samples, but some OTUs (M2, M7, and M10) were isolated from one collection site. It is reported that site-specific factors have a stronger impact on *Methylobacterium* community composition than plant-specific factors (Knief et al. 2010). Our results also support this conclusion. Although it is not clear where the distribution and dominance of PPFM occur, it is reported that suitable growth temperatures differ among *Methylobacterium* species. The growth of most species occurs at 15°C-30°C. Some species such as *M. komagatae* and *M. salsuginis* cannot grow at extreme temperatures such as 4°C or 40°C (Kato et al. 2008, Wang et al. 2007); however, *M. marchantiae* can grow at 4°C (Schauer et al. 2011). Temperature may be one factor influencing the distribution of PPFM OTUs at different sites.

Genus *Methylobacterium* comprised only one species, *M. organophilum*, when approved lists of bacterial names were defined in 1980 (Patt et al. 1976, Skerman et al. 1980). However, novel species were proposed later, and 54 species including synonyms are currently validated. Many species were isolated from plants (Anda et al. 2011, Idris et al. 2006, Madhaiyan & Poonguzhali 2014, Mizuno et al. 2012, Schauer et al. 2011, Tani et al. 2012, Van Aken et al. 2004). We also previously detected *Methylobacterium* in leaves and stems of potato plants grown in the Hokkaido area. However, the distribution of *Methylobacterium* on leaves of potato plants cultivated at different field sites was not reported. Multiple species of *Methylobacterium* are distributed in tissues of plants such as soybean, rice, and barley (Anda et al. 2001, Lai et al. 2020, Tani et al. 2015). The OTUs M1, M2, M5, M6, M7, M8, M9, and M11—which were closely related to *M. goesingense*, *M. brachiatum*, *M. phyllostachyos*, *M. aerolatum*, *M. komagatae*, *M. thiocyanatum*, and *M. salsuginis*—were also isolated from plants (Fig. 2). The OTU M4, which was related to *M. hispanicum*, was isolated from drinking

Table 1. Phylogenetic distribution of operational taxonomic units (OTUs) of pink-pigmented facultative methylotrophs (PPFM) isolated from the leaf of potato plants cultivated in various locations across Japan[a]

	Collection site							
OTU	"Hokkaido"	"Yamagata"	"Ibaraki"	"Hiroshima"	"Nagasaki"	Closest known species[b]	Acc. No.	Identity (%)
M 1	9.2	-	1.1	-	-	*Methylobacterium goesingense*	NR_115219	99
M 2	5.1	-	-	-	-	*Methylobacterium marchantiae*	NR_116549	99
M 3	1.0	1.0	36.2	-	-	*Methylobacterium marchantiae*	NR_116549	99
M 4	-	5.0	1.1	-	-	*Methylobacterium hispanicum*	NR_112613	99
M 5	-	27.3	19.1	47.4	58.2	*Methylobacterium fujisawaense*	NR_112232	99
M 6	-	-	-	8.3	3.1	*Methylobacterium brachiatum*	NR_041032	99
M 7	-	-	-	-	7.1	*Methylobacterium aerolatum*	NR_044130	99
M 8	-	-	-	1.0	25.5	*Methylobacterium komagatae*	NR_041441	98
M 9	84.7	66.7	31.9	43.3	4.1	*Methylobacterium populi*	NR_074257	99
M10	-	-	8.5	-	-	*Methylobacterium suomiense*	NR_041030	99
M11	-	-	2.1	-	2.0	*Methylobacterium salsuginis*	NR_044038	98
Total	100	100	100	100	100			

[a] Relative abundance (%) of isolates belonging to each OTU
[b] The results of a pairwise BLAST analysis between a representative sequence and its closest type strain

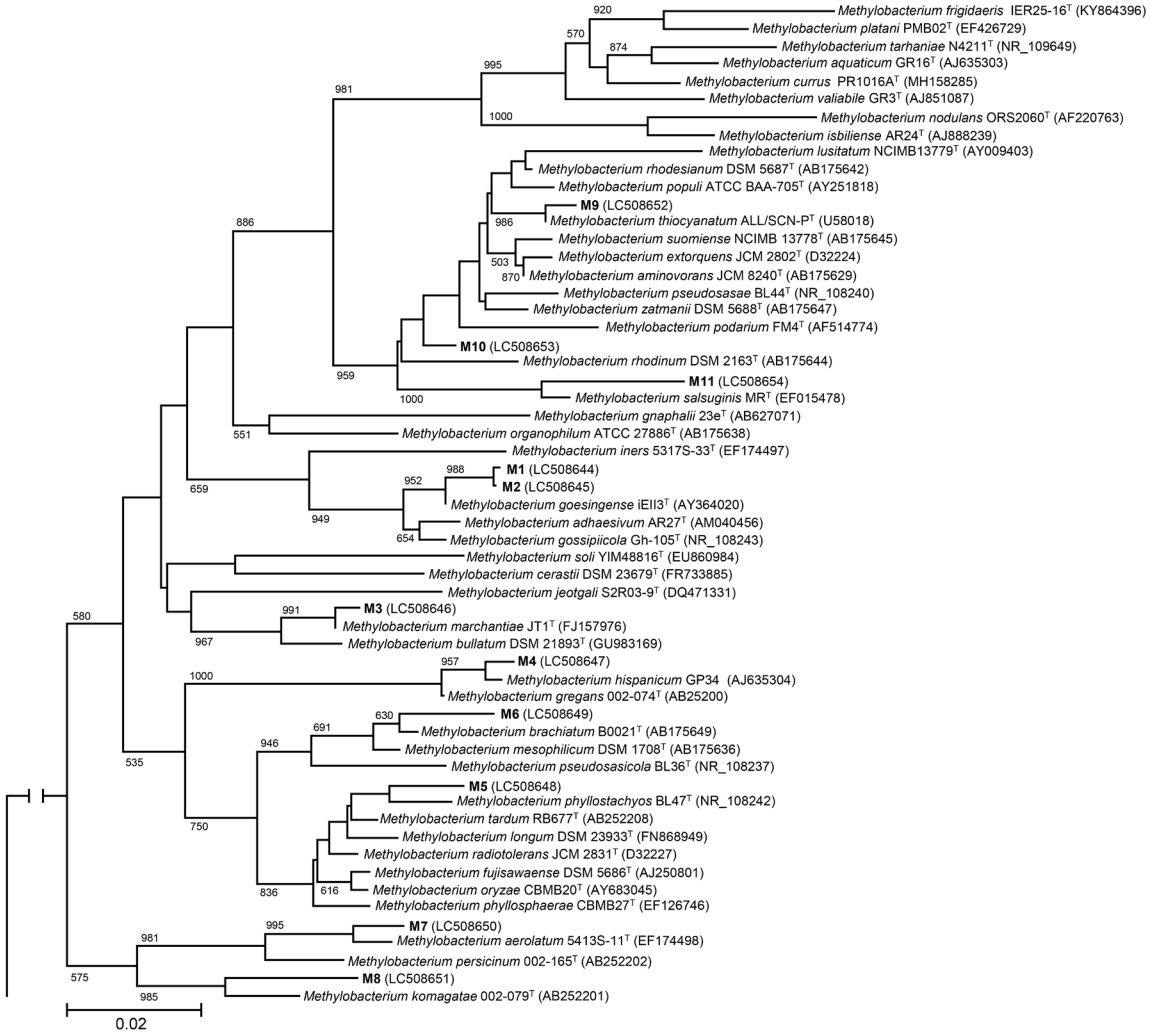

Fig. 2. Phylogenetic of 16S rRNA genes based on the representative sequences of operational taxonomic units (OTUs) for potato-associated *Methylobacterium* (M1-M11)
The tree was constructed using the neighbor-joining method. The scale represents 0.02 substitutions per site. Numbers at the nodes are proportions of 1,000 bootstrap resamplings, and values < 500 are not shown.

water (Wang et al. 2007). Our data revealed that many *Methylobacterium*, including unreported species from plant samples, also colonized potato leaves.

Methylobacterium is one of the dominant genera among plant symbionts and has various beneficial interactions, including carbon cycling, plant growth-promoting, and induction of disease resistance of host plants (Indiragandhi et al. 2008, Sakai & Yurimoto 2013). *Methylobacterium* were detected from potato leaves in our previous study (Someya et al. 2013), but their distribution and dominance were not determined in detail. Our present results revealed the distribution and dominant species of *Methylobacterium* from potato leaves grown at different field sites using the same seed tubers. In a further study, we will focus on the difference in ecological and functional roles of different *Methylobacterium* species with the host plants.

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