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Research Article

Analysis of 16S rRNA and *mxa*F 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 *mxa*F (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 *mxa*F 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, methylotrofics, 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 cellulose), 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 genushas 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 *mxa*F 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 urophyla*) (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 $MgCl_2$, 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 *mxa*F gene fragments were sequenced with two primers (mxa1003f and mxa1561r).

Sequence analysis

All the cromatograms 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 *mxa*F 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 *mxa*F 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 *mxaF* genes.

Isolate	Host	Identification*	Phylogenetic groups	
			16 S *rRNA*	*mxa*F
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	*Methylobacterium sp.*	5	VII
TC3-13	Coffee	*M. extorquens*	4	II
TC3-14	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
TP4-2	Sweet pepper	*M. hispanicum*	2	I
TP5	Sweet pepper	*M. hispanicum*	2	I
TP7	Sweet pepper	*Uncultured methylotrophic bacterium*	7	VI
TP8	Sweet pepper	*M. hispanicum*	2	V
MP2-3	Sweet pepper	*M. hispanicum*	2	V
Aw04	*Borreria verticillata*	*Methylobacterium sp.*	2	III
Aw05	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw06	*Borreria verticillata*	*Methylobacterium sp.*	7	VI
Aw08	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw09	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw10	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw11	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw12	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw13	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw15	*Borreria verticillata*	*M. radiotolerans*	7	VI
Aw16	*Borreria verticillata*	*M. hispanicum*	2	I
Aw18	*Borreria verticillata*	*M. radiotolerans*	7	VI
R1E	Eucalyptus	*Methylobacterium spp.*	3	III
R2E	Eucalyptus	*Methylobacterium spp.*	7	III
R3E	Eucalyptus	*Methylobacterium spp.*	1	VII
R10E	Eucalyptus	*Methylobacterium spp.*	7	VII
R12E	Eucalyptus	*Methylobacterium spp*	6	VII
R14E	Eucalyptus	*Methylobacterium spp.*	5	VII
R16E	Eucalyptus	*Methylobacterium spp.*	3	VII

*Identification based on the RDP database (http://simo.marsci.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 *mxa*F phylogenetical trees was incomplete. Comparative analysis of *mxa*F 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

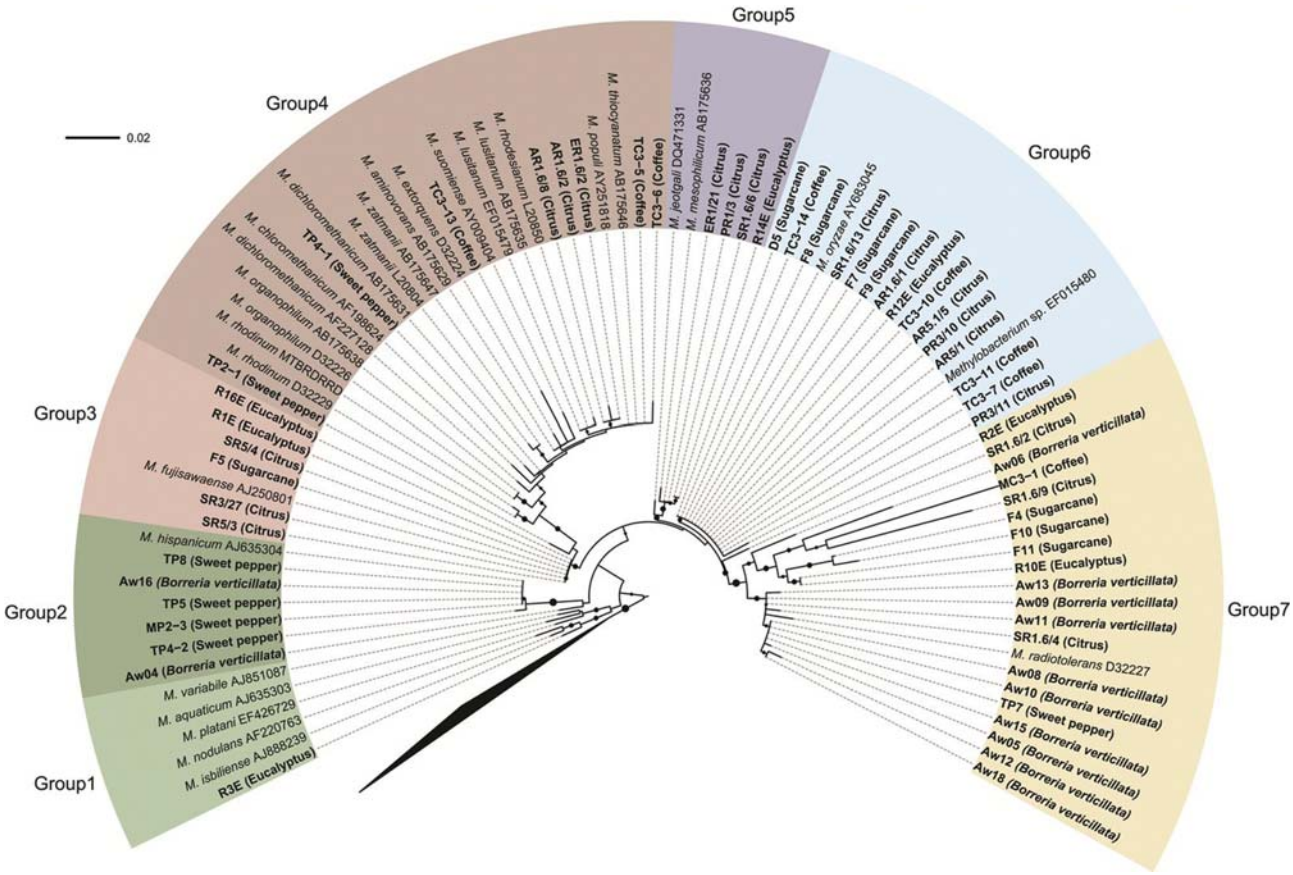

Figure 1 - Phylogenetic analysis of the 16S *rRNA* gene. Bootstrap values (1000 repetitions) above 50% are represented by solid circles next to tree branches. There were 642 nucleotide positions in the final dataset. *Beijerinckia mobilis, Methylocella silvestris* and *Methylosinus trichosporium* were used as outgroup. The layout of trees was designed using the online application "Interactive Tree Of Life" (iTOL) (http://itol.embl.de/).

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. verticilata*, 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-evolutive 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 *mxa*F phylogeny analysis suggests the role of plant species in the selection of *Methylobacterium* species for establishing an endophytic interaction. As previously de-

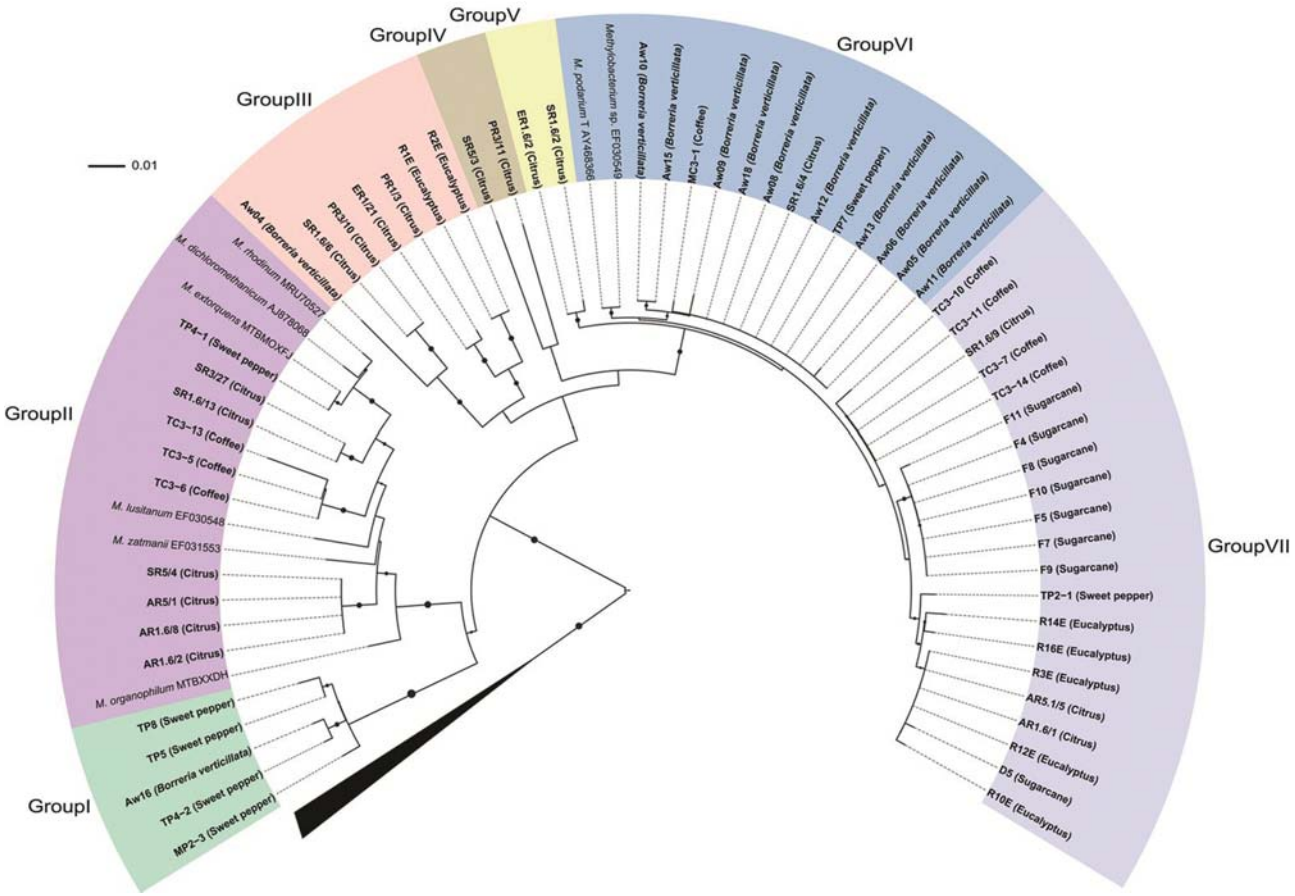

Figure 2 - Phylogenetic analysis of the *mxaF* gene. Bootstrap values (1000 repetitions) above 50% are represented by solid circles next to tree branches. There were 423 nucleotide positions in the final dataset. *Beijerinckia mobilis, Methylocella silvestris* and *Methylosinus trichosporium* were used 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 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

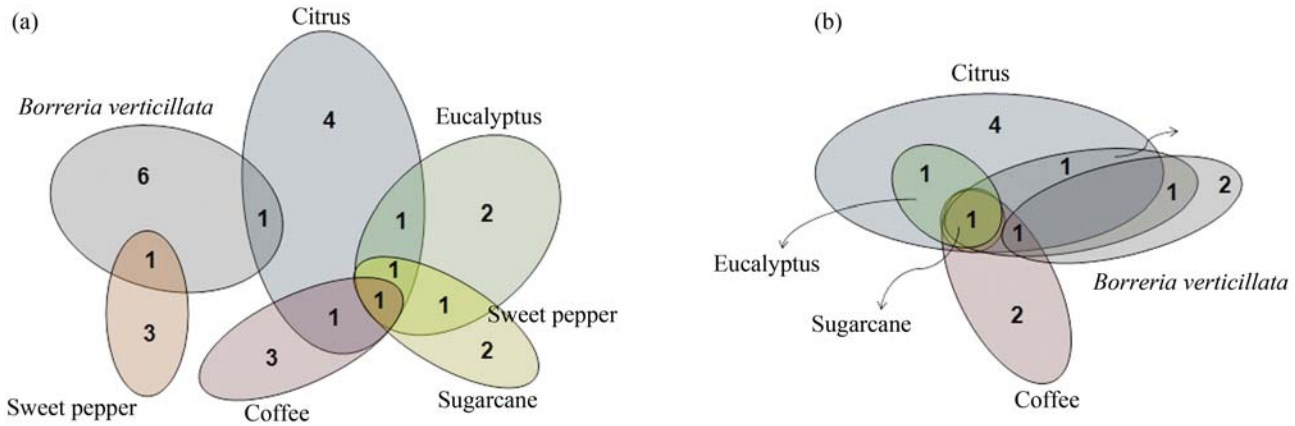

Figure 3 - Venn diagrams of operational taxonomic units (OTUs) assigned at 97% sequence similarity. (a) Venn diagram for 16S *rRNA* gene analysis with 27 OTUs, and (b) Venn diagram for *mxaF* gene analysis with 13 OTUs.

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 *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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References

Andreote FD, Lacava PT, Gai CS, Araújo WL, Maccheroni Jr. W, van Overbeek LS, van Elsas JD and Azevedo JL (2006) Model plants for studying the interaction between *Methylobacterium mesophilicum* and *Xylella fastidiosa*. Can J Microbiol 52:419-426.

Andreote FD, Carneiro RT, Salles JF, Marcon J, Labate CA, Azevedo JL and Araújo WL (2009) Culture-independent assessment of Rhizobiales-related Alphaproteobacteria and the diversity of *Methylobacterium* in the rhizosphere and rhizoplane of transgenic eucalyptus. Microbial Ecol 57:82-93.

Araújo WL, Saridakis HO, Barroso PAV, Aguilar-Vildoso CI and Azevedo JL (2001) Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can J Microbiol 47:229-236.

Araújo WL, Marcon J, Maccheroni Jr. W, van Elsas JD and Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plant. Appl Environ Microbiol 68:4906-4914.

Ardley JK, O'Hara GW, Reeve WG, Yates RJ, Dilworth MJ, Tiwari RP and Howieson JG (2009) Root nodule bacteria isolated from South African *Lotononis bainesii*, *L. listii* and *L. solitudinis* are species of *Methylobacterium* that are unable to utilize methanol. Arch Microbiol 191:311-318.

Aslam Z, Lee CS, Kim KH, Im WT, Ten LN and Lee ST (2007) *Methylobacterium jeotgali* sp. nov., a non-pigmented, facultatively methylotrophic bacterium isolated from jeotgal, a traditional Korean fermented seafood. Int J Syst Evol Microbiol 57:566-571.

Cao YR, Wang Q, Jin RX, Tang SK, Jiang Y, He WX, Lai HX, Xu LH and Jiang CL (2011) *Methylobacterium soli* sp. nov. a methanol-utilizing bacterium isolated from the forest soil. Antonie Van Leeuwenhoek 99:629-634.

Cervantes-Martinez J, Lopez-Diaz S and Rodriguez-Garay B (2004) Detection of the effects of *Methylobacterium* in *Agave tequilana* Weber var. azul by laser-induced fluorescence. Plant Sci 166:889-892.

Chanprame S, Todd JJ and Widholm JM (1996) Prevention of pink-pigmented methylotrophic bacteria (*Methylobacterium mesophilicum*) contamination of plant tissues cultures. Plant Cell Rep 16:222-225.

Ferreira A, Quecine MC, Lacava PT, Oda S, Azevedo JL and Araújo WL (2008) Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by *Pantoea agglomerans.* FEMS Microbiol Lett 287:8-14.

Gallego V, Garcia MT and Ventosa A (2005a) *Methylobacterium hispanicum* sp. nov. and *Methylobacterium aquaticum* sp. nov., isolated from drinking water. Int J Syst Evol Microbiol 55:281-287.

Gallego V, Garcia MT and Ventosa A (2005b) *Methylobacterium isbiliense* sp. nov., isolated from the drinking water system of Sevilla, Spain. Int J Syst Evol Microbiol 55:2333-2337.

Gallego V, Garcia MT and Ventosa A (2006) *Methylobacterium adhaesivum* sp. nov., a methylotrophic bacterium isolated from drinking water. Int J Syst Evol Microbiol 56:339-342.

Heuer H, Krsek M, Baker P, Smalla K and Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S *rRNA* and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233-3241.

Heyer J, Galchenko VF and Dunfield PF (2002) Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. Microbiology 148:2831-2846.

Inagaki F, Tsunogai U, Suzuki M, Kosaka A, Machiyama H, Takai K, Nunoura T, Nealson KH and Horikoshi K (2004) Characterization of C_1-metabolizing prokaryotic communities in methane seep habitats at the Kuroshima Knoll, Southern Ryukyu Arc, by analyzing *pmoA*, *mmoX*, *mxaF*, *mcrA* and 16S *rRNA* genes. Appl Environ Microbiol 70:7445-7455.

Jayashree S, Lalitha R, Vadivukkarasi P, Kato Y and Seshadri S (2011) Cellulase production by pink pigmented facultative methylotrophic strains (PPFMs). Appl Biochem Biotechnol 164:666-680.

Kang YS, Kim J, Shin HD, Nam YD, Bae JW, Jeon CO and Park W (2007) *Methylobacterium platani* sp. nov., isolated from

a leaf of the tree *Platanus orientalis*. Int J Syst Evol Microbiol 57:2849-2853.

Kato Y, Asahara M, Goto K, Kasai H and Yokota A (2008) *Methylobacterium persicinum* sp. nov., *Methylobacterium komagatae* sp. nov., *Methylobacterium brachiatum* sp. nov., *Methylobacterium tardum* sp. nov. and *Methylobacterium gregans* sp. nov., isolated from freshwater. Int J Syst Evol Microbiol 58:1134-1141.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-120.

Konstantinidis KT, Ramette A and Tiedje JM (2006) Toward a more robust assessment of intraspecific diversity, using fewer genetic markers. Appl Environ Microbiol 72:7286-7293.

Lacava PT, Araújo WL, Marcon J, Maccheroni Jr W and Azevedo JL (2004) Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria *Xylella fastidiosa*, casual agent of Citrus Variegated Chlorosis. Lett Appl Microbiol 39:55-59.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML and Pace NR (1985) Rapid-determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955-6959.

Liu Z, Lozupone C, Hamady M, Bushman FD and Knight R (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Res 35:120-130.

Madhaiyan M, Poonguzhali S, Sundaram SP and Sa TM (2006a) A new insight into foliar applied methanol influencing phylloplane methylotrophic dynamics and growth promotion of cotton (*Gossypium hirsutum* L.) and sugarcane (*Saccharum officinarum* L.). Environ Exp Bot 57:168-176.

Madhaiyan M, Reddy BVS, Anandham R, Senthilkumar M, Poonguzhali S, Sundaram SP and Sa TM (2006b) Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. Curr Microbiol 53:270-276.

Madhaiyan M, Kim BY, Poonguzhali S, Kwon SW, Song MH, Ryu JH, Go SJ, Koo BS and Sa TM (2007) *Methylobacterium oryzae* sp. nov., an aerobic, pink-pigmented, facultatively methylotrophic, 1-aminocyclopropane-1-carboxylate deaminase-producing bacterium isolated from rice. Int J Syst Evol Microbiol 57:326-331.

Madhaiyan M, Poonguzhali S, Kwon SW and Sa TM (2009) *Methylobacterium phyllosphaerae* sp. nov., a pink-pigmented, facultative methylotroph from the phyllosphere of rice. Int J Syst Evol Microbiol 59:22-27.

Madhaiyan M, Poonguzhali S, Senthilkumar M, Lee JS and Lee KC (2011) *Methylobacterium gossipiicola* sp. nov., a pink-pigmented facultative methylotrophic bacteria isolated from cotton phyllosphere. Int J Syst Evol Microbiol (Epub).

McDonald IR, Kenna EM and Murrell JC (1995) Detection of methanotrofic bacteria in environmental samples with the PCR. Appl Environ Microbiol 61:116-121.

Moosvi SA, Mcdonald IR, Pearce DA, Kelly DP and Wood AP (2005) Molecular detection and isolation from Antarctica of methylotrophic bacteria able to grow with methylated sulfur compounds. Syst Appl Microbiol 28:541-554.

Pirttilä AM, Laukkane H, Pospiech H, Myllylã R and Hohtola A (2000) Detection of intracellular bacteria in buds of scoth pine (*Pinus sylvestris* L.) by *in situ* hybridization. Appl Environ Microbiol 66:3037-3077.

Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, *et al.* (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541.

Skovran E, Crowther GJ, Guo X, Yang S and Lidstrom ME (2010) A systems biology approach uncovers cellular strategies used by *Methylobacterium extorquens* AM1 during the switch from multi-to single-carbon growth. PLoS One 24:e14091.

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Bivin-Masson C, *et al.* (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214-220.

Sy A, Timmers ACJ, Knief C and Vorholt JA (2005) Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. Appl Environ Microbiol 71:7245-7252.

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software ver. 4.0. Mol Biol Evol 24:1596-1599.

Toyama H, Anthony C and Lidstrom ME (1998) Construction of insertion and deletion *mxa* mutants of *Methylobacterium extorquens* AM1 by electroporation. FEMS Microbiol Lett 166:1-7.

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.

Weon HY, Kim BY, Joa JH, Son JA, Song MH, Kwon SW, Go SJ and Yoon SH (2008) *Methylobacterium iners* sp. nov. and *Methylobacterium aerolatum* sp. nov., isolated from air samples in Korea. Int J Syst Evol Microbiol 58:93-96.

Yates RJ, Howieson JG, Reeve WG, Nandasena KG, Law IJ, Bra UL, Ardley JK, Nistelberger HM, Real D and O'Hara GW (2007) *Lotononis angolensis* forms nitrogen fixing, lupinoid nodules with phylogenetically unique, fast-growing, pink-pigmented bacteria, which do not nodulate *L. bainesii* or *L. listii*. Soil Biol Biochem 39:1680-1688.

Van Aken B, Peres CM, Doty SL, Yoon JM and Schnoor JL (2004) *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, metane-utilizing bacterium isolated from poplar trees (*Populus deltoids* x *nigra* DN34). Microbiology 54:1191-1196.

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