



Analysis of 16S rRNA and *mxoF* 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 *mxoF* (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 inhabitation was higher in the *mxoF* 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, methylotrophs, 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). 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 Kuroshima 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 cellulase), as yet, PPFMs have not been described as plant-pathogens, thereby indicating their additional capacity of offering host plant protection by inducing

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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 (Andreote *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 *mxoF* 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 *mxoF* genes, to so 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 culture collection 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), *Borreria 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 *E. 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 0.1 IU/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 *mxoF* gene was obtained with *mxo1003f* and *mxo1561r* 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 *mxoF* gene fragments were sequenced with two primers (*mxo1003f* and *mxo1561r*).

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 *mxoF* 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 *mxoF* 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 *mxoF* 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 *mxoF* 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. isbiliense* 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

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

Isolate	Host	Identification*	Phylogenetic groups		Isolate	Host	Identification*	Phylogenetic groups	
			16 S <i>rRNA</i>	<i>mxoF</i>				16 S <i>rRNA</i>	<i>mxoF</i>
TC3-5	Coffee	<i>M. populi</i>	4	II	TP4-2	Sweet pepper	<i>M. hispanicum</i>	2	I
TC3-6	Coffee	<i>Methylobacterium</i> sp.	4	II	TP5	Sweet pepper	<i>M. hispanicum</i>	2	I
TC3-7	Coffee	<i>Methylobacterium</i> sp.	5	VII	TP7	Sweet pepper	Uncultured methylo-trophic bacterium	7	VI
TC3-10	Coffee	<i>Methylobacterium</i> sp..	5	VII	TP8	Sweet pepper	<i>M. hispanicum</i>	2	V
TC3-11	Coffee	<i>Methylobacterium</i> sp.	5	VII	MP2-3	Sweet pepper	<i>M. hispanicum</i>	2	V
TC3-13	Coffee	<i>M. extorquens</i>	4	II	Aw04	<i>Borreria verticillata</i>	<i>Methylobacterium</i> sp.	2	III
TC3-14	Coffee	<i>Methylobacterium</i> sp.	5	VII	Aw05	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
MC3-1	Coffee	<i>Methylobacterium</i> sp.	7	VI	Aw06	<i>Borreria verticillata</i>	<i>Methylobacterium</i> sp.	7	VI
F4	Sugarcane	<i>Methylobacterium</i> sp.	7	VII	Aw08	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F5	Sugarcane	<i>M. fujisawaense</i>	3	VII	Aw09	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F7	Sugarcane	<i>Methylobacterium</i> sp.	5	VII	Aw10	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F8	Sugarcane	<i>Methylobacterium</i> sp.	5	VII	Aw11	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F9	Sugarcane	<i>Methylobacterium</i> sp.	6	VII	Aw12	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F10	Sugarcane	<i>Methylobacterium</i> sp.	7	VII	Aw13	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
F11	Sugarcane	<i>Methylobacterium</i> sp.	7	VII	Aw15	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
D5	Sugarcane	<i>Methylobacterium</i> sp.	5	VII	Aw16	<i>Borreria verticillata</i>	<i>M. hispanicum</i>	2	I
AR1.6/1	Citrus	<i>Methylobacterium</i> sp.	6	VII	Aw18	<i>Borreria verticillata</i>	<i>M. radiotolerans</i>	7	VI
AR1.6/2	Citrus	<i>Methylobacterium</i> sp.	4	II	R1E	Eucalyptus	<i>Methylobacterium</i> spp.	3	III
AR1.6/8	Citrus	<i>Methylobacterium</i> sp.	4	II	R2E	Eucalyptus	<i>Methylobacterium</i> spp.	7	III
AR5/1	Citrus	<i>Methylobacterium</i> sp.	5	II	R3E	Eucalyptus	<i>Methylobacterium</i> spp.	1	VII
AR5.1/5	Citrus	<i>Methylobacterium</i> sp.	6	VII	R10E	Eucalyptus	<i>Methylobacterium</i> spp.	7	VII
ER1/21	Citrus	<i>M. mesophilicum</i>	5	III	R12E	Eucalyptus	<i>Methylobacterium</i> spp.	6	VII
ER1.6/2	Citrus	<i>Methylobacterium</i> sp.	4	V	R14E	Eucalyptus	<i>Methylobacterium</i> spp.	5	VII
SR1.6/2	Citrus	<i>Methylobacterium</i> sp.	7	V	R16E	Eucalyptus	<i>Methylobacterium</i> spp.	3	VII
SR1.6/4	Citrus	<i>M. radiotolerans</i>	7	VI					
SR1.6/6	Citrus	<i>Methylobacterium</i> sp.	5	III					
SR1.6/9	Citrus	<i>Methylobacterium</i> sp.	7	VII					
SR1.6/13	Citrus	<i>Methylobacterium</i> sp.	4	II					
SR3/27	Citrus	<i>Methylobacterium</i> sp.	3	II					
SR5/3	Citrus	<i>M. fujisawaense</i>	3	IV					
SR5/4	Citrus	<i>M. fujisawaense</i>	3	II					
PR1/3	Citrus	<i>M. mesophilicum</i>	5	III					
PR3/10	Citrus	<i>Methylobacterium</i> sp.	5	III					
PR3/11	Citrus	<i>Methylobacterium</i> sp.	5	IV					
TP2-1	Sweet pepper	<i>M. fujisawaense</i>	4	VII					
TP4-1	Sweet pepper	<i>Methylobacterium</i> sp.	4	II					

*Identification based on the RDP database (http://simo.marisci.uga.edu/public_db/rdp_query.htm) and phylogenetic analysis in this study (Figure 1).

indicating the occurrence of species, as yet not described for this genus.

Congruency between the 16S *rRNA* and *mxoF* phylogenetical trees was incomplete. Comparative analysis of *mxoF* partial gene sequences by BLASTn against the nr/nt database at GenBank, classified most isolates as “uncultured methylo-trophic bacterium or *Methylobacterium* sp.” (Table 1 and Figure 2). This was a possible outcome of the limited number of *mxoF* sequences available in the database. In addition, phylogenetic analysis with the *mxoF* 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 *mxoF* 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

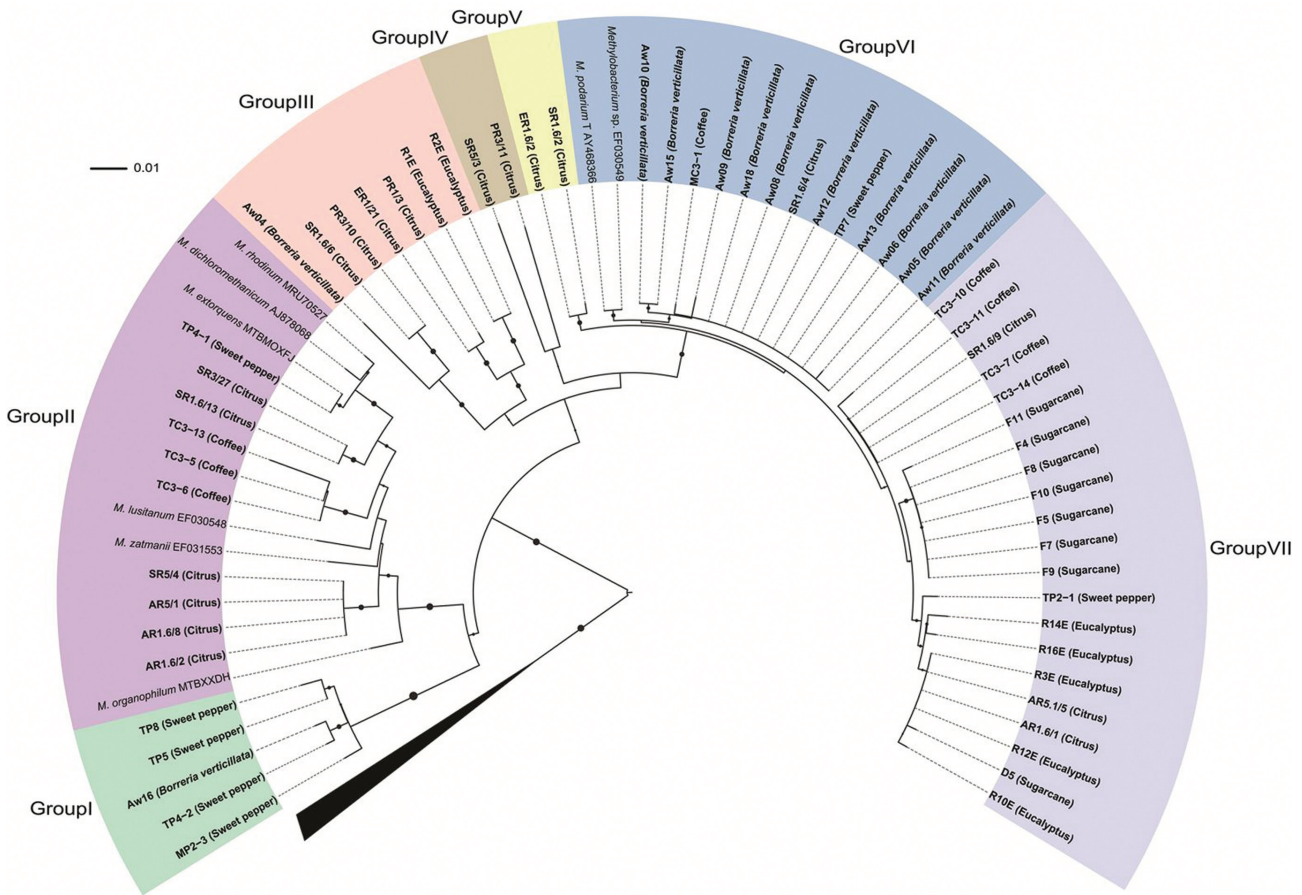


Figure 2 - Phylogenetic analysis of the *mxoF* gene. Bootstrap values (1000 repetitions) above 50% are represented by solid circles next to tree branches. There were 423 nucleotide positions in the final dataset. *Beijerinckia mobilis*, *Methylocella silvestris* and *Methylosinus trichosporium* were used as outgroup. The layout of trees was designed using the online application “Interactive Tree Of Life” (iTOL) (<http://itol.embl.de/>).

scribed, epiphytic colonization is the first stage towards developing such an association (Andreote *et al.*, 2006). Under like circumstances, the methylophilic 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 *mxoF* 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

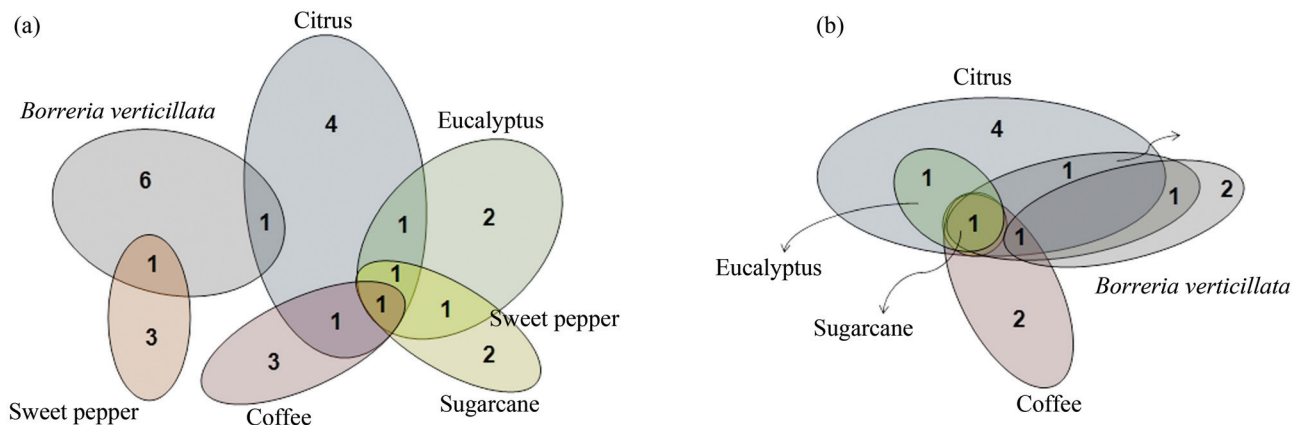


Figure 3 - Venn diagrams of operational taxonomic units (OTUs) assigned at 97% sequence similarity. (a) Venn diagram for 16S *rRNA* gene analysis with 27 OTUs, and (b) Venn diagram for *mxoF* gene analysis with 13 OTUs.

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 *mxoF* phylogeny) are present in group 2 (16S *rRNA* phylogeny), whereas isolates in group VI (*mxoF* 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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