TRANSMISSION OF *METHYLOBACTERIUM MESOPHILICUM* BY *BUCEPHALOGONIA XANTHOPHIS* FOR PARATRANSGENIC CONTROL STRATEGY OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

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ABSTRACT

Methylobacterium mesophilicum, originally isolated as an endophytic bacterium from citrus plants, was genetically transformed to express green fluorescent protein (GFP). The GFP-labeled strain of *M. mesophilicum* was inoculated into *Catharanthus roseus* (model plant) seedlings and further observed colonizing its xylem vessels. The transmission of this endophyte by *Bucephalogonia xanthophis*, one of the insect vectors that transmit *Xylella fastidiosa (Xf)* subsp. *pauca*, was verified by insects feeding from fluids containing the GFP bacterium followed by transmission to plants and isolating the endophyte from *C. roseus* plants. Forty-five days after inoculation, the plants exhibited endophytic colonization by *M. mesophilicum*, confirming this bacterium as a nonpathogenic, xylem-associated endophyte. Our data demonstrate that *M. mesophilicum* not only occupy the same niche of *Xf* subsp. *pauca* inside plants but also may be transmitted by *B. xanthophis*. The transmission, colonization, and genetic manipulation of *M. mesophilicum* is a prerequisite to examining the potential use of symbiotic control to interrupt the transmission of *Xf* subsp. *pauca*, the bacterial pathogen causing citrus variegated chlorosis by insect vectors.

LAYPERSON SUMMARY

We report the localization of the endophytic bacterium, *M. mesophilicum*, in *Catharanthus roseus* model plant and the transmission of this endophyte by *Bucephalogonia xanthophis.* The results indicated that *M. mesophilicum* as a good candidate for a symbiotic control strategy to reduce the spread of *Xylella fastidiosa* (*Xf*) subsp. *pauca*. The transgenic endophytic *M. mesophilicum* tested has most of the prerequisites, for a successful strategy using paratransgenesis, that is, *M. mesophilicum* that colonize citrus plants and *B. xanthophis* is amenable to isolation, culture, and transformation with foreign genes. Also, we used the green fluorescent protein gene, which does not affect the fitness of the bacteria, as a marker gene makes transfer of bacteria and the plasmid traceable, was done with success in *M. mesophilicum*. The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf* subsp. *pauca* makes it possible to makes *M. mesophilicum* an interesting candidate for the symbiotic control of the citrus variegated chlorosis agent, e.g., through a paratransgenesis approach.

INTRODUCTION

Citrus variegated chlorosis (CVC) is a disease of sweet orange (*Citrus sinensis* (L.)) trees caused by *Xylella fastidiosa* (*Xf*) subsp. *pauca* (Schaad et al., 2004). In Brazil, CVC is responsible for losses of US $100 million per year to the citrus industry (Della-Coletta et al., 2001). Endophytes colonize an ecological niche similar to that of phytopathogens, and this fact might favor them as candidates for biocontrol agents (Hallmann et al., 1997) because they have access to and could interact with phytopathogens (Azevedo et al., 2000). Many endophytic bacteria have been isolated from sweet orange (Araújo et al., 2002), but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *Xf* subsp. *pauca* in the xylem vessels of plants (Araújo et al., 2002; Lacava et al., 2006). The genus *Methylobacterium* is described as a main player in the interaction between the endophytic community and the pathogen *Xf* subsp. *pauca* (Araújo et al., 2002; Lacava et al., 2004). *Catharanthus roseus* (L.) G. Don has been shown to be an excellent experimental host for *Xf* subsp. *pauca* (Monteiro et al., 2001). *Catharanthus roseus* has also been used to study the interactions between *Xf* subsp. *pauca* and endophytic bacteria (Lacava et al., 2007). Xylem-feeding leafhoppers (Homoptera: Cicadellidae, tribes Cicadellini and Proconiini) are unique organisms in terms of their nutritional ecology; they are able to feed from xylem fluid, which is difficult to access and a nutritionally dilute food (Young, 1968). A clear association has been observed between Cicadellinae leafhoppers xylem-feeding habit and ability to transmit *Xf* (Almeida and Purcell, 2003). In Brazilian citrus groves, *Dilobopterus costalimai* Young, *Oncometopia facialis* (Signoret), and *Acrogonia citrina* Marucci & Cavichioli are the most common sharpshooters found, whereas *Bucephalogonia xanthophis* (Berg) is the most commonly trapped in citrus nurseries and young groves (Redak et al., 2004). A new genetic transformation tool, called paratransgenesis, has been used to prevent the transmission of pathogens by insect vectors to humans (Beard et al., 1998). Paratransgenesis means genetic alteration of symbiotic microbes that are carried by insects. The overall strategy of disease prevention is called symbiotic control and is a variation on the theme of symbiotic therapy (Ahmed, 2003). The key to symbiotic control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen (Miller, 2007).

OBJECTIVES

In this work, we report the localization of the endophytic bacterium, *M. mesophilicum*, in *C. roseus* model plant and the transmission of this endophyte by *B. xanthophis*. Also, we propose *M. mesophilicum* as a candidate for a symbiotic control strategy to reduce the spread of *Xf* subsp. *pauca*.

RESULTS

When the pCM88 was introduced into the strain M. mesophilicum SR1.6/6, up to 102 transformants per µg of plasmid DNA were obtained (now called SRGFP), indicating a high efficiency of transformation. The analysis of randomly selected SRGFP transformants revealed that pCM88 was stably maintained in medium without antibiotic, expressing both the resistance to tetracycline and the gfp gene, after 20 generations in 120 h, 95%, decreasing the stability on 0.25% per generations approximately (**Table 1**). To evaluate the bacterial community of insect heads, five insects were used. After isolation, a total of 2.14×10^3 bacteria with an average of $3.56\times10^2\pm23.2$ bacteria per insect head were isolated. The original bacterial community of *B. xanthophis* was comprised of five groups: *Methylobacterium* sp., Actinomycetes, *Curtobacterium* sp., *Sphingomonas* sp., and *Bacillus* sp. (**Figure 1**). The *Methylobacterium* genus occurred naturally in *B. xanthophis*. The ecological niche occupied by the endophytic bacterium *M. mesophilicum* on *C. roseus* plants was determined by visualization with fluorescent microscope, of in vitro cultivated plants, 45 days after bacterium inoculation. A preferential colonization of plant xylem by this bacterium is clearly observed in fluorescence microscopy (**Figures 2C and D**). **Figures 2A and B** show vessels from control plants, where no fluorescent cell can be observed. The insects used in transmission experiments were monitored for the presence of the SRGFP strain 24 h after acquisition. Bacteria isolation from insect heads revealed the average population density of *M. mesophilicum* of 1.64×102 ±11.33 CFU/insect head suggesting that the bacteria are capable of colonizing the foregut of the insect as they were not washed way by the sap flux. The ability of the sharpshooter *B. xanthophis* in transmitting *M. mesophilicum* was accessed by insect acquisition of endophytic strain SRGFP and further feeding in C. roseus plants cultivated in greenhouse. Forty-five days after the insect feeding on plants, leaves on which insects were trapped, were submitted to bacterial isolation. The population density of *M. mesophilicum* found in *C. roseus* leaves 45 days after insect transmission presented an average of 2.8×103 CFU/g of fresh tissue. In analyzing inoculated plants, from 45 plants used in insect traps, six presented the SRGFP strain colonizing inner tissues endophytically. It indicates that *B. xanthophis* is able to transmit the endophytic bacteria in the same way it transmits *Xf*, with an efficiency of transmission of 13.3% (**Table 2**).

CONCLUSIONS

The results from this study suggest that the pCM88 plasmid was stably maintained in planta and sharpshooter for at least 180 generations in the transmission assay. As shown in this study, the transgenic endophytic *M. mesophilicum* has most of the prerequisites, for a successful strategy using paratransgenesis. The use of GFP, which does not affect the fitness of the bacteria, as a marker gene makes transfer of bacteria and the plasmid traceable, was done with success in *M. mesophilicum* in this work. Many aspects can influence the transmission efficiency, such as phytopathogen populations in feeding plant and the interaction between bacterial communities residing vector foregut and inoculated plant. Furthermore, bacteria community present in plants and insects could influence disease development by reducing the insect transmission efficiency due to competition with pathogens or by symbiotic control of *Xf*. The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf* subsp. *pauca* makes it possible to study the potential interactions between these bacteria in the insect body and makes *M. mesophilicum* an interesting candidate for the symbiotic control of the CVC agent, e.g., through a paratransgenesis approach.

Table 1. Plasmidial stability of pCM88 on *Methylobacterium mesophilicum*. The percent of reaming colonies caring out the pMC88 was obtained from randomly collected samples after 24, 48, 72, and 120 h of culture cells of strain SR1.6/6 growing without antibiotic tetracycline.

Generation number	Reaming colonies caring out pCM88 (%)	SD[a]
0 (0 h)	100	0
4 (24 h)	99	1
8 (48 h)	98	3.21
12 (72 h)	95	2.08
20 (120 h)	95	1

[a] SD for four replicates

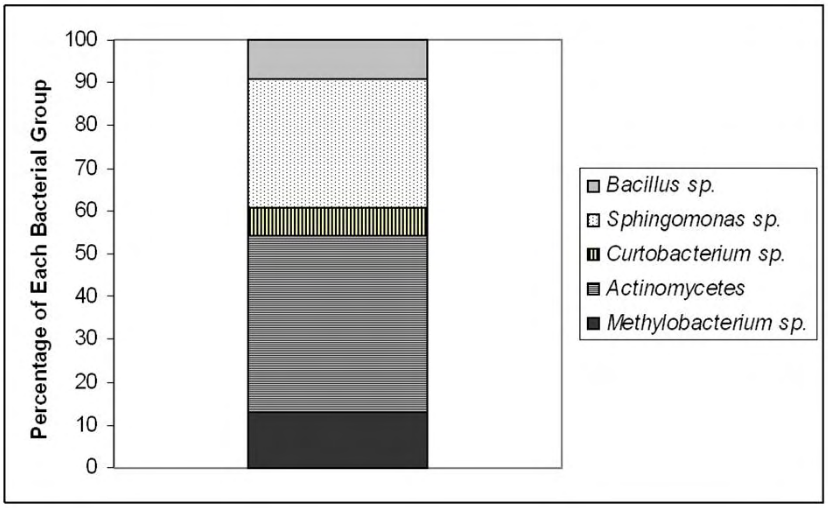

Figure 1. Most dominant group of bacteria isolated from *Bucephalogonia xanthophis*.

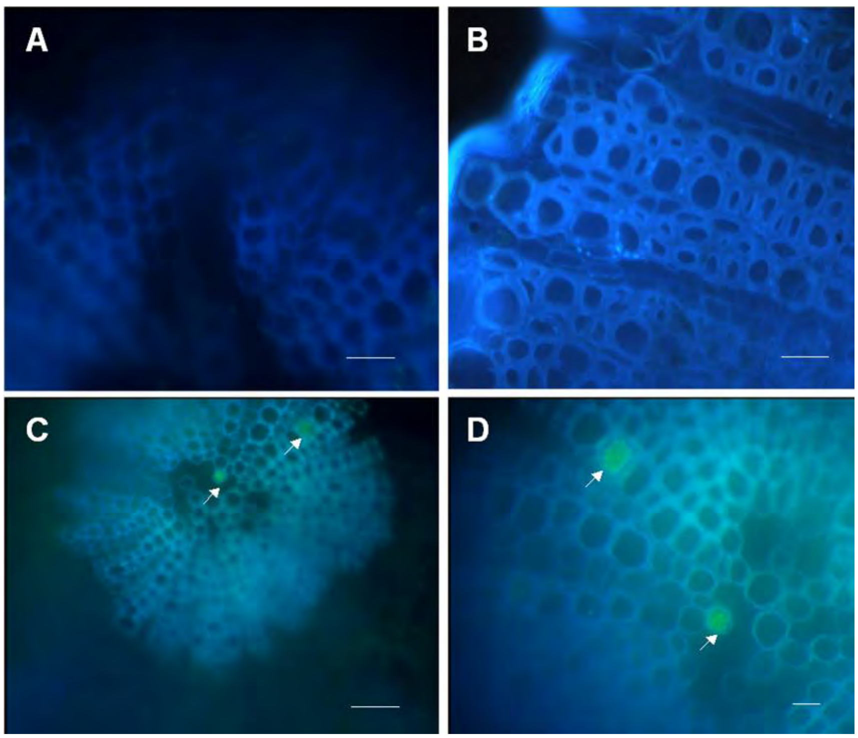

Figure 2. Fluorescent microscopy evidencing the ecological niche occupied by endophytic *Methylobacterium mesophilicum*, expressing GFP in *Catharanthus roseus* plants. Xylem vessels observed under a fluorescence microscope (Leica MZ FLIII) 45 days after inoculation. Images are the result of the overlay of images produced using filters DAPI and GFP. A and B) Xylem vessels of a control plant, scale bar=10 µm. C and D) Colonized xylem vessel, scale bar=10 µm and 5 µm.

Table 2. Evidence of the transmission of *M. mesophilicum* expressing GFP (SRGFP) to healthy plants (*C. roseus*) by insects (*B. xanthophis*). Plants were inoculated by insects, which acquired the fluorescent bacteria from membrane system. Endophytic bacteria were isolated from inoculated plants after 45 days of inoculation and fluorescent bacteria were counted. The average of SRGFP in inoculated plants was calculated as colony forming unit (CFU)/g of fresh tissue.

Number of inoculated plants	Number of plants positive to presence of SRGFP	Transmission rate	SRGFP in plants (CFU/g fresh tissue)
45	**6**	**13.3%**	**2.8 x 10^3**

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