ANALYSIS OF THE BACTERIAL COMMUNITY ASSOCIATED WITH SHARPSHOOTERS, INSECT VECTORS OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

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ABSTRACT

Xylella fastidiosa subsp. *pauca* (*Xfp*) causes citrus variegated chlorosis (CVC) disease in Brazil, resulting in significant production problems in the citrus industry. *Xfp* is mainly transmitted by three species of sharpshooters (Hemiptera: Cicadellidae) in Brazil, *Dilobopterus costalimai* (Young), *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis* (Signoret). Endophytic bacteria have been defined as those that do not visibly harm the host plant but can be isolated from surface-disinfected plant tissue or the inner parts of plants, showing potential benefits in the biocontrol of pathogens causing diseases. Some endophytes colonize the same niche of phytopathogens, such as *Xfp*, allowing interaction with the phytopathogen during plant colonization and transmission. We evaluated the bacterial communities associated with the heads of the insect vectors of *Xfp* that were collected from CVC affected citrus groves in Brazil. Bacteria were isolated from the heads of three insect species (*O. facialis, D. costalimai* and *A. citrina*). Total DNA of insect heads was analyzed by denaturing gradient gel electrophoresis (DGGE). The compositon of the microbial community was found to be characteristic of the insect species and period of evalution. Specific polymerase chain reactions (PCRs) for detection of two important citrus endophytes, *Curtobacterium flaccumfaciens* and *Methylobacterium mesophilicum* were performed, and the highest frequency of detection was 89.6% for *C. flaccumfaciens*, which has been described as a citrus endophyte that interacts with *Xfp*.

INTRODUCTION

Citrus variegated chlorosis (CVC) was first reported in Brazil in 1987 (Rossetti et al. 1990) and has spread over at least 90% of the orchards in Brazil (Lambais et al. 2000) and is caused by the xylem-limited gram-negative bacterial pathogen, *Xylella fastidiosa* subsp. *pauca* (*Xfp*) (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). Endophytic microorganisms, not visibly harmful to the host plant, can be isolated from surface-disinfected plant tissue or the inner parts of plants (Hallmann et al. 1997). Endophytes were reported to be contributing to host plant protection and ultimately survival (Azevedo et al. 2000). Furthermore, endophytes can colonize an ecological niche similar to that of phytopathogens, which makes them possible candidates as biocontrol agents (Hallmann et al. 1997). Araújo et al. (2002) found that the endophytic bacteria *Methylobacterium* spp. and *Curtobacterium flaccumfaciens* were present in asymptomatic citrus trees. Lacava et al. (2004) reported that the growth of *Xfp* was inhibited by endophytic *C. flaccumfaciens* and stimulated by *Methylobacterium* sp. and Lacava et al. (2007) demonstated that *C. flaccumfaciens* reduced the severity of CVC symptoms when co-inoculated with *Xfp in planta*. Cicadellinae leafhoppers, commonly named sharpshooters, are xylem-feeders (Young 1968). In Brazilian citrus groves, *Dilobopterus costalimai*, *Oncometopia facialis* and *Acrogonia citrina* are the most common sharpshooters found (Lopes et al. 1996). After acquisition of *Xf* by the insects, colonies of bacterial cells were visible in the cibarium and pre-cibarium of transmitting insects attached to the foregut walls (Purcell & Finlay 1979; Newman et al. 2003). Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xfp* cells in the citrus plants (Almeida et al. 2003) and the low number of colonized vessels in affected plants (Alves et al. 2003). The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions, could occur.

OBJECTIVES

The aims of this work were:

1. Access the bacterial population associated with the main sharpshooters responsible for the transmission of *Xfp* in citrus.
2. Compare the bacteria collected from insects to endophytic bacteria collected from citrus by denaturing gradient gel electrophoresis (DGGE).

RESULTS

A total of 17,230 bacteria were isolated and they were classified according to morphological groups. The morphological groups found during isolation and the results of the sequencing of one representative of each group were: G1) actinomycetes, G2) dark pink colonies (*Curtobacterium* sp.), G3) light pink colonies (*Methylobacterium* sp.), G4) yellow colonies (*Sphingomonas* sp.), G5) white colonies (*Bacillus* sp.), and G6) transparent colonies (*Microbacterium* sp.). From the heads analyzed, 51.7% of insects from the species *O. facialis* were positive for the presence of the endophytic bacteria *M. mesophilicum*, 8.7% of the *D. costalimai* and 20% of the *A. citrina*. *C. flaccumfaciens* was found in 89.6% of *O. facialis*, 39.1% of *D. costalimai* and 70% of *A. citrina*. A summary of the results comparing to previous data of *Xpf* transmission by the insect vectors and the presence of *Curtobacterium* sp. is presented in **Table 1**. The DGGE analysis showed considerable variability between the different insect species and also between sampling periods. **Figure 1** shows that the samples from March are more similar to samples from May than the ones from July. The bacteria isolated from insects are represented by the code IAB (Insect Associated Bacteria) described on **Table 2**. In the present study, *Curtobacterium* sp. was the most important bacteria colonizing insect heads. *Curtobacterium flaccumfaciens* was implicated in playing an important role in the prevention of CVC symptoms in citrus trees. The presence of the citrus endophyte, *Curtobacterium* sp., colonizing the insect heads could explain why the transmission efficiency of *Xfp* by vectors is low (5 to 10%), when compared to the transmission of *Xf* subsp. *piercei* by GWSS, which transmit Pierce's disease (PD) (45%). **Table 1** illustrates that *Curtobacterium*, can play an important role in the transmission of *Xfp*, as it could be influencing pathogen adhesion to the vector foregut or inhibiting growth of the pathogen.

CONCLUSIONS

The bacterial communities associated with insects appear to change with changes in environmental conditions. Endophytic bacteria could influence disease development by reducing the insect transmission efficiency due to competition with pathogens in host plants and also in insect foreguts. In addition the bacterial communities in the foregut of insect vectors of *Xfp* changed with time, environmental conditions and in different insect species. However, since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *Xfp*, they maybe candidates for biological control of *Xfp*, which requires endophytic bacteria that can colonize both the insect vectors of CVC and citrus plants.

Table 1. Resume of the results of the present work, comparing to previous data of *Xfp* transmission by the insect vectors and the presence of *Curtobacterium* sp.

	O. facialis	*A. citrina*	*D. costalimai*
Curtobacterium sp. frequency of isolation (G2)	1.01×10^3	2.16×10^2	4.33×10^1
Curtobacterium sp. positive specific PCR	89.6%	70%	39.1%
Presence of Haplotype 1 (*Curtobacterium* sp.) from 120 total isolates	45	17	0
Transmission rate of *Xfp* (Krügner et al., 2000)	1%	2%	5%

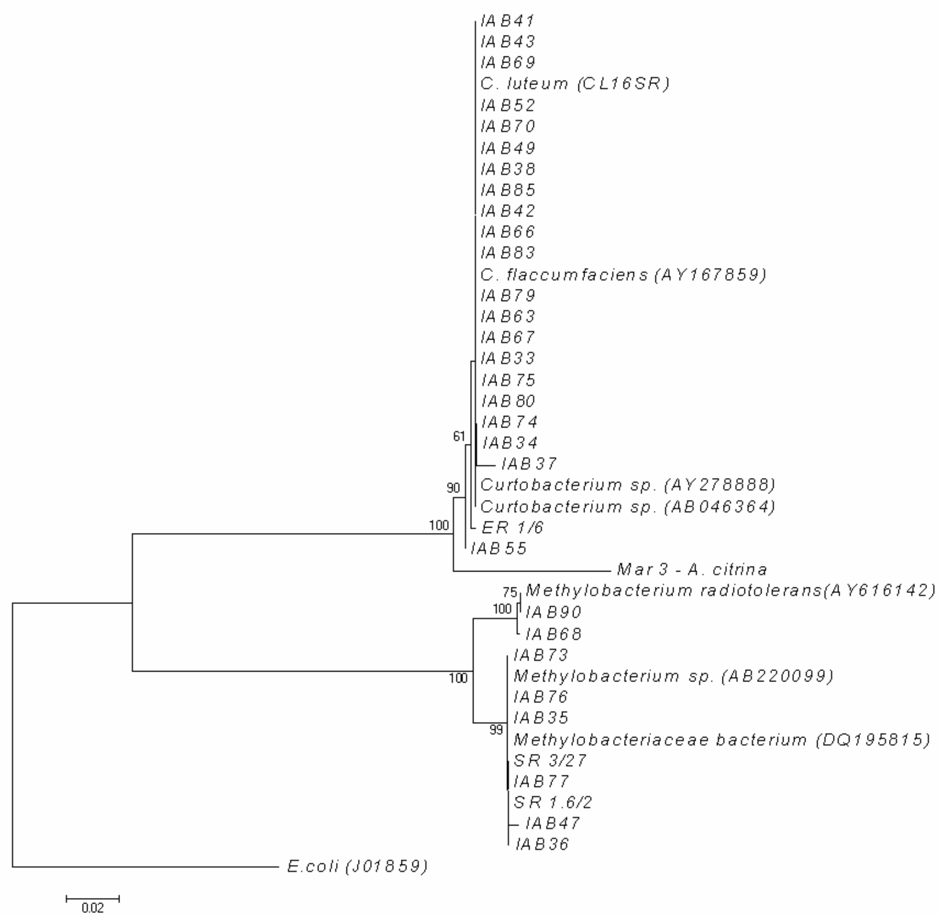

Figure 1. Dendogram representing the insect associated bacteria clustered with citrus endophytes. Bootstraps of 1,000 repetitions.

Table 2. Average of the number of colony forming units per head of insect (CFU/head) found in each isolation experiment. Groups: G1) actinomycetes, G2) dark pink (*Curtobacterium* sp.), G3) light pink (*Methylobacterium* sp.), G4) yellow (*Sphingomonas* sp.), G5) white (*Bacillus* sp.), G6) transparent (*Microbacterium* sp.).

Vector insect	Isolation	G1	G2	G3	G4	G5	G6
O. facialis	March	100,4 (±44,6)	1225,7 (±253,2)	51 (±11,1)	627,7 (±176,3)	185,13 (±33,8)	48 (±21,4)
	May	23 (±11,1)	0	101,7 (±46,5)	2,75 (±1,2)	82,25 (±36,3)	0
	June	12,73 (±5,03)	1,5 (±0,47)	0,4 (±0,10)	0,8 (±0,26)	1,6 (±0,5)	3,1 (±1,38)
A. citrina	March	0	16,2 (+5,18)	2 (±0,89)	0	0	0
	May	0	0	0	1 (±0,35)	6,5 (±1,16)	176,9 (±48,1)
	June	0	0,6 (±0,17)	0	1,5 (±0,5)	0,2 (±0,08)	0,4 (±0,17)
D. costalimai	March	0,2 (±0,08)	584,6 (±258,8)	1 (±0,44)	121,1 (±49,6)	8,5 (±3,8)	4 (±1,78)
	May	0	1 (±0,35)	0,25 (±0,12)	1,625 (±0,65)	1,5 (±0,47)	1,25 (±0,37)
	June	0,4 (±0,1)	0	0	0	0,2 (±0,08)	0

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