Analysis of Gene Expression in Two Growth States of *Xylella fastidiosa* and Its Relationship with Pathogenicity

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Xylella fastidiosa is a plant pathogen responsible for diseases of economically important crops. Although there is considerable disagreement about its mechanism of pathogenicity, blockage of the vessels is one of the most accepted hypotheses. Loss of virulence by this bacterium was observed after serial passages in axenic culture. To confirm the loss of pathogenicity of *X. fastidiosa*, the causing agent of citrus variegated chlorosis (CVC), freshly-isolated bacteria (first passage [FP]condition) as well as bacteria obtained after 46 passages in axenic culture (several passage [SP]condition) were inoculated into sweet orange and periwinkle plants. Using real time quantitative polymerase chain reaction, we verified that the colonization of FP cells was more efficient for both hosts. The sequence of the complete *X. fastidiosa* genome allowed the construction of a DNA microarray that was used to investigate the total changes in gene expression associated with the FP condition. Most genes found to be induced in the FP condition were associated with adhesion and probably with adaptation to the host environment. This report represents the first study of the transcriptome of this pathogen, which has recently gained more importance, since the genome of several strains has been either partially or entirely sequenced.

Xylella fastidiosa is a gram-negative and xylem-inhabiting bacterium responsible for diseases of economically important crops such as plum, almond, peach, coffee, grapevine, and citrus (Lima et al. 1998, Purcell and Hopkins 1996) as well as ornamental plants (Barnard et al. 1998). 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 sequenced the genome of the CVC strain 9a5c of *X. fastidiosa*, which became the first plant pathogen to have its complete genome sequenced (Simpson et al. 2000).

There is considerable disagreement about the mechanisms of pathogenicity of *X. fastidiosa*. However, the vascular occlusion by bacterial aggregates and the production of extracellular polysaccharides (EPS) leading to water stress is the most accepted cause of the disease (Machado et al. 2001). The high

water stress observed in leaves affected with CVC is characterized by a drastic decrease in the water potential, inducing leaf wilting even in plants grown in humid soil. The water deficiency symptoms of CVC-affected plants occur due to the increase in the water flow resistance throughout the xylem vessels (Machado et al. 1994). Ultrastructural studies of *X. fastidiosa* strains in xylem vessels showed cellular aggregates immersed in an electron-dense region probably composed by EPS (Chagas et al. 1992). Aggregated colonies appeared to be attached to the xylem vessels by extracellular strands produced by the bacteria, which seemed to be related to pathogenicity.

Hopkins (1985) observed that virulent strains of *X. fastidiosa* causing Pierce's disease (PD) could partially or totally lose their virulence after successive transfers in culture medium. The cells also lost their aggregation ability, suggesting that there may be a connection between aggregation and pathogenicity of *X. fastidiosa*. A similar observation was made for the CVC strain inoculated into periwinkle (*Catharanthus roseus*), an alternative host. Inoculation with cells at the eighth transfer in culture medium was able to infect a greater number of plants and induce symptoms faster than inoculation using cells at the 58th passage (Monteiro et al. 2001).

Loss of virulence after several passages in axenic culture in either solid or liquid medium is observed in several pathogenic bacteria with wide host spectrum (Behr et al. 1999; Hu et al. 1991; Levine et al. 1990; McCutchan et al. 1976; Somerville et al. 2002). The major biological property lost in avirulent strains after several passages was the ability to attach to tissues (Masuzawa et al. 1994). We quantified the ability of *X. fastidiosa* to colonize sweet orange (*Citrus sinensis* L. Osb.) and periwinkle plants after being exposed to both growth conditions by real time quantitative polymerase chain reaction (qPCR).

We hypothesize that the loss of the aggregation ability of *X. fastidiosa* after several passages in culture medium is associated with suppression of gene expression and that the reactivation of these genes might be slow or no longer attained, which could be reflected in the delay or absence of symptom development. This led us to start a comparative study, using microarray technology, of the gene expression profiles upon first passage in culture and after serial subcultures, opening the possibility of identifying genes associated with pathogenicity.

This report represents the first transcriptome study of this bacterial pathogen that has recently gained more importance

since the entire genome of isolates from citrus (Simpson et al. 2000) and grape (ONSA website) have been completely sequenced and those of almond and oleander strains (U.S. Department of Energy microbial genomics webpage) have been partially sequenced.

RESULTS

Concentration of *X. fastidiosa* in plant tissue, estimated by real time qPCR.

The ability of *X. fastidiosa* to colonize plant tissues was estimated by determining the number of bacterial cells in infected tissues using qPCR. For the standard curve, serial dilutions of *X. fastidiosa* DNA, ranging from 100,000 to 1 estimated copy number, were done and used as template for the analyses (Fig. 1). “Threshold cycle” (C_T) values increased in each dilution, ranging from 23.35 to 40 cycles. Therefore, under the PCR conditions tested, the quantification was shown to be linear from 100 to 100,000 initial copies of template DNA per reaction. The straight line calculated by logarithmic regression is represented by the equation $y = -4.12972 \times \ln(\text{number of copies}) + 44.397$, with $R^2 = 0.9975$. Samples with a fluorescent signal above a preset threshold ($C_T < 36.29$) after 40 PCR cycles were considered positive, which corresponds to >100

Fig. 1. Standard curve of *Xylella fastidiosa* cells determined by real-time quantitative polymerase chain reaction (PCR). C_T is plotted against the log number of *X. fastidiosa* cells in samples of 10-fold dilutions of template DNA (only one template per cell). The straight line calculated by logarithmic regression was $y = -4.12972 \times \ln(\text{number of copies}) + 44.397$, with $R^2 = 0.9975$. The samples with an increase of fluorescent signal above a present threshold within 40 PCR cycles were considered positive when C_T values were lower than 36.29, which corresponds to >100 initial copies of template.

initial copies of template. The high value of R^2 , obtained for the standard curve, and the good reproducibility of the repetitions within each point of the serial dilution of the DNA confirm the validity of the assay for the quantification of the target DNA.

The estimated populations of *X. fastidiosa* in plant tissues are shown in Table 1. Three measurements of a single DNA sample were carried out. The percentage of coefficient variation (CV%) of the intrameasures ranged from 0.00 to 2.91%, with an overall average of 0.82% (data not shown). Intermeasures of the C_T values (mean of 10 plants) showed a CV% ranging from 0.18 to 18%. However, values of C_T from either citrus or periwinkle inoculated with *X. fastidiosa* grown under several passage (SP) and first passage (FP) conditions were significantly different ($P < 0.001$, according to analysis of t test). The average of C_T for both plant species was always lower when cells of the FP condition were used for the inoculation (lower C_T , more template). For both hosts, the population of *X. fastidiosa* in FP conditions kept increasing for 60 to 180 days, reaching 3.9×10^4 (sweet orange) and 7.7×10^4 (periwinkle) DNA copies per reaction. In contrast, SP bacterial populations increased for only 120 days reaching 0.9×10^3 (sweet orange) and 2.6×10^3 (periwinkle) DNA copies per reaction and then declined after 180 days. The concentration of *X. fastidiosa* on periwinkle was always higher than in sweet orange for both growth conditions. Development of symptoms was observed only in sweet orange and periwinkle plants inoculated with cells from the FP condition, which is in agreement with the number of cells present in the plants.

Construction of *X. fastidiosa* DNA microarray and analysis.

We constructed DNA microarrays carrying representative sequences from approximately 2,200 open reading frames (ORF) from the genome of *X. fastidiosa* strain 9a5c. Total RNA obtained from cells grown under FP and SP conditions was labeled and used in a competitive hybridization. The data obtained from the experiments (published as supporting information on the Centro APTA Citros website) were used for statistical analyses with SAM (significance analysis of microarrays), which calculated the fold change and the significance of the differences in expression. SAM calculates the fold change in gene expression level by the ratio of average fluorescent intensity for each condition. The fold change cutoff used in the analysis was 2.0, together with $\Delta 0.40469$ and 1,000 permutations. These parameters resulted in a false significant number (FSN) of 0.79 and a false discovery rate (FDR) of less than 2%, meaning that less than one so-called statistically significant ORF could be a false positive in our experiment. To increase confidence, we have picked only ORFs that showed a

Table 1. Summary of results of real-time polymerase chain reaction detection of *Xylella fastidiosa* in citrus and periwinkle plants^a

Inoculum	60 dai ^b				120 dai				180 dai			
	C_T ^c	SD	CV%	Copy no. ^d	C_T	SD	CV%	Copy no.	C_T	SD	CV%	Copy no.
Citrus												
SP	39.98	0.07	0.18	ND ^e	32.64	1.28	3.81	88	40.00	0.00	0.00	ND
FP	34.18	1.96	5.51	532	30.61	3.37	10.50	1,035	30.2	5.59	18.00	39,775
t test ^f	0.0001				0.006				0.004			
Periwinkle												
SP	32.55	0.81	2.40	827	31.59	2.65	8.21	2,635	34.97	0.67	1.80	200
FP	28.74	1.03	3.46	6,218	27.77	2.44	8.30	10,158	25.97	4.45	16.63	77,700
t test	0.0001				0.0003				0.0001			

^a Citrus and periwinkle plants were inoculated with *X. fastidiosa* grown under several passage (SP) and first passage (FP) conditions.

^b dai = days after inoculation.

^c Average of 10 plants.

^d Copy number of DNA estimated from C_T values

^e ND = Not determined, below detection limit (less than 100 cells).

^f Statistical t test between samples from SP and FP conditions.

significant difference in expression in both Cy3 and Cy5 labeling (in independent SAM analyses) and displayed an average background-subtracted fluorescent intensity above 500 units, since control experiments showed that weaker signals were likely to produce unreliable results (data not shown). To verify whether specific *X. fastidiosa* sequences could be associated with preferential incorporation of one fluorophore, DNA from the 9a5c strain was labeled with Cy3-dCTP and Cy5-dCTP in two separate reactions, and equimolecular amounts of the labeled DNAs were mixed and hybridized to a microarray. Two independent hybridizations were performed (in triplicate), and the results showed that all spots provided similar hybridization intensities with both fluorophores. SAM analysis was performed on the data obtained from these experiments, returning no significant changes (Costa de Oliveira et al. 2002). Similar results were obtained using labeled cDNA derived from total RNA from *X. fastidiosa* cells grown in liquid Periwinkle wilt (PW) medium (data not shown). No fluorescence was observed in the chip when the total RNA was labeled using Klenow, showing absence of DNA in the total RNA preparation (data not shown).

The induction or repression of the genes is shown in a scatter plot (Fig. 2). Expression levels for the majority of genes did not differ between the growth conditions. Of this subset, 18

genes showed a statistically significant increase in the levels of transcripts, while 21 genes were repressed in the FP condition. The genes differentially expressed are distributed throughout the different categories, as summarized in Table 2. It is important to mention that the function of genes was assigned only by homology with proteins from other organisms, since no gene from *X. fastidiosa* has been functionally characterized so far.

Many of the differentially expressed genes encode hypothetical proteins, reflecting the large number of ORFs encoding unknown proteins found in the genome of *X. fastidiosa* and housekeeping genes. Interestingly, genes potentially involved in pathogenicity, virulence, and adaptation were induced only in the FP condition.

Induction of genes in the FP condition encoding pathogenicity-related proteins.

Seven genes encoding pathogenicity-related proteins are expressed mainly in the FP condition. Three of these genes are related to adhesion in other organisms. They encode a putative fimbrial protein similar to the FimA precursor of *Xanthomonas hyacinthi* and two nonpili adhesins similar to UspA1 from *Moraxella catharralis* and Hsf from *Haemophilus influenzae*. Although the ratio for the *msrA* gene is below the cutoff limit, its higher expression in the FP condition is worth mentioning,

Table 2. Expression ratio of functional groups of genes in first passage (FP) condition

Functional group ^a	Gene ^a	ORF number ^a	Description	Ratio of gene expression ^b
Intermediary metabolism	<i>orf111</i>	XF0357	Esterase	3.17
	<i>orf111</i>	XF2151	Esterase	2.74
	AF0343	XF1133	Tryptophan repressor binding protein	-2.01
	<i>korA</i>	XFa0057	Transcriptional regulator	-2.23
	<i>gloA</i>	XF1399	Lactoylglutathione lyase	-2.07
Biosynthesis of small molecules	SCH10.14c	XF0356	Cytochrome P-450 hydroxylase	2.17
	<i>orfU1</i>	XF1441	Phosphohydrolase	-3.65
	<i>folC</i>	XF1946	Folypolyglutamate synthase dihydrofolate synthase	-2.38
Macromolecule metabolism	<i>proS</i>	XF0445	Prolyl-tRNA synthetase	2.02
	<i>mag1</i>	XF1326	DNA-3-methyladenine glycosidase	-3.51
	<i>miaA</i>	XF0090	tRNA delta(2)-isopentenylpyrophosphate transferase (35.5 kDa)	-2.12
	<i>traC</i>	XF2025	DNA primase	-2.02
Cell structure	<i>lyc</i>	XF2392	Autolytic lysozyme	2.23
	<i>trbE</i>	XF2053	Conjugal transfer protein	-2.16
	<i>dmt</i>	XF0612	Dolichol-phosphate mannosyltransferase	-2.94
Cellular process	<i>ape2426</i>	XF1398	Na ⁺ /H ⁺ exchange protein	-2.17
	<i>secB</i>	XF1801	Protein-export protein	-2.64
Pathogenicity, virulence, and adaptation	<i>fimA</i>	XF2539	Fimbrial protein	2.20
	<i>msrA</i>	XF1940	Peptide methionine sulfoxide reductase	1.76
	<i>uspA1</i>	XF1516	Surface-exposed outer membrane protein	10.15
	<i>hsf</i>	XF1529	Surface protein	3.68
	<i>cvaC</i>	XF0263	Colicin V precursor	6.51
	<i>acrA</i>	XF2093	Precursor of drug resistance protein	2.07
	<i>xpsE</i>	XF1517	General secretory pathway protein E	2.00
	...	XF1255	...	8.04
Hypothetical protein	...	XF0531	...	8.18
	...	XF2076	...	2.83
	...	XF1434	...	2.55
	...	XF0265	...	2.06
	...	XF2662	...	3.57
	...	XF2252-53-54 ^c	...	-2.42
	...	XF1853	...	-2.36
	...	XF0544-45-46 ^c	...	-2.05
	...	XF1307	...	-2.29
	...	XF1792	...	-2.23
	...	XF2317	...	-2.45
	...	XF0583	...	-5.50
	...	XF1417	...	-2.11
	...	XF2720	...	-2.25

^a Based on AEG's *Xylella fastidiosa* genome project.

^b Positive and negative values represent induction and repression in FP condition, respectively.

^c This pair of primers covers three ORFs.

since the MsrA protein is important for the maintenance of adhesion in pathogenic bacteria (Wizemann et al. 1996).

Two other genes identified in our analysis are involved in adaptation to the environment. One of these genes encodes a protein with similarity to Colicin V in its C-terminal portion, while the other one is homologous to *acrA*, a multidrug, resistance-protein encoding gene. Pfam analysis indicates that it belongs to the HlyD secretion protein family. The *acrB* gene located downstream of *acrA* also showed a significant higher expression in FP, although with a fold change less than 2.

The last gene possibly involved in pathogenicity is *xpsE*. This ORF encodes one of the components of the type II secretion system (general secretory pathway) that is responsible for secretion of degrading enzymes and toxins.

Fig. 2. Identification of genes with significant changes in expression. Scatter plot of the observed relative difference vs. the expected relative difference calculated by significance analysis of microarrays. The dashed lines indicate the cutoff for twofold induction and repression. The potentially significant induced or repressed genes for $\Delta = 0.40469$ are represented by $\circ$ and $\square$, respectively.

Confirmation of microarray data with reverse transcription (RT)-PCR analysis.

In order to validate the results from the microarray study, we performed RT-PCR analysis for the *fimA*, *msrA*, *uspA1*, *hsf*, *cvaC*, *acrA*, and *xpsE* genes (Fig. 3). For these genes, expression ratios obtained in the microarray analysis were consistent with the RT-PCR ratios (Table 2, Fig. 3A), even though the data from the RT-PCR results from a single experiment, while in microarray analysis, the expression ratio for each transcript was calculated from the average expression ratios based on multiple measurements.

The ratios observed in the experiment in planta for all seven genes were different from those obtained for the condition utilized in the microarray. This difference was mainly observed for the *msrA* gene, which did not show any expression, even after 35 amplification cycles. All other genes showed a ratio between 1.27 and 1.70; nevertheless, as obtained in the microarray experiment, induction was always observed for the FP cells (Table 2, Fig. 3B).

DISCUSSION

X. fastidiosa cells grown under the SP condition were transferred in culture for one year, resulting in 46 passages. In this growth condition, *X. fastidiosa* and other microorganisms lose virulence or exhibit a significant reduction of it. Focusing on the virulence of *X. fastidiosa*, we used real-time PCR for quantification of bacteria within the xylem vessel. Calibration experiments using known amounts of *X. fastidiosa* DNA demonstrated that the detection was possible for C_T values less than 36.29, which is equivalent to 100 cells (Fig. 1). Intrameasure reproducibility among replications from the same DNA source was high (mean CV of 0.82%). Even though intermeasures showed CV% of 18, the statistical difference between the treatments inoculated with FP and SP conditions of the bacteria was significant in Student *t* test (Table 1). Cells of *X. fastidiosa* grown under the FP condition had greater ability to colonize plant tissue than those grown under the SP condition in all the

Fig. 3. Reverse transcription-polymerase chain reaction of first passage (FP)-induced genes encoding pathogenicity factors in *Xylella fastidiosa*. The ratios of the normalized quantitated band densities are presented as the FP value divided by the several passage (SP) value. **A**, Experiment using the condition utilized in the microarray experiment. **B**, Experiment in planta, evaluated 15 days after inoculation. nd = not determined.

evaluated points. The growth condition seems to affect multiplication and, consequently the movement within the hosts. This effect was more evident in the treatments with SP cells when a significant decrease of cell number, 15 cm above the inoculation point, was observed 180 days after inoculation (dai). On the other hand, a significant increase was observed in treatments with FP cells (Table 1). The more efficient colonization of FP cells within the plants allows development of symptoms in both sweet orange and periwinkle hosts. Conversely, symptoms were not observed in either plant species inoculated with SP cells, probably due to the low cell number observed in these plants, which could be insufficient for obstructing vessels. Vascular occlusion by cell aggregates leading to water stress is the most accepted cause of the disease in plants infected with *X. fastidiosa* (Hopkins 1989). The degree of the occlusion might be related to the pathogen population within the xylem.

Hopkins (1985) suggests that colonization and pathogenicity of the PD strains of *X. fastidiosa* seem to be dependent on systemic movement within the xylem vessels. Virulence seems to be related to multiplication and movement in the host tissue, since avirulent or weakly virulent strains obtained after weekly serial transfers multiplied at a very slow rate and rarely moved from the inoculation point into the xylem vessels. In the present paper, inoculum of *X. fastidiosa* from the SP condition could not colonize the xylem vessels of either host as efficiently as FP. Colonization can be understood as the ability of the cells to adhere and multiply and, consequently, fill the vessels along the xylem. These results are in accordance to the hypothesis that successive passages could reduce bacterial virulence and affect the ability of colonization of the host (Hopkins 1985), as pointed out for other pathogenic bacteria (Masuzawa et al. 1994) and recently shown by Monteiro and associates (2001). In our experiments, we did not observe symptom development with the SP bacteria even 180 dai. Indeed, the population verified at this timepoint showed a decrease in relation to that at 120 dai, suggesting that the SP population is avirulent. It has been shown for other bacteria that mutations are associated with phenotypic changes after several passages in culture, including loss of virulence (Behr et al. 1999; Somerville et al. 2002). Nevertheless, the reason for the loss of virulence in *X. fastidiosa* remains unclear.

A comparison of the gene expression profiles in FP and SP conditions of *X. fastidiosa* revealed that only FP cells showed induction of genes related to pathogenicity, virulence, and adaptation, strengthening the idea that these genes could be related to the higher infectivity of bacteria in this condition, as determined by qPCR. In the assay using bacteria grown in planta, all the genes but *msrA* showed the same trend of higher expression in FP than in SP, although with a lower ratio as compared with the other experiments. The RT-PCR experiment in planta revealed no expression of *msrA* in either condition. The evaluation of gene expression 15 dai in the plant could be the reason explaining the absence of transcripts of *msrA*, since this gene could be necessary in more advanced stages of colonization, and for this reason, a differential expression was observed after isolation of bacteria from symptomatic plants.

All seven genes detected in the microarray have very specific functions in pathogenicity. The *msrA* gene encodes a protein that catalyzes the reduction of methionine sulfoxide back to methionine (Weissbach et al. 2002). It is required for the functioning of adhesins in three mammalian pathogens, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, and *Escherichia coli* (Wizemann et al. 1996). More recently, the importance of this gene in the virulence of the plant pathogen *Erwinia chrysanthemi* was characterized. *msrA* mutants are more sensitive to oxidative stress, are incapable of systemic invasion, and ex-

hibited reduced virulence on chicory leaves (Mohammed et al. 1999). The hypothesis to explain the alterations observed in these mutants is related to their incapacity to survive in plant tissue, leading to the decline of the population. The direct cause of this is thought to be oxidative damage caused by different factors, which, in the absence of the peptide methionine sulfoxide repair function, limits bacterial survival throughout the plant.

Thiol groups present in the bacterial cell surface could be mediating the adhesion of *X. fastidiosa* (Leite et al. 2002). The sulfur residue in its reduced form allows adhesion, and MsrA enzyme keeps these residues in this state (Lowther et al. 2000). Alternatively, the structures necessary for adhesion depend on some proteins that are sensitive to oxidative stress, and the role of MsrA is to maintain these proteins in their active form.

Another gene involved in adhesion of *X. fastidiosa* that was detected in the FP condition is *fimA*, similar to the one described for the plant pathogen *X. hyacinthi* (van Doorn et al. 2001). This protein is an adhesin of the type-IV fimbriae, similar to others found in numerous bacterial species that infect not only plants but also animals and humans (Ojanen-reuhs et al. 1997). They are considered key mediators of adhesion and motility, being an important virulence determinant. In *X. campestris* pv. *vesicatoria*, a plant pathogen, *fimA*, contributes to cell-to-cell aggregation. Moreover, the *fimA* mutant poorly colonized the trichomes of tomato leaves, suggesting that adhesiveness to these plant structures involves FimA, similar to the type IV fimbriae of human pathogens (van Doorn et al. 2001). Therefore, the *X. fastidiosa* *fimA* can be involved in cell aggregation and the ability to form adherent colonies on surfaces.

Two other genes involved in the adhesion process in human pathogens were also highly expressed in *X. fastidiosa* grown under the FP condition. The *uspA1* and *hsf* genes show homology to *uspA1* of *M. catharralis* and *hsf* of *Haemophilus influenzae*, respectively. A Blast search of the NCBI database showed homologies to several outer membrane proteins and adhesins important for attachment to host tissues (Cope et al. 1999; Hoiczky et al. 2000; Peak et al. 2000; St. Geme et al. 1996). The C-terminal regions of both adhesins in *X. fastidiosa* are similar and exhibit characteristics of autotransporter proteins.

In *M. catharralis*, *UspA1* is structurally related to the *UspA2* protein, although with different biological functions. *UspA1* is essential for cell attachment, while *UspA2* mediates serum resistance (Aebi et al. 1998; Lafontaine et al. 2000). No homologue of *uspA2* was found in the *X. fastidiosa* genome. *UspA1* has been shown to be essential for the attachment of *M. catharralis* to Chang conjunctival tissue in vitro (Lafontaine et al. 2000). Recently a *uspA1* homologue, named *xadA*, was characterized in *Xanthomonas oryzae* pv. *oryzae* (Ray et al. 2002). *xadA* mutants show reduced virulence and altered colony and lawn morphology. The XadA protein appears to be expressed in minimal medium but not in rich medium. As the authors point out, xylem sap is likely be more akin to minimal medium than to rich medium, suggesting that *xadA* is indeed being expressed in planta. Similarly, we observed that *X. fastidiosa* *uspA1* is also differentially expressed in our growth conditions, in which freshly isolated bacteria were compared with bacteria grown in a very rich medium. Interestingly, the high expression of this gene in FP shows that one culture passage was not sufficient to turn off *uspA1* expression.

The *hsf* gene of *X. fastidiosa* is similar to the *hsf* and *hia* genes of *H. influenzae*. The *hsf* gene product plays an important role in the process of respiratory tract colonization by *H. influenzae* type b, while Hia mediates adhesion of the nontypeable *H. influenzae*. Hsf and Hia are 81% similar to each other and appear to recognize the same cell receptor; however, Hsf protein is associated with expression of short surface fibrils. In

contrast, Hia has not been associated with fibril expression (St. Geme et al. 1996). These proteins could be part of a novel class of adhesins, like YadA of *Yersinia* spp. and UspA of *M. catharralis* (Hoiczky et al. 2000). These adhesins follow the same architecture, forming a novel class of surface projections. The surface projections may be suitable to fulfill multiple functions, including the mediation of adherence to host cell surface structures, which may explain the wide occurrence of these adhesins among pathogens and free-living microorganisms. The expression of both adhesin genes in *X. fastidiosa*, together with *msrA* e *fimA* in FP, could produce an efficient adhesion mechanism, not only among cells but also with the surface of xylem vessels, resulting in a more efficient colonization. Based on bioinformatics analysis, these adhesins were recently suggested to be pathogenicity-related factors in *X. fastidiosa* (Bhattacharyya et al. 2002).

Scanning electron microscopy was recently used for studies on the biofilm formation in *X. fastidiosa* (Marques et al. 2002). The authors stress the necessity of identification of genes related to formation of the biofilm and their role in the infection and development of the disease. We show in this work that genes possibly related to disease development are involved in adhesion, which is an essential step for biofilm formation. Our results agree with the hypothesis that biofilm formation may be a key element in the pathogenicity of *X. fastidiosa*, resulting in an efficient capacity of colonization of the xylem vessels. Since the insect vector inoculates the bacteria inside the xylem vessel, a highly turbulent and negative pressure environment, an efficient adhesion mechanism could be essential for survival.

Other factors could also contribute with the more efficient colonization of FP cells, increasing the capacity of adaptation and competitiveness in the habitat to be colonized. Secretion of toxins and exoenzymes that depend on the type II secretion machinery could also be increased by a higher expression of components of this system, as observed for *xpsE*. Production of proteins, like colicin V, an antibacterial toxin produced by *E. coli* that acts against closely related sensitive bacteria (Havarstein et al. 1994), can also be an advantage factor for competition, since they could act against naturally found endophytes of the xylem. Studies suggest that colicins provide a competitive edge in nutrient-poor environments (Riley and Gordon 1999).

Moreover, some other gene products could act as detoxifying agents against a set of different drugs, represented here by AcrA and AcrB. Blast search using AcrA shows high similarity to other proteins present in several human and plant pathogens. It is thought to bring the outer and inner membranes closer and acts together with AcrB, a proton antiporter, composing a multidrug efflux system (Nikaido and Zgurskaya 2001). These different factors are likely to make *X. fastidiosa* an organism that is efficient for inhabiting the xylem vessels.

The genes that are possibly related to the higher colonization capacity in FP, identified as differentially expressed in the microarray experiment, can be a fraction of the genes actually expressed inside the plant. This is because the FP condition involves one step of growth in medium, which could turn off some of the pathogenicity genes in this initial condition. Moreover, by using the parameters defined in SAM, we detected only a percentage of the genes that could be observed using a less stringent setup but that could also increase the number of false positives. Still, we were able to detect some genes possibly involved in the colonization of the xylem vessels. Whiteley and associates (2001), using a fold change >2, detected few differentially expressed genes when evaluating biofilms of *Pseudomonas aeruginosa*. In a universe of 5,500 genes, only 34 were activated and 39 were repressed in biofilm populations representing approximately 1% of the genome. Similar results were found for *Caenorhabditis elegans*, where 1.5% of the ge-

nome was differentially expressed in different developmental stages, growth conditions, or worm strains (Jiang et al. 2001).

Typical plant pathogens secrete effectors and enzymes used for the penetration and colonization of plant tissues (Barras et al. 1994; Lahaye and Bonas 2001). However, *X. fastidiosa* resembles some human bacterial pathogens whose pathogenicity depends on biofilm formation, leading to the occasional obstruction of the colonized area (Lafontaine et al. 2000; Murphy and Kirkham 2002).

Further studies aiming to produce mutants for the genes identified in this work are crucial in order to better characterize the mechanism utilized by the bacterium, especially in biofilm formation.

MATERIALS AND METHODS

X. fastidiosa growth conditions.

Cells of *X. fastidiosa* strain 9a5c were grown in PW broth (Davis et al. 1981) for 4 to 5 days, reaching 10^8 colony forming units (CFU) per ml. The cells were collected by centrifugation and were resuspended in phosphate-buffered saline (PBS). A total volume of 40 μ l of the suspension was applied as a drop on the main shoot of periwinkle plants. We then pierced the spots where the drops had been deposited with a syringe needle, until the plant tissue absorbed all the suspension. Control plants were inoculated similarly with PBS free of bacteria. The plants were maintained in greenhouse conditions. Four months later, the plants showed symptoms such as stunting, short internodes, narrow limbs, small leaves, and chlorosis. Petioles and stems were aseptically ground in PBS, and the suspension was spread on PW medium. The first colonies were observed between 10 to 15 dai. This condition of *X. fastidiosa* was named first passage (FP). To obtain the *X. fastidiosa* after several passages (SP), the FP cells were transferred 46 times (once a week) to fresh PW plates.

Pathogenicity assays.

Plants of sweet orange cv. Pêra and periwinkle were grown in 2l pots containing a mixture of soil, sand, and manure (2:1:1) and were maintained in a greenhouse under moderate water stress. Both FP and SP inoculum of *X. fastidiosa* were harvested and suspended in PBS at a final concentration of 10^8 CFU/ml. A total volume of 40 μ l of the concentrated suspension of bacteria or of PBS as a control was inoculated in drops at the main shoot. The colonization in the plants was monitored by qPCR at 60, 120, and 180 dai.

qPCR assays.

Primers and the TaqMan probe (Applied Biosystems, Foster City, CA, U.S.A.) were developed for 5' nuclease PCR assays of *X. fastidiosa* (Oliveira et al. 2002). The 5' nuclease PCR with a fluorescent probe was performed in a 25- μ l volume, using the TaqMan core PCR reagent kit (Applied Biosystems) as follows: 12.5 μ l TaqMan Universal PCR Master Mix (5 μ l of 10 $\times$ TaqMan buffer, 10 mM Tris [pH 8.3], 50 mM KCl, 5 μ l of a deoxynucleoside triphosphate solution [2 mM each dATP, dCTP, and dGTP and 4 mM dUTP], 0.5 U of Amp Erase uracil N-glycosylase, and 0.25 U of AmpliTaq Gold), 525 nM of each primer, 500 nM of the probe, 1 μ l of DNA solution (10 ng), and 6.87 μ l of water. The TaqMan buffer contains a passive reference dye, ROX, which is used to normalize for volume variations and to standardize the reaction (Heid et al. 1996). The amplifications were performed as described in the ABI PRISM 7700 User's Manual, using the recommended universal thermal cycler protocol as follows: all cycles began with 2 min at 50°C, 10 min at 95°C, followed by 40 two-step cycles of 15 s at 95°C and 1 min at

60°C. The determination of the fluorescent intensity of each dye, data acquisition, and data analysis were carried out in an ABI 7700 Prism Sequence Detector (Applied Biosystems). Fluorescent intensities were calculated using the Sequence Detector Software 1.6 application software (Applied Biosystems), as described by the manufacturer. Briefly, a normalized emission intensity of the reporter dye (R_n) is defined for each reaction tube, and ΔR_n , an indication of the signal magnitude generated by PCR, is calculated. The first statistically significant increase in ΔR_n for a given sample is denominated the C_T value, which is the fractional cycle number of PCR, calculated as the average standard deviation of R_n for the early cycles, in which no fluorescence is observed. Quantification of samples with unknown DNA amounts is accomplished by interpolation of their C_T value obtained from a standard curve (C_T plotted against log target DNA) run simultaneously with the experimental samples. The standard curve was constructed according to the method previously described (Oliveira et al. 2002). Briefly, known amounts of *X. fastidiosa* and sweet orange total DNA were mixed to obtain a range of 1×10^0 to 1×10^5 copies of *X. fastidiosa* DNA per reaction mixture and then were used to make standard curves (i.e., C_T values plotted against the logarithm of the DNA copy number). In all experiments, appropriate negative controls containing no template DNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each sample was tested in triplicate, which was used for checking the standard deviation that was used for accepting or rejecting the measurement. The statistical analyses of the values obtained from the plants were carried, using the ANOVA procedure. The significance of the differences between the FP and SP treatments was verified by the Student *t* test.

Plant tissue extracts.

For qPCR analyses, total DNA was extracted from 150 mg of xylem-rich tissues from leaves (petiole and midrib) collected from seedlings of sweet orange and periwinkle inoculated with bacteria at both FP and SP conditions. Total DNA was extracted, based on modifications of the CTAB-Sarcosyl extraction method described previously (Machado et al. 1996). The DNA was diluted to 10 ng/ μ l and was stored at -20°C.

Microarray construction.

Specific pairs of primers were designed using Primer3 software (provided by S. Rozen and H. J. Skaletsky, Whitehead Institute, Cambridge, MA, U.S.A. and Howard Hughes Medical Institute, Chevy Chase, MD, U.S.A.) for the amplification of the ORFs found in the genome of *X. fastidiosa* strain 9a5c. We have simultaneously evaluated approximately 2,200 ORFs, including ORFs present in the pXf 51 plasmid. Many ORFs present in the 9a5c genome were actually duplications scattered throughout the chromosome or small ORFs encoding hypothetical proteins. These could not be efficiently and specifically amplified, due to their reduced size and chromosomal location (closely flanked by larger ORFs). Our estimation is that these analyses cover more than 90% of the most relevant ORFs present in the *X. fastidiosa* 9a5c genome.

To maximize the homogeneity of the microarray hybridizations, amplified fragments had an average GC content around 50 to 60%. Such PCR products were purified with the aid of the QiaQuick 96 PCR purification system (Qiagen, Valencia, CA, U.S.A.), were dried in a Savant speed-vac, were resuspended in 50% dimethyl sulfoxide at a final concentration of 100 to 200 ng/ μ l, and were spotted onto CMT-GAPS silane-coated slides (Corning Glass Co., Corning, NY, U.S.A.), using an GMS 417 arrayer (Affimetrix Inc., Santa Clara, CA, U.S.A.) according to the manufacturer's instructions.

RNA isolation and labeling.

Cells from the FP and SP conditions were scraped from plates and were washed twice with diethylpyrocarbonate (DEPC)-treated water. Total RNA was isolated as described by V. Rhodius on the University of California at San Francisco website. Further details are available on the Centro APTA Citros website.

RNA obtained from *X. fastidiosa* cells was labeled by reverse transcription with either Cy3 or Cy5 (both labeling for each condition). Briefly, 30 μ g of total bacterial RNA was mixed with 16 μ g of random hexamers (Gibco BRL, Gaithersburg, MD, U.S.A.) in a 30- μ l final volume. Annealing was accomplished by incubation for 2 min at 75°C, 2 min at 55°C, 2 min at 45°C, 2 min at 37°C, and 2 min at 22°C, followed by addition of 6 μ l of SuperScript II reaction buffer (Gibco BRL), 3 μ l of 0.1 M of dithiothreitol, 1 μ l dNTP mix (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM dCTP), 2 μ l of 1.0 mM Cy3- or Cy5-labeled dCTP (Amersham Biosciences, Little Chalfont, U.K.), and 2 μ l of SuperScript II reverse transcriptase (200 U/ μ l) (Gibco BRL) to the reaction. Reactions without SuperScript, using Klenow, were done in parallel to verify presence of contaminating DNA. The cDNA synthesis was carried at 42°C for 2 h. After labeling, the RNA was hydrolyzed in a 0.1 N NaOH treatment and was neutralized by the addition of 0.1 N HCl. The labeled cDNA was diluted to 500 μ l with Tris-EDTA, purified, and concentrated using a Microcon-30 (Amicon, Bedford, MA, U.S.A.) to 10 μ l.

Hybridization and analysis.

Arrays were hybridized overnight (42°C) in a GeneTac hybridization station (Genomic Solutions, Inc., Ann Arbor, MI, U.S.A.), in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 0.25 mg of sheared salmon sperm DNA per ml, 0.5% sodium dodecyl sulfate (SDS), and a mixture of the two labeled cDNA samples. After hybridization, slides were washed twice (42°C) in 0.5 $\times$ SSC and 0.01% SDS, followed by two washes in 0.06 $\times$ SSC and 0.01% SDS, and two final washes in 0.06 $\times$ SSC. All washing steps consisted of 1 min of flow, followed by 5 min of incubation. Slides were then snap-dried and subjected to fluorescence detection with an GMS 418 Array Scanner (Affimetrix Inc.). Images were analyzed with Affymetrix Jaguar v. 2.0, using the easy threshold and variable circle size algorithm. Normalization between the intensities in the two channels was achieved with the Jaguar All Spots option. Three independent experiments were performed with nine individual comparisons. These data were consolidated into a GATC database with Affymetrix MicroDB v. 2.0, and the data was used for final statistical analysis with SAM, a statistical technique developed for finding significant genes in a set of microarray experiments (Tusher et al. 2001) available on Stanford University's SAM webpage.

RT-PCR.

The same total RNA used for the microarrays was reverse-transcribed with SuperScript II (Invitrogen, Carlsbad, CA, U.S.A.) by using 1 μ g of the R-specific primer for each of the analyzed genes (Table 3). The RNAs were normalized, using ribosomal RNA content. We carried analyses for all seven pathogenicity genes (*fimA*, *msrA*, *uspA1*, *hsf*, *cvaC*, *acrA*, and *xpsE*) that were amplified with specific primers (Table 3). A 25 μ l master mix contained the following components: 2.5 μ M dNTP, Taq polymerase reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 100 ng of specific primers, 2.5 units of Taq DNA polymerase, and 2.5 μ l of first-strand cDNA template. The PCR cycle profile was 94°C for 3 min for initial denaturation, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, and

Table 3. The nucleotide sequences of the primers used for reverse transcriptase-polymerase chain reaction

Gene name	Primers sequence	Fragment size (in base pairs)
<i>jimA</i>	F—5' CCCTCGAGCCAAAATTATGTCGCCAGA 3' R—5' CACTCGAGGTGACGGTGGAGGAGCAG 3'	350
<i>msrA</i>	F—5' AATCTCGAGTGGGACGTAGTGAAC 3' R—5' AAGCTCGAGCCGGATGGAGTA 3'	498
<i>uspA1</i>	F—5' AACTCGAGAGCAGGCCGCCGGTGATAGCAGTA 3' R—5' AGACGCTCGAGCCCCGCCGCAAGAT 3'	1,122
<i>hsf</i>	F—5' CGCTCGAGGGGTCTTTGTATGT 3' R—5' GGCTCGAGACGCTGTGAGGTTTC 3'	647
<i>cvaC</i>	F—5' GTCTCGAGGCGACCTTGCTAC 3' R—5' AGCAGCCTCGAGACCACAGATAC 3'	194
<i>acrA</i>	F—5' CTCTCGAGCACGCGTGGCTGGAATA 3' R—5' AGCTCGAGCGCCTTCTTTGACTTTT 3'	919
<i>xpsE</i>	F—5' GTCTCGAGTTGTTGGCGGAAGTATGAA 3' R—5' CCCTCGAGCCAGTGACCAGCAAAATG 3'	1,101

72°C for 1 min. PCR products were run on agarose gels, quantified densitometrically using EagleSight software v. 3.2 (Stratagene, La Jolla, CA, U.S.A.).

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 from either FP or SP conditions, in order to evaluate the gene expression of all seven pathogenicity genes detected in microarray. The inoculation was performed as described above for the pathogenicity assays but using several inoculation points. 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. After 15 days, a period of time sufficient for the colonization of the vessels (Almeida et al. 2001), the leaf petioles and midribs were excised from all the plants. For extraction of cells from the xylem, the bacteria were forced from the petioles or stems, using a DEPC-treated water wash using a syringe attached to a rubber tube. The resultant bacterial cell suspension was centrifuged at 4,000 × g for 10 min at 4°C, and total RNA was extracted using TRIZOL reagent (Invitrogen).

The RT-PCR was performed as described above. However, due to the low RNA concentration, we performed 35 amplification cycles. Reactions without SuperScript II were done in parallel to rule out the possibility of amplification from contaminating DNA.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Agronomical and Environmental Genomes (AEG), an ONSA program, *Xylella* database: aeg.lbi.ic.unicamp.br/xf
- Centro APTA Citros Sylvio Moreira *X. fastidiosa* webpage: www.centrodecitricultura.br/miame_xf.htm
- Organization for Nucleotide Sequencing and Analysis (ONSA); onsona.lbi.ic.unicamp.br/world/xf-grape/
- Stanford University Department of Statistics Significant Analysis of Microarrays webpage: www-stat.stanford.edu/~tibs/SAM/index.html
- United States Department of Energy's Genome Institute microbial genomics webpage: www.jgi.doe.gov/JGI_microbial/html
- University of California at San Francisco Department of Biochemistry and Biophysics microarrays and software website: www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf
- Washington University in St. Louis database of protein families (Pfam): pfam.wustl.edu/
- The Whitehead Institute and Massachusetts Institute of Technology Primer3 software: www-genome.wi.mit.edu/genome_software/other/primer3.html

Erratum, Vol. 16, No. 10, 2003

A literature citation in the article by de Souza et al. was published incorrectly. The article was cited at the top of the second column on page 871. Mohammed et al. 1999 should be Hassouni et al. 1999 and the citation should be as follows.

Hassouni, M. E., Chambost, J. P., Expert, D., Van Gijsegem, F., and Barras, F. 1999. The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen *Erwinia chrysanthemi*. Proc. Natl. Acad. Sci. U.S.A. 96:887-892.