

DETECTION OF SIDEROPHORES IN THE ENDOPHYTIC BACTERIA *METHYLOBACTERIUM* SPP. ASSOCIATED WITH *XYLELLA FASTIDIOSA*

Project Leaders:

Paulo T. Lacava
University of São Paulo/ ES ALQ
Piracicaba, SP 13400-970
ptlacava@esalq.usp.br

Maria E. Silva Stenico
University of São Paulo/CENA
Piracicaba, SP 13400-970

Flávia T. H. Pacheco
University of São Paulo/CENA
Piracicaba, SP 13400-970

Cooperators:

Wellington L. Araújo
University of São Paulo/ESALQ
Piracicaba, SP 13400-970

Ana V. C. Simionato
University of São Paulo/IQSC
São Carlos, SP 13560-970

Emanuel Carrilho
University of São Paulo/IQSC
São Carlos, SP 13560-970

Siu Mui Tsai
University of São Paulo/CENA
Piracicaba, SP 13400-970

João Lúcio Azevedo
University of São Paulo/ESALQ
Piracicaba, SP 13400-970

Reporting Period: The results reported here are from work conducted August 20, 2004 to January 20, 2006.

ABSTRACT

We analyzed the production of siderophores by endophytic bacteria *Methylobacterium* spp., which occupy the same niche as *Xylella fastidiosa* (*Xf*) in citrus plants. All strains of *Methylobacterium* spp. tested were CAS-positive for siderophore production. *Methylobacterium* spp. produced hydroxamate-type, but not catechol-type siderophores. Specific primers for pyoverdinin, a hydroxamate type ferrisiderophore receptor gene were used to amplify this gene from *Methylobacterium* strains. The growth of *Xf* was stimulated by the presence of a supernatant-siderophore of endophytic *Methylobacterium mesophilicum*. If *Xf* is able to use heterologous siderophores during its establishment inside the host plant, it may benefit from production of siderophores by endophytic symbionts.

INTRODUCTION

Endophytes colonize the living, internal tissues of plants without causing any immediate, over negative effects (Hallmann et al., 1997; Azevedo et al., 2000). Research has shown that endophytic microorganisms isolated from surface disinfected plant tissues exhibit a potential as biocontrol agents against phytopathogens (Sturz et al., 1998) and insects (Azevedo et al., 2000) as well as increasing plant growth and hastening plant development (Lodewyckx et al., 2002). However, synergistic interactions between endophytes and phytopathogens have not been studied yet.

Bacterial siderophores are low-molecular-weight compounds with high iron (III) chelating affinity (Sharma and Johri, 2003) that are responsible for the solubilization and transport of iron (III) into bacterial cells. Iron is an essential mineral and its sequestration by specific bacterial siderophores may induce the development of plant disease (Nachin et al., 2003; Etchegaray et al., 2004). Acquisition of iron from siderophores produced by other microbial species has already been described for *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990), *Actinobacillus pleuropneumoniae* (Diarra et al., 1996), *Streptomyces* sp. (Imbert et al., 1995), and *Arthrobacter flavescens* (Winkelmann, 1991).

Xylella fastidiosa (*Xf*) is the causal agent of Citrus Variegated Chlorosis (CVC), which is an important disease of citrus species (Hartung et al., 1994). In Brazil, over 70 million sweet orange trees (38%) are affected, and CVC is responsible for losses of US\$ 100 million per year to the Brazilian citrus industry, affecting all commercial sweet orange varieties (de Souza et al., 2005). *Xf* was the first plant pathogen to have its genome completely sequenced and putative genes for membrane receptors, including siderophores, were found (Simpson et al., 2000).

The genus *Methylobacterium*, which occupies the same ecological niche as *X. fastidiosa*, was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*Citrus sinensis*). Recently, an interaction between *Methylobacterium* species and *Xf* was strongly indicated (Araújo et al., 2002; Lacava et al., 2004).

OBJECTIVES

The aim of this study was to evaluate the ability of *Methylobacterium* spp., isolated as citrus endophytic bacteria (Araújo et al., 2002), to produce siderophores and to investigate the capacity of *Xf* to use siderophores produced by *Methylobacterium mesophilicum* (*M. mesophilicum*) for growth and development.

RESULTS

All strains of *Methylobacterium* spp. tested were CAS-positive for siderophores production (Table 1), and the siderophores production tested by the CAS-agar assay revealed that 66% of CVC-symptomatic, 55% of uninfected, 20% of asymptomatic and 10% of tangerine strains of *Methylobacterium* spp., showed very high production. Also, all strains of *Methylobacterium*

spp. were negative in the Arnow assay, which means that, these strains are negative for catechol-type siderophores (Table 1). However, all strains of *Methylobacterium* spp were able to product hydromate-type to varying degrees as shown by the by Csák assay (Table 1).

Only three strains of endophytic *Methylobacterium* (AR5.1/5, AR5.1/6 and AR1.6/2) were PCR positive (Table 1), but these three strains were isolated from plants that showed CVC symptoms (Araújo et al., 2002). The strains AR5.1/5 and AR5.1/6 also produced very high concentrations of siderophore in the CAS-agar assay. These primers were developed to detect pyoverdine, a siderophore hydroxamate-type receptor, in *X. fastidiosa* (Pacheco et al., 2001) and these same primers recognized at least in three strains of *Methylobacterium* the same amplicon detected in *Xf*.

Growth of the *Xf* in PW broth medium was stimulated by the presence of *M. mesophilicum* supernatants that contained siderophores (Figure 1) and inhibition of this same strain was observed in the negative control (PW broth medium without a source of iron) (Figure1).

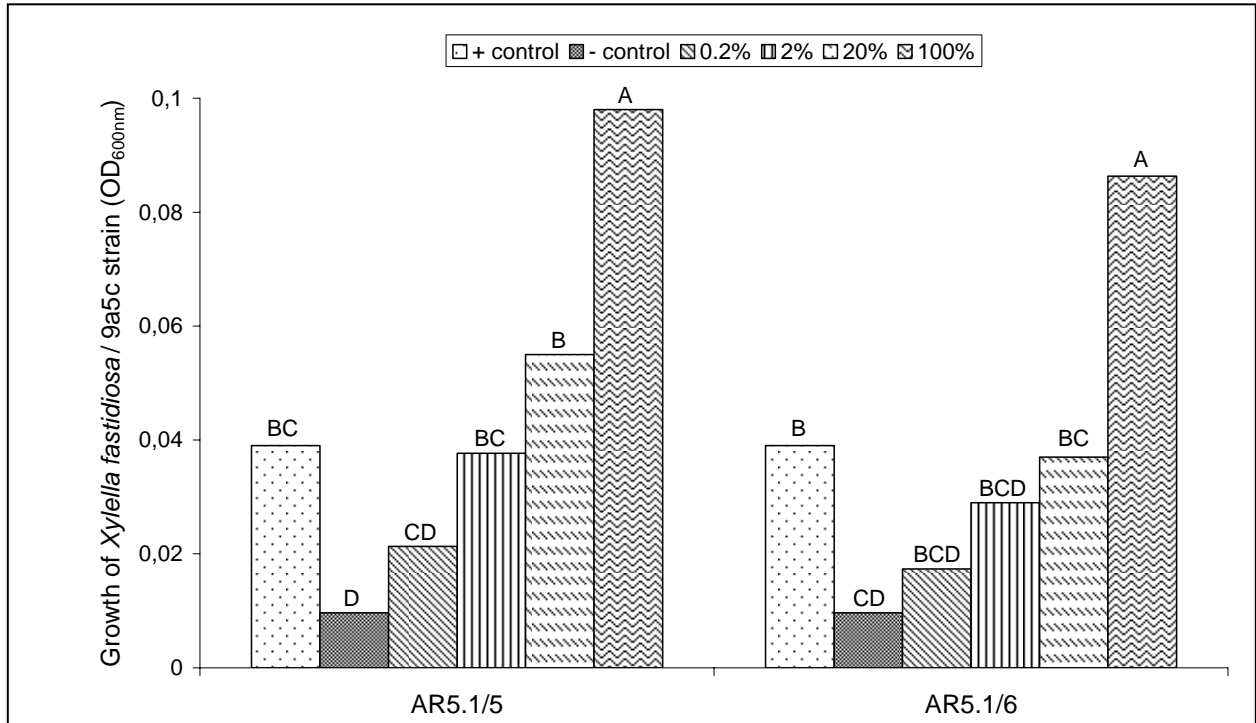


Figure 1. Effect of cell-free supernatants of the endophytic bacteria *M. mesophilicum* (AR5.1/5 and AR5.1/6 strains) with siderophore-production on the growth of *Xf* in PW broth medium. Negative control: PW broth medium without a source of iron and positive control: standard PW broth medium. Different letters on bars for same treatment means statistic difference by Tukey's test at 5% of significance.

Table 1. Siderophore production by endophytic *Methylobacterium* strains.

Strains	CAS-Agar Univesal test*	Csák test* (Hydroxamate-type)	Arnow test* (Catechol-type)	PCR**
AR1.6/1	+	+	-	-
AR1.6/6	+++	++	-	-
AR3/20	++	+	-	-
AR4/19	+	+	-	-
AR5/1	++	+	-	-
AR5.1/4	+++	+	-	-
AR5.1/5	+++	+++	-	+
AR5.1/6	+++	++	-	+
AR1.6/2	+	+	-	+
AR1.6/3	+++	+	-	-
AR1.6/8	+++	+	-	-
AR1.6/11	++	+	-	-
AR1.6/4	+	+	-	-
ER1/21	+	+	-	-
ER1.6/1	+	+	-	-
ER1.6/4	++	++	-	-
ER1.6/5	+++	+	-	-
ER5/2	++	+	-	-
SR1.6/6	+++	+	-	-
SR1.6/13	++	++	-	-
SR3/27	++	++	-	-
SR1.6/1	+++	+	-	-
SR1.6/15	++	+	-	-
SR5/4	++	++	-	-
SR1.4/10	+++	++	-	-
SR1.6/4	+++	+	-	-
SR1.6/2	+++	+	-	-
PR1/3	+	+	-	-
PR1.4/10	+++	+	-	-
PR2/2	+	+	-	-
PR3/5	+	+	-	-
PR3/11	++	++	-	-
PR3/15	+	+	-	-
PR5/4	+	+	-	-
PR5.1/1	++	+	-	-
PR3/8	++	++	-	-
PR3/17	++	+	-	-

* Intensity: -, none; +, low; ++, high; +++, very high

**Presence (+) or absence (-) of PCR product

CONCLUSIONS

The present data corroborates the hypotheses that there is a relationship between *Xf*, causal agent of CVC, and the endophytic bacteria *Methylobacterium* (Araújo et al., 2002; Lacava et al., 2004). In addition, our results indicated that *Xf* was able to use *Methylobacterium* siderophores *in vitro* as a source of iron (Figure 1), and suggested that in some instances *Methylobacterium* could help the growth of *Xf*, particularly under environmental conditions where iron sources are limited. Iron-siderophore complexes are taken up by specific transport systems, but some microorganisms have also developed transport systems for heterologous siderophores produced by other species (Raaijmakers et al., 1995; Howard, 1999).

Endophytes must compete with plant cells for iron supply, and therefore siderophore production may be highly important for endophytic growth (Idris et al., 2004). Additionally, the production of siderophores has been reported to be one of the mechanisms to outcompete pathogens (O'Sullivan and O'Gara, 1992; Schippers et al., 1987) and may have the same function in endophytes. The present study suggested that *Xf* can use molecules produced by endophytic bacteria as siderophores. In this context, as a factor influencing the symptom of CVC (Pacheco et al., 2001; Silva-Stenico et al., 2005), the genus *Methylobacterium* could help *Xf* to survive inside the xylem vessels because competition for iron in the environment has an important role in microbial systems.

REFERENCES

- Araújo, W.L., Marcon, J., Maccheroni Jr., W., van Elsas, J.D., van Vuurde, J.W.L., Azevedo J.L., 2002 Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Appl. Environ. Microbiol.* 68, 4906-4914.
- Azevedo, J.L., Maccheroni Jr., W., Pereira, J.O., Araujo, W.L., 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electron. J. Biotech.* 3 (1) ISSN 0717-3458.
- de Souza, A.A., Takita, M.A., Pereira, E.O., Coletta-Filho, H.D., Machado, M.A., 2005. Expression of pathogenicity-related genes of *Xylella fastidiosa* *in vitro* and *in planta*. *Curr. Microbiol.* 50, 223-228.
- Diarra, M.S., Mckee, J.A., Dolence, E.K., Darwish, I., Miller, M.J., Malouin, F., Jacques, M., 1996. Growth of *Actinobacillus pleuropneumoniae* is promoted by exogenous hydroxamate and catechol siderophores. *Appl. Environ. Microbiol.* 62, 853-859.
- Etchegaray, A., Silva-Stenico, M.E., Moon, D.H., Tsai, S.M., 2004. *In silico* analysis of nonribosomal peptide synthetases of *Xanthomonas axonopodis* pv. *citri*: identification of putative siderophore and lipopeptide biosynthetic genes. *Microbiol. Res.* 159, 425-437.
- Hallmann, J., Quadt-Hallman, A., Mahaffee, W.F., Kloepper, J.W., 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43, 895-914.
- Hartung, J.S., Beretta, J., Brlansky, R.H., Spisso, J., Lee, R.F., 1994. Citrus variegated chlorosis bacterium: axenic culture, pathogenicity, and serological relationships with other strains of *Xylella fastidiosa*. *Phytopathology* 84, 591-597.
- Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl. Environ. Microbiol.* 70, 2667-2677.
- Imbert, M., Bechet, M., Blondeau, R., 1995. Comparison of the main siderophore produced by some species of *Streptomyces*. *Curr. Microbiol.* 31, 129-133.
- Lacava, P.T., Araújo, W.L., Marcon, J., Maccheroni, W.Jr, Azevedo, J.L., 2004. Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria *Xylella fastidiosa*, causal agent of citrus-variegated chlorosis. *Lett. Appl. Microbiol.* 39, 55-59.
- Lodewyckx, C., Vangronsveld, J., Porteus, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., Lelie, D.V., 2002. Endophytic bacteria and their potential applications. *Crit. Rev. Plant Sci.* 21, 586-606.
- Martinez, J.L., Delgado-Iribarren, A., Baquero, F., 1990. Mechanisms of iron acquisition and bacterial virulence. *FEMS Microbiol. Rev.* 75, 45-56.
- Meidanis, J., Braga, M.D.V., Verjovski-Almeida, S., 2002. Whole-genome analysis of transporters in the plant pathogen *Xylella fastidiosa*. *Microbiol. Mol. Biol. Rev.* 66, 272-299.
- Nachin, L., Loiseau, L., Expert, D., Barras, F., 2003. SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *EMBO J.* 22, 427-437.
- O'Sullivan, D.J., O'Gara, F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol. Rev.* 56, 662-676.
- Pacheco, F.T.H., Silva, M.E., Rodrigues, J.L.M., Tsai, S.M., 2001. Molecular characterization of putative iron receptor genes in *Xylella fastidiosa*. In: *Rhizosphere Control Points: Molecules to Food Webs*, June 1–3. University of California, Davis, USA.
- Schippers, B., Bakker, A.W., Bakker, P.A.H.M., 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu. Rev. Phytopathol.* 25, 339–358.
- Sharma, A., Johri, B.N., 2003. Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. *Microbiol. Res.* 158, 243-248.
- Silva-Stenico, M.E., Pacheco, F.T.H., Rodrigues, J.L.M., Carrilho, E., Tsai, S.M., 2005. Growth and siderophore production of *Xylella fastidiosa* under iron-limited conditions. *Microbiol. Res.* 160, 429-436.
- Simpson, A.J.G., Reinach, F.C., Arruda, P., and 112 other authors, 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406, 151-157.
- Sturz, A.V., Christie, B.R., Matheson, B.G., 1998. Association of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can. J. Microbiol.* 44, 162-167
- Winkelmann, G., 1991. Specificity of iron transport in bacteria and fungi. In: Winkelmann, G., (Ed.), *CRC handbook of microbial iron chelates*. CRC Press, Boca Raton, Florida, pp. 65-105.

FUNDING AGENCIES

Funding for this project was provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

MULTI-LOCUS SIMPLE SEQUENCE REPEATS AND SINGLE NUCLEOTIDE POLYMORPHISM MARKERS FOR GENOTYPING AND ASSESSING GENETIC DIVERSITY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

Project Leader:

Hong Lin
Crop Disease, Pest, &
Genetics Research Unit
SJVAC, ARS-USDA
Parlier, CA 93648
hlin@fresno.ars.usda.gov

Cooperators:

Harshavardhan Doddapaneni	Andrew Walker	Edwin Civerolo	Russell L. Groves
Dept. of Vitic. & Enology	Dept. of Vitic. & Enology	SJVAC, ARS-USDA	Department of Entomology
University of California,	University of California	Parlier, CA 93648	University of Wisconsin
Davis, CA 95616	Davis, CA 95616		Madison, WI 53706

Reporting Period: The results reported here from work conducted October 2004 to September 2006

ABSTRACT

In California, information regarding the population structure and genetic diversity as well as the genetic evolutionary and epidemiological relationships among *Xylella fastidiosa* (*Xf*) strains in agricultural populations is not clear. To develop effective management strategies, we need to understand pathogen population structure and genetic diversity in the agricultural ecosystem. Here we report development and utilization of two multilocus marker systems, Simple Sequence Repeats (SSR) and single nucleotide polymorphism (SNP) for genotyping and assessing genetic diversity of *Xf* in California. Strain diversity studies using SSRs on samples from different geographic populations and/or from different hosts demonstrated that host selection plays an important role in *Xf* genetic differentiation among agricultural populations in California. Whole-genome comparison of four sequenced strains identified 12,754 potential SNPs in coding sequences and 20,779 SNPs in non-coding regions across four *Xf* strains. Small scale validation (16 loci) tests showed that SNP genotype is tightly linked to the hosts from which the strains were derived. Together, SNP and SSR marker systems appear to be useful tools for pathogen detection and population genetic analyses.

INTRODUCTION

Host plant resistance is a critical component of integrated crop management. However, durability of resistant grape plants depends upon the variability and adaptability of the pathogen population as well as disease resistance gene(s). Population genetics research demonstrates that the evolutionary potential of a pathogen is reflected in its genetic diversity and its genetic structure. Pathogen populations with higher evolutionary potential are more likely to overcome host resistance than pathogen populations with a lower evolutionary potential (MacDonald and Linde, 2002). The resulting changes in population structure or virulence can lead to host resistance breakdown. Therefore, understanding pathogen genetic diversity is critical in developing an effective disease control strategy. To characterize population structures of *Xylella fastidiosa* (*Xf*) and to understand genetic diversity of *Xf* in agricultural systems, sensitive and accurate marker system(s) are required. The goal of this project is to develop a reliable marker system(s) that unambiguously identifies *Xf* strains from various geographic locations and host plants and further to understand the pathogen dynamics. Previously, we reported the development of multilocus simple sequence repeats (SSR) markers for *Xf* population genetic analysis (Lin et al., 2005). This marker system appeared to be sensitive in detection and powerful in discriminating *Xf* genotypes. This marker system also provides high throughput capability for a large scale population sample analyses. Recently, we developed a new marker system; single nucleotide polymorphism (SNP). We performed whole-genome sequence analysis of CVC, PD, ALS and OLS strains and identified potential SNP loci in both coding and non-coding regions (Doddapaneni et al., 2006). This marker system has proven to be powerful and reliable for distinguishing genetic relatedness. This marker system is very sensitive, has a high degree of specificity, and is quite powerful in detecting genetic polymorphism. Further, adaptability to high a through-put diagnostic platform makes this system an ideal tool for large scale studies of *Xf* population genetics and epidemiological risk assessment analyses.

OBJECTIVES

1. Analyze *Xf* seasonal population dynamics; spatial and temporal disease development and genetic diversity.
2. Comparative whole genome analyses of the *X. fastidiosa* strains to identify SNP loci and develop SNP based marker system for fingerprinting *Xf* strains.

RESULTS AND DISCUSSION

Objective 1.

Previously, we analyzed genetic diversities and geographic population structures of *Xf* in California vineyards (Napa, Sonoma and Kern and Riverside counties). Results based on multi-locus SSR marker systems and hierarchical sampling