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Análise comparativa da expressão de genes de *Xylella fastidiosa* associados à patogenicidade e formação de biofilme

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Índice

1. Resumo

O presente trabalho tem como objetivo detectar e estudar a expressão de genes possivelmente envolvidos com patogenicidade da estirpe 9a5c de *X. fastidiosa*. Para desenvolvimento do trabalho foram utilizadas bactérias imediatamente após o isolamento da planta sintomática, denominado aqui isolamento primário (IP) e bactérias após 46 repicagens (SR) sucessivas em meio de cultura. Uma possível perda de virulência da *X. fastidiosa* submetida às condições de SR foi verificada inoculando-se plantas de laranja doce (*Citrus sinensis*) e vinca (*Cataranthus roseus*) com as bactérias obtidas nestas condições. Através do uso de PCR quantitativo em tempo real, foi verificado que a colonização das células originadas de SR foi menos eficiente em ambos hospedeiros. A tecnologia de DNA `microarray` foi utilizada para investigar as mudanças da expressão gênica associadas com a condição IP. Verificou-se que muitos dos genes diferencialmente expressos codificam para proteínas hipotéticas. Genes potencialmente envolvidos com patogenicidade, virulência e adaptação foram induzidos apenas na condição IP. Três destes genes (*fim*A, *hsf* e *usp*A1) foram associados com adesão na superfície do hospedeiro e quatro (*msr*A, *acr*A, *cva*C e *xps*E) com a capacidade de adaptação do patógeno no ambiente do hospedeiro.

A indução destes genes na condição IP foi confirmada por RT-PCR tanto na condição *in vitro* quanto na condição *in planta* 15 dias após inoculação, período este que corresponde ao início da colonização. Contudo, 90 dias após inoculação, período de colonização mais avançada com o surgimento dos primeiros sintomas, o nível de expressão dos genes de adesão foi similar em ambas condições de crescimento. Entretanto, uma maior expressão foi observada na condição IP para os genes envolvidos com adaptação no ambiente do hospedeiro. Estes resultados sugerem que a adesão é importante para o início da formação do biofilme. Por outro lado, os genes relacionados com adaptação são essenciais para manutenção do biofilme *in planta*.

Também, a expressão destes genes durante a formação de biofilme *in vitro* em *X. fastidiosa* foi avaliada por RT-PCR semi-quantitativo após 3, 5, 10, 20 e 30 dias de crescimento em superfície de vidro na interface líquido-ar. A expressão dos genes na condição *in vitro* foi similar à condição *in planta*, onde os genes de adesão tiveram uma maior indução nas etapas inicias de formação de biofilme. Estes resultados indicam que estes genes podem estar envolvidos com a adesão em diferentes superfícies. Entretanto, apenas alguns genes relacionados à adaptação (*xps*E, *acr*A) se comportaram de forma similar ao observado *in planta*, ou seja, com

maior indução na etapa de biofilme maduro. Isto pode ser resultado dos diferentes ambientes experimentais utilizados, uma vez que, a expressão destes genes pode ser regulada de acordo com o ambiente pela qual a bactéria é exposta.

Análises de microscopia ótica em diferentes fases do biofilme formado em lâminas de vidro revelaram que a formação de biofilme em *X. fastidiosa* apresenta pelo menos cinco fases distintas, sendo aos 20 dias a fase de maior densidade celular. Vários são os estudos que visam detectar os genes expressos em diferentes fases e ambientes da formação de biofilme, principalmente em bactérias que causam doenças em humanos, uma vez que, a formação do biofilme tem sido atribuída como a causa de sérias doenças. Contudo, há poucas informações em relação à expressão de genes envolvidos na formação de biofilme em patógenos de plantas. Por este motivo, e como a formação de biofilme indica ser o mecanismo primário de patogenicidade de *X. fastidiosa*, foi utilizada a tecnologia do DNA ´microarray´ para avaliar os genes expressos na fase de biofilme maduro comparado ao crescimento planctônico. Um total de 202 genes (9,18%) foram significativamente induzidos, enquanto que 32 genes (1,45%) foram reprimidos na condição de biofilme. A maioria dos genes diferencialmente expressos codifica para proteínas ainda hipotéticas. Na condição de crescimento em biofilme foi verificado um aumento da expressão de genes 'housekeeping' que codificam funções metabólicas. Também foi detectado um grande número de genes do mega plasmídio pXF51 sendo diferencialmente expresso, o que poderia provavelmente estar associado com transferência horizontal de genes em *X. fastidiosa* em biofilme. Em relação a categoria de patogenicidade, a maioria dos genes expressos na condição de biofilme foram associados com produção e detoxificação de toxinas e adaptação para crescimento em condições atípicas.

A expressão destes genes associados com adaptação pode conferir características competitivas à *X. fastidiosa* no habitat a ser colonizado, dando uma clara indicação da importância destes fatores no estabelecimento do biofilme. Tais afirmações demonstram que a propriedade fisiológica do biofilme de *X. fastidiosa* é similar ao observado em bactérias patogênicas em humanos, indicando que a formação de biofilme como mecanismo de patogenicidade de bactérias de diferentes hospedeiros apresentam características comuns.

2. Abstract

The main goal of this study is to survey the expression of genes possibly involved in the pathogenicity of strain 9a5c of *X. fastidiosa*. Freshly-isolated bacteria from symptomatic plants (first passage condition or FP) as well as bacteria obtained after 46 transfers to axenic culture (several passage condition or SP) were utilized in this work. A possible lost of virulence of the *X. fastidiosa* in the SP condition was verified after inoculation into sweet orange and periwinkle plants. Using real-time quantitative PCR, we verified that the colonization of SP cells was less efficient in both hosts. The DNA microarray technology was used to investigate the global changes in gene expression associated with the pathogenic FP condition. Most of the differentially expressed genes encode hypothetical proteins. Genes potentially involved with pathogenicity, virulence and adaptation were induced in the FP condition. Three of these genes (*fim*A, *hsf* and *usp*A1) are associated with adhesion to the host surfaces and four (*msr*A, *acr*A, *cva*C e *xps*E) with adaptation in the host environment.

The induction of these genes in the FP condition was confirmed by RT-PCR both *in vitro* and *in planta* 15 days after inoculation, period in which the initial colonization of the vessels was taking place. Ninety days after inoculation, when the colonization had reached to more advanced stages and the first symptoms were developed, the expression levels of the adhesion genes were similar although a higher expression was observed for genes related to adaptation in the pathogenic condition. These results suggest that the adhesion is important at the beginning of the biofilm formation, whereas the genes related to adaptation are essential for the maintenance of this biofilm *in planta*.

We also evaluated the expression of these genes *in vitro* during biofilm formation by semiquantitative RT-PCR after 3, 5, 10, 20 and 30 days of growth at the medium-air interface in a glass flask. The gene expression observed under *in vitro* condition was similar to that observed *in planta* for the adhesion genes whose expression occurred mainly at the initial step of biofilm formation. These results indicate that these genes can be involved in adhesion to different attachment surfaces. In relation to the adaptation-related genes, *xps*E and *acr*A showed and expression pattern similar to that observed *in planta*, with over-expression in the mature biofilm. On the other hand, the expression of *cva*C and *msr*A did not show the same pattern as found in the *in planta*. These genes showed little differences in relation to the stages of biofilm development. This difference could have resulted from the different experimental designs

utilized since the genetic reprogramming of gene expression of the bacterial biofilm depends on the changes in multiple environmental conditions to which the bacterium is exposed.

Light transmission microscopy analysis of different phases of the biofilm formed in glass covers revealed that the *X. fastidiosa* biofilm development presented five distinct stages. At the 20^{th} day, the biofilm showed high cell density. A considerable increase in the gene expression number studies involving biofilm formation has been observed, mainly for bacteria causing human diseases, since the biofilm formation has been associated with serious diseases. However, limited information is available concerning gene expression involved in biofilm formation in plant-pathogen interactions. For this reason and because biofilm formation seems to be the main pathogenicity mechanism in *X. fastidiosa*, we utilized the microarray technology to access changes in gene expression in mature biofilm cells when compared with planktonic cells. A total of 202 genes (9.18%) were significantly up-regulated, while 32 genes (1.45%) were down-regulated in the mature biofilm. The majority of the differentially expressed genes encodes hypothetical proteins. Under the biofilm condition we observed an increase in the expression of some housekeeping genes responsible for metabolic functions. We also found a large number of genes from the pXF51 plasmid being differentially expressed. This could possibly be associated with lateral gene transfer in the *X. fastidiosa* biofilm. Moreover most of the pathogenicity-related genes over-expressed in the biofilm condition were associated with toxin production, detoxification and adaptation to atypical conditions.

The expression of genes associated with adaptation and competitiveness in the habitat to be colonized gives a clear indication of the importance of such factors in the established biofilm of *X. fastidiosa*. This demonstrates that the physiological properties of this biofilm are similar to the ones observed in human pathogens, indicating that the pathogenicity mechanisms of bacteria of different host may show common characteristics.

3. Introdução Geral

3.1. *Xylella fastidiosa*: Características gerais

A *Xylella fastidiosa* é uma bactéria Gram negativa, com formato de bastonete e de tamanho variável, podendo chegar até 0,7 μm de diâmetro e 20 μm de comprimento. Não apresenta motilidade, nem flagelos e não são pigmentadas (Wells et al., 1987). Caracteriza-se pelo crescimento lento em meio de cultura, sendo suas colônias circulares, discretas, medindo até 0,6 mm de diâmetro após 10 dias de incubação a 28ºC, podendo alcançar 1,5 mm depois de 30 dias, com variações decorrentes do meio de cultura utilizado (Coletta-Filho, 2002a).

A *X. fastidiosa* é limitada ao xilema das plantas hospedeiras e ao lúmen do canal alimentar de insetos vetores (cigarrinhas), responsáveis pela transmissão da bactéria entre as plantas (Hopkins, 1995). Possui uma ampla gama de hospedeiros, incluindo membros de pelo menos 28 famílias de plantas mono e dicotiledôneas (Hopkins & Adlerz, 1988). Está associada a doenças em culturas economicamente importantes como ameixa, uva, café e citros (Purcell & Hopkins, 1996; Lima et al., 1998). No Brasil causa principalmente a clorose variegada dos citros (CVC), doença que acarreta prejuízos a economia citrícola do país. As estimativas dos danos econômicos causados pela CVC na primeira década do século XXI é da ordem de 286 – 322 milhões de dólares (Fernandes-Jr, 2003). Dada a importância econômica e social da citricultura para o Estado de São Paulo e para o Brasil e aos danos provocados pela CVC nos pomares, foi realizado um extensivo programa de pesquisa envolvendo o sequenciamento completo do genoma da *X. fastidiosa* estirpe 9a5c (Simpson et al., 2000).

Após sequenciamento foi verificado que seu genoma apresenta um conteúdo GC de 52,7% e é composto de um cromossomo principal de 2.679.305 pares de bases (pb) e dois plasmídeos, um magaplasmídeo contendo 51.158 pb e um miniplasmídeo com 1.285 pb. Um total de 2.904 ORFs (fase aberta de leitura) foram anotadas, sendo, deste total, 48 % semelhante a proteínas já descritas em outros organismos e o restante correspondendo a proteínas ainda não caracterizadas. As proteínas homólogas foram categorizadas em diferentes grupos funcionais (www.aeg.lbi.unicamp.br/xf). Dentre as diferentes categorias funcionais, 147 proteínas foram associadas como envolvidas com patogenicidade, virulência e adaptação. Curiosamente os genes responsáveis pela especificidade planta-patógeno, geralmente encontrados em bactérias fitopatogênicas, não foram encontrados no genoma da *X. fastidiosa*. Esses genes correspondem

aos genes de avirulência (*avr*) presentes no patógeno, cujos produtos interagem com proteínas de resistência no hospedeiro (R) (Coletta-Filho et al., 2002b).

A disfunção do xilema decorrente da adesão e colonização pela *X. fastidiosa* aparenta ser o mecanismo primário de patogenicidade nas plantas infectadas (Machado et al., 2001a). A combinação de outros fatores como a absorção de nutrientes e produção de toxinas, também parece contribuir para patogenicidade (Machado et al., 2001b). Contudo, apesar do sequenciamento genético e de inferências quanto a patogenicidade, apenas os estudos funcionais poderão levar a um completo entendimento dos mecanismos de patogenicidade desta bactéria.

3.2. Mecanismo de patogenicidade X formação de biofilme

Os principais sintomas da CVC decorrentes da colonização da *X. fastidiosa* em plantas de laranja doce podem assim ser descritos: *i.* inicialmente há o aparecimento de manchas cloróticas na parte dorsal das folhas correspondendo a pontos marrons com aspecto de goma na superfície ventral; *ii.* posteriormente, as plantas severamente atacadas apresentam um aspecto de debilidade geral com ramos envassourados, uma superbrotação das gemas e folhas murchas nas horas quentes do dia, mesmo em condições de umidade, finalmente há uma redução drástica no tamanho dos frutos, tornando a planta economicamente inviável (Fig. 1). Estes sintomas sugerem haver um entupimento no xilema ocasionando principalmente o bloqueio do transporte de água e nutrientes, resultado, provavelmente, de uma eficiente capacidade de aderência e multiplicação da *X. fastidiosa* nestes vasos condutores (Machado et al., 1994; McElrone et al., 2001; Machado et al., 2001a; Medina, 2002).

No genoma da bactéria foram encontrados vários genes responsáveis pela adesão da bactéria na superfície do hospedeiro, inclusive genes anteriormente detectados apenas no processo de adesão de patógenos em humanos (Simpson et al., 2000). Entretanto, pouco ou nada se sabe sobre a expressão e interação destes genes e quais estão relacionados à adesão com a planta ou com o vetor.

A capacidade de adesão em superficies sólida seguida de multiplicação e colonização bacteriana é características de formação de biofilme. O termo biofilme descreve a habilidade das bactérias em aderir a superfícies sólidas e estabelecer, em conseqüência, uma comunidade microbiana. Na formação do biofilme, uma população de bactérias adere em superfícies ou interfaces formando uma densa matriz composta principalmente por exopolissacarídeos

(Costerton et al., 1995) que consiste em uma importante estratégia de sobrevivência para as bactérias na natureza (De Kievit & Iglewski 1999; Marques et al., 2002).

A formação de biofilme é composta por diferentes estágios iniciando-se pela adesão na superfície, proliferação bacteriana dentro de microcolonias e expansão das microcolonias, formando estruturas altamente organizadas. Sauer (2003) dividiu a formação de biofilme em cinco diferentes estágios (Fig. 2). O estádio 1, correspondente a adesão reversível das células na superfície; o estádio 2 é referente à adesão irreversível mediada principalmente pela produção de substâncias exopoliméricas; no estádio 3 inicia-se a primeira fase de maturação do biofilme caracterizada pelo início do desenvolvimento da arquitetura do biofilme; a segunda fase de maturação, estádio 4, corresponde ao biofilme totalmente maduro, com alta densidade celular, e a arquitetura do biofilme apresenta-se de forma complexa; o estádio 5 é referente à fase de dispersão das células do biofilme.

Quando as células atingem o estágio de biofilme maduro, é ativado um sistema de comunicação intercelular denominado "quorum sensing" (Sauer 2003). Esta sinalização permite que as bactérias regulem a expressão de genes específicos como, por exemplo, associados a fatores de virulência, resistência a compostos antimicrobianos, respostas de defesa do hospedeiro, condições de deficiência nutricional, produção de antibióticos e transferência de plasmídio por conjugação (De Kievit & Iglewski, 1999; Davey and O´Toole, 2000; Rahmati et al., 2002; Molin and Tolker-Nielsen, 2002). Estas características permitem que as células em biofilme apresentem grande vantagem adaptativa e competitiva no hospedeiro (Davey and O´Toole, 2000). Tem sido demonstrado, por exemplo, que células em biofilme são 500 vezes mais resistentes a compostos antimicrobianos que em crescimento planctônico (Costerton et al., 1995). Em *X. fastidiosa*, até o momento, nenhum gene foi caracterizado como envolvido com "quorum-sensing". Contudo, estudos recentes sugeriram que o gene *rpf*F, pertencente a um "cluster" de genes envolvidos com a regulação de fatores de patogenicidade, provavelmente está associado com a sinalização celular em *X. fastidiosa* (Scarpari et al., 2003; Dow et al., 2003).

Atualmente, o número de estudos da expressão de genes envolvidos na formação de biofilme tem aumentado principalmente em bactérias que causam doenças em humanos, uma vez que, a formação de biofilme, está associada a doenças como fibrose cística, periodontia, otite média, endocardite etc. (Dolan and Costerton, 2002). Contudo, há poucas informações sobre a expressão de genes envolvidos na formação de biofilme em patógeno de planta.

Recentemente, a caracterização da formação de biofilme foi feita em *X. fastidiosa* por microscopia de varredura. Foi demonstrado que *X. fastidiosa* pode formar biofilme em diferentes superfícies e que a morfologia do biofilme parece variar de acordo com a estirpe testada e as condições ambientais analisadas (Marques et al., 2002). Porém os autores mencionam a necessidade da identificação de genes associados com a formação de biofilme e seu papel na infecção e desenvolvimento da doença.

Em *X. fastidiosa* há relatos de perda de virulência após sucessivas repicagens em meio de cultura tanto para a linhagem da doença de 'Pierce' de videira (PD), quanto da CVC (Hopkins, 1985; Monteiro et al., 2001). Hopkins (1985) menciona que estirpes virulentas de *X. fastidiosa* causadora de PD são obtidas após isolamento da bactéria, enquanto que estirpes fracamente virulentas e avirulentas são obtidas após um ano de repicagens semanais. Após sucessivas repicagens as células perdem a capacidade de formar agregados, sugerindo que pode haver uma relação entre adesão (biofilme) e a patogenicidade da bactéria (Hopkins, 1985). Observação semelhante foi feita em *X. fastidiosa* da CVC onde a estirpe 9a5c, após oito repicagens, quando inoculadas em plantas de *Catharantus roseo*, foi capaz de expressar sintomas mais rapidamente e em um maior número de plantas do que a mesma estirpe com 58 repicagens (Monteiro et al., 2001). A perda de virulência bacteriana após muito tempo de cultivo *in vitro* é uma característica comumente observada em bactérias patogênicas de uma grande faixa de hospedeiro (McCutcham et al., 1976; Levine et al., 1990; Hu et al., 1991).

Figura 1. Sintomas de CVC em plantas de laranja doce. A) Manchas cloróticas na parte dorsal das folhas correspondendo a pontos marrons com aspecto de goma na superfície ventral. B) Aspecto de super brotamento de ramos. C) diferença entre um fruto sadio (esquerda) e outro com CVC (direita) apresentando tamanho reduzido, endurecidos e com maturação precoce. (Figuras A e C, retiradas da página do Fundecitrus. http://www.fundecitrus.com.br/cvc.html; Figura B cortesia do Dr. Marcos A. Machado).

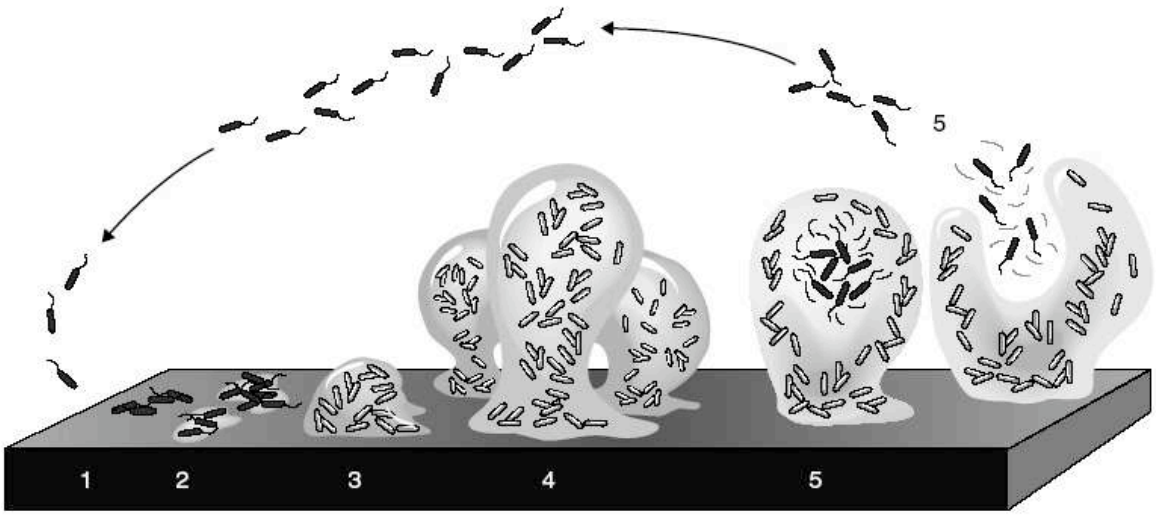


Figura 2. Modelo dos estágios de desenvolvimento de biofilme bacteriano. 1) Neste estágio as células bacterianas aderem de forma reversível na superfície. 2) No estágio 2 as células aderem na superfície de forma irreversível, uma etapa mediada principalmente por substâncias exopoliméricas. 3) Estágio correspondente ao início da maturação do biofilme, indicado pelo desenvolvimento inicial da arquitetura do biofilme. 4) Estágio de total maturação do biofilme, indicado por uma complexa arquitetura do biofilme. 5) Estágio de dispersão com o aparecimento de células móveis que deixam as microcolônias.

3.3. Genes relacionados à patogenicidade detectados no genoma da *X. fastidiosa*

Baseado nos sintomas da CVC, pode-se estabelecer algumas hipóteses sobre os mecanismos de patogenicidade da bactéria e os genes associados a eles. Os principais mecanismos envolvem bloqueio de vasos do xilema, produção de exo-enzimas e fatores de competição que permitem uma melhor adaptação no ambiente colonizado.

Em relação à hipótese envolvendo o bloqueio dos vasos de xilema, vários genes provavelmente envolvidos com a adesão bacteriana, tanto no hospedeiro quanto no vetor, foram encontradas no genoma da *X. fastidiosa* (Simpson *et al.*, 2000). Um cluster de genes codificando EPS foi detectado com alta similaridade ao cluster "*gum*" de *Xanthomonas campestris* pv. campestris. Entretanto, não foram detectados os genes *gum*I, *gum*L e *gum*G, sugerindo que provavelmente a goma produzida por *X. fastidiosa* apresente viscosidade diferente daquela de *X. c.* campestris. Atualmente são conhecidos 36 genes relacionados à biossíntese e função de filamentos de fímbrias, destes 26 foram detectados no genoma de *X. fastidiosa*. Outros genes relacionados a adesão, porém anteriormente só detectados em bactérias causando doenças em humanos, também foram detectados. Estes genes apresentam similaridade com *uspA1* de *Moraxella catarrhalis*, dois *hsf* de *Haemophilus influenzae*, sendo este relacionado a adesão da bactéria às células epiteliais do trato respiratório (St. Geme *et al*., 1996), e três com alta similaridade a hemaglutinina (*psp*A) de *Neisseria meningitidis.* Entretanto em *X. fastidiosa* este gene apresenta aproximadamente 1.000 resíduos de aminoácidos a mais que em *N. meningitidis*. Supõe-se que todos estes genes atuando em conjunto promovam um forte agregado bacteriano resultando na formação de biofilme. Contudo apenas estudos funcionais poderão elucidar se todos eles estão de fato envolvidos na adesão bactéria-bactéria e com a planta hospedeira, ou se alguns estão relacionados apenas com a adesão no vetor.

A colonização da *X. fastidiosa* nos feixes vasculares sugere que ela se movimente através dos vasos pela degradação das pit-membranas, que são compostas basicamente de celulose. Foi demonstrado que esta movimentação está associada à patogenicidade de *X. fastidiosa* causadora da doença de 'Pierce' e de CVC (Hopkins, 1985; Almeida et al., 2001). Os produtos dos principais genes relacionados à degradação da parede celular vegetal encontrados no genoma são similares a enzimas celulolíticas, sendo três endo-1,4-β-glucanases e uma celobiohidrolase.

Além dos genes envolvidos no processo de adesão e degradação de parede, outros fatores de adaptação também podem estar contribuindo para uma melhor interação da bactéria com o

hospedeiro. A expressão destes genes pode conferir vantagens competitivas no ambiente colonizado. Dentre estes, podem ser destacados vários genes codificando toxinas, como as hemolisinas, pertencente a família das toxinas RTX (repetições em toxina), que representam importantes fatores de virulência, com uma ampla disseminação em bactérias Gram-negativas (Coote, 1992). Também alguns genes envolvidos com resistência a drogas e biossíntese de compostos antimicrobianos foram encontrados, sugerindo que a bactéria possa apresentar um mecanismo de defesa contra toxinas ou antibióticos produzidos por endofíticos comumente encontrados no xilema. Por outro lado, as toxinas produzidas pela *X. fastidiosa* devem representar um importante papel na sua capacidade competitiva frente a outros microrganismos.

Também foram encontrados genes relacionados com resistência a estresse osmótico, estresse oxidativo e um eficiente sistema de absorção de nutrientes. Genes como *msr*A e *mdo*H apresentam mais de uma função e podem desempenhar importante papel na interação da *X. fastidiosa* com o hospedeiro. O produto do gene *msr*A é necessário para o funcionamento de adesinas de três patógenos de humanos, *Streptococcus pneumoniae*, *Neisseria gonorrhaea* e *Escherichia coli* (Wizemann et al., 1996). Recentemente, a importância deste gene na virulência de um patógeno de planta, foi caracterizado em *Erwinia chrysanthemi*. Células carregando mutação no gene *msr*A são mais sensíveis a estresse oxidativo, são incapazes de invadir o hospedeiro de forma sistêmica e exibem reduzida virulência em folhas de chicória (Hassouni et al., 1999). O gene *mdo*H é necessário para biossíntese do esqueleto glicosídico de glucanas periplasmáticas osmoreguladas (Loubens et al., 1993). Também em *Erwinia chrysanthemi* foi demonstrado que este gene é necessário para patogenicidade, uma vez que, mutantes para este gene apresentaram completa perda de virulência e foram incapazes de crescer dentro do hospedeiro (Page et al., 2001). A competição por nutrientes deve representar um componente importante na eficiência da interação desta bactéria, uma vez que o xilema é um ambiente pobre em nutrientes e sintomas de deficiência mineral são comuns em plantas com CVC. Muitos genes relacionados à absorção de nutrientes da seiva foram identificados no genoma da *X. fastidiosa* inclusive genes envolvidos com estresse nutricional.

De forma geral a *X. fastidiosa* além de apresentar genes que podem promover um eficiente mecanismo de adesão, necessário para colonização no hospedeiro, também necessita adaptar-se a várias condições de estresse provenientes do ambiente colonizado, por este motivo os genes relacionados com adaptação que conferem resistência a estresse nutricional, osmótico,

oxidativo e compostos antimicrobianos assim como produção de toxinas são essenciais para manutenção da população bacteriana dentro do hospedeiro.

3.4.Tecnologia do microarray no estudo da expressão gênica

Os projetos genoma tem gerado grande quantidade de dados de seqüência de nucleotídeos. Um passo importante no uso dos dados de seqüências genômicas é a identificação das funções das diferentes ORFs presentes no genoma. Muitas das ORFs identificadas nos genomas de vários organismos não tem homologia com seqüências depositadas em banco de dados. Por outro lado, quando as ORFs apresentam homologia significativa com seqüências depositadas no banco de dados isto não tem sido suficiente para determinar o papel biológico do produto deste gene.

Atualmente uma valiosa ferramenta que vem sendo usado no estudo da expressão de genes é a tecnologia de "microarray" (Rhodius et al., 2002). "Microarray" são pequenos ensaios do gene fragmentado aderido a "chips" de vidro. Estes "biochips" são usados para determinar a atividade do gene usando hibridização entre a seqüência do "microarray" e a amostra fluorescente. Após hibridização os "chips" são lidos com detectores de fluorescência e a intensidade de cada "spot" revela a identidade e a quantidade de cada seqüência presente na amostra. Os dados são então analisados usando ferramentas de bioinformática. Devido à possibilidade de milhares de genes estarem presentes em um único "microarray", dados de genomas inteiros podem ser obtidos em um único experimento (Shena, 2000).

A modulação da expressão gênica em função de mudanças ambientais em bactérias, principalmente patogênicas em humanos, tem sido extensiva e intensivamente estudada utilizando a técnica de "microarray" (Rhodius et al., 2002). Em fitobactéria esta técnica foi recentemente utilizada em *Erwinia chrysanthemi* para detecção de genes expressos durante infecção na planta (Okinaka et al., 2002). Os autores chamam atenção que os genes responsáveis pela interação com o patógeno, esperados para terem sua expressão alterada, não foram detectados, e que maior expressão foi de genes "housekeeping". Outro grupo de genes induzido na condição de infecção na planta foi associado com adaptação do patógeno no ambiente do hospedeiro.

Assim, a disponibilidade de seqüências genômicas completas de bactérias, junto com o desenvolvimento de "microarrays", pelo qual a expressão de genomas inteiro de um organismo

dentro de duas condições pode ser avaliada, tem iniciado segundo Sauer (2003) a era pós-genômica das pesquisas de biofilme e gerado novas informações.

Em diferentes estudos, a mudança da expressão gênica em células crescendo em biofilme, em comparação com o crescimento planctônico, tem sido sempre observada alterações no nível de expressão gênica com genes induzidos e reprimidos, sendo uma maior quantidade de genes induzidos na condição de biofilme (Whiteley et al., 2001, Stanley et al., 2003, Schembri et al., 2003). Contudo, a quantidade de genes induzidos tem variado de 1 a 38 % do genoma total. Tal discrepância é observada dependendo das condições experimentais utilizadas e estringência das análises (Sauer, 2003).

Estudos de expressão gênica utilizando técnicas como "microarray" tem permitido a identificação de genes envolvidos em diferentes fases da formação de biofilme. Em *Escherichia coli* e *Pseudomonas aeruginosa* genes que codificam para proteínas de membrana externa, fímbrias, flagelos e adesinas foram associadas como envolvidas com as etapas iniciais de formação de biofilme (O´Toole & Kolter, 1998; Otto et al., 2001; Schembri et al., 2003). Genes envolvidos com resistência a antibiótico e transferência horizontal de genes têm sido expressos em biofilme maduro (Whiteley et al., 2001; Molin and Tolker-Nielsen, 2002). Nesta etapa a expressão de determinados grupos de genes são regulados pelos mecanismos de "quorum sensing" (Schuster et al., 2003; Wagner et al., 2003). Contudo, nenhum gene regulado em resposta ao "quorum sensing" foi encontrado no estudo de formação de biofilme em *E. coli* e *P. aeruginosa* realizados por Schembri et al. (2003) e Whiteley et al. (2001), respectivamente. As análises de biofilme por "microarray" realizadas por Schembri et al. (2003) também revelaram a expressão diferencial de genes em condições de pouco oxigênio e nutrientes. Dependendo das condições experimentais utilizadas como fase de crescimento, condições de estresse e superfície de adesão, ocorre reprogramação da expressão gênica das células em biofilme (Pringent-Combaret et al., 1999).

A tecnologia de "microarray" foi recentemente usada para estudo do genoma e transcriptoma de *X. fastidiosa* (Costa de Oliveira et al., 2002, Nunes et al., 2003). Contudo, até o presente trabalho, nenhum estudo utilizando "microarray" foi realizado visando detectar genes relacionados à patogenicidade e formação de biofilme em *X. fastidiosa*.

4. Hipóteses

O caráter fastidioso do padrão de crescimento da bactéria *Xylella fastidiosa* representa dificuldade adicional nos estudos de expressão gênica e de sua interação com o hospedeiro ou com o vetor. Para superar essas dificuldades devem ser buscados modelos que, sem se distanciarem das condições da bactéria dentro da planta, possibilitem uma abordagem de entendimento dos mecanismos de patogenicidade dessa bactéria.

Evidências de que a estirpe de *X. fastidiosa* causadora da doença de 'Pierce' perde a virulência e a capacidade de formar agregados após vários sub-cultivos; de que a bactéria forma agregados no interior do xilema e a identificação, no genoma desta bactéria, de uma grande quantidade de genes relacionados à capacidade de adesão em superfície levaram à formulação das seguintes hipóteses visando um melhor entendimento do mecanismo de patogenicidade desta bactéria:

- A expressão de genes em condições de isolamento primário da bactéria de plantas hospedeiras aproxima-se das condições de interação *X. fastidiosa* com a planta.
- A patogenicidade dessa bactéria estaria também associada à sua capacidade de formar biofilme em superfícies.

5. Objetivos

Gerais

- Estabelecer um modelo geral de experimentação para avaliar patogenicidade da bactéria.
- Analisar a capacidade de colonização e expressão diferencial de genes de *X. fastidiosa* no modelo experimental estabelecido.
- Avaliar a expressão diferencial de genes em condições que favoreçam ou não a formação de biofilme.

Específicos

- Avaliar a taxa de colonização da bactéria em condições de isolamento primário e sucessivas repicagens.
- Avaliar a expressão diferencial de genes em condições de isolamento primário e após sucessivas repicagens.

- Avaliar a expressão diferencial de genes de *X. fastidiosa* crescendo em comunidades sésseis (biofilme) comparando-as com crescimento planctônico (vida livre).

6. Modelo experimental

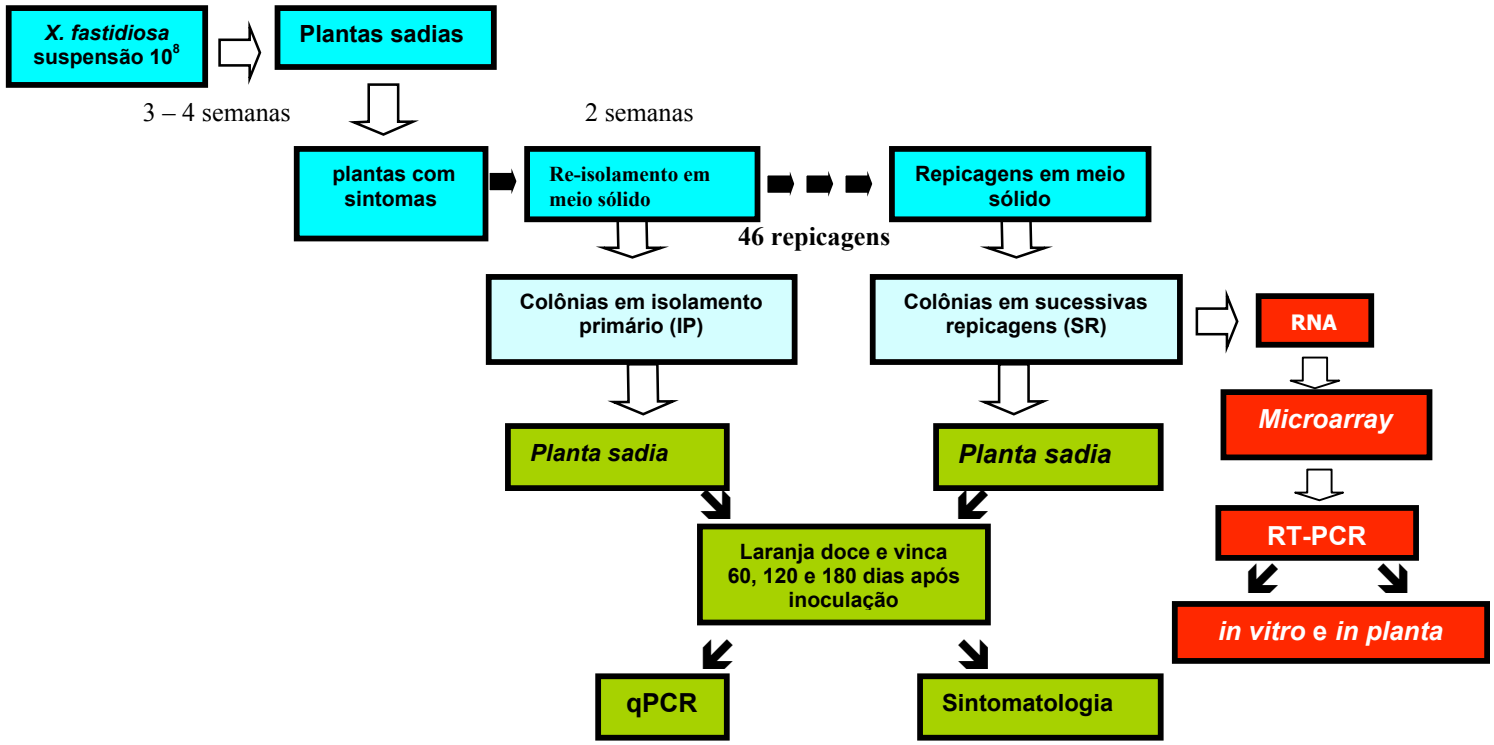
O estudo funcional do genoma é essencial para o entendimento dos processos biológicos de um organismo. No caso da *X. fastidiosa*, o maior enfoque nos estudos de genoma funcional foi sempre o de buscar um melhor entendimento dos mecanismos de patogenicidade, contudo, há grandes dificuldades no estudo da interação *X. fastidiosa*-hospedeiro.

Por ser um organismo fastidioso, o surgimento das primeiras colônias após isolamento em meio sólido leva de 10 – 15 dias. Há uma baixa eficiência de inoculação, e quando esta é conseguida, a colonização é muito lenta e extremamente dependente das condições ambientais, levando a uma demora no surgimento de sintomas em citros. Além disso, a dificuldade de obtenção da seiva do xilema de plantas de citros é muito grande, e somado à lenta colonização bacteriana, a quantidade de massa celular obtida é muito baixa, dificultando os avanços no entendimento da interação desta bactéria com o hospedeiro. Por este motivo, modelos *in vitro* que mimetizem o crescimento *in vivo* são de grande importância para estudos de genoma funcional da *X. fastidiosa* com enfoque em mecanismos de patogenicidade.

Desta forma, neste estudo, foram desenvolvidos alguns modelos experimentais utilizando células recém isoladas de plantas com sintomas de CVC visando detectar genes diferencialmente expressos na condição experimental de interesse. Os modelos desenvolvidos nos três capítulos da tese encontram-se esquematizados nas figuras abaixo:

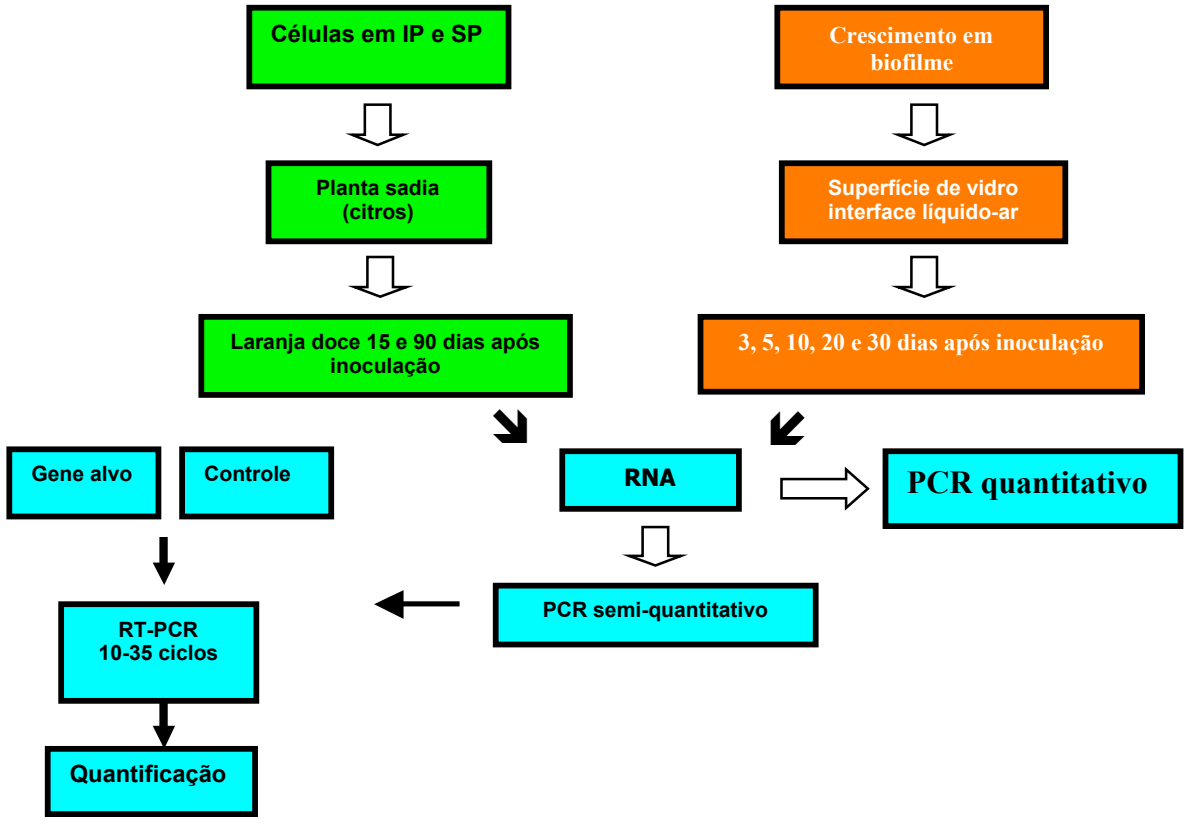
6. Modelo Experimental

6.1. Capítulo 1. Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity



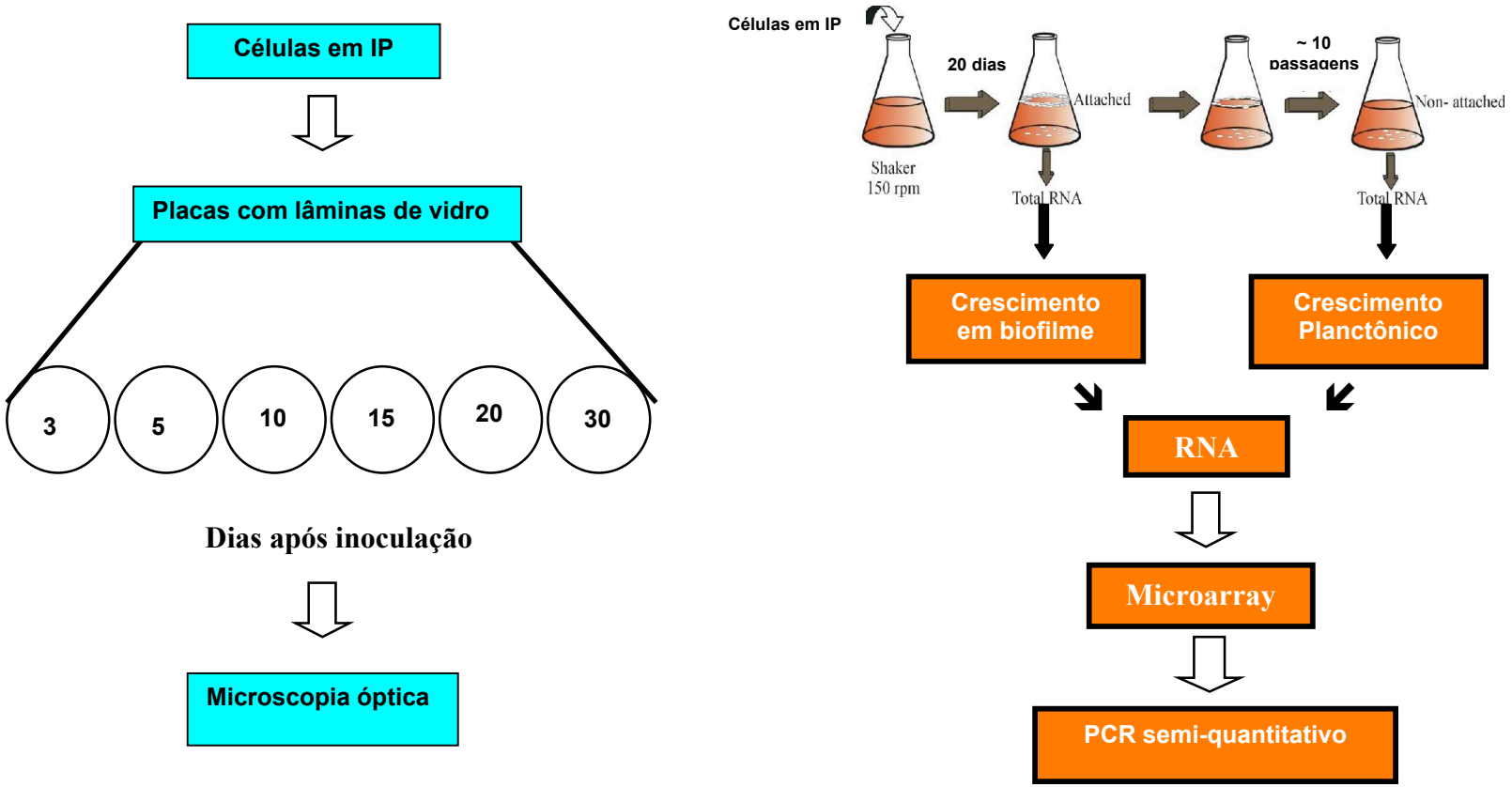
Esquema experimental do trabalho: Representado em azul, células de *X. fastidiosa* na 8ª repicagem foram inoculadas em plantas de vinca. Após surgimento dos sintomas as células foram re-isoladas, sendo as primeiras colônias obtidas na placa denominadas de células em isolamento primário (IP). Algumas destas colônias foram repicadas por 46 vezes, denominando-se células após sucessivas repicagens (SR). Na representação em verde, células em IP e SR foram inoculadas em plantas de vinca e laranja doce e a colonização e sintomatologia analisadas após 60, 120 e 180 dias por PCR quantitativo em tempo real e visualização dos sintomas. Para análise da expressão diferencial, representação em vermelho, foi extraído RNA total das células em IP e SR e submetidas a técnica de 'microarray'. Alguns genes foram validados por RT-PCR tanto *in planta* quanto *in vitro*.

6.2. Capítulo 2. Expression of pathogenicity-related genes of *Xylella fastidiosa in planta* and *in vitro*



Esquema experimental do trabalho: A expressão *in vivo* dos genes detectados no 'microarray' foram avaliados 15 e 90 dias após inoculação na planta, representação em verde, e *in vitro* após 3, 5, 10, 20 e 30 dias após inoculação em frascos de vidro, representação em vermelho. Para avaliação da expressão foram utilizadas as técnicas de PCR semi-quantitativo e PCR quantitativo em tempo real, representados em azul.

6.3. Capítulo 3. Gene expression profile of the plant pathogen *X. fastidiosa* during biofilm formation *in vitro.*



Esquema experimental do trabalho: Células em IP foram inoculadas em lamínulas de vidro e a formação do crescimento em biofilme acompanhada por microscopia óptica após 3, 5, 10, 15, 20 e 30 dias (representação em azul). O biofilme aderido na superfície da interface líquido-ar e as células planctônicas obtidas após 10 passagens foram coletadas e o RNA extraído e utilizado para as análises de 'microarray'. Alguns genes detectados no 'microarray' foram validados por PCR semi-quantitativo (representação em vermelho).

7. CAPÍTULOS

CAPÍTULO 1

Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity.

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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 agroindustry. 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

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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 $y = -4.12972 \times \text{Ln}$ (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 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.

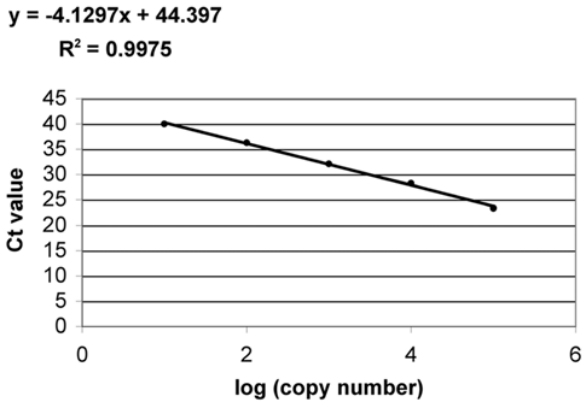

Fig. 1. Standard curve of *Xylella fastidiosa* cells determined by real-time quantitative polymerase chain reaction (PCR). C_T is plotted against the log number of *X. fastidiosa* cells in samples of 10-fold dilutions of template DNA (only one template per cell). The straight line calculated by logarithmic regression was $y = -4.12972 \times \text{Ln}$ (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.

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 Δ0.40469 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]

	60 dai[b]				120 dai				180 dai			
Inoculum	**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 *Haemofilus influenzae*. Although the ratio for the *msr*A 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	*orf*111	XF0357	Esterase	3.17
	*orf*111	XF2151	Esterase	2.74
	AF0343	XF1133	Tryptophan repressor binding protein	–2.01
	*kor*A	XFa0057	Transcriptional regulator	–2.23
	*glo*A	XF1399	Lactoylglutathione lyase	–2.07
Biosynthesis of small molecules	SCH10.14c	XF0356	Cytochrome P-450 hydroxylase	2.17
	*orf*U1	XF1441	Phosphohydrolase	–3.65
	*fol*C	XF1946	Folylpolyglutamate synthase dihydrofolate synthase	–2.38
Macromolecule metabolism	*pro*S	XF0445	Prolyl-tRNA synthetase	2.02
	*mag*I	XF1326	DNA-3-methyladenine glycosidase	–3.51
	*mia*A	XF0090	tRNA delta(2)-isopentenylpyrophosphate transferase (35.5 kDa)	–2.12
	*tra*C	XF2025	DNA primase	–2.02
Cell structure	*lyc*	XF2392	Autolytic lysozyme	2.23
	*trb*E	XF2053	Conjugal transfer protein	–2.16
	dmt	XF0612	Dolichol-phosphate mannosyltransferase	–2.94
Cellular process	*ape*2426	XF1398	Na^+/H^+ exchange protein	–2.17
	*sec*B	XF1801	Protein-export protein	–2.64
Pathogenicity, virulence, and adaptation	*fim*A	XF2539	Fimbrial protein	2.20
	*msr*A	XF1940	Peptide methionine sulfoxide reductase	1.76
	*usp*A1	XF1516	Surface-exposed outer membrane protein	10.15
	hsf	XF1529	Surface protein	3.68
	*cva*C	XF0263	Colicin V precursor	6.51
	acrA	XF2093	Precursor of drug resistance protein	2.07
	*xps*E	XF1517	General secretory pathway protein E	2.00
Hypothetical protein	…	XF1255	…	8.04
	…	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 *acr*A, a multidrug, resistance-protein encoding gene. Pfam analysis indicates that it belongs to the HlyD secretion protein family. The *acr*B gene located downstream of *acr*A also showed a significant higher expression in FP, although with a fold change less than 2.

The last gene possibly involved in pathogenicity is *xps*E. 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.

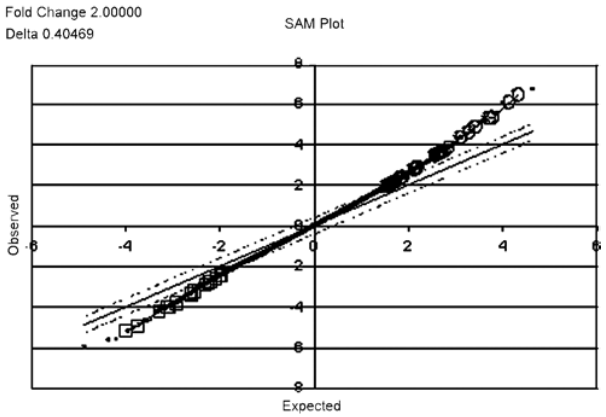

Fig. 2. Identification of genes with significant changes in expression. Scatter plot of the observed relative difference vs. the expected relative difference calculated by significance analysis of microarrays. The dashed lines indicate the cutoff for twofold induction and repression. The potentially significant induced or repressed genes for $\Delta = 0.40469$ are represented by ○ and □, 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 *fim*A, *msr*A, *usp*A1, *hsf*, *cva*C, *acr*A, and *xps*E 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 *msr*A 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

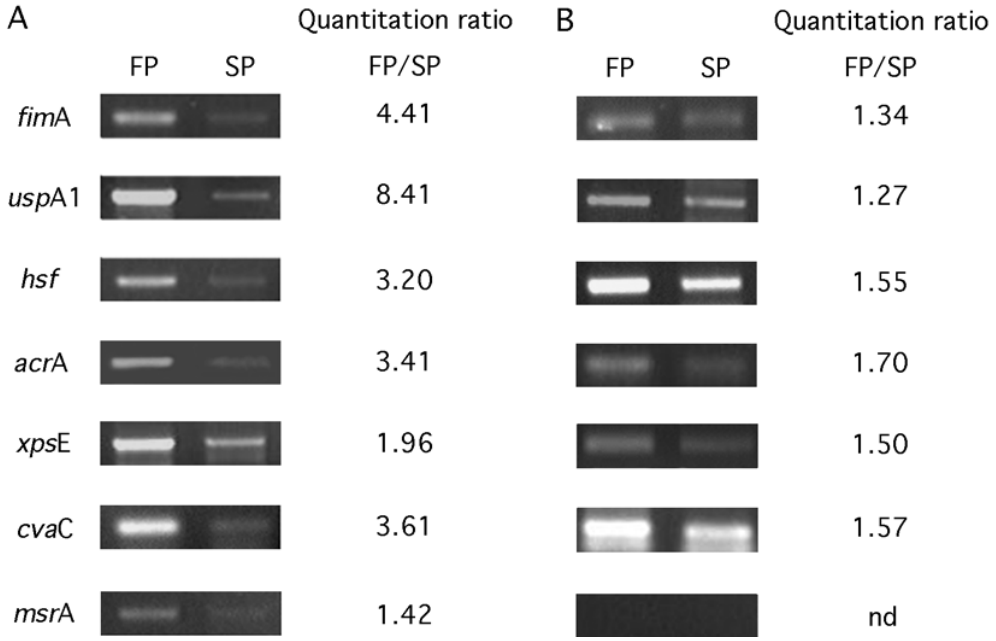

Fig. 3. Reverse transcription-polymerase chain reaction of first passage (FP)-induced genes encoding pathogenicity factors in *Xylella fastidiosa*. The ratios of the normalized quantitated band densities are presented as the FP value divided by the several passage (SP) value. **A,** Experiment using 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 *msr*A 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 *msr*A in either condition. The evaluation of gene expression 15 dai in the plant could be the reason explaining the absence of transcripts of *msr*A, 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 *msr*A 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 gonorrhaea*, 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. *msr*A mutants are more sensitive to oxidative stress, are incapable of systemic invasion, and exhibited 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 *fim*A, 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, *fim*A, 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 fim*A 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 *usp*A1 and *hsf* genes show homology to *usp*A1 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; Hoiczyk 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 *usp*A2 was found in the *X. fastidiosa* genome. UspA1 has been shown to be essential for the attachment of *M. catharralis* to Chang conjuctival tissue in vitro (Lafontaine et al. 2000). Recently a *usp*A1 homologue, named *xad*A, was characterized in *Xanthomonas oryzae* pv. *oryzae* (Ray et al. 2002). *xad*A 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 *xad*A is indeed being expressed in planta. Similarly, we observed that *X. fastidiosa usp*A1 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 *usp*A1 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* (Hoiczyk 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 *msr*A e *fim*A 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 *xps*E. 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 genome 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 µl 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 µl 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 fluoregenic probe was performed in a 25-µl volume, using the TaqMan core PCR reagent kit (Applied Biosystems) as follows: 12.5 µl TaqMan Universal PCR Master Mix (5 µl of 10× TaqMan buffer, 10 mM Tris [pH 8.3], 50 mM KCl, 5 µl 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 µl of DNA solution (10 ng), and 6.87 µl 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/µl and was stored at –20ºC.

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/µl, 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 µg of total bacterial RNA was mixed with 16 µg of random hexamers (Gibco BRL, Gaithersburg, MD, U.S.A.) in a 30-µl final volume. Annealing was accomplished by incubation for 2 min at 75ºC, 2 min at 55ºC, 2 min at 45ºC, 2 min at 37ºC, and 2 min at 22ºC, followed by addition of 6 µl of SuperScript II reaction buffer (Gibco BRL), 3 µl of 0.1 M of dithiothreitol, 1 µl dNTP mix (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM dCTP), 2 µl of 1.0 mM Cy3- or Cy5-labeled dCTP (Amersham Biosciences, Little Chalfont, U.K.), and 2 µl of SuperScript II reverse transcriptase (200 U/µl) (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 µl with Tris-EDTA, purified, and concentrated using a Microcon-30 (Amicon, Bedford, MA, U.S.A.) to 10 µl.

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× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× 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× SSC and 0.01% SDS, followed by two washes in 0.06× SSC and 0.01% SDS, and two final washes in 0.06× 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 µg 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 (*fim*A, *msr*A, *usp*A1, *hsf*, *cva*C, *acr*A, and *xps*E) that were amplified with specific primers (Table 3). A 25 µl master mix contained the following components: 2.5 µM 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 µl 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, 50ºC 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)
fimA	F—5′ CCCTCGAGCCAAAATTATGTCGCCAGA 3′	350
	R—5′ CACTCGAGGTGACGGTGGAGGAGCAG 3′	
msrA	F—5′ AATCTCGAGTGGGACGTAGTGAAC 3′	498
	R—5′ AAGCTCGAGCCGGATGGAGTA 3′	
uspA1	F—5′ AACTCGAGAGCAGGCCGCCGGTGATAGCAGTA 3′	1,122
	R—5′ AGACGCTCGAGCCCCCGCCGCAAGAT 3′	
hsf	F—5′ CGCTCGAGGGGTCTTTGTATGT 3′	647
	R—5′ GGCTCGAGACGCTGTGAGGTTC 3′	
cvaC	F—5′ GTCTCGAGGCGACCTTGCTAC 3′	194
	R—5′ AGCAGCCTCGAGACCACAGATAC 3′	
acrA	F—5′ CTCTCGAGCACGCGTGGCTGGAATA 3′	919
	R—5′ AGCTCGAGCGCCTTCTTTGACTTTT 3′	
xpsE	F—5′ GTCTCGAGTTGTTGGCGGAAGTATGAA 3′	1,101
	R—5′ CCCTCGAGCCAGTGACCAGCAAAATG 3′	

72ºC 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 4ºC, 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

CAPÍTULO 2

Expression of pathogenicity-related genes of *Xylella fastidiosa in planta* and *in vitro*

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Expression of pathogenicity-related genes of *Xylella fastidiosa in planta* and *in vitro*

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Abstract

Xylella fastidiosa is responsible for several economically important plant diseases. The most well accepted cause of the disease is a vascular occlusion resulting from biofilm formation. Microarray technology was previously used to examine the global gene expression profile of *X. fastidiosa* in two growth states, showing different pathogenicity profiles. In the present study, we evaluated some of the pathogenicity-related genes for their expression *in planta* and *in vitro* by RT-PCR. The results suggest that adhesion is important in the beginning of the biofilm formation, while the genes related to adaptation are essential for the maintenance of this biofilm *in planta*. Similar results were observed i*n vitro* mainly for the adhesion genes. The pattern of expression observed suggests that the adhesion modulates the biofilm formation while the expression of some adaptation genes may be related to the environment in which the organism is living.

Keywords: CVC, biofilm, RT-PCR, Citrus

1. Introduction

Xylella fastidiosa is a Gram-negative and xylem-inhabiting bacterium responsible for economically important diseases in different crops (Lima et al. 1998, Purcell and Hopkins 1996; Barnard et al. 1998). In Brazil, it is responsible for the 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 (Simpson et al. 2000).

The most well accept cause of CVC is a vascular occlusion leading to water stress. This occlusion seems be due to biofilm formation into the vessel, since it was previously demonstrated that the plant-pathogen *X. fastidiosa* can grow in biofilm (Marques et al., 2002) and that this biofilm could be an important factor for its pathogenicity (Souza et al., 2003). Biofilms are defined as matrix-enclosed microbial population adherent to each other and to surfaces or interfaces (Costerton et al., 1995). The adhesion surface can be biotic (host) or abiotic, which can be subdivided into nonnutritive (glass, plastic, metal, etc) and nutritive (e.g. chitin) (Davey and O´Toole, 2000). Growth in biofilm results in phenotypic adaptations that allow the formation of highly organized and structured sessile communities, which possess enhanced resistance to antimicrobial treatments and host defense responses (Schembri et al., 2003), characteristics that confer advantages to the population in respect to the survival in the environment.

Genetic studies of single-species biofilms have shown that they are formed by multiple steps (Davey and O´Toole, 2000). The different steps of biofilm formation involve several genes which altered expression in each phase of development. The reprogramming of gene expression of the bacteria biofilm results from changes in multiple environmental

physicochemical conditions, environment to which the bacterium is exposed and attachment surface (Prigent-Combaret et al., 1999, O´Toole et al., 2000).

In a previous study Souza et al (2003) used DNA microarray technology to examine the global gene expression of *X. fastidiosa* in two growth states, showing different pathogenicity profiles. Seven of the genes encode putative pathogenicity factors expressed only in the pathogenic condition. Three 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 RT-PCR *in planta* 15 days after inoculation (Souza et al., 2003), period of initial colonization of the vessels (Almeida et al., 2001). In this study, we used these genes for evaluation of their expression *in planta* 90 days after inoculation, period when the blockage of vessel had already occurred probably due to the formation of a mature biofilm by *X. fastidiosa*. We also evaluated the expression of these genes *in vitro* during different phase of biofilm formation by semiquantitative RT-PCR. The results show an interesting trend with the expression of adhesion genes in the beginning of the colonization process followed by expression of the adaptation genes.

2. Materials and methods

2.1. Bacterial strain and biofilm 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 PBS. The suspension was distributed on plates containing PW medium. The first colonies were observed between 10 to 15 days after plating. To obtain cells in biofilms, 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 1L flask containing 300 mL of PW broth. A total of 3 flasks was used in each analysis. After 3 days growing at 28ºC in a rotary shaker at 120 rpm a thin biofilm was observed attached to the glass at the medium-air interface. Cells adhering to the glass surface were collected at 3, 5, 10, 20 and 30 days after inoculation. The biofilm layer was scraped from the flask and washed by centrifugation at 8,000 Xg for 5 min at 4ºC with DEPC-treated water. After collecting the samples, total RNA was extracted as described by Rhodius (www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf). The pellet was store at –80 °C until required.

2.2. 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 two different growth states, freshly-isolated bacteria (first passage-FP) and obtained after 46 passages in axenic culture (several passage-SP). The inoculation was performed as described by Souza et al (2003). The plants were maintained in a growth chamber at 28 ºC with a photoperiod of 12 h light and 12 h dark. Three plants were inoculated with FP and three others with SP conditions. 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 (2003). Total RNA was extracted using the TRIZOL reagent (Invitrogen).

2.3. Semiquantitative reverse transcription (RT)-PCR analysis.

Semiquantitative RT-PCR was used to monitor the expression of some genes involved with patogenicity (Table 1) in different phase of biofilm formation and *in planta* 90 days after inoculation

The cDNA was prepared from 500 ng of total RNA for the *in vitro* and 100 ng for the *in planta* condition, respectively. The RNA was reverse transcribed with SuperScript II (Invitrogen) and random hexamers primers (Invitrogen). To rule out the possibility of amplification from contaminating DNA, reactions using the RNA were done in parallel. The RNAs were normalized using ribosomal RNA content. We carried analyses for some genes up-regulated in the pathogenic condition (Souza et al., 2003) 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 Polymerase, and 2.5 μl of first strand cDNA template. The PCR cycle profile was 94ºC for 3 min for initial denaturation followed by cycles of 94ºC for 1 min, 50ºC for 1 min, and 72ºC 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.

2.4. Real-Time Reverse transcription-PCR assay to *usp*A1 in different steps of biofilm formation *in vitro*.

Fifthy nanograms of the same total RNA used for the semiquantitative RT-PCR were reverse transcribed with SuperScript II and random hexamers primers (Invitrogen). For the relative quantification, 1μl of the cDNA was utilized in real-time PCRs using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The 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 compared with planktonic growth (data not show). The probe was labeled with FAM and also carried non-fluorescent quencher and a Tm enhancer. The PCR thermal cycling conditions were as follows: an initial step at 50ºC for 2 min; 10 min at 95ºC; and 40 cycles, with 1 cycle consisting of 15 s at 95ºC and 1 min at 60ºC.

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_ts 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: $\Delta C_t = C_t$ (target gene) - C_t (endogenous control). The fold increased of the target gene in the different days of the biofilm formation was determined by the equation: $\Delta\Delta C_t = \Delta C_t$ (sample) - ΔC_t (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 $2^{-\Delta\Delta Ct}$.

3. Results

3.1. Examination of pathogenicity related-genes *in planta* by semiquantitative RT-PCR.

In order to evaluate the expression level of pathogenicity-related genes *in planta* 90 days after inoculation with *X. fastidiosa* in FP and SP conditions we used semiquantitative RT-PCR. The ratio of the normalized quantitated signals is showed in Figure 1. These results were obtained after 35 amplification cycles.

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

3.2. Examination of pathogenicity related-genes of biofilm formation by semiquantitative RT-PCR.

In order to verify changes in the expression level of pathogenicity-related genes in different phase of the biofilm development in *X. fastidiosa* we used semiquantitative reversed transcription polymerase chain reaction. For this experiment, total RNA was extracted from three independent experiments and converted to cDNA. The figure 2 shows the results of the analyses for adhesion (*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 steps of the biofilm formation. A statistically significative increase in the expression level of *fim*A was observed during 3, 5 and 10 days, followed by a decrease after 20 days. Two non-fimbriae adhesions, *hsf* and *usp*A1, were also up-regulated in the initial steps of biofilm

formation, however only significative increase in the expression level was observed after 5 days of biofilm formation. These results suggest that all these adhesins are involved in the attachment to the abiotic surface.

The *msr*A and *cvaC* did not show significative differences in the expression level in all the steps of the biofilm formation. *acr*A showed significative up-regulation mainly in the later steps of the biofilm formation. Expression of *xps*E was similar in the initial time points. At 20 days there was a significative increase in the expression, which decreased at 30 days (Fig. 2).

3.3. Relative transcripts levels of *usp*A1 gene in *X. fastidiosa* biofilm

We utilized real-time RT-PCR in order to validate the results obtained in the semiquantitative RT-PCR for the expression level of *usp*A1 in the different phases of the biofilm formation *in vitro*. The transcription of *usp*A1 was over-expressed at 5 and 10 days of biofilm formation (Fig. 3). The results of expression level obtained at the initial steps of biofilm formation for this gene was similar with what was observed for the semiquantitative RT-PCR.

4. Discussion

The colonization of *X. fastidiosa* in the SP condition is delayed compared to FP and a decrease in the SP population is observed at 180 days contrary to the cells in the FP condition, which kept growing *in planta* and induced development of symptoms (Souza et al., 2003).

Souza et al (2003) detected over-expression 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 it was observed development of the symptoms, probably due the vessel blockage (Almeida et al.,

2001). The expression levels of the adhesion genes after 90 days were similar in both states condition. These results suggest that the over-expression of these genes in the FP condition compared to SP at 15 days could be an important factor to promote an efficient colonization during the development of the biofilm in the vessels. The delayed expression of these genes in SP could also be correlated to the poor colonization observed right after inoculation.

In later stages of the colonization (90 days), the expression levels of the adhesion genes are similar both in FP and SP conditions. However the expression level of adaptation-related genes is low even at 90 days in SP condition. The *msr*A gene that was not detected after 15 days (Souza et al., 2003) showed expression at 90 days only in the FP condition (Fig. 2), suggesting that MsrA is necessary in more advanced stages of the colonization. The low expression level of adaptation-related genes in the SP condition in later stages of the colonization in the plant and the observed decline of 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.

To evaluate the expression of these genes in different steps of the biofilm formation we utilized glass as an abiotic surface and analyzed the expression of the genes by semiquantitative RT-PCR in different stages of the biofilm formation. According to Sauer (2003) the biofilm development occurs in five distinct stages: (1) reversible attachment to the surface, (2) irreversible attachment, (3) first maturation, early development of the biofilm architecture, (4) fully mature biofilm, complex biofilm architecture and (5) dispersion of biofilm cells. The expression of adhesion genes was observed mainly in the initial steps of the biofilm formation. The *fim*A gene was up-regulated from the third days of biofilm development suggesting that in *X. fastidiosa* this gene can be involved in the very initial stage of biofilm formation, which agrees with other models of biofilm

development in gram-negative bacteria (Davey and O´Toole et al., 2000). The *usp*A1 and *hsf* genes encode adhesins important for the attachment to the host tissues (Cope et al; 1999; Hoiczyk et al. 2000; St. Geme et al. 1996). In *X. fastidiosa* these genes were up-regulated at 5-10 days of the biofilm development suggesting a possible role in the initial attachment as well as in the biofilm architecture formation.

These adaptation-related genes can confer advantages to cells in biofilm promoting higher resistance to antimicrobial compounds (*cva*C, *acr*A) and defense response of the host (*msr*A, *xps*E). Genes that confers these characteristics are expressed after a high cell density when a cell-cell communication begins, a mechanism named quorum-sensing. The quorum-sensing has been best characterized in human pathogen (Kievit and Iglewski, 2000). The up-regulation of these genes in later stages of biofilm formation characterized by high cell densities suggests that *X. fastidiosa* biofilm development could be similar to human pathogens where it was recently demonstrated that AcrAB can mediate cell-cell communication in response to cell density (Rahmati et al., 2002). In this work we observed high expression of *acr*A at day 20 of the biofilm formation, period of high cell density. The *acr*B gene also showed a significant higher expression in pathogenic condition in microarray analyses, however the fold change was lower than 2 (Souza et al., 2003). The *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.

The gene expression observed in the *in vitro* condition was similar to that observed *in planta*, where the adhesion genes were mainly expressed in the initial step of biofilm formation. 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 understand of the initial steps of the biofilm development in *X. fastidiosa*.

Some differences were observed in the expression of adaptation-related genes *in planta* and *in vitro*. These differences were mainly observed for the *msr*A and *cva*C genes. *msr*A did not show any expression in the initial colonization *in planta* but showed expression in all stages of biofilm formation *in vitro*, however, a signal was observed after 35 cycles, suggesting the presence of a low amount of mRNA. The *cva*C gene showed high expression in later stages of the colonization *in planta*, however, similar expression levels were observed in all steps of the biofilm formation *in vitro*. The difference observed in the expression of the adaptation-related genes *in vitro* and *in planta* could have resulted from the different experimental designs utilized since the expression of the adaptation-related genes is possibly dependent on the environmental condition to which the bacterium is exposed (Prigent-Combaret et al., 1999, O´Toole et al., 2000). The high expression of these genes in *X. fastidiosa* was associated with increase the capacity of adaptation and competitiveness in the habitat (Souza et al., 2003). The expression of these genes *in vitro* suggests that the expression of adaptation genes as well as adhesion genes modulate the biofilm formation stages.

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

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

[1] Based in *Xylella* database (http:// aeg.lbi.ic.unicamp.br/xf)

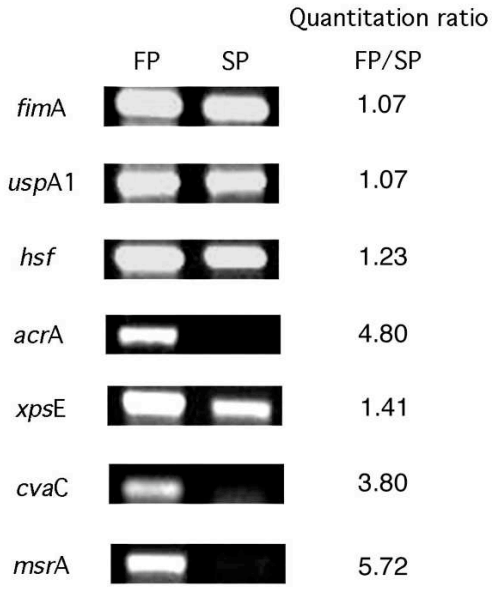

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) of FP induced genes. Expression of genes encoding pathogenicity factors in *X. fastidiosa* were evaluated *in planta* 90 days after inoculation. The ratios of the normalized quantitated band densities are presented as the FP value divided by the SP value.

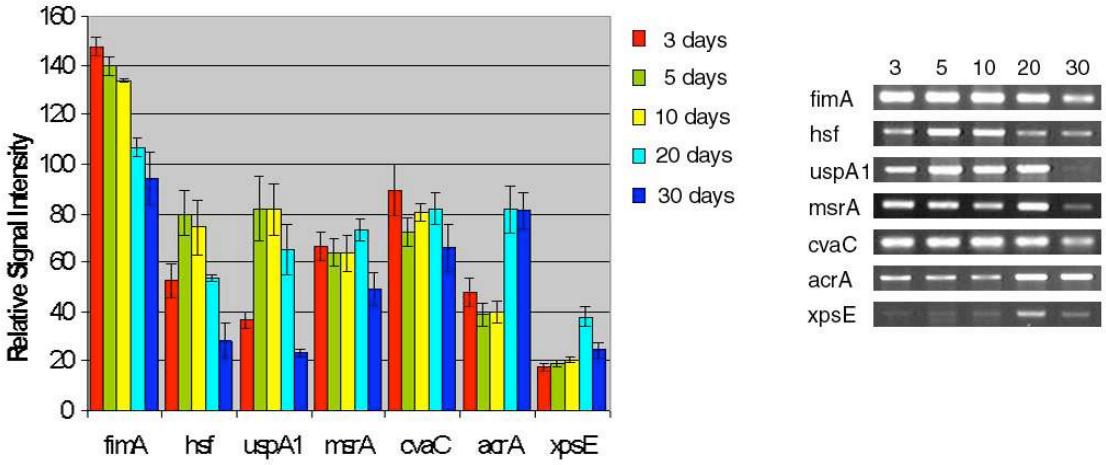

Figure 2. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). The mRNA levels of *fim*A, *hsf*, *usp*A1, *msr*A, *cva*C, *acr*A, and *xps*E in *X. fastidiosa* were evaluated 3, 5, 10, 20 and 30 days after inoculation. Data were normalized using ribosomal RNA content. The values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

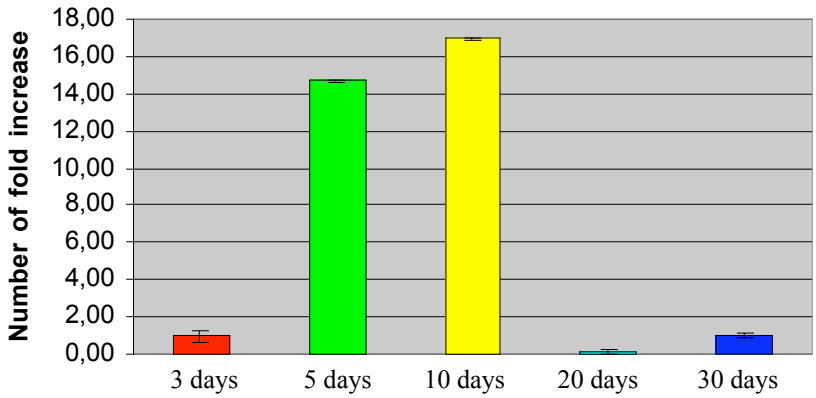

Figure 3. Relative quantitation of *usp*A1 from growth of *X. fastidiosa* at 3, 5, 10, 20 and 30 days of the biofilm formation. The measures were normalized using the C_ts obtained for the amplifications of the endogenous control run in the same plate. The values represent the fold increase in the gene expression compared to values obtained for 30 days (calibrator). The results are averages of two repetitions.

CAPÍTULO 3

Gene expression profile of the plant pathogen *Xylella fastidiosa* during biofilm formation *in vitro*

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Gene expression profile of the plant pathogen *Xylella fastidiosa* during biofilm formation *in vitro*.

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Abstract

A Biofilm is a community of microorganisms attached to a solid surface. Cells within biofilms differ from planktonic cells, showing higher resistance to biocides, detergent, antibiotic treatments and host defense responses. Even though there are a number of gene expression studies in bacterial biofilm formation, limited information is available concerning plant pathogen. It was previously demonstrated that the plant pathogen *Xylella fastidiosa* could grow as a biofilm, a possibly important factor for its pathogenicity. In this study we utilized analysis of microarrays to specifically identify genes expressed in *Xylella fastidiosa* cells growing in a biofilm, when compared to planktonic cells. About half of the differentially expressed genes encode hypothetical proteins, reflecting the large number of ORFs with unknown functions in bacterial genomes. However, under the biofilm condition we observed an increase in the expression of some housekeeping genes responsible for metabolic functions. We also found a large number of genes from the pXF51 plasmid being differentially expressed. Some of the overexpressed genes in the biofilm condition encode

proteins involved in attachment to surfaces. Other genes possibly confer advantages to the bacterium in the environment that it colonizes. This study demonstrates that the gene expression in the biofilm growth condition of the plant pathogen *X. fastidiosa* is quite similar to other characterized systems.

Keywords: microarray, mature biofilm

1. Introduction

Xylella fastidiosa has been associated with diseases of economically important crops including citrus, grapevine, plum, almond, peach, and coffee [1,2]. In citrus, it is responsible for citrus variegated chlorosis (CVC), a disease that has caused million-dollar damages to the Brazilian citrus industry. The economic importance of the citrus industry in Brazil and the high level of damage caused by CVC in Brazilian orchards have resulted in an extensive research program starting with the sequencing of the entire genome of *X. fastidiosa* [3].

This Gram-negative bacterium is a fastidious organism that is able to colonize the xylem vessel of several host plants and the cibarium of sharpshooter leafhopper vectors [1]. The ability to colonize the host is associated with the efficient capacity of adherence and multiplication in the vessel resulting in blockage of the xylem and consequent water stress symptoms [4, 5].

The capacity of adherence in solid surface, multiplication and colonization are characteristics of biofilm formation. Most bacteria can attach to solid surfaces and form biofilms, which are defined as matrix-enclosed microbial populations adhering to each other and to surfaces or interfaces [6]. Recently, the characterization of biofilm formation

in *X. fastidiosa* was performed by scanning electron microscopy. It was demonstrated that this organism could form biofilm in different solid surfaces and the biofilm morphology seems to vary according to the strain tested and the micro environmental conditions analyzed [7].

Cells grown on solid surfaces show physiological differences from planktonic ones. Bacteria within biofilm show higher resistance to biocides, detergent and antibiotic treatments [8]. A considerable increase in the number of gene expression studies involving biofilm formation has been observed, mainly for bacteria causing human diseases [9]. However, limited information is available about gene expression involved in biofilm formation of plant pathogens. The goal of this study was to identify genes associated with biofilm formation in *X. fastidiosa*. For this purpose we utilized microarray technology to access changes in gene expression in cells grown in mature biofilm compared with planktonic cells. Some genes detected by microarray were chosen to confirm by RT-PCR the expression level during biofilm growth. This transcriptome study opens new insights in the understanding of biofilm growth of *X. fastidiosa* in abiotic surfaces.

2. Material and Methods

2.1. *X. fastidiosa* growth condition

Citrus sinensis plants were inoculated with strain 9a5c of *X. fastidiosa* and when CVC symptoms were visible, petioles and stems were collected and aseptically ground in PBS. The suspension was distributed on plates containing PW medium [10]. The first colonies were observed between 10 to 15 days after plating. To obtain cells in biofilm, 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 transferred to a

1L flask containing 300 mL of PW broth, previously described to promote *X. fastidiosa* biofilm formation *in vitro* [7]. A total of three flasks were used in each analysis. After three days of growth at 28ºC in a rotary shaker at 120 rpm a thin biofilm formation was observed attached to the glass at the medium-air interface. The sample was collected after 20 days when the most abundant layer of biofilm formation was observed. The biofilm layer was scraped from flask and washed by centrifugation at 8,000 xg for 5 min at 4ºC with DEPC-treated water.

To obtain *X. fastidiosa* in planktonic growth, cells not attached to the glass were weekly transferred to another flask until they completely lost the capacity to adhere in glass surfaces. This characteristic is obtained after approximately ten passages. The cells were collected after five days of growth (exponential phase) and washed with DEPC-treated water by centrifugation at 8,000 xg for 5 min at 4ºC.

2.2. Microscopy and image analysis

To determine the time when *X. fastidiosa* forms a mature biofilm, we incubated the bacterium at 28ºC on 1.2 cm diameter glass cover slips immersed in PW medium in 'Nunclon delta SI Multidish 24 wells' (Nunc A/S, Roskilde, Denmark). Five individual colonies were transferred to a microcentrifuge tube containing 1 ml of PW broth. The tubes were vortexed and the cell suspension was transferred to the wells. The biofilm of *X. fastidiosa* strain 9a5c was analyzed at 3, 5, 10, 15, 20 and 30 days after inoculation using a light microscope (Olympus BX50, Olympus America Inc., Melville, NY) and a 40x magnification PL40XPH objective lens. Three glass cover slips were analyzed for each time point. The images were processed further for display using PHOTOSHOP 7.0 (Adobe Systems Inc., San Jose, CA).

2.3. RNA isolation and cDNA labeling

Total RNA of the biofilm and planktonic cells was isolated as described by Rhodius (www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf). RNA obtained from *X. fastidiosa* cells was labeled by reverse transcription with either Cy3 or Cy5 (both labels for each condition) [11].

2.4. Microarray construction

The *X. fastidiosa* microarray was constructed as previously described to Costa de Oliveira and collaborators [12]. The array contained 2200 PCR-amplified ORFs found in the genome of *X. fastidiosa* 9a5c strain, including the ones present in the pXf 51 plasmid.

The PCR products were purified with the QiaQuick 96 PCR purification system (Qiagen), dried in a Savant speed-vac, resuspended in 50% DMSO at a final concentration of 100 - 200 ng/ul and spotted onto CMT-GAPS silane-coated slides (Corning), using an Affymetrix 417 arrayer, according to the manufacturer's instructions.

2.5. DNA microarray and analysis

Arrays were hybridized overnight (42^{o}C) in a GeneTac Hybridization Station (Genomic Solutions, Inc., Ann Arbor, MI), in 6 X SSC, 5 X Denhardt´s solution, 0.25 mg/ml sheared salmon sperm DNA, 0.5% SDS, and a mixture of the two labeled cDNA samples. After hybridization, slides were washed twice (42^{o}C) in 0.5 X SSC, 0.01% SDS, followed by two washes in 0.06 X SSC, 0.01% SDS and two final washes in 0.06 X SSC. All washing steps consisted of 1 min of flow, followed by 5 min of incubation. Slides were then dried and subjected to fluorescence detection with a GMS 418 Array Scanner (Affymetrix Inc., Santa Clara, CA). 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. Two independent experiments were performed with six individual comparisons (independently grown and prepared sample). 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 (Significance Analysis of Microarrays) [13, http://www-stat.stanford.edu/~tibs/SAM/index.html).

2.6. RT-PCR analysis

RT-PCR was used to confirm DNA microarray gene expression. Cells adhering to the glass surface were collected 20 days after biofilm formation. Total RNA was prepared as described above. For RT-PCR, cDNA was prepared from 100 ng of total RNA with SuperScript II reverse transcriptase (Invitrogen). Reactions without SuperScript II were done in parallel to rule out the possibility of amplification from contaminating DNA. The RNAs were normalized using ribosomal RNA content. We carried out analyses for five genes (*pil*A, *pil*C, *mdo*H, *het*I, *spo*J) up-regulated in the biofilm condition that were amplified with specific primers (Table 1). A 25µl 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 Polymerase, and 2.5 µl of first strand cDNA template. The PCR cycle profile was 94ºC for 3 min for initial denaturation followed by cycles of 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min. Cycles were repeated in a series of every five cycles: from 10 – 30 for all the gene, in order to estimate the relative template concentration. PCR products were run on 1% agarose gel, and

densitometrically quantified using EagleSight software v. 3.2 (Stratagene Corp., La Jolla, CA).

3. Results

3.1. Biofilm Formation

To verify the morphology of mature biofilm we analyzed the growth of *X. fastidiosa* from 3 to 30 days after inoculation in glass cover under light transmission microscopy. Between 3-5 days after inoculation, planktonic bacteria adhered to the glass substratum (Fig. 1A). At day 10, cell clusters had begun their development, but small clusters were still present free from the biofilm (Fig. 1B). An early development of the biofilm architecture was observed 15 days after inoculation (Fig. 1C). Nevertheless it had small dimension compared with that observed after 20 days, when the cell clusters reached their maximum dimensions (Fig. 1D). This time point was chosen for the microarray analysis. After 30 days, alterations in the biofilm structure were observed due to a dispersion of cell clusters from the biofilm (Fig. 1E).

3.2. DNA array analysis of *X. fastidiosa* of CVC biofilm

The *X. fastidiosa* 9a5c chip used in this study was previously described by Costa de Oliveira and collaborators [12]. We examined the global gene expression of *X. fastidiosa* in biofilm compared with planktonic growth (Table 2).

The data obtained from the experiments were used for statistical analyses with SAM (Significance Analysis of Microarrays), which calculates the fold change and the significance of the differences in expression [13]. The fold change cutoff used in the analysis was 1.5 and a threshold Δof 1.28. These parameters resulted in a False Significant Number (FSN) of 0.46422 and a False Discovery Rate (FDR) of less than 1 %, meaning

that less than three statistically significant called ORF could be a false positive in our experiment. 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 gene expression is shown in a scatter plot (Fig. 2). The differentially expressed genes are distributed throughout the different categories into functional groups according to the Xylella database (http://aeg.lbi.ic.unicamp.br/xf) as summarized in Table 2. In this subset, a total of 202 genes (9.18%) were significantly up-regulated, while 32 genes (1,45%) were down-regulated in the mature biofilm. Approximately half (108/234) of the differentially expressed genes encode hypothetical proteins, reflecting the large number of ORFs with unknown functions typically observed in bacterial genomes.

Under the mature biofilm condition, we observed an increase in the expression of genes involved in energy metabolism, regulatory functions, protein metabolism, plasmid maintenance and the biosynthesis of amino acids, cofactors, prosthetic groups, carries, DNA, RNA, surface polysaccharides, lipopolysaccharides, antigens and transport proteins. Based on the genome annotation, the majority of genes expressed in biofilm condition from the pathogenicity category, were present in the subcategory that comprises toxin production, detoxification and adaptation to atypical conditions.

3.3. Genes associated with biofilm

Among the genes related to attachment, we detected overexpression of pili (*pil*A, or *fim*A and *pil*C), *slp* and *psp*A (Table 2) that are reported to be involved in the attachment of the bacteria and/or biofilm formation [14, 15, 16, 17]. We were also able to detect up-regulation of *ton*B, *het*I, *lin*C, *czc*A and *mex*A that are probably related to defense or

resistance of the biofilm [18, 19, 20, 21, 22]. The other genes possibly involved with adaptation and/or pathogenicity that were overexpressed in *X. fastidiosa* biofilm are *sac*1, *mdo*H, *xps*D, *xps*H, *eng*XCA and *muc*D.

A large number of genes from the conjugative plasmid pXF51 were differentially expressed in the biofilm condition, a phenomenon that could be related to conjugation, which is an active mechanism in biofilms for transferring genes within or between populations [14].

3.4. Confirmation of microarray data of the biofilm-growth regulated genes by RT-PCR.

In order to validate the results from the microarray study, we performed RT-PCR analysis for the *pil*A, *pil*C, *mdo*H, *het*I and *spo*J genes (Fig. 3). For these genes, expression ratios obtained in the microarray analysis were consistent with the RT-PCR ratios (Table 2, Fig. 3) even though the data from the RT-PCR resulted from a single experiment while, in the microarray analysis, the expression ratio for each transcript was calculated from the average expression ratios based on multiple measurements. These results were obtained using 20 amplification cycles.

4. Discussion

Biofilm formation is a key element in the pathogenicity of different bacteria [14, 9]. It was previously demonstrated that *X. fastidiosa* can grow in biofilm [7], and that this biofilm could be an important factor for its pathogenicity [11]. In this study, we developed a system using glass cover slips as adhesion surfaces for the bacteria, where we were able to verify different stages of the biofilm development, which is in accordance with other current models of biofilm formation [23]. In *X. fastidiosa,* however, biofilm development is

slower than in other organisms, in which it may take place within a few hours or days [23, 24]. This characteristic is probably a consequence of the fastidious growth typically observed for this organism.

In order to analyze gene expression in *X. fastidiosa* during biofilm formation, we decided to evaluate the changes that occur in the mature biofilm, when it displays several characteristics known to confer advantages to the bacterial population, including resistance to antimicrobial agents.

To analyze the changes in gene expression that occur in biofilm and planktonic growth of *X. fastidiosa*, we utilized the bacteria attached to a glass surface in the medium-air interface (biofilm growth) and cells that lost the capacity of adhesion in surface (planktonic growth), which can be obtained after several passages *in vitro*. This loss is also known in other bacterial systems [9]. In *X. fastidiosa* the biofilm formation in the medium-air interface seems also to be dependent on the medium composition [25].

To compare the gene expression profiles in biofilm and planktonic growth of *X. fastidiosa* we used DNA microarrays harboring sequences from approximately 2,200 ORFs from the genome of *X. fastidiosa* strain 9a5c including ORFs present in the pXf 51 plasmid. DNA microarray technology has been recently used to study *X. fastidiosa* genomes and transcription profiles [12, 26, 11]. Our microarray data revealed that *X. fastidiosa* gene expression in biofilm is different from that observed during planktonic growth. Many genes (approximately 9.18%) were up-regulated in biofilms and these ORFs were distributed in several functional categories. The number of biofilm specific genes is similar to other organisms [15, 24] but higher than *Pseudomonas aeruginosa* where a small amount of biofilm induced genes (0.5%) were detected [27]. In our study some of the differentially expressed genes could have resulted from the passages of the bacteria in culture until a

planktonic condition was obtained, nevertheless we were able to identify up-regulation of genes involved in biofilm development in other organisms.

Among the genes involved in biofilm formation, overexpression of *pil*A and *pil*C was detected. These adhesins are known to be required for the initial attachment to surfaces [15], however it has recently been reported that these appendages act also in biofilm development [28].

Gene expression in *X. fastidiosa* biofilm revealed the induction of several genes related to adaptation, strengthening the idea that these genes are expressed in biofilm showing high cell density and could be conferring advantages to the bacterial population. Among these genes, *ton*B is involved in the uptake of ferric iron [29], which is recognized as one of the key steps in the development of several pathogens inside their hosts [30]. In *Staphylococcus epidermis,* the production of iron chelators was generally 2-50 times higher in biofilms compared to the planktonic population [31].

The nature of the biofilm structure and the physiological attributes of microorganisms in a biofilm confer an inherent resistance to environmental factors that could harm the biofilm [9]. In this sense, we observed biofilm-associated overexpression of some genes that are likely related with resistance to antimicrobial agents (*lin*C, *mex*E) and heavy metal (*czc*A), as well as toxin production (*het*I). These characteristics have also been observed in other organisms growing inside biofilms [15]. In the mature biofilm of *X. fastidiosa* we also observed the up-regulation of *xps*D, *xps*H, *sac*1 and *mdo*H. The *xps*D and *xps*H genes belong to an operon involved in the general secretion pathway (GSP). Gram-negative bacteria with pathogenic potential use the GSP to deliver virulence factors into the extracellular environment for interaction with the host. Extracellular toxins and enzymes, pili, curli, autotransporters, and crystaline S-layers are well-studied examples of

virulence determinants secreted by the GSP [32]. In our analyses we found genes that encode pathogenicity factors like exoenzymes and pili that could be secreted by GSP.

In *C. reinhardtii* the *sac*1 mutant has altered responses to sulfur limitation, does not take up sulfate as rapidly as wild-type cells, and does not synthesize periplasmic proteins that normally accumulate during sulfur-limited growth. The *sac*1 gene of *C. reinhardtii* is similar to an *E. coli* gene that may be involved in the response of *E. coli* to nutrient deprivation [33]. In *X. fastidiosa* this gene could be associated with nutrient deprivation, since the condition of biofilm utilized was the medium-air interface.

The *mdo*H gene identified in *X. fastidiosa* is similar to *mdo*H from *E. coli* that, together with *mdo*G, forms an operon necessary for the biosynthesis of the glucose backbone of osmoregulated periplasmic glucans (OPGs) [34]. *mdo*G and *mdo*H homologues (*opg*G and *opg*H) were characterized in *Erwinia chrysanthemi*, a phytopathogenic bacterium that causes soft rot disease in a wide range of plants. The *opg* mutants exhibit a pleiotropic phenotype, including overproduction of exopolysaccharides, reduced motility, bile salt hypersensitivity, reduced protease, cellulase, and pectate lyase production, and complete loss of virulence. The authors demonstrated that OPGs present in the periplasmic space of the bacteria are necessary for growth inside the host [35]. Contrary to *E. coli* and *E. chrysanthemi* where these genes form an operon, in *X. fastidiosa* they are separated in the genome. Interestingly it was recently demonstrated that the *P. aeruginosa* gene *ndv*B, which encodes a glycosyltransferase required for the synthesis of periplasmic glucans in this organism, is responsible for resistance to some antibiotics in biofilm cells [36].

Up-regulation of genes that encode extracellular enzymes was also observed in biofilme cells. These genes, *eng*XCA and *muc*D, encode a cellulase and a perisplasmic

protease, respectively and seems to be important for the pathogenicity of *Xanthomonas campestris* pv. campestris [37] and *P. aeruginosa* [38]. On the other hand, we found no evidence for overexpression of genes involved in exopolysaccharide (EPS) production and the *rpf* genes, which regulate the production of exoenzymes and EPS in *Xanthomonas campestris* pv. campestris [39]. However analysis of EPS by Fourier transform infrared (FITR) spectroscopy of planktonic and biofilm cells of *X. fastidiosa* showed no difference (data not shown).

We also observed biofilm-associated overexpression of a large number of genes from plasmid pXF51, a conjugative plasmid that could be important for lateral gene transfer in this bacterium [40]. Plasmids seem to be an important factor in biofilm not only because of conjugation [41] but also because biofilm development seems to be induced by the plasmids [42]. The induction of plasmid genes observed in our experiment could probably be associated with lateral gene transfer in *X. fastidiosa*, a phenomenon that seems to play a major role in the adaptability of *X. fastidiosa* strains to infect a wide range of plant species [29].

Genes like *mdo*H, *xps*K and *gac*C, up-regulated in the biofilm condition (Table 2), have recently been shown not to be differentially expressed in an experiment comparing growth in high (stationary phase) and low cell density (early log) [43]. Since gene expression is dependent on the environmental condition to which the bacterium is exposed [44, 45], the difference in gene regulation observed between the works may be a result of the different experimental conditions utilized.

Some of the genes identified in this work showed a small but statistically significative fold change. Depending on mRNA stability, post-translational modifications, and protein function, a small change may be biologically important for many genes. A

correlation analysis of mRNA and protein abundance was previously done where it was demonstrated that 1 mRNA copy can generate in avarege 4000 molecules in yeast [46]. So a small variation in mRNA could represent a significative variation in protein levels and depending on the function, this could result in an important biological response.

This study is the first report that uses DNA microarray analysis to compare global gene expression between planktonic and biofilm growth of a plant pathogen. The expression profile observed in the biofilm of *X. fastidiosa* does not differ from biofilms of other bacteria and corroborates the idea that genes associated with adaptation and competitiveness are importance factors in the development and maintenance of biofilms.

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Table 1. Primers sequence used for RT-PCR

Gene name	Primers sequence	Fragment size (in base pairs)
*pil*A	F -5′ GTCCGGTACGATTTTGAGCAGTG 3’	480
	R - 5′ CGGCCCCGCATTGGTAA 3’	
*pil*C	F – 5’ ATACCAGCCCGCCGTTGACTT 3’	414
	R – 5’ CAGCCCTGACCAGATTGCGATAA 3’	
*mdo*H	F - 5′ CTTGGCGGAGGAGCGGGAGTATG 3’	343
	R - 5′ CCAAGCGACACCGTAGGCAATGAT 3’	
*het*I	F - 5′ CGCAGGCACGGCTTGTCTTC 3’	463
	R - 5′ GCCTGCCACTCATCTATCCTCCAC 3’	
*spo*J	F – 5’ GGGCGCACAGTATTCCTTGATTCC 3’	664
	R – 5’GCTGCGGCCTTTCGGTGTCTT 3’	

Table 2- Expression ratio of functional groups of genes inducted in biofilm condition

Functional Group	Gene*	ORF number*	Gene product	Ratio of gene expression†
				Biofilm
I. Intermediary Metabolism				
I.A. Degradation	*bgl*X	XF0439	Beta-glucosidase	1.63
	cdh	XF1825	NAD(P)H steroid dehydrogenase	2.44
I.B. Central metabolism intermediary	*acs*	XF2255	acetyl coenzyme A synthetase	2.56
I.C. Energy metabolism, Carbon	*nuo*E	XF0309	NADH-ubiquinone oxidoreductase, NQO2 subunit	1.75
	*yne*N	XF1990	Thioredoxin	3.41
	*etf*A	XF0253	Electron transfer flavoprotein alpha subunit	2.42
	-	XF0826	Fructose-bisphosphate aldolase	1.73
	*lpd*A	XF0868	dihydrolipoamide dehydrogenase	8.70
	*sdh*B	XF1073	succinate dehydrogenase iron-sulfur protein	1.65
	icd	XF2700	isocitrate dehydrogenase	1.64
	atpH	XF1146	ATP synthase, delta chain	1.72
	*cyc*J	XF2459	c-type cytochrome biogenesis protein	1.64
I.D. Regulatory Functions	*pho*R	XF2592	two-component system, sensor protein	1,54
	*prs*X	XF0216	transcriptional regulator (MarR family)	1,69
	*rpo*D	XF1350	RNA polymerase sigma-70 factor	1,85
	*rpo*H	XF2691	RNA polymerase sigma-32 factor	2,44
	AF0343	XF1133	tryptophan repressor binding protein	2,33
	*gac*A	XF2608	transcriptional regulator (LuxR/UhpA family)	1,59
	*kor*B	XF0061	transcriptional repressor	1.91
	*rel*A	XF1316	ATP:GTP 3'-pyrophosphotranferase	2,04
	*kor*A	XFa0057	transcriptional regulator	4,17
II. Biosynthesis of small molecules				
II.A. Amino acids biosynthesis	*pro*C	XF2712	pyrroline-5-carboxylate reductase	2,00
	*ilv*G	XF1821	acetolactate synthase isozyme II, large subunit	5,11
	HP0757	XF2443	beta-alanine synthetase	4,31
	*met*B	XF0864	cystathionine gamma-synthase	4,08
	*trp*F	XF1374	N-(5'-phosphoribosyl) anthranilate isomerase	1,83
	*aro*G	XF0026	phospho-2-dehydro-3-deoxyheptonate aldolase	2,28
	MTH1640	XF2338	chorismate mutase	2,14
	*aro*Q	XF2325	P-protein	1,96
	*aro*K	XF1335	shikimate kinase	1,89
	*thr*C	XF2223	threonine synthase	-1.72
	*ser*C	XF2326	phosphoserine aminotransferase	-2.03
	*trp*E	XF0210	anthranilate synthase component I	-2.62
	*his*D	XF2219	histidinol dehydrogenase	-1.50
II.B. Nucleotides biosynthesis	SCF55.27	XF0560	GMP synthase	1,86
	*pur*L	XF1423	phosphoribosylformylglycinamidine synthetase	2,24
	*pyr*E	XF0153	orotate phosphoribosyl transferase	2,22
	*nrd*A	XF1196	ribonucleoside-diphosphate reductase alpha chain	4,32
II.C. Sugars and sugar nucleotides biosynthesis	SCF11.04	XF1297	gluconolactonase precursor	1,99
II.D. Cofactors, prosthetic groups, carriers biosynthesis	*bio*B	XF0064	biotin synthase	2,87
	*isp*A	XF0661	geranyltranstransferase (farnesyl-diphosphate synthase)	4,62
	*cys*G	XF0832	siroheme synthase	1,64
	*fol*C	XF1946	folylpolyglutamate synthase/dihydrofolate synthase	5,55
	*pan*C	XF0230	pantoate--beta-alanine ligase	2,97
	SCE9433c	XF1924	L-aspartate oxidase	1,59
	*thi*C	XF1888	thiamine biosynthesis protein	6,10
II.E. Fatty acid and phosphatidic acid biosynthesis	DRB0080	XF2269	3-alpha-hydroxysteroid dehydrogenase	-1.61
III. Macromolecule metabolism				
III.A. DNA metabolism	*top*A	XF0920	DNA topoisomerase I	4,44
	DR0420	XF1229	ATP-dependent helicase	2,60
	*dna*A	XF0001	chromosomal replication initiator	2,89
	*top*A	XFa0003	topoisomerase I	1,55
	rin	Xfa0019	site-specific recombinase	1,82
	*mut*Y	XF1909	A/G-specific adenine glycosylase	2,32
	nth	XF0647	endonuclease III	1,71
	*lla*IIA	XF0935	methyltransferase	1,55
	*mut*X	XF1262	7,8-dihydro-8-oxoguanine-triphosphatase	-2.70
III.B. RNA metabolism	*rpm*A	XF2423	50S ribosomal protein L27	5,41
	*rpl*L	XF2634	50S ribosomal protein L7/L12	2,82
	*rpl*V	XF1157	50S ribosomal protein L22	4,02
	*rps*A	XF2438	30S ribosomal protein S1	1,61
	rpsG	XF2630	30S ribosomal protein S7	4,55
	*rps*H	XF1166	30S ribosomal protein S8	3,60
	*rps*O	XF0238	30S ribosomal protein S15	3,85
	*rlu*D	XF0939	ribosomal large subunit pseudouridine synthase D	1,88
	*rbf*A	XF0236	ribosomal-binding factor A	1,77
	*rps*F	XF2561	30S ribosomal protein S6	-1.78

III. C. Protein metabolism	Rv0198c	XF0576	metallopeptidase	5,62
	TM0911	XF2553	initiation factor eIF-2B, alpha subunit-related	6,24
	*ate*1	XF1018	arginine-tRNA-protein transferase	1,73
	*grp*E	XF2341	heat shock protein	1,53
	*clp*X	XF1188	ATP-dependent Clp protease ATP binding subunit Clpx	4,38
	*muc*D	XF2241	periplasmic protease	1,50
	*clp*B	XF0381	ATP-dependent Clp protease subunit	4,14
IV. Cell structure				
IV.A. Membrane components	*dc*14	XF0103	membrane protein	7,27
	slp	XF1811	outer membrane protein Slp precursor	2,63
	pcp	XF1547	peptidoglycan-associated outer membrane lipoprotein precursor	-1.89
IV.B. Murein sacculus, peptidoglycan	*mre*D	XF1311	rod shape-determining protein	1,57
	*mrd*A	XF1312	penicillin-binding protein 2	1,60
	*pbp*4	XF1614	penicillin binding protein	1,76
IV.C. Surface polysaccharides, lipopolysaccharides and antigens	*rfb*B	XF0611	dTDP-glucose 4-6-dehydratase	5,56
	*rfb*B	XF0255	dTDP-glucose 4,6-dehydratase	1,97
	*pil*A	XF0080	fimbrial adhesin precursor	2,47
	*pil*C	XF2538	fimbrial assembly protein	2,78
V. Cellular Process				
V.A. Transport	*glt*P	XF1937	proton glutamate symport protein	2,31
	*yba*L	XF2140	cation:proton antiporter	1,52
	*mnt*H2	XF1015	manganese transport protein	1,87
	HI0561/560	XF2261	oligopeptide transporter	1,68
	DR1357	XF0874	ABC transporter permease protein	2,07
	*btu*E	XF1604	ABC transporter vitamin B12 uptake permease	2,72
	f451	XF1728	transport protein	6,20
	*msb*A	XF1081	ABC transporter ATP-binding protein	2,73
V.B. Cell division	*min*C	XF1322	Cell division inhibitor	7,21
VI. Mobile Genetics Elements				
VI.A. Phage related functions and prophage	*noh*A	XF2501	phage-related protein	8,62
	*lyc*V	XF1669	phage-related lysozyme	1,67
	-	XF1875	phage-related protein	2,86
	-	XF2526	phage-related protein	-1.58
	gpj	XF2488	phage-related baseplate assembly protein	-1.63
	gpu	XF2481	phage-related tail protein	-1.62
VI.B. Plasmid maintenance protein	*pem*K	XFa0027	plasmid maintenance protein	2,29
	*tra*G	XFa0016	conjugal transfer protein	1,72
	*tra*E	XFa0012	conjugal transfer protein	1,95
	trbE	XF2053	conjugal transfer protein	1,57
	*trb*I	XFa0040	conjugal transfer protein	2,50
	*trb*F	XFa0043	conjugal transfer protein	2,16
	*trb*N	XFa0036	conjugal transfer protein	1,63
	*par*E	XFa0029	plasmid stabilization protein	2,27
	*tax*C	Xfa0047	nickase	1,66
	*inc*C	XFa0060	plasmid replication protein	1,75
	*spo*0J	XFa0059	plasmid replication/partition protein	2,51
	*ccg*AII	XF1759	conserved plasmid protein	6,02
VI.C. Transposon- and intron-related functions	-	XF0535	transposase OrfA	2,06
VII. Pathogenicity, virulence, and adaptation				
VII. C. Toxin production and detoxification	*ton*B	XF2287	TonB protein	3,12
	*het*I	XF1934	HetI protein	2,12
	*lin*C	XF1726	2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	2,08
	*mex*E	XF2084	component of multidrug efflux system	1,55
	*czc*A	XF2083	cation efflux system protein	2,19
VII.D. Host cell wall degradation	*eng*XCA	XF0810	extracellular endoglucanase precursor	1,84
VII.F. Surface proteins	*psp*A	XF0889	hemagglutinin-like secreted protein	1,73
VII.G. Adaptation, atypical conditions	*sac*1	XF0785	sulfur deprivation response regulator	1,86
	*mdo*H	XF1623	periplasmic glucan biosynthesis protein	1,69
	*hsp*A	XF2234	low molecular weight heat shock protein	-1.83
	*vap*D	XFa0052	virulence-associated protein D	2,17
	*xps*H	XF1520	general secretory pathway protein H precursor	1,80
	*xps*K	XF1523	general secretory pathway protein K	2,79
	*vac*B	XF1987	VacB protein	-1.55
VIII. Hypothetical				
VIII.A. Conserved Hypothetical Protein		XFa0025		2,32
		XF2257		3,98
		XF1080		2,35
		XF1560		2,19
		XF0449		1,57
		XF1708		2,04
		XF2490		2,03
		XF0948		2,66
		XF1405		1,53
		XF1231		2,67
		XF0096		1,77
		XF1504		1,61

	XF2619	1,70
	XF1482	2,23
	XF1230	5,88
	XF2573	2,40
	XFa0050	1,86
	XF1919	3,55
	XF2008	2,88
	XF1410	2,16
	XF0086	1,82
	XF1294	2,49
	XF1649	1,51
	XF0250	2,92
	XF0404	2,21
	XFa0045	3,09
	XF2357	1,53
	XF1901	-1.96
	XF1278	-4.39
	XF2450	-2.09
	XF0497	-1.68
	XF2010	-1.54
VIII.B. Hypothetical Protein	XF2107	1,83
	XF1662	1,73
	XF2665	2,54
	XF0546	1,55
	XF1980	1,95
	XF1138	1,62
	XF1416	4,01
	XF1664	1,66
	XF0583	5,44
	XF1877	1,67
	XF1861	2,58
	XF1710	2,24
	XF1306	1,70
	XF2117	5,64
	XF1244	3,48
	XFa0020	1,86
	XF1635	4,67
	XF1963	2,61
	XF2098	2,28
	XF2316	2,06
	XF2289	6,93
	XF2519	1,97
	XF0561	1,70
	XF1598	2,45
	XF1832	1,68
	XF0629	1,64
	XF1233	6,31
	XF1100	1,94
	XF1009	4,43
	XF2192	1,72
	XFa0028	3,72
	XF0247	2,03
	XFa0051	3,59
	XF1836	3,11
	XF0403	1,84
	XF0772	1,88
	XF1667	1,58
	XFa0049	3,09
	XF0862	1,57
	XF1249	3,73
	XF2250	2,00
	XF0867	2,85
	XF0865	1,62
	XFa0048	3,07
	XF1756	1,75
	XF0070	4,90
	XF0473	2,48
	XF1977	3,48
	XF0936	1,69
	XFa0004	4,08
	XFa0026	3,61
	XF2125	2,27
	XF2075	1,82
	XF0655	1,87
	XFa0031	2,09
	XF2779	1,59
	XF2720	2,44
	XF0035	5,22
	XF0632	1,63
	XF1629	3,38

	XF1542		1,78
	XF0342		-1.76
	XF2129		-1.68
	XF1069		-1.57
	XF1784		-2.44
	XF1793		-1.78
	XF2449		-1.63
	XF2136		-1.54
	XF2738		-2.10
	XF0735		-1.61
	XF2688		-1.52
	XF0016		-1.58
	XF1770		-1.82
	XF1787		-1.76
	XF0532		-1.65
IX. Other	XF1213	GTP-binding elongation factor protein	1,90

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

† Positive and negative values represent induction and repression in biofilm condition, respectively.

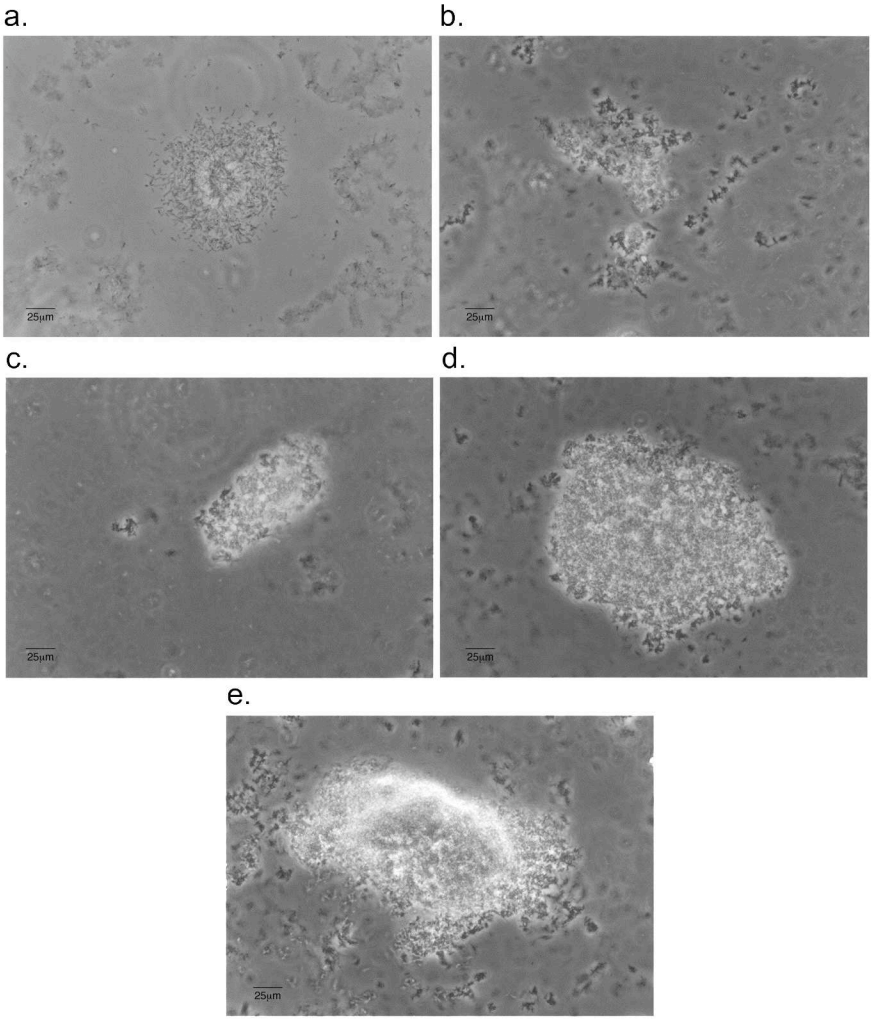


Figure 1. Biofilm in *Xylella fastidiosa*. Cells grown on glass covers were visualized at different stages of the biofilm formation under light microscopy at 5 (a), 10 (b), 15 (c), 20 (d) and 30 (e) days after inoculation.

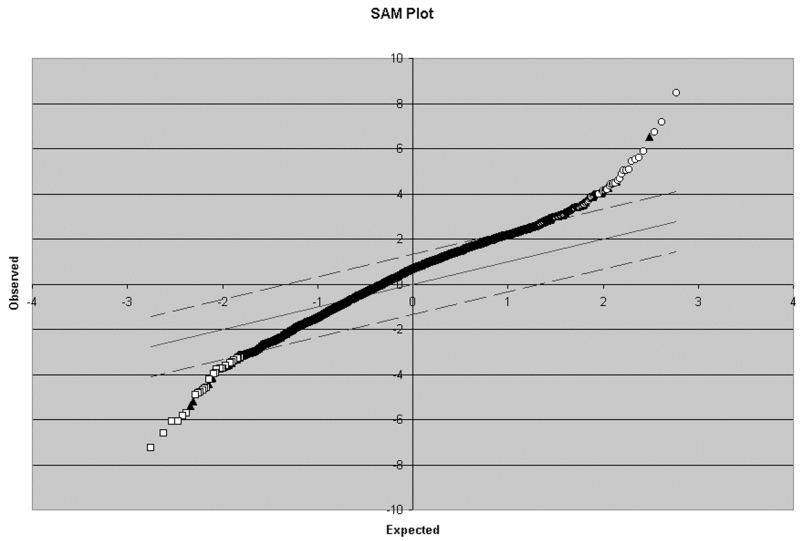


Figure 2. Identification of genes with significant changes in expression. Scatter plot of the observed relative difference versus the expected relative difference calculated by SAM. The dashed lines indicate the statistic cutoff for induction and repression. The potentially significant induced or repressed genes for $\Delta = 1.28$, are represented by ○ and □, respectively.

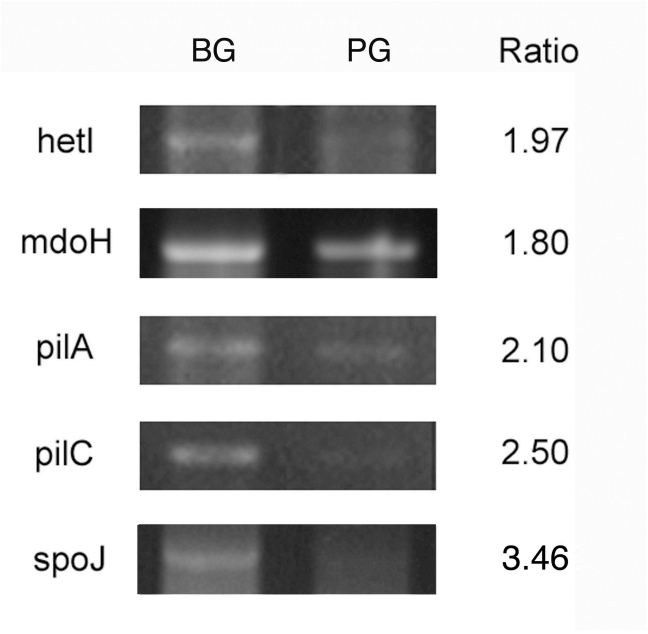


Figure 3. Reverse transcription-polymerase chain reaction (RT-PCR) of biofilm-growth induced genes in *X. fastidiosa*. The ratios of the normalized quantitated band densities are presented as the biofilm growth (BG) value divided by the planktonic growth (PG) value.

8. Conclusões gerais

Para testar as hipóteses formuladas neste estudo as seguintes estratégias foram estabelecidas: 1. A avaliação da colonização e virulência das células em IP e SR foram efetuadas através de PCR quantitativo em tempo real e observação visual dos sintomas. 2. A expressão diferencial dos genes de *X. fastidiosa* induzidos na condição IP e no crescimento em biofilme foi monitorada utilizando-se a técnica de ´microarray´ e RT-PCR semi-quantitativo.

No trabalho visando a detecção de genes envolvidos com patogenicidade (Cap.1), células de *X. fastidiosa* recém isoladas de planta com sintomas e , denominadas células de isolamento primário (IP), foram mais virulentas do que células repicadas 46 vezes em meio de cultura. A maior virulência das células após IP foi deduzida pelo maior número de células e mais rápida colonização nas plantas hospedeiras, quer seja laranja doce ou vinca, o que pôde ser demonstrado por PCR quantitativo em tempo real (Anexo 10.2) e desenvolvimento de sintomas apenas com as células nesta condição (Anexo 10.3). Estes resultados corroboram com a hipótese que as células em IP apresentam padrão similar de crescimento dentro da planta. A tecnologia de ´microarray´ foi eficiente para detectar genes induzidos e reprimidos na condição IP. Dentre os genes induzidos, sete foram associados com categoria de patogenicidade, sendo três (*fim*A, *usp*A1, *hsf*) envolvidos com adesão e quatro (*acr*A, *msr*A, *xps*E e *cva*C) com adaptação e competitividade no ambiente do hospedeiro. O gene *fim*A tem homologia com fimbria do tipo IV, que são consideradas fatores de virulência em vários patógenos de humanos e animais, por mediar a adesão bacteriana nas células epiteliais do hospedeiro. Em patógeno de planta um homólogo deste gene foi detectado em *X. campestris.* pv. vesicatoria e está envolvido na agregação célula-célula. Utilizando a seqüência de aminoácidos da proteina codificada por *fim*A de *X. fastidiosa* detectada pelo 'microarray' foi feita uma busca por similaridades com outras seqüências no genoma da *X. fastidiosa* utilizando a ferramenta 'blastp', sendo detectado cinco outros genes (XF2542, XF0487, XF0539, XF0538, XF1791) cujos produtos apresentam homologia a FimA. Os genes *hsf*, com duas cópias no genoma (XF1528, XF1981), e *usp*A1 estão relacionados a adesão de bactérias patogênicas de humanos às células epiteliais do trato respiratório. A proteína MsrA tem sido associada com adesão e com adaptação. Foi demonstrado que MrsA é importante para manter o estado adesivo de

três bactérias patogênicas de humanos e também várias são as referências relatando a importância de MsrA na proteção contra o estresse oxidativo. Os outros genes detectados estão associados a resistência a drogas (*acr*A), secreção de fatores de virulência (*xps*E) e uma provável bacteriocina (*cva*C). A eficiência de colonização na condição IP pode estar associada a uma maior capacidade de adesão destas bactérias no xilema, evidenciada pela maior expressão dos genes *hsf*, *usp*A1, *fim*A e *msr*A, todos envolvidos na adesão de bactérias em superfícies do hospedeiro. Os genes envolvidos com adaptação podem estar contribuindo com a maior eficiência da colonização das células na condição IP, possivelmente por aumentar a capacidade de adaptação e competição da bactéria no xilema.

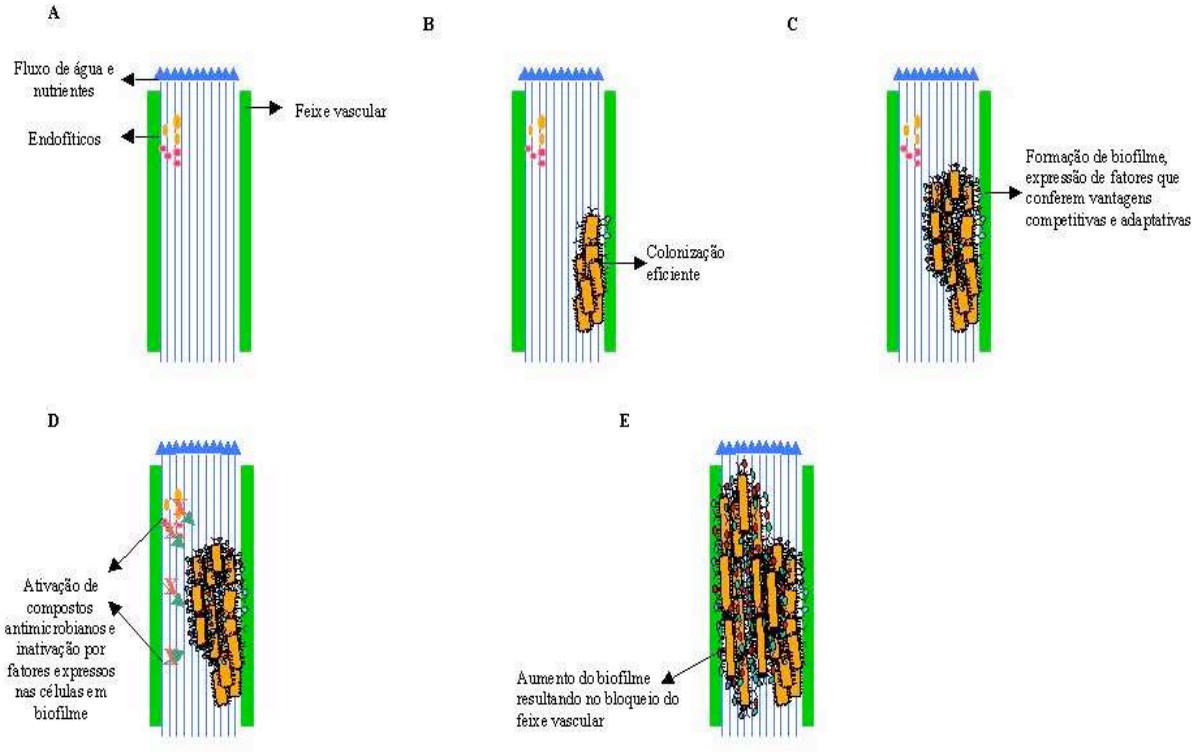
A maior expressão dos genes de patogenicidade foi confirmada por RT-PCR tanto na condição *in vitro* quanto *in planta* 15 dias após inoculação. No trabalho apresentado no Capítulo 2 a expressão dos genes relacionados com a categoria de patogenicidade detectados por ´microarray´ foram avaliados através de RT-PCR semi-quantitativo na condição *in planta*, 90 dias após inoculação (estádio avançado de colonização), e na condição *in vitro* em diferentes fases da formação de biofilme em superfície abiótica. Após 90 dias de colonização foi verificado que os genes de adesão apresentaram expressão similar tanto na condição IP quanto na SR, entretanto os genes de adaptação foram induzidos apenas na condição IP. Este resultado demonstra que os genes de adesão são importantes para o início da colonização, uma vez que aos 15 dias eles também são induzidos em IP, porém os genes de adaptação são necessários para a manutenção do biofilme.

Na condição *in vitro*, a expressão dos genes de adesão foi similar ao observado *in planta*, quando genes associados ao processo de adesão foram induzidos nos estágios iniciais de formação de biofilme, evidenciando o envolvimento destes genes em diferentes superfícies de adesão. Devidos às dificuldades dos estudos da *X. fastidiosa in planta*, estes resultados indicam que superficies abióticas podem ser usadas para mimetizar a adesão *in vivo*. Alguns genes de adaptação (*acr*A e *xpsE*) também apresentaram expressão similar ao observado *in planta*, ou seja, maior indução nos estágios avançados da formação de biofilme, porém outros (*msr*A e *cva*C) se comportaram de forma diferente ao observado *in planta*, sugerindo que a expressão dos genes de adaptação pode ser modulada de acordo com o ambiente ao qual a bactéria é exposta.

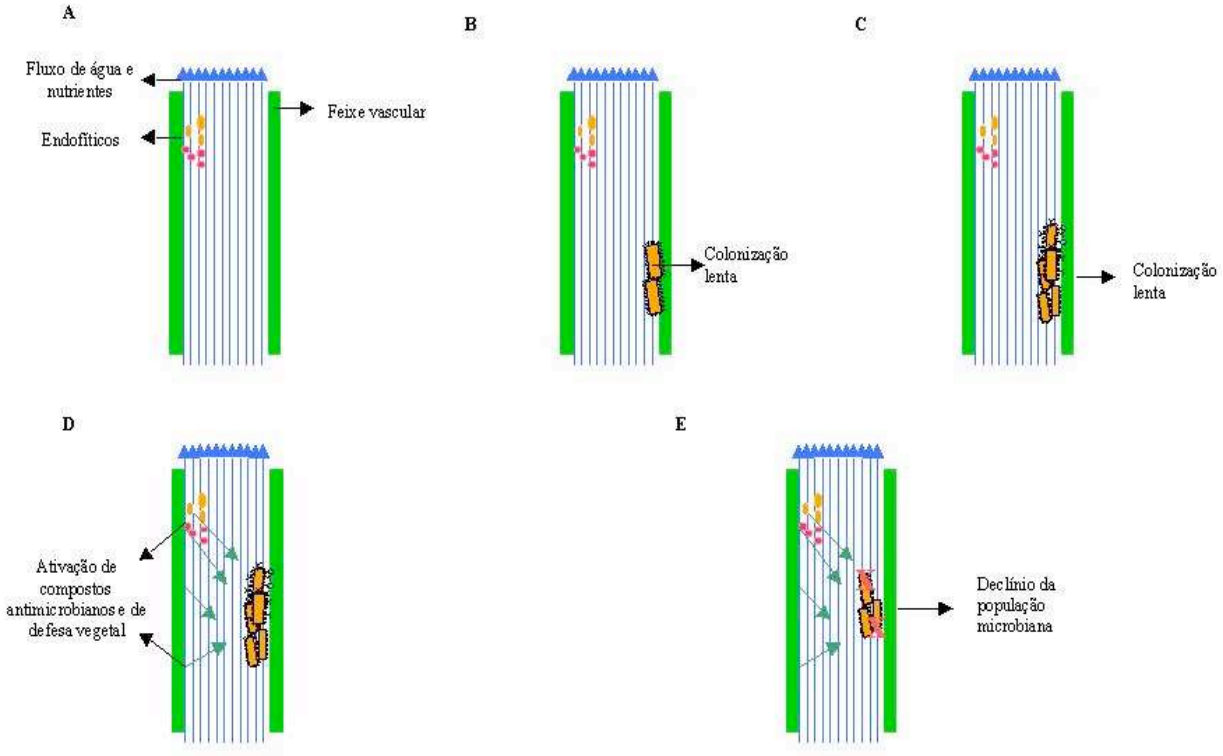
Os resultados do trabalho apresentado no Capítulo 3 demonstram que em *X. fastidiosa* a formação do biofilme sobre superfície abiótica pode ser bem visualizada em pelo menos cinco fases distintas. A fase de biofilme maduro foi observada 20 dias após inoculação. A tecnologia de ´microarray´ foi utilizada para análise comparativa dos genes expressos na condição de crescimento em biofilme maduro em comparação com a condição de crescimento planctônico. Aproximadamente 9% dos genes foram induzidos e 1,5% foram reprimidos na condição de biofilme. A maioria dos genes induzidos codifica para proteínas com funções essenciais para manutenção celular e para proteínas de função ainda desconhecidas. Dentre os genes induzidos associados com crescimento em biofilme, foram detectados genes associados a adesão (*pil*A, *pil*C, *slp*, *psp*A), defesa ou resistência (*het*I, *lin*C, *czc*A, *mex*A, *mdo*H), adaptação e/ou patogenicidade (*ton*B, *sac*1, *xps*D, *xps*H, *eng*XCA *e muc*D) e conjugação (plasmído pXF51). Os genes relacionado a adesão são similares a genes também encontrados em outros microrganismos tanto em estágios iniciais quanto avançado da formação de biofilme. Os genes de defesa ou resistência estão provavelmente envolvidos com produção de toxinas (*het*I), resistência a compostos antimicrobianos (*lin*C, *mex*A), metais pesados (*czc*A) e estresse osmótico (*mdo*H). Em relação aos genes envolvidos com as categorias adaptação e/ou patogenicidade, foram detectados genes associados com absorção de nutrientes (*ton*B, *sac*1), proteases (*muc*D) e exo-enzimas (*eng*XCA), consideradas fatores de virulência, e genes responsáveis pela secreção de fatores de virulência (*xps*D, *xps*H). A grande quantidade de genes expressos referente ao plasmídeo pXF51 indica a possível ocorrência de transferência horizontal de genes dentro do biofilme.

As proteínas codificadas pelos genes expressos no biofilme de *X. fastidiosa* apresentam funções similares às observadas em biofilme de bactérias que causam doenças em humanos, onde uma grande quantidade dos genes expressos proporcionam vantagens adaptativas e competitivas no ambiente colonizado. Estes resultados indicam que a formação de biofilme enquanto mecanismo associado a patogenicidade de bactérias de diferentes hospedeiros apresentariam características comuns.

Baseados nos trabalhos desenvolvidos propõe-se o seguinte modelo de interação da *X. fastidiosa* com o hospedeiro:



Modelo de interação eficiente das células de *X. fastidiosa* na condição IP com o hospedeiro. A. Feixe vascular contendo endofíticos e seiva do xilema. B. Colonização eficiente das células em IP proporcionada pela rápida expressão de adesinas fimbriais e afimbriais. C. Formação de biofilme e ativação de genes que conferem vantagens adaptativas e competitivas para a comunidade microbiana. D. Inativação dos compostos antimicrobianos e de defesa vegetal. E. Aumento do biofilme resultando no bloqueio do feixe vascular.



Modelo de interação ineficiente das células de *X. fastidiosa* na condição SR com o hospedeiro. A. Feixe vascular contendo endofíticos e seiva do xilema. B. Colonização lenta das células em SR decorrente da expressão tardia de adesinas fimbriais e afimbriais. C. Devido à lenta colonização não há formação de biofilme. D. Ativação de compostos antimicrobianos e de defesa vegetal. E. Como não foi formado o biofilme, não há ativação dos genes que conferem vantagens adaptativas e competitivas para célula resultando em declínio da população.

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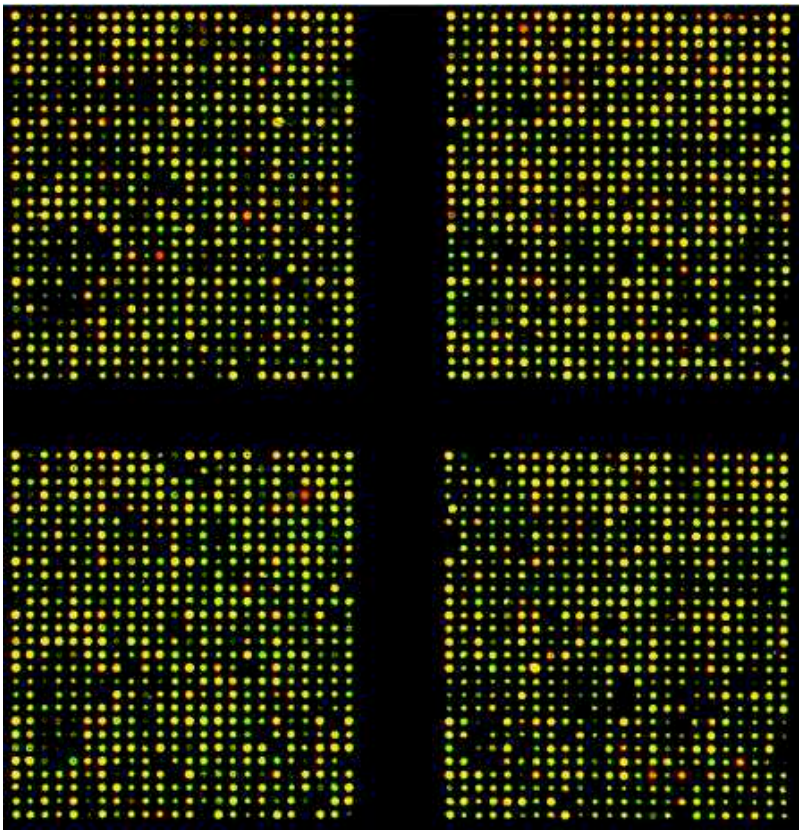
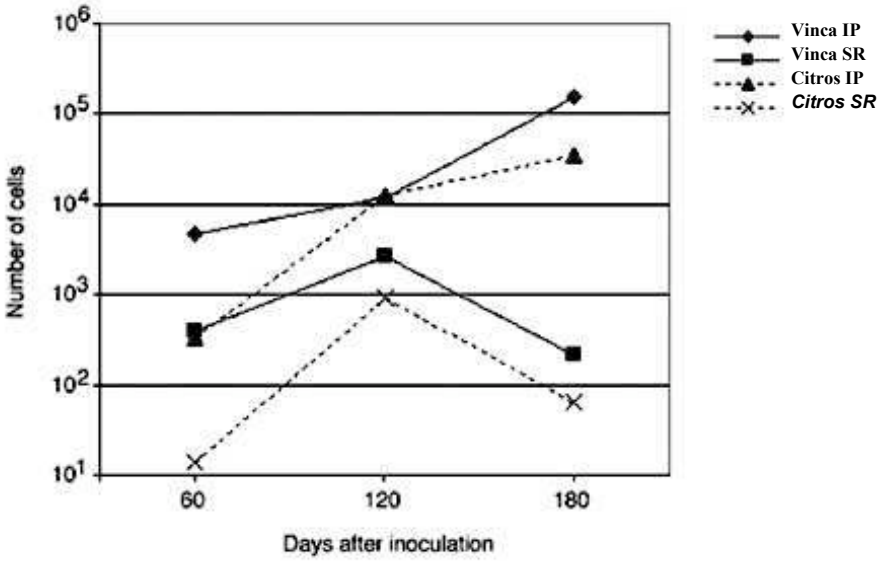
10. Apêndices

Apêndice 10.1. ‘Microarray’ contendo aproximadamente 2.200 ORFs da *X. fastidiosa*



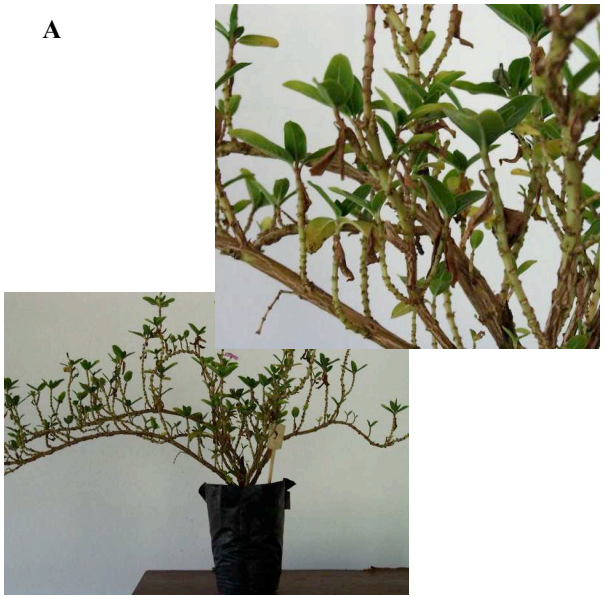
Microarray de *X. fastidiosa* contendo 4 subquadrantes com ~2,200 ORFs. cDNA de IP (verde) e SR (vermelho) foi hibridizado no ‘chip’. A intensidade por ‘spot’ corresponde a expressão relativa da ORF entre as duas condições de crescimento.

Anexo 10.2. Análise quantitativa de *X. fastidiosa* por qPCR. Plantas de laranja doce e vinca inoculadas com *X. fastidiosa* na condição IP e SR foram amostradas aos 60, 120 e 180 dias. O gráfico representa a média do número de células de 10 plantas.

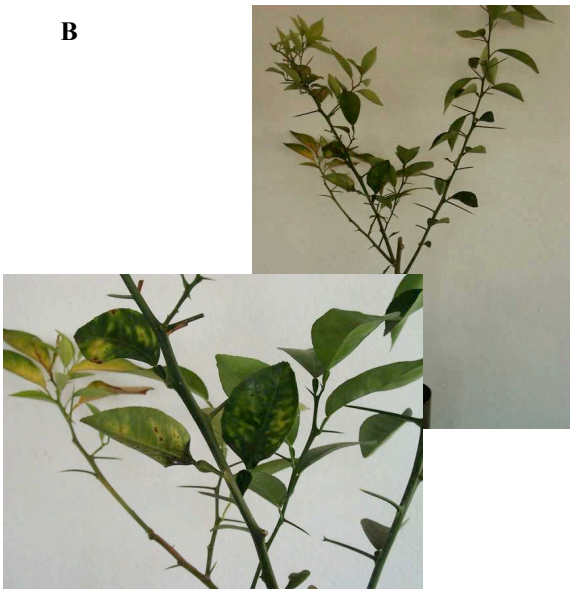


Anexo 10.3. Sintomas decorrentes da colonização de *X. fastidiosa* na condição IP. A. Plantas de vinca apresentando amarelecimento foliar e encurtamento dos internódios. B. Plantas de citros apresentando folhas cloróticas com lesões necróticas.

A



B



Anexo 10.4. Teste *t* da média de 3 repetições da intensidade de amplificação dos genes *fim*A, *hsf*, *usp*A1, *msr*A, *cva*C, *acr*A, *xps*E por RT-PCR semi-quantitativo nas diferentes fases da formação de biofilme da *X. fastidiosa in vitro.*

	*fim*A	*hsf*	*usp*A1	*msr*A	*cva*C	*acr*A	*xps*E
g3xg5	0,146573	0,038684	0,032859	0,378513	0,125445	0,144642	0,310963
g3xg10	0,030777	0,092996	0,020186	0,378205	0,242699	0,167579	0,105701
g3xg20	0,000905	0,439583	0,048292	0,199236	0,297689	0,069209	0,011895
g3xg30	0,014727	0,03439	0,018468	0,168346	0,087809	0,053112	0,076628
g5xg10	0,089014	0,360834	0,485943	0,48161	0,169271	0,436946	0,222799
g5xg20	0,001726	0,049124	0,178494	0,127402	0,170607	0,03402	0,013372
g5xg30	0,018726	0,00626	0,022076	0,196459	0,302427	0,019984	0,108886
g10xg20	0,010657	0,102709	0,15771	0,159612	0,413111	0,037349	0,019744
g10xg30	0,031351	0,01691	0,014397	0,201971	0,135257	0,022325	0,177329
g20xg30	0,127704	0,032673	0,025761	0,122828	0,123235	0,422502	0,029472

Apêndice 10.5. Atividade científica da candidata durante a vigência do curso de doutorado

(fevereiro de 2001 a julho de 2004)

Artigos e trabalhos científicos completos publicados em revistas com comitê editorial

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