Current Microbiology Vol. 50 (2005), pp. 223–228
DOI: 10.1007/s00284-004-4447-8

Current Microbiology
An International Journal
© Springer Science+Business Media, Inc. 2005

Expression of Pathogenicity-Related Genes of *Xylella fastidiosa* In Vitro and In Planta

Alessandra A. de Souza,[1,2] Marco A. Takita,[2] Eridan O. Pereira,[2,3] Helvécio D. Coletta-Filho,[2] Marcos A. Machado[2]

[1]Embrapa Recursos Genéticos e Biotecnologia, 70770-900 Brasília-DF, Brazil
[2]Centro APTA Citros Sylvio Moreira, Rod. Anhanguera Km 158 CP, 04 13490-970 Cordeirópolis, SP Brazil
[3]UNESP – Instituto de Biociências, Distrito de Rubião Júnior s/n, CEP: 18618-000 Botucatu, SP Brazil

Received: 1 September 2004 / Accepted: 4 November 2004

Abstract. *Xylella fastidiosa* is responsible for several economically important plant diseases. It is currently assumed that the symptoms are caused by vascular occlusion due to biofilm formation. Microarray technology was previously used to examine the global gene expression profile of *X. fastidiosa* freshly isolated from symptomatic plants or after several passages by axenic culture medium, and different pathogenicity profiles have been obtained. In the present study the expression of some pathogenicity-related genes was evaluated in vitro and in planta by RT-PCR. The results suggest that adhesion is important at the beginning of biofilm formation, while the genes related to adaptation are essential for the organism's maintenance in planta. Similar results were observed in vitro mainly for the adhesion genes. The pattern of expression observed suggests that adhesion modulates biofilm formation whereas the expression of some adaptation genes may be related to the environment in which the organism is living.

Xylella fastidiosa is a Gram-negative and xylem-inhabiting bacterium responsible for economically important diseases in different crops [2, 8, 12]. In Brazil, it is responsible for citrus variegated chlorosis (CVC), a disease that causes annual losses of about $100 million to the citrus agro-industry. For this reason, a Brazilian consortium carried out the sequencing of the genome of the CVC strain 9a5c of *X. fastidiosa*, which became the first plant pathogen to have its complete genome sequenced [14].

The generally accepted cause of the symptoms induced by *X. fastidiosa* is the occurrence of vascular occlusion inside the vessel leading to water stress. It was previously demonstrated that the bacterium is able to grow as a biofilm [9] and this biofilm could be an important factor for pathogenicity [15]. Biofilms are defined as a matrix-enclosed microbial population adherent to each other and to surfaces or interfaces [4].

Correspondence to: M.A. Machado; *email:* marcos@centrodecitricultura.br

Genetic studies of single-species biofilms have shown that they are formed by multiple steps regulated by several genes with altered expression in each phase of development [5].

The initial steps of biofilm formation involve the attachment of the bacteria to the surface. When the biofilm reaches the mature stage, with high cell density, an intercellular signaling system (quorum sensing) is activated, allowing the bacteria to regulate the expression of a specific set of genes such as virulence factors and those associated with resistance to antimicrobial compounds and the host defense mechanisms [5, 13]. These characteristics give the cells in the biofilm an advantage in adaptation and competition in the host environment [5].

The loss of virulence after several passages in axenic culture is a well-known phenomenon. And this phenomenon led us to previously compare the global gene expression of *X. fastidiosa* in two growth states: a pathogenic condition (FP, first passage after isolation) and a nonpathogenic condition (SP, several passages in axenic culture) [15]. Seven genes possibly associated

Table 1. Genes evaluated and nucleotide sequences of the primers used for RT-PCR

ORF number[a]	Gene name	Primer sequence	Fragment size (in base pairs)
XF2539	*fim*A	F: 5′-CCCTCGAGCCAAAATTATGTCGCCAGA-3′ R: 5′-CACTCGAGGTGACGGTGGAGGAGCAG-3′	350
XF1940	*msr*A	F: 5′-AATCTCGAGTGGGACGTAGTGAAC-3′ R: 5′-AAGCTCGAGCCGGATGGAGTA-3′	498
XF1516	*usp*A1	F: 5′-AACTCGAGAGCAGGCCGCCGGTGATAGCAGTA-3′ R: 5′-AGACGCTCGAGCCCCCGCCGCAAGAT-3′	1,122
XF1529	*hsf*	F: 5′-CGCTCGAGGGGTCTTTGTATGT-3′ R: 5′-GGCTCGAGACGCTGTGAGGTTC-3′	647
XF0263	*cva*C	F: 5′-GTCTCGAGGCGACCTTGCTAC-3′ R: 5′-AGCAGCCTCGAGACCACAGATAC-3′	194
XF2093	*acr*A	F: 5′-CTCTCGAGCACGCGTGGCTGGAATA-3′ R: 5′-AGCTCGAGCGCCTTCTTTGACTTTT-3′	919
XF1517	*xps*E	F: 5′-GTCTCGAGTTGTTGGCGGAAGTATGAA-3′ R: 5′-CCCTCGAGCCAGTGACCAGCAAAATG-3′	1,101

[a]Based on the *Xylella* database (http:// aeg.lbi.ic.unicamp.br/xf).

with pathogenicity showed higher expression in FP. Three of these encode adhesins (*fim*A, *usp*A1, and *hsf*) and the other four are related to adaptation to the environment (*msr*A, *cva*C, *xps*E, and *acr*A). The expression of the genes was confirmed by reverse transcription polymerase chain reaction (RT-PCR) in planta 15 days after inoculation [15], which corresponds to the period of initial colonization of the vessels [1]. Interestingly, the bacteria in the nonpathogenic condition showed the same expression pattern for all the genes but with lower levels. The only exception was *msr*A, which was not expressed in either of the conditions. Cells in SP were able to initially colonize the host but, contrary to FP, a decline in the population was observed after 120 days. In the present study, we analyzed the expression of these genes in planta in advanced stages of colonization (90 days) in order to determine which of the genes are suppressed in SP that could be related to the decline in the population.

The expression analysis of these genes in vitro, using different stages of the bacterial growth attached to a glass surface, was also performed by RT-PCR. The results showed an interesting trend, with the expression of the adhesion genes at the beginning of the colonization process followed by expression of the adaptation genes.

Materials and Methods

Bacterial strain and in vitro growth condition. *Citrus sinensis* plants were previously inoculated with 9a5c strain of *X. fastidiosa* and when CVC symptoms were visible, petioles and stems were collected and aseptically ground in phosphate-buffered saline (PBS). The suspension was distributed on plates containing Periwinkle Wilt (PW) medium [6]. The first colonies were observed between 10 and 15 days after plating. To obtain cells attached to glass surface, several individual colonies were transferred to a polypropylene tube containing 3 mL of PW broth. When the OD_{600nm} reached 0.3 the tubes were vortexed and the cells were transferred to a 1 L flask containing 300 mL of PW broth. A total of three flasks was used in each analysis. After 3 days of growth at 28°C in a rotary shaker at 120 rpm a thin biofilm was observed attached to the glass at the medium-air interface. Cells attached to the glass surface were collected at 3, 5, 10, 20, and 30 days after inoculation. The cell layer was scraped from the flask and washed by centrifugation at 8000 *g* for 5 min at 4°C with DEPC-treated water. After collection of the samples, total RNA was extracted as described by Rhodius (www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf). The pellet was stored at −80°C until required.

Plant inoculation and cell harvest. *Citrus sinensis* plants propagated by seeds and grown in an insect-proof greenhouse were used for inoculation of *X. fastidiosa* 9a5c in the two different growth states: freshly isolated bacteria (FP) and bacteria obtained after 46 passages in axenic culture (SP). The inoculation was performed as described by Souza et al. [15]. The plants were maintained in a growth chamber at 28°C with a photoperiod of 12 h light and 12 h dark. Three plants were inoculated with FP and three others with SP bacteria. After 90 days the leaf petioles and midribs were excised from all the plants. The *X. fastidiosa* cells from the xylem were extracted as described by Souza et al. [15].

RT-PCR analysis. Total RNA was extracted using the TRIZOL reagent (Invitrogen). RT-PCR was used to monitor the expression of the seven genes involved in pathogenicity (Table 1), as previously described [15], at different growth stages in vitro and in planta, 90 days after inoculation.

The cDNA was prepared from 500 ng of total RNA for the in vitro condition and 100 ng for the in planta condition. The RNA was reverse transcribed with SuperScript II (Invitrogen) and random hexamers primers (Invitrogen). To rule out the possibility of amplification from contaminating DNA, PCR using the RNA as template was done in parallel. The RNAs were normalized using the ribosomal RNA content. We carried out analyses for some genes up-regulated in the pathogenic condition [15] that were amplified with specific primers (Table 1). A 25 μL master mix contained the following components: 2.5 μM dNTP, *Taq* DNA polymerase reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 100 ng of specific primers, 2.5 units of *Taq* DNA poly-

merase, and 2.5 μL of first strand cDNA template. The PCR cycle profile was 94°C for 3 min for initial denaturation followed by cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Samples were collected every five cycles from cycle 10 to 30 for all the genes, in order to estimate the relative concentration. PCR products were run on 1% agarose gel, and quantified densitometrically using EagleSight software v.3.2 (Stratagene). The experiments were performed three times using independent cDNA samples.

Real-time RT-PCR assay for *usp*A1 in different stages of growth in vitro. Fifty nanograms of the same total RNA used for the semiquantitative RT-PCR was reverse transcribed with SuperScript II and random hexamer primers (Invitrogen). For the relative quantification, 1 μL of the cDNA was utilized in real-time PCRs using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). Primerexpress software (Applied Biosystems) was used to design primers to amplify and quantify *usp*A1 (target gene) and XF0244 (endogenous control). The endogenous control was chosen based on the similar expression levels observed in microarray analyses of the *X. fastidiosa* growing in biofilm in vitro compared with planktonic growth (data not shown). The probe was labeled with FAM and also carried nonfluorescent quencher and a Tm enhancer. The PCR thermal cycling conditions were as follows: an initial step at 50°C for 2 min; 10 min at 95°C; and 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C.

Each sample was processed in duplicate and an appropriate negative control containing no template cDNA was also subjected to the same procedure to exclude or detect any possible contamination or carryover. The endogenous control was used to normalize the samples for differences in the amounts of cDNA added to each reaction mixture. The results were normalized by using the C_t values obtained for the endogenous control present in the same plate. C_t is defined as the first amplification cycle at which fluorescence indicating PCR products is detectable above the threshold. For normalization, we utilized the equation: $\Delta C_t = C_t(\text{target gene}) - C_t(\text{endogenous control})$. The fold increased of the target gene on the different days of biofilm formation was determined by the equation: $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{calibrator})$. The calibrator is the value obtained for one specific sample. Therefore the fold increase is always obtained in relation to the specific calibrator utilized. We chose the values obtained for 30 days of the biofilm formation as the calibrator. The relative quantification was obtained by $2^{-\Delta\Delta Ct}$.

Results and Discussion

Examination of pathogenicity-related genes in planta by RT-PCR. RT-PCR was used to evaluate the expression level of pathogenicity-related genes in planta 90 days after inoculation with the bacterium in FP and SP conditions. The ratio of the normalized quantitated signals was obtained after 35 amplification cycles (Fig. 1).

The expression levels of the adhesion genes (*fim*A, *usp*A1, *hsf*) were similar in the two conditions. However, the expression level of the adaptation-related genes (*acr*A, *xps*E, *cva*C, *msr*A) was in general higher in FP. Moreover amplification of *acr*A and *msr*A genes was obtained only for the FP condition.

The colonization of *X. fastidiosa* in the SP condition was delayed compared with FP and a decrease in the SP population was observed after 120 days, in contrast to the cells in the FP condition, which kept growing in planta and induced development of symptoms [15].

Souza et al. [15] detected overexpression of the adhesion- and adaptation-related genes in *X. fastidiosa* in the FP condition 15 days after inoculation in planta. In this work we evaluated the expression of the same genes 90 days after inoculation, when development of the symptoms was previously reported, probably due to the blockage of vessels [1]. The expression levels of the adhesion genes after 90 days were similar in both conditions. These results indicate that the higher expression of these genes in the FP condition compared with SP at 15 days could be an important factor promoting efficient colonization during the development of the biofilm in the vessels. The delayed expression of these genes in SP could also be correlated with the poor colonization observed immediately after inoculation.

On the other hand, in later stages of the colonization (90 days), the expression level of adaptation-related genes is low in the SP condition. The *msr*A gene, which was not detected after 15 days [15], showed expression after 90 days only in the FP condition (Fig. 1), suggesting that MsrA is necessary in the more advanced stages of colonization. The low expression level of adaptation-related genes in the SP condition in later stages of plant colonization and the observed decline in the population suggest that the expression of these genes could be essential for the maintenance of the biofilm in planta by promoting competitive advantages in the host environment.

Examination of pathogenicity-related genes in vitro by RT-PCR. In order to verify changes in the expression level of pathogenicity-related genes in different stages of *X. fastidiosa* grown in vitro we used RT-PCR. For this purpose, total RNA was extracted from three independent experiments. Figure 2 shows the results of the analyses for adhesion-related (*fim*A, *usp*A1, *hsf*) and adaptation-related genes (*msr*A, *cva*C, *acr*A, *xps*E). The statistical analysis ($p \leq 0.05$) was done using the values obtained after 25 PCR cycles, except for *msr*A where the best signal was obtained after 35 cycles. The genes involved in adhesion were up-regulated mainly during the initial stages of growth on the abiotic surface. A statistically significant increase in the expression level of *fim*A was observed on the third, fifth, and tenth days, followed by a decrease on the twentieth day, suggesting that, in *X. fastidiosa*, this gene could be involved in the very first stage of adhesion, which agrees with other models of biofilm development in Gram-negative bacteria [5]. Two non-fimbriae adhesins, *hsf* and *usp*A1, were also up-regulated in

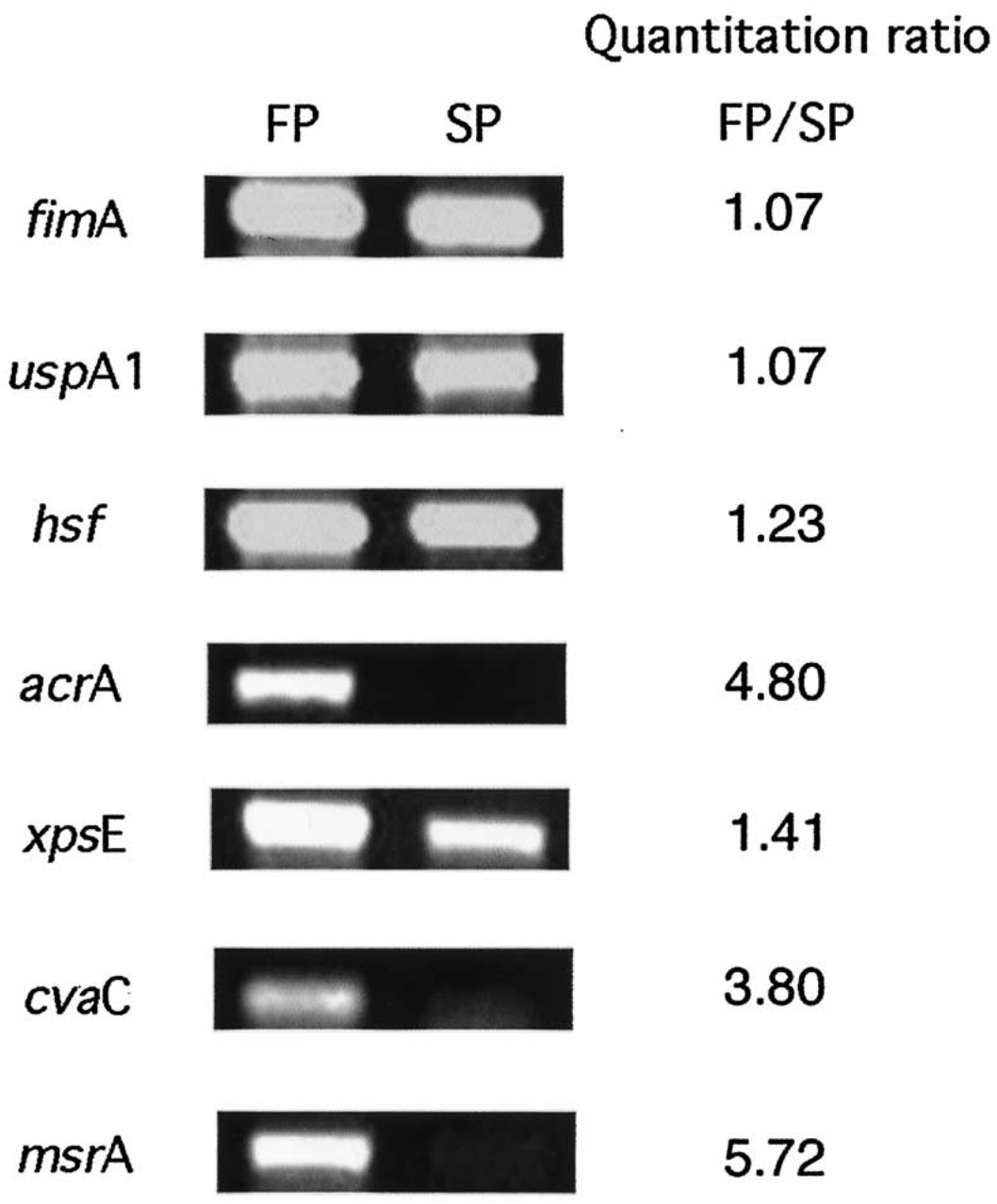

Fig. 1. Reverse transcription polymerase chain reaction of genes induced in the first passage (FP) of *X. fastidiosa* after isolation from the host. Expression of genes encoding pathogenicity factors in *X. fastidiosa* were evaluated in planta 90 days after inoculation. The ratios of the normalized quantitated band densities are presented as the FP value divided by the value obtained for the gene expression of cells after several passages in axenic culture (SP).

these initial stages; however, the only time points that showed a significant increase in the expression level were the fifth and tenth days, suggesting a possible role in initial adhesion as well as in biofilm architecture formation. In other organisms these genes encode adhesins, which are important for the attachment to the host tissues [3, 7, 16].

We utilized real-time RT-PCR in order to validate the results obtained in the RT-PCR for *usp*A1 in the different states of in vitro growth. The transcription of *usp*A1 was up-regulated on the fifth and tenth days (Fig. 3). The change in expression observed in the initial stages of attachment on an abiotic surface confirmed the RT-PCR results.

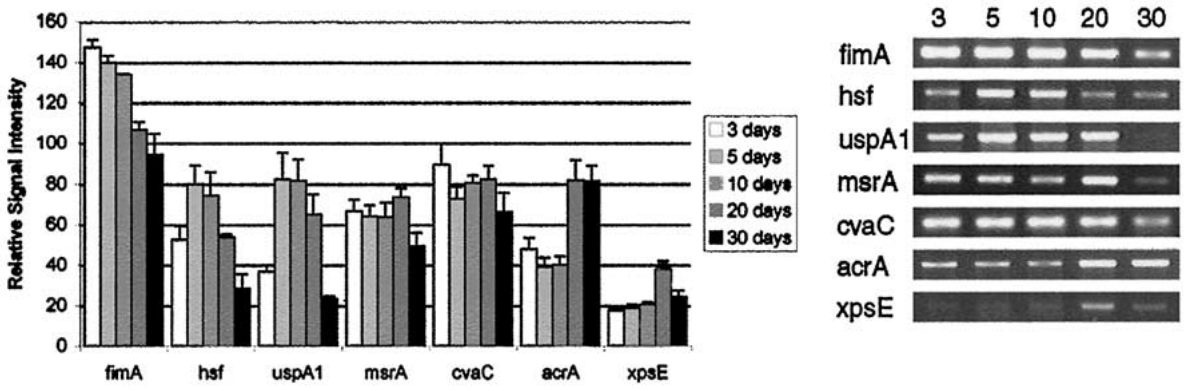

Fig. 2. Reverse transcription polymerase chain reaction. The mRNA levels of *fim*A, *hsf*, *usp*A1, *msr*A, *cva*C, *acr*A, and *xps*E in *X. fastidiosa* were evaluated 3, 5, 10, 20, and 30 days after inoculation. Data were normalized using ribosomal RNA content. The values correspond to the average of three repetitions. T-bars indicate the standard error.

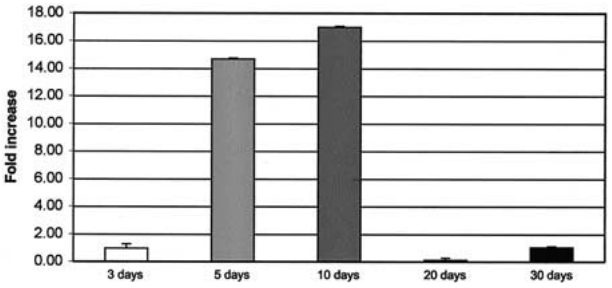

Fig. 3. Relative quantitation of *usp*A1 in real-time quantitative polymerase chain reaction from in vitro growth of *X. fastidiosa* at 3, 5, 10, 20, and 30 days after inoculation. cDNAs were prepared from the samples and used for the quantitation in the ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The measures were normalized using the threshold cycles (C_ts) obtained for the amplifications of the endogenous control run in the same plate. The values represent the fold increase in gene expression compared with values obtained for 30 days (calibrator) and the results are averages of two repetitions.

The expression of adhesion genes observed in the in vitro condition was similar to that observed in planta, where the genes were mainly expressed in the initial attachment stages. These results indicate that these genes can be involved in adhesion to different attachment surfaces. Due to the methodological difficulties in measuring expression of *X. fastidiosa* genes in planta, this is an important finding since it could simulate the conditions for attachment, helping the understanding of the initial steps of biofilm development in *X. fastidiosa*.

The *msr*A and *cvaC* did not show significant differences in expression level at any stage of growth on the abiotic surface. *acr*A showed significant up-regulation mainly in the later stages of growth on the abiotic surface. Expression of *xps*E was similar at the initial time points. On the twentieth day there was a significant increase in the expression, which decreased on the thirtieth day (Fig. 2).

Expression of adaptation-related genes can confer advantages to cells, promoting higher resistance to antimicrobial compounds (*cva*C, *acr*A) and the defense response of the host (*msr*A, *xps*E). It was recently demonstrated that AcrAB can mediate cell-cell communication in response to cell density [13]. In this work we observed high expression of *acr*A at day 20 of growth on an abiotic surface, a period of high cell density. The *acr*B gene also showed a significantly higher expression in the pathogenic condition in microarray analyses; however, the fold change was lower than 2 [15]. *xps*E also showed high expression at this time point. This gene is one of the components of the type II secretion system (general secretory pathway, GSP), which is responsible for secretion of degrading enzymes and toxins [17].

Some differences were observed when comparing the expression of adaptation-related genes in planta and in vitro. These differences were mainly observed for the *msr*A and *cva*C genes. No expression of the *msr*A gene was verified in the initial colonization in planta, contrary to the growth in vitro where expression was observed at all the time points. However, the amount of *msr*A mRNA seems to be low since 35 cycles were necessary for visualization of the bands. The *cva*C gene showed high expression in the later stages of colonization in planta and, like *msr*A, its expression level was similar in all stages of in vitro growth. The difference observed in the expression of the adaptation-related genes in vitro and in planta could have resulted from the different environments in which the cells were grown, since the expression of the adaptation-related genes is possibly dependent on the environmental conditions to which the

bacterium is exposed [10, 11]. The high expression of these genes in *X. fastidiosa* was associated with an increase in the capacity of adaptation and competitiveness in the habitat [15]. The pattern of expression observed suggests that adhesion genes modulate biofilm formation while the expression of some adaptation genes may be related to the environment in which the organism is living.

The pathogenicity of *X. fastidiosa* is a result of biofilm formation inside the vessels leading to blockage and consequent water stress. This pathogenicity mechanism differs from those of the other phytopathogenic bacteria, which depends on the type III secretion system, constituting a new way of causing disease in plants.

ACKNOWLEDGMENTS

We thank Yoko B. Rosato for critical review of the manuscript. This work was supported by research grants from FAPESP (Process number 99/04266-6) and MCT-CNPq (Institutos do Milênio Process number 62.0054/01-8). M.A.T. is a FAPESP Posdoctoral Fellow and E.O.P. is an M.Sc. student supported by FAPESP. H.D.C.F. and M.A.M are partially supported by CNPq.

Literature Cited

1. Almeida RPP, Pereira EF, Purcell AH, Lopes JRS (2001) Multiplication and movement of a citrus strain of *Xylella fastidiosa* within sweet orange. Plant Dis 85:382–386
2. Barnard EL, Ash EC, Hopkins DL, McGovern RJ (1998) Distribution of *Xylella fastidiosa* in oaks in Florida and its association with growth decline in *Quercus laevis*. Plant Dis 82:569–572
3. Cope L, Lafontaine ER, Slaughter CA, Hasemann CA Jr, Aebi C, Henderson FW, et al. (1999) Characterization of the *Moraxella catarrhalis usp*A1 and *usp*A2 genes and their encoded products. J Bacteriol 181:4026–4034
4. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lapin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49:711–745
5. Davey ME, ÓToole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867
6. Davis MJ, French WJ, Schaad NW (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr Microbiol 6:309–314
7. Hoiczyk E, Roggenkamp A, Reichenbecher M, Lupas A, Heesemann J (2000) Structure and sequence analysis of *Yersinia* YadA and *Moraxella* UspAs reveal a novel class of adhesins. EMBO J 19:5989–5999
8. Lima JEO, Miranda VS, Hartung JS, Brlansky RH, Coutinho A, Roberto SR, et al. (1998) Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison with *Xylella fastidiosa* of citrus. Plant Dis 82:94–97
9. Marques LLR, Ceri H, Manfio GP, Reid DM, Olson ME (2002) Characterization of biofilm formation by *Xylella fastidiosa* in vitro. Plant Dis 86:633–638
10. O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79
11. Prigent-Combaret C, Vidal O, Dorel C, Lejeune P (1999) Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J Bacteriol 181:5993–6002
12. Purcell AH, Hopkins DL (1996) Fastidious xylem-limited bacterial plant pathogens. Annu Rev Phytopathol 34:131–151
13. Rahmati S, Yang S, Davidson AL, Zechiedrich EL (2002) Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. Mol Microbiol 43:677–685
14. Simpson AJG, Reinach FC, Arruda P, et al. (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151–157
15. Souza AA, Takita MA, Coletta-Filho HD, Caldana C, Goldman GH, Yanai GM, et al. (2003) Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity. Mol Plant-Microb Interact 16: 867–875
16. St. Geme JW, Cutter D, Barenkamp SJ (1996) Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. J Bacteriol 178:6281–6287
17. Stathopoulos C, Hendrixson DR, Thanassi DG, Hultgren SJ, St. Geme JW, Curtiss R (2000) Secretion of virulence determinants by the general secretory pathway in gram-negative pathogens: An evolving story. Microbes Infect 2:1061–1072