Analysis of 16S rRNA and mxaF genes revealing insights into Methylobacterium niche-specific plant association

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Abstract

The genus Methylobacterium comprises pink-pigmented facultative methylotrophic (PPFM) bacteria, known to be an important plant-associated bacterial group. Species of this group, described as plant-nodulating, have the dual capacity of producing cytokinin and enzymes, such as pectinase and cellulase, involved in systemic resistance induction and nitrogen fixation under specific plant environmental conditions. The aim hereby was to evaluate the phylogenetic distribution of Methylobacterium spp. isolates from different host plants. Thus, a comparative analysis between sequences from structural (16S rRNA) and functional mxaF (which codifies for a subunit of the enzyme methanol dehydrogenase) ubiquitous genes, was undertaken. Notably, some Methylobacterium spp. isolates are generalists through colonizing more than one host plant, whereas others are exclusively found in certain specific plant-species. Congruency between phylogeny and specific host inhabitance was higher in the mxaF gene than in the 16S rRNA, a possible indication of function-based selection in this niche. Therefore, in a first stage, plant colonization by Methylobacterium spp. could represent generalist behavior, possibly related to microbial competition and adaptation to a plant environment. Otherwise, niche-specific colonization is apparently impelled by the host plant.

Key words: phylogenetic diversity, methylotrophics, PPFM, plant-bacteria interaction.

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Introduction

The Methylobacterium genus, which belongs to the class Alphaproteobacteria, is described as pink-pigmented facultative methylotrophic (PPFM) bacteria. Interestingly, this bacterial group presents the ability to metabolize one-carbon compounds as carbon sources (Toyama et al., 1998; Skovran et al., 2010).

A wide variety of Methylobacterium species have been isolated from plants (Pirttilä et al., 2000; Sy et al., 2001; Araújo et al., 2002; Yates et al., 2007; Ferreira et al., 2008; Andreote et al., 2009; Madhaiyan et al., 2011), the soil (Cao et al., 2011), cold lands, such as Antarctica (Moosvi et al., 2005), and the bottom of the Kuroshio Knoll sea in Japan (Inagaki et al., 2004). On considering bacteria-plant association, it has been shown that this genus can establish a beneficial interaction with the hosts, by fixing nitrogen (Sy et al., 2001; Yates et al., 2007), producing cellulase (Jayashree et al., 2011), or interacting with other plant pathogens (Araújo et al., 2002, Lacava et al., 2004, Madhaiyan et al., 2006a, 2006b). Curiously, in spite of the specific capacity for synthesizing hydrolytic enzymes (i.e. pectinase and cellulose), as yet, PPFMs have not been described as plant-pathogens, thereby indicating their additional capacity of offering host plant protection by inducing
systemic resistance during the colonization process (Madhaiyan et al., 2006a, b). Additionally, a high level of PPFM inoculation can modulate the composition of the bacterial community associated with the host plant (Andréote et al., 2006), thereby implying that some competition may occur during this phase.

According to the most recent analysis, 34 species of the genus have been described to date (Kato et al., 2008; Weon et al., 2008; Madhaiyan et al., 2009), half of which (17) within the last five years, a clear indication that only a minor part of the diversity of this genus has been described so far. Thus, further studies of plant-associated members of the Methylobacterium genus will furnish additional knowledge on their distribution and ecology, thereby leading to research towards developing strains capable of enhancing plant fitness.

Since methylotrophic metabolism conferred by the mxaF gene is advantageous for Methylobacterium extorquens during plant colonization (Sy et al., 2005), it is plausible that the evolution of Methylobacterium-plant interaction has led to the selection of methylotrophic species/genotypes. Thus, in the present study, the genetic diversity of 60 Methylobacterium spp. strains obtained from eight different host plants was assessed by sequence analysis of 16S rRNA and mxaF genes, to facilitate comprehension of the distribution of the Methylobacterium species in various host plants.

Material and Methods

Strains of Methylobacterium spp. and plant-species origins

Endophytic bacterial isolates (Table 1), obtained from the collection culture of the Laboratory of Microbial Genetics (ESALQ/USP, Piracicaba, Brazil), were isolated from previous studies of surface-disinfested Citrus spp. (18 isolates) (Araújo et al., 2002), eucalyptus (Eucalyptus grandis x Eucalyptus urophylla) (7 isolates) (Ferreira et al., 2008), Saccharum spp. (8 isolates) (Rossetto, 2008, Doctoral thesis, Universidade de São Paulo, Piracicaba), Coffea arabica (8 isolates), Borreiera verticillata (12 isolates) and Capsicum annuum (7 isolates).

DNA extraction and sequencing methodology

After cultivation, bacterial DNA was extracted according to previously described methodology (Araújo et al., 2002). A partial sequence of the 16S rRNA gene (27-1401, according to Escherichia coli position) was amplified with the primers R1378 (Heuer et al., 1997) and P027F (Lane et al., 1985). PCRs were performed in 50 µL of a reaction containing 1 X enzyme buffer, 3.75 mM of MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.1U/µL of Taq DNA Polymerase (Invitrogen, Brazil). Initial denaturation was carried out at 94 °C for 4 min, followed by 35 thermal cycles of 30 s at 94 °C, 1 min at 62.5 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. Partial amplification of the mxaF gene was obtained with mxa1003f and mxa1561r primers (McDonald et al., 1995). All PCR amplification was checked through electrophoresis on agarose gel (1.5% w/v agarose) and UV visualization of the ethidium bromide stained gels, after which, PCR products were purified (PureLink, Invitrogen). The 16S rDNA fragments were sequenced using internal primers for both strains in an automated sequencer (MegaBACE 1000), whereas mxaF gene fragments were sequenced with two primers (mxa1003f and mxa1561r).

Sequence analysis

All the chromatograms were first trimmed for high quality bases (80% of bases with quality > 20) by means of Phred software and the trimmed sequences used for comparison in the Ribosomal Data Project (for 16S rRNA gene) and the GenBank database (nr/nt) (for the mxaF gene). The best hits of well-characterized strains of the Methylobacterium genus were retrieved from the databases, and subsequently used for alignment and phylogeny analysis with MEGA 4.0 version software (Tamura et al., 2007). Evolutionary history was inferred through the Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary distances were computed by the Kimura 2-parameter method (Kimura, 1980). All the sequences obtained here were assigned to operational taxonomic units (OTUs) using MOTHUR (Schloss et al., 2009), at the frequency of 97% sequence similarity. Furthermore, Venn diagrams were constructed for 16S rRNA and mxaF gene analysis to cross-compare and visualize the distribution of these OTUs in plant species.

Nucleotide sequence accession numbers

120 DNA sequences of partial 16S rRNA and mxaF genes were deposited in the GenBank database under accession numbers EU789466 to EU789518 and EU789406 to EU789465, respectively.

Results

Phylogenetic analysis was carried out with partial 16S rRNA and partial mxaF gene sequences from isolates obtained in both the present study and from the GenBank and RDP databases. In the present study, phylogeny based on the 16S rRNA partial gene sequence with V6 and V7 regions generated 7 groups (Figure 1 and Table 1). Of these, group 1 presented only one eucalyptus isolate, similar to sequences from M. ishiiense and M. nodulans, whereas group 7, comprised of isolates obtained from all the hosts used here, was similar to those from M. radiotolerans. The other groups (2, 3, 4, 5 and 6) consisted of isolates from two to four different hosts. Although group 7 was close to M. radiotolerans, analysis revealed certain isolates, such as R2E, SR1.6/2, AW06, MC3-1, SR1.6/9, F4, F10, F11 and R10E, to be divergent from the main group, thus possibly
indicating the occurrence of species, as yet not described for this genus.

Congruency between the 16S rRNA and mxaF phylogenetical trees was incomplete. Comparative analysis of mxaF partial gene sequences by BLASTn against the nr/nt database at GenBank, classified most isolates as “uncultured methylotrophic bacterium or Methylobacterium sp.” (Table 1 and Figure 2). This was a possible outcome of the limited number of mxaF sequences available in the database. In addition, phylogenetic analysis with the mxaF gene sequences also revealed the formation of seven groups (Figure 2). Groups I, II, III and IV presented isolates from two or three hosts, groups IV and V only from citrus and group VI mainly from B. verticillata (except for TP7 and MC3-1). On the other hand, group VII contained isolates from all the hosts, with the exception of B. verticillata.

We observed that the clusters obtained by mxaF gene sequence analysis, revealed a certain association with host plants, since isolates from B. verticillata were located in group VII, those from sugarcane mainly in group VI (only two belonged to groups I and III), those from eucalyptus mainly in group VII (only two in group III), and those from

### Table 1 - Identification of Methylobacterium spp. isolated from different hosts by the partial sequence of the 16S rRNA and mxaF genes.

| Isolate | Host      | Identification* | Phenylogenetic groups |
|---------|-----------|-----------------|-----------------------|
|         |           | 16S rRNA | mxaF   |                           |                          |
| TC3-5   | Coffee    | *M. populi* | 4 | II   |
| TC3-6   | Coffee    | Methylobacterium sp. | 4 | II   |
| TC3-7   | Coffee    | Methylobacterium sp. | 5 | VII  |
| TC3-10  | Coffee    | Methylobacterium sp. | 5 | VII  |
| TC3-11  | Coffee    | M. extorquens  | 4 | II   |
| TC3-13  | Coffee    | Methylobacterium sp. | 5 | VII  |
| MC3-1   | Coffee    | Methylobacterium sp. | 7 | VI   |
| F4      | Sugarcane | Methylobacterium sp. | 7 | VII  |
| F5      | Sugarcane | *M. fujisawaense* | 3 | VII  |
| F7      | Sugarcane | Methylobacterium sp. | 5 | VII  |
| F8      | Sugarcane | Methylobacterium sp. | 5 | VII  |
| F9      | Sugarcane | Methylobacterium sp. | 6 | VII  |
| F10     | Sugarcane | Methylobacterium sp. | 7 | VII  |
| F11     | Sugarcane | Methylobacterium sp. | 7 | VII  |
| D5      | Sugarcane | Methylobacterium sp. | 5 | VII  |
| AR1.6/1 | Citrus    | Methylobacterium sp. | 6 | VII  |
| AR1.6/2 | Citrus    | Methylobacterium sp. | 4 | II   |
| AR1.6/8 | Citrus    | Methylobacterium sp. | 4 | II   |
| AR5/1   | Citrus    | Methylobacterium sp. | 5 | II   |
| AR5.1/5 | Citrus    | Methylobacterium sp. | 6 | VII  |
| ER1/21  | Citrus    | *M. mesophilicum* | 5 | III  |
| ER1.6/2 | Citrus    | Methylobacterium sp. | 4 | V    |
| SR1.6/2 | Citrus    | Methylobacterium sp. | 7 | V    |
| SR1.6/4 | Citrus    | *M. radiotolerans* | 7 | VI   |
| SR1.6/6 | Citrus    | Methylobacterium sp. | 5 | III  |
| SR1.6/9 | Citrus    | Methylobacterium sp. | 7 | VII  |
| SR1.6/13| Citrus    | Methylobacterium sp. | 4 | II   |
| SR3/27  | Citrus    | Methylobacterium sp. | 3 | II   |
| SR5/3   | Citrus    | *M. fujisawaense* | 3 | IV   |
| SR5/4   | Citrus    | *M. fujisawaense* | 3 | II   |
| PR1/3   | Citrus    | *M. mesophilicum* | 5 | III  |
| PR3/10  | Citrus    | Methylobacterium sp. | 5 | III  |
| PR3/11  | Citrus    | Methylobacterium sp. | 5 | IV   |
| TP2-1   | Sweet pepper | *M. fujisawaense* | 4 | VII  |
| TP4-1   | Sweet pepper | Methylobacterium sp. | 4 | II   |
| TP4-3   | Sweet pepper | Methylobacterium sp. | 7 | VI   |
| TP4-4   | Sweet pepper | Methylobacterium sp. | 7 | VI   |
| TP4-5   | Sweet pepper | Methylobacterium sp. | 7 | VI   |
| TP4-6   | Sweet pepper | Methylobacterium sp. | 7 | VI   |
| TP4-7   | Sweet pepper | Methylobacterium sp. | 7 | VI   |
| TP4-8   | Sweet pepper | Methylobacterium sp. | 7 | VI   |

*Identification based on the RDP database (http://simo.marsci.uga.edu/public_db/rdp_query.htm) and phylogenetic analysis in this study (Figure 1).
sweet pepper mainly in group I (three in groups II, VI and VII). However, the bacterial population isolated from citrus plants was found in four of the seven groups (II, IV, V, VII).

This was confirmed by a Venn diagram, obtained using 97% similarity in 16S rRNA gene sequences (Figure 3a). The analysis showed that 74% (20) of OTUs were found to be exclusive to one host plant (six to B. verticillata, four to citrus, three to sweet pepper, three to coffee, two to eucalyptus, and two to sugarcane). Additionally, only 26% (7) of OTUs were found in two host plants, and only one in four. A similar analysis, using mxaF gene sequences (Figure 3b), revealed 13 OTUs, of which, 61.5% (eight) were exclusive to only one host plant, and 38.5% (5) to two.

Discussion

The genus Methylobacterium is commonly found in natural environments, such as soil, air, dust, ocean and lake waters, and sediments, as well as urban environments (Van Aken et al., 2004). A remarkable niche of this group is its association with plants, where it is capable of colonizing leaf surfaces (Chanprame et al., 1996; Madhaiyan et al., 2011), inner tissues (Pirttilä et al., 2000; Araújo et al., 2001, 2002; Andreote et al., 2006; Yates et al., 2007), and nodules (Sy et al., 2001; Yates et al., 2007). These features could possibly have arisen from an intimate co-evolution process between Methylobacterium spp. and host plants. An example of this co-evolutionary process is the bacterial capacity to mediate high photosynthetic activity in the host, by the induction of a higher number of stomata, increased chlorophyll concentration and greater amount of malic acid (Cervantes-Martinez et al., 2004). Moreover, mxaF gene associated with methylotrophic metabolism is responsible for increasing M. extorquens fitness during plant epiphytic colonization under competitive conditions (Sy et al., 2005). All together, it is assumed that plants are the main niche for assessing the diversity of the genus Methylobacterium.

As diversity in the genus Methylobacterium has not been fully explored, e.g. 17 new species of Methylobacterium were only described quite recently (Gallego et al., 2005a, b, 2006; Aslam et al., 2007; Kang et al., 2007; Madhaiyan et al., 2007; Wang et al., 2007; Kato et al., 2008; Weon et al., 2008), the present study constitutes a significant contribution to the description of diversity in this ubiquitous bacterial group.

The mxaF phylogeny analysis suggests the role of plant species in the selection of Methylobacterium species for establishing an endophytic interaction. As previously de-
scribed, epiphytic colonization is the first stage towards developing such an association (Andreote et al., 2006). Under like circumstances, the methylotrophic metabolism state is advantageous for *M. extorquens* under competitive conditions (Sy et al., 2005). This advantage is associated to the ability to use, as a carbon source, methanol produced during plant-growth. However, some isolates affiliated by 16S rRNA genes to the *Methylobacterium* genus, through not having *mxaF* genes, were incapable of colonizing or nodulating *Lotononis* spp. (Ardley et al., 2009), thereby implying that the capacity to use methanol produced by the plant itself is an important characteristic determining selection.

All the groups containing isolates from two or more different hosts (except group 1, with only one isolate) show...
species ability in colonizing various hosts. Thus, the host plant is not able to completely select the bacterial genotypes. Controversially, *Borreria verticillata* isolates were found mainly in group 7 (except for two isolates in group 2), thus indicating that part of *Methylobacterium* spp. diversity inside the host plant could be determined by specific association, although random events may occur.

Notably, all the isolates observed in group I (from mxaF phylogeny) are present in group 2 (16S rRNA phylogeny), whereas isolates in group VI (mxaF phylogeny) are so in group 7 (16S rRNA phylogeny). However, exceptions occurred, such as eventual changes in positioning. On comparing the two phylogenetic trees, this variable allocation could be attributed to (i) ecological differentiation of the isolate in the environment where it develops (Konstantinidis et al., 2006), or (ii) the occurrence of horizontal gene transfer (HGT) (Heyer et al., 2002).

The results obtained in the present work show the genetic diversity of the *Methylobacterium* spp. community associated with plants, with the inference that this specific diversity inside the host plant could be impelled not only by the host plant itself, but also by the generalist behavior of some strains for using certain plant compounds, such as alcohols produced during plant metabolism. If so, *B. verticillata* is the strongest plant species when selecting *Methylobacterium* spp. endophytes. It can also be concluded that it is possible to acquire additional knowledge on *Methylobacterium* spp. phylogeny through studies using distinct plant species. In summary, it is assumed that, although, in a first step of plant colonization, the generalist behavior of *Methylobacterium* species plays a pivotal role in niche occupation, afterwards, niche-specific-association may be driven by the host plant.

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