Universidade Estadual de Campinas
Instituto de Biologia

Expressão diferencial de *Xanthomonas axonopodis* pv. *citri* na interação com *Citrus sinensis* utilizando eletroforese bidimensional de proteínas e cDNA RDA ("Representational Difference Analysis")

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Orientadora: Profa. Dra. Yoko Bomura Rosato

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ÍNDICE

RESUMO

No presente estudo, a expressão diferencial de *X. axonopodis* pv. *citri* em resposta a diferentes condições de cultura foi analisada através de eletroforese bidimensional (2-DE) de proteínas e cDNA RDA ("Representational Difference Analysis"). Para a análise de proteínas, a bactéria foi cultivada no meio basal MM1 e na presença de extrato de folhas de uma planta suscetível (*Citrus sinensis*) assim como de uma resistente (*Citrus reticulata*) e uma não hospedeira (*Passiflorae edulis*). O perfil de proteínas revelou 12 "spots" diferenciais no tratamento com extrato de citros (hospedeiro suscetível) quando comparado àquele do MM1. O perfil da bactéria cultivada em meio complexo NYG foi também analisado e a comparação com aquele do meio MM1 revelou 36 proteínas diferenciais. Cinco proteínas dos diferentes tratamentos tiveram a região N-terminal seqüenciada, incluindo 2 proteínas constitutivamente expressas, 2 induzidas na presença de extrato de citros e 1 induzida no meio complexo. Um método para a recuperação da bactéria de folhas da planta hospedeira foi também desenvolvido e validado através da análise de proteínas e RNA. O perfil de proteínas obtido através de 2-DE foi semelhante ao obtido pela bactéria cultivada na presença de extrato de folhas de *Citrus sinensis*. RNA total foi analisado através de eletroforese em gel de agarose e reações de RT-PCR utilizando primers para genes de *X. axonopodis* pv. *citri* confirmaram a qualidade do RNA obtido. O emprego desta técnica permitirá estudos de expressão *in planta* de diversas espécies de fitopatógenos. Com a técnica de cDNA RDA foram identificados cDNAs expressos na presença de extrato de folhas da planta hospedeira, assim como *in vivo* após hibridizações subtrativas contra cDNA da bactéria cultivada em MM1. Uma hibridização subtrativa de

cDNAs da bactéria cultivada no meio NYG contra cDNAs expressos em MM1 foi também efetuada. Um total de 37 genes distintos foi identificado através de busca de homologia no genoma de *X. axonopodis* pv. *citri*, incluindo genes que codificam proteínas hipotéticas (12), genes relacionados ao metabolismo (13), processos celulares (6) e patogenicidade (4), e elementos genéticos móveis (2).

ABSTRACT

The present study reports the differential expression of *X. axonopodis* pv. *citri* in response to different growth conditions by two-dimensional gel electrophoresis (2-DE) of proteins and cDNA RDA (Representational Difference Analysis). For the protein analysis, the bacterium was cultured in the basal medium MM1 and in the presence of leaf extracts from a susceptible host plant (sweet orange) as well as a resistant (ponkan) and a non-host plant (passion fruit). The protein profiles revealed 12 differential spots in the citrus extract treatment (susceptible host) when compared to that of MM1. The 2-DE protein profile of the bacterium grown in the complex medium NYG was also obtained and the comparison with that of MM1 revealed 36 differential spots. Five proteins from the different treatments were successfully N-terminally sequenced and included 2 constitutively expressed proteins, 2 proteins up-regulated in the presence of citrus extracts and 1 up-regulated in the complex medium. A method for the recovery of bacterial cells from the host plant leaves was also developed and validated by the analysis of protein and RNA extracted from the recovered cells. The protein pattern obtained by 2-DE was similar to that obtained for the bacterium grown in the presence of leaf extract of *Citrus sinensis*. Total RNA was analysed by agarose gel electrophoresis and RT-PCR reactions using specific primers for *X. axonopodis* pv. *citri* genes confirmed the quality of the RNA obtained. This method will be useful for *in planta* expression studies of several phytopathogenic species. In the cDNA RDA technique, cDNAs expressed in the presence of leaf extract of the host plant, as well as *in vivo* were identified after subtractive hybridizations against cDNAs of the bacterium cultured in the basal medium MM1. Also, a subtractive hybridization of cDNAs of the

bacterium grown in complex media against cDNAs expressed in the minimal medium was performed. A total of 37 distinct genes were identified by homology searches in the genome of *X. axonopodis* pv. *citri*, including genes that encode hypothetical proteins (12), genes involved in metabolism (13), cellular processes (6) and pathogenicity (4), and mobile genetic elements (2).

INTRODUÇÃO

O cancro cítrico é uma doença considerada potencialmente destrutiva para a citricultura em diversas regiões produtoras de citros ao redor do mundo (Rossetti, 1977; Stall & Seymour, 1983). A doença é causada por *Xanthomonas axonopodis* pv. *citri* e é caracterizada por lesões erupescentes em ramos folhas e frutos. Linhagens de *X. axonopodis* pv. *citri* possuem uma ampla gama de hospedeiros e causam a doença em praticamente todas as variedades de laranja (Stall & Seynour 1983). Outras *Xanthomonas* que causam sintomas semelhantes em citros são linhagens de *X. axonopodis* pv. *aurantifolii* dos grupos B, C e D porém apresentam menor importância econômica devido ao número limitado de hospedeiros e a ausência de severidade dos sintomas. *X. axonopodis* pv. *citrumelo*, anteriormente classificada como *X. campestris* pv. *citrumelo,* é responsável pelo "citrus bacterial spot", mas foi inicialmente associada com uma forma de cancro cítrico (grupo E) devido a similaridade dos sintomas (Civerolo, 1984).

O aumento da incidência do cancro cítrico no Brasil, ocorrido nos dois últimos anos, levou ao desenvolvimento do projeto genoma de *X. axonopodis* pv. *citri* financiado pela FAPESP, que representa o segundo projeto genoma desenvolvido no Brasil. Este projeto já está concluído e um grande número de informações está disponível para análise.

Uma das abordagens para o estudo do genoma funcional envolve a análise da expressão gênica diferencial. Muitas técnicas têm sido utilizadas para a análise da expressão gênica baseadas em "mRNA differential display" e "expressed sequence tags" (EST). Entretanto, essas técnicas são trabalhosas e em geral um grande número de seqüências deve ser gerado para a obtenção dos resultados esperados. A técnica de RDA

("Representational Difference Analysis") de cDNA tem sido considerada promissora para o estudo da expressão diferencial. Este método foi inicialmente desenvolvido para eucariotos e tem identificado com sucesso vários genes diferencialmente expressos em diferentes tecidos (Cooper *et al.*, 2000; Kim *et al.*, 2000). Em bactérias, cDNA RDA está sendo utilizada em poucos laboratórios no mundo e apenas dois estudos foram registrados na literatura (Bowler *et al.*, 1999; Becker *et al.*, 2001).

Outra técnica muito utilizada no estudo da expressão gênica é a análise de proteínas diferencialmente expressas através de eletroforese bidimensional (2-DE) em gel de poliacrilamida. Com esta técnica, são elaborados mapas 2-D e a comparação dos produtos protéicos de diferentes tratamentos pode levar à identificação de proteínas diferencialmente expressas. O seqüenciamento NH_2-terminal leva a determinação de uma pequena seqüência de aminoácidos, suficiente para permitir a dedução da provável função da proteína.

Os conhecimentos gerados a partir da análise do genoma funcional de *X. axonopodis* pv. *citri* poderá melhor informar sobre os genes e proteínas induzidos especificamente. O gênero *Xanthomonas* possui muitas espécies e afeta várias culturas como a do feijão, arroz, maracujá, entre outras. Como as espécies são semelhantes, o estudo da bactéria responsável pelo cancro cítrico poderá também contribuir para o conhecimento das outras espécies de *Xanthomonas*.

OBJETIVO

O presente trabalho teve por objetivo analisar a expressão diferencial de *X. axonopodis* pv. *citri* em diferentes condições de cultura para identificar possíveis genes envolvidos na interação planta-patógeno. Para atingir este objetivo foram realizadas as seguintes etapas:

- Análise de proteínas diferencialmente expressas através de eletroforese bidimensional em gel de poliacrilamida

- Identificação das proteínas diferencialmente expressas assim como algumas proteínas constitutivas através de seqüenciamento NH_2-terminal

- Padronização da técnica de cDNA RDA ("Representational Difference Analysis") para *X. axonopodis* pv. *citri*

- Obtenção de produtos diferenciais após hibridizações subtrativas seguidas de amplificação por PCR

- Identificação dos genes diferencialmente expressos nas diferentes condições de cultura

- Confirmação da expressão diferencial por RT-PCR utilizando primers específicos

REVISÃO DE LITERATURA

X. axonopodis pv. *citri* e o cancro cítrico

X. axonopodis pv. *citri*, originalmente classificada como *X. campestris* pv. *citri* (grupo A), foi reclassificada como pertencente à espécie *X. axonopodis* baseada em análises de hibridização DNA:DNA e outras características fenotípicas (Vauterin *et al.*, 1995). A bactéria *X. axonopodis* pv. *citri* é caracterizada por colônias amarelas e crescimento na maioria dos meios rotineiramente utilizados no laboratório. Estudos de diversidade genética de linhagens responsáveis pelo cancro cítrico de várias regiões do mundo revelaram uma alta uniformidade entre as linhagens baseados em "fingerprinting" de DNA (Hartung & Civerolo, 1987), "restriction fragment length polymorphism" (RFLP) (Gabriel *et al.*, 1988) e anticorpos monoclonais (Alvarez *et al.*, 1991).

As células bacterianas sobrevivem no solo e nas superfícies de ervas daninhas por períodos curtos (Stall & Civerolo, 1983) e são dispersadas por curtas distâncias pela chuva juntamente com o vento e equipamentos mecânicos (Serizawa *et al.*, 1969; Stall *et al.*, 1980). A transmissão a longas distâncias geralmente ocorre através de material propagativo contaminado.

O cancro cítrico, causado por *X. axonopodis* pv. *citri*, foi observado inicialmente em espécies herbárias de *Citrus medica* na Índia em 1927-1931, mas somente após sua introdução nos EUA, foi considerada como uma nova doença (Stall & Civerolo, 1993). A ocorrência do cancro cítrico já foi registrada em praticamente todas as principais regiões citrícolas do mundo. A doença somente não foi constatada em áreas localizadas em regiões áridas, como por exemplo, os estados do Arizona, Novo México e Califórnia, nos Estados

Unidos, e a região Mediterrânea. O cancro cítrico normalmente ocorre de forma severa em regiões onde há coincidência de períodos chuvosos com temperaturas elevadas (Peltier & Frederich, 1926).

O cancro cítrico A ou cancro cítrico Asiático é a forma mais importante da doença. Essa forma de cancro cítrico tem sua origem provavelmente na região do Sudeste Asiático, mas se encontra atualmente disseminado em várias regiões produtoras de citros da Ásia, África, Oceania e América. O cancro cítrico A é causado pela bactéria *X. axonopodis* pv. *citri*, considerada a mais agressiva e a que causa maiores danos aos citros. A bactéria é patogênica para todas as espécies do gênero *Citrus* e também para outras plantas da família Rutaceae (Stall & Seymour, 1983). Os sintomas incluem a formação de lesões erupescentes em folhas, ramos e frutos, causados pela hipertrofia de células do parênquima no centro das lesões. Em seguida, há uma multiplicação intensa da bactéria e exsudação de massa bacteriana (Koizumi, 1976). As lesões causadas pelo cancro cítrico são geralmente circulares, podendo aumentar irregularmente, mas não interferem na qualidade dos frutos que podem ser processados. Entretanto, a comercialização do fruto *in natura* pode ser comprometida e dependendo da severidade da doença, pode ocorrer abscisão de folhas e frutos afetados, levando à menor produtividade.

Para o manejo da doença tem sido proposto a combinação de espécies/cultivares resistentes e utilização de produtos químicos (Civerolo, 1984). A adoção de medidas preventivas como implantação de quebra-ventos, uniformes para colhedores, instalação de arco rodolúvel nas entradas das propriedades, entre outras, tem sido considerada eficiente no controle da doença e tem diminuído a incidência do cancro cítrico. Entretanto, essa doença ainda representa uma ameaça para a citricultura no país.

Expressão diferencial de proteínas

Para a investigação da expressão diferencial, uma das técnicas a ser utilizada é a análise de proteínas expressas em diferentes condições de cultura através da eletroforese bidimensional (2-DE) em gel de poliacrilamida. Esta técnica tem sido considerada a mais eficiente para a análise de proteomas e envolve a separação de proteínas pelo seu ponto isoelétrico (p*I*) na primeira dimensão, e separação de acordo com a massa molecular (M_r) na segunda. Essa combinação é extremamente eficiente, permitindo a separação de milhares de proteínas em cada gel. O interesse no estudo de proteomas tem aumentado recentemente com o aumento de seqüências disponíveis resultantes de análises de seqüenciamento de genomas. No gel, são observados em torno de 1000 a 3000 "spots", cada um representando, em geral, uma única proteína. A análise do genoma através das proteínas leva a obtenção de informações importantes sobre a expressão, regulação gênica e modificações na transcrição ou tradução do gene (Jungblut & Wittmann-Liebold, 1995).

A eletroforese 2-D permite também uma análise subtrativa que consiste na comparação de produtos protéicos produzidos de um grupo celular exposto a diferentes condições biológicas. A comparação entre os dados gerados leva à identificação de proteínas de expressão diferencial que são induzidas ou reprimidas sob diferentes condições. Recentemente, os novos métodos de caracterização de proteínas e o aumento na sensibilidade dos métodos existentes têm aumentado a taxa com que proteínas podem ser caracterizadas nos géis (Wilkins *et al.*, 1996). Anteriormente as proteínas eram caracterizadas predominantemente através do seqüenciamento e "Western immunoblotting", técnicas lentas e trabalhosas. Consequentemente, poucas proteínas podiam ser identificadas a cada ano. Para alcançar os projetos de seqüenciamento de

genomas, estas técnicas teriam que identificar um número muito maior de proteínas. O desenvolvimento de técnicas mais sensíveis e rápidas para o seqüenciamento da região NH_2-terminal assim como de membranas de transferência e imobilização estável do tipo PVDF (polivinildifluoreto) tem permitido o seqüenciamento com quantidades mínimas de proteínas, na faixa de picomoles (Matsudaira, 1987). A espectrometria de massa é outro método eficiente e rápido para a identificação de proteínas, utilizado em combinação com a eletroforese bidimensional. Esta abordagem baseia-se na digestão de proteínas e análise das massas dos peptídeos gerados através de espectrometria de massa. A utilização deste método tem permitido a identificação de várias proteínas expressas em diferentes condições. Em *Helicobacter pylori*, por exemplo, foram identificadas 26 das 33 proteínas secretadas que podem estar envolvidas na interação hospedeiro-patógeno (Bumann *et al.*, 2002).

A análise de proteínas diferencialmente expressas tem sido utilizada em *E. coli*, *Bacillus subtilis* e espécies de *Listeria*, para identificação de proteínas induzidas por respostas adaptativas devido a variações ambientais, principalmente a temperatura e estresse (Yura *et al.*, 1993; Jones & Inouye, 1994; Volker *et al.*, 1994). Os resultados obtidos têm ampliado as perspectivas de estudos gerando as já conhecidas proteínas de choque de temperatura (HSP, "heat shock proteins" e CSP, "cold shock proteins") e as proteínas de estresse (GSP, "general stress proteins"). Em bactérias de importância para a agricultura ou que interagem com plantas, poucos estudos análogos têm sido registrados na literatura. Guerreiro *et al.* (1997) detectaram através de 2-DE cerca de 1700 proteínas constitutivas, que representam cerca de 30% do genoma de *Rhizobium leguminosarum*, e

analisaram 20 dessas proteínas utilizando sequenciamento NH_2-terminal. A indução com flavonóis permitiu a identificação de quatro proteínas diferenciais (Guerreiro *et al.*, 1997).

cDNA RDA

Com os avanços dos projetos genoma, um grande número de seqüências encontra-se disponível para análise. A função dos genes seqüenciados tem sido analisada principalmente através do estudo da expressão diferencial seja em diferentes tecidos ou estágios de desenvolvimento, ou em tecidos sadios ou doentes. Entretanto, até recentemente, as análises limitavam-se ao estudo de um gene de cada vez. A técnica de RDA ("Representational Difference Analysis") de cDNA tem sido considerada promissora para o estudo de um maior número de genes expressos ao mesmo tempo. A técnica de RDA foi desenvolvida inicialmente para a análise entre dois genomas (Lisitsyn *et al.*, 1993) e baseia-se no enriquecimento de fragmentos diferenciais através de PCR, após hibridização subtrativa de duas populações de DNA. Esta técnica foi adaptada para possibilitar o isolamento de genes com expressão alterada em diferentes tratamentos (cDNA RDA) e possui várias vantagens sobre outras abordagens, incluindo o isolamento de poucas bandas falso positivas e a capacidade de detecção de transcritos raros. Assim, cDNA RDA é um método eficiente e sensível, capaz de detectar genes que são expressos em pequena porcentagem na célula. O esquema geral da técnica está apresentado na Fig. 1.

A técnica de cDNA RDA consiste inicialmente na extração do RNA dos tratamentos de interesse e controle. A partir do RNA obtido, é realizada a síntese de cDNA de fita simples utilizando hexanucleotídeos randômicos. Em seguida, a segunda fita de cDNA é sintetizada e os cDNAs são clivados com uma enzima de restrição, que cliva os

transcritos pelo menos uma vez. Oligonucleotídeos adaptadores são então ligados aos fragmentos digeridos que são posteriormente amplificados por PCR. Os produtos de PCR do organismo submetido ao tratamento de interesse são denominados de amplicons do "tester", e os produtos obtidos do organismo no tratamento controle são denominados de amplicons do "driver". Os adaptadores são removidos por clivagem e apenas os amplicons do "tester" são ligados a novos adaptadores na extremidade 5'. Em seguida, é realizada uma hibridização subtrativa com excesso de "driver". É feita amplificação por PCR de maneira que apenas os fragmentos que forem diferenciais serão amplificados, pois apenas estes terão adaptadores nas duas fitas. Este processo é repetido duas ou três vezes (2-3 hibridizações) e os fragmentos obtidos são clonados e seqüenciados.

A técnica de cDNA RDA foi inicialmente desenvolvida para eucariotos e tem sido utilizada na análise de células de vários tipos de tecidos como células pulmonares, tumorais, de fígado e células de linfoma cutâneo (Cooper *et al.*, 2000; Welford *et al.*, 1998; Kim *et al.*, 2000; Hansen-Hagge *et al.*, 2001). Cooper *et al.* 2000 identificaram 16 genes diferenciais ao analisar a expressão de células de pulmão adulto e fetal utilizando cDNA RDA. Similarmente, Welford *et al.* (1998) observaram 173 produtos diferenciais ao combinar cDNA RDA com a técnica de microarray na análise de células tumorais. Modificações da técnica também têm sido feitas para diminuir a quantidade de RNA necessária e para aumentar a eficiência na amplificação dos fragmentos diferenciais (Pastorian *et al.*, 2000). Em bactérias, existem poucos estudos utilizando cDNA RDA para a análise da expressão diferencial. Algumas adaptações têm sido feitas para sua aplicação em procariotos e genes diferencialmente expressos têm sido identificados com sucesso (Bowler, *et al.*, 1999).

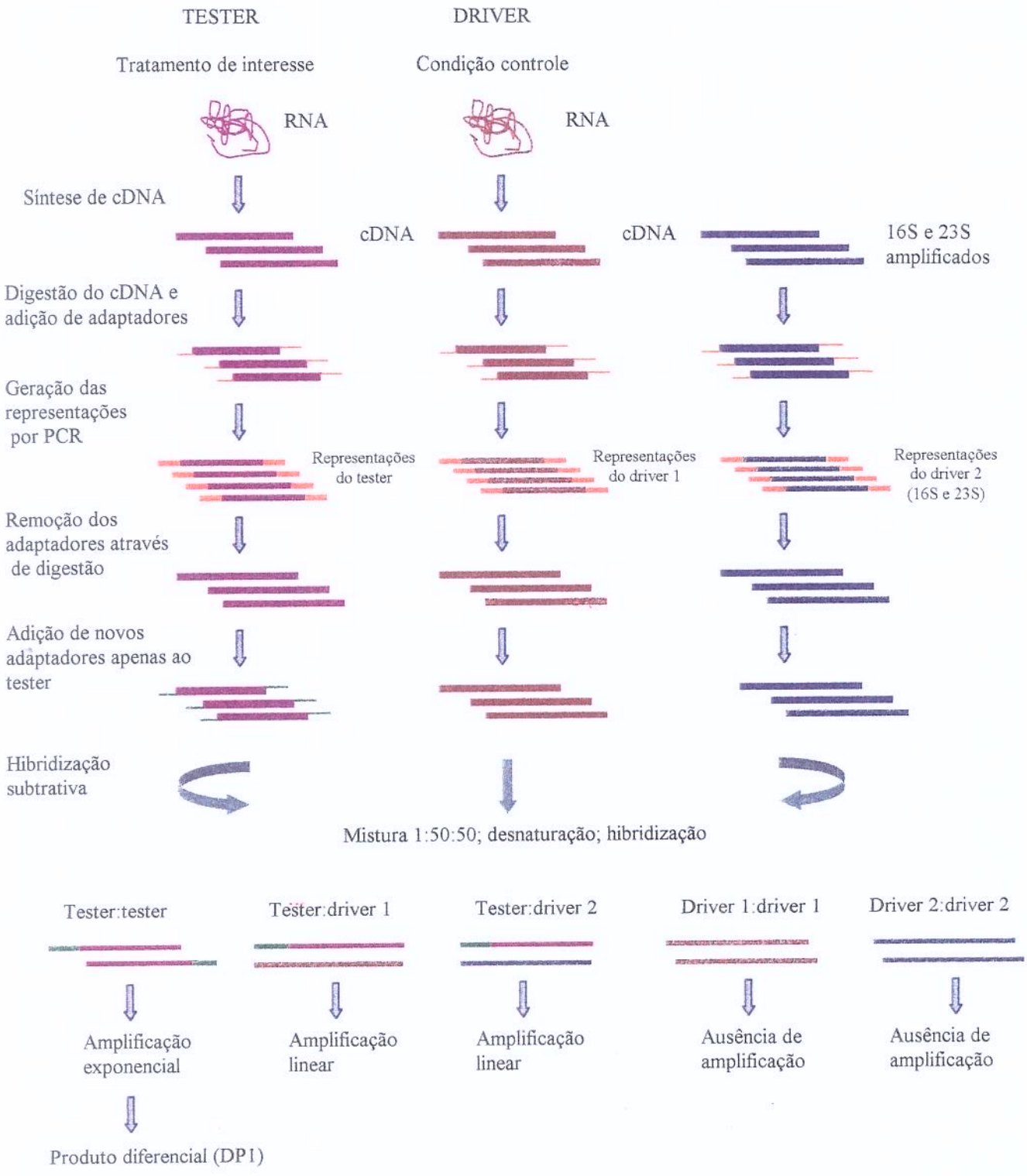

Fig. 1. Esquema geral da técnica de cDNA RDA ("Representational Difference Analysis"), conforme Bowler *et al.* (1999).

ARTIGO I

DIFFERENTIALLY EXPRESSED PROTEINS IN THE INTERACTION OF *Xanthomonas axonopodis* pv. *citri* WITH LEAF EXTRACT OF THE HOST PLANT

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Differentially expressed proteins in the interaction of *Xanthomonas axonopodis* pv. *citri* with leaf extract of the host plant

The present study reports the expression of proteins of *Xanthomonas axonopodis* pv. *citri* in response to different growth conditions. The bacterium was cultured in the basal medium MM1 and in the presence of leaf extracts from a susceptible host plant (sweet orange) as well as a resistant (ponkan) and a nonhost plant (passion fruit). The protein profiles were analyzed by two-dimensional gel electrophoresis (2-DE). Twelve differential spots (induced, up- and down-regulated and repressed) were observed in the protein profiles of the bacterium cultivated in citrus extract (susceptible host) when compared to that of MM1. The 2-DE profile of the bacterium cultured in the complex medium nutrient yeast glycerol was also obtained and the comparison with that of MM1 revealed 36 differential spots. Five proteins from the different treatments were successfully *N*-terminally sequenced and the putative functions were assigned by homology searches in databases. Two constitutively expressed proteins, B4 and B5, were identified as pseudouridine synthase and elongation factor P, respectively. The large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase and a sulfate binding protein were found as specifically up-regulated in the presence of citrus extracts. Finally, the heat shock protein G was found exclusively in the complex medium and repressed in all other media.

Keywords: *Xanthomonas axonopodis* pv. *citri* / Two-dimensional electrophoresis / Proteins / *N*-terminal sequencing

PRO 0084

1 Introduction

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri,* is considered a serious disease which affects the production of citrus in several areas around the world [1, 2]. The pathogen induces the formation of raised lesions on branches, leaves and fruits. Strains of *X. axonopodis* pv. *citri* group A are the most aggressive and infect all orange varieties [2]. Other *Xanthomonas* that cause similar symptoms in citrus are strains of *X. axonopodis* pv. *aurantifolii* groups B, C and D, however, these diseases are not economically important due to the limited number of hosts and lack of severity of the symptoms. *X. campestris* pv. *citrumelo* is the causal agent of "citrus bacterial spot", but was initially associated with a form of citrus canker (group E) due to the similarity of the symptoms [3].

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Abbreviations: MM1, medium containing NaCL, $(NH_4)_2$ SO_4, CaCL, KH_2 PO_4, K_2HPO_4, sucrose; **MMC**, MM1 + leaf extract of sweet orange; **MME**, MM1 + leaf extract of passion fruit; **MMP**, MM1 + leaf extract of ponkan; **NYG**, nutrient yeast glycerol

Intensive studies have been conducted in order to elucidate the mechanisms involved in the plant-pathogen interaction and several genes involved in pathogenicity have been isolated in xanthomonads. The *hrp* (hypersensitive response and pathogenicity) genes, which determine pathogenicity in host plants and hypersensitivity in nonhost plants, are organized in a large cluster comprising six complementation groups [4]. The expression of *hrp* genes is controlled by environmental signals and the identification of such signals has led to the development of media mimicking *in planta* conditions. It has been reported that the *hrp* genes of *X. campestris* pv. *vesicatoria* are suppressed in complex medium and induced in the host or conditioned media [5, 6]. The avirulence (*avr*) genes comprise another group of basic pathogenicity genes which have been cloned and characterized in many pathovars of *X. campestris* including *vesicatoria*, *campestris*, *malvacearum*, *citri*, as well as *X. oryzae* [7]. In addition to these two basic gene categories, the *rpf* (regulation of pathogenicity factors) cluster contains several genes that influence the disease and the severity of the symptoms [8]. These regulatory genes are located outside the *hrp* cluster, and have also been extensively analyzed [9, 10, 11].

Although a large repertoire of genes involved in pathogenicity have been described in xanthomonads, there are no reports of proteins differentially expressed in the

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interaction with the host or in environmentally controlled conditions. One of the approaches for the study of such products involves the analysis of proteins by 2-DE. With this technique, 2-DE maps of the bacterium cultivated in different conditions are elaborated and the use of subtractive analysis can lead to the identification of differentially expressed proteins. Further characterization of these proteins by different strategies including *N*-terminal sequencing could allow the identification of the corresponding function. This strategy has been employed in several bacterial species but rarely in agriculturally important bacteria. One of the few reports in such bacteria described four differential proteins induced by flavonoids in *Rhizobium leguminosarum* [12].

The present study reports the expression of proteins of *X. axonopodis* pv. *citri* in response to different growth conditions, analyzed by 2-DE. The MM1 medium [6], described as a noninducing medium of the *hrp* genes in *X. campestris*, was used as a basal medium. *X. axonopodis* pv. *citri* was grown in MM1 and in the presence of leaf extracts from a susceptible host (sweet orange) as well as a resistant (ponkan) and a nonhost plant (passion fruit). Five proteins were *N*-terminally sequenced and the putative functions were assigned by homology searches in current databases.

2 Materials and methods

2.1 Bacterial strains and culture conditions

X. *axonopodis* pv. *citri* 306, obtained from the culture collection of plant pathogenic bacteria of IAPAR (Instituto Agronômico do Paraná, PR-Brazil), was used in this study. This strain was cultured in nutrient yeast glycerol (NYG) medium [13] with or without agar, at 28°C. For the induction experiments, the bacterium was grown in NYG medium until an A_{600} = 1.2 was reached in order to obtain cellular mass. The cells were centrifuged, resuspended in distilled water and added to the different media. The initial A_{600} in all the media was of approximately 0.3. The modified MM1 medium [6] containing 20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4 and 10 mM sucrose was used as the basal medium.

2.2 Plant leaf extract preparation

Leaves of sweet orange (*Citrus sinensis*), ponkan (*C. reticulata*) and passion fruit (*Passiflorae edulis*) were used in this study. The leaf extract was prepared by triturating 10 g of fresh leaves without the midribs in 100 mL of MM1 medium. The mixture was homogenized (Politron – Superohm, Piracicaba, Brazil) and centrifuged twice at 3840 *g* for 20 min. The supernatant was recovered, filtered in Millipore 0.2 μm and maintained at –20°C. Appropriate volumes of the prepared suspension were then added to the MM1 basal medium, MM1 + leaf extract of sweet orange (MMC), MM1 + leaf extract of ponkan (MMP) and MM1 + leaf extract of passion fruit (MME).

2.3 Protein extraction

The extraction of total proteins was performed as described by de Mot and Vanderleyden [14] with some modifications. Cultures of *X. axonopodis* pv. *citri* were centrifuged and the precipitate washed in phosphate buffer (K_2HPO_4 1.24 g/L; KH_2PO_4 0.39 g/L; NaCl 8.8 g/L; pH 7.2). The pellet was suspended in 0.75 mL of extraction buffer (0.7 M sucrose, 0.5 M TrisHCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM DTT) and incubated for 15 min at room temperature. The same volume of phenol was added and after 15 min of agitation the suspension was centrifuged at 10 000 *g* for 3 min and the supernatant was recovered. This procedure was repeated twice. The proteins were precipitated by the addition of five volumes of 0.1 M ammonium acetate in methanol. The precipitate was washed once with acetone 80%, dried and solubilized in 75 μL of lysis buffer (9.8 M urea, 0,2% v/v NP-40 (Sigma, St. Louis, MO, USA), 100 mM DTT and 2% v/v of a mixture of carrier ampholytes of pH 5–7 and pH 3.5–10 (Bio-Rad, Hercules, CA, USA) in a ratio of 5:1). The samples were maintained at –70°C.

2.4 2-D PAGE

The first dimension electrophoresis was performed according to the method described by de Mot and Vanderleyden [14]. Gel strips of 3.6% acrylamide, 0.21% bisacrylamide, 7.2% ampholytes pH 5–7 and 3.5–10 in the proportion of 5:1 v/v; 2% of NP-40 and 55% of urea were prepared. Protein concentration was determined as described by Bradford [15] and approximately 150 μg were applied to the gel after a prerun. The isoelectric focusing was performed at 400 V for 18 h, using 20 mM NaOH in the upper reservoir and 10 mM H_3PO_4 in the lower. Each gel strip of the first dimension was placed onto the stacking gel of the SDS-PAGE [16]. The electrophoresis was performed at 120 V until the sample reached the resolution gel and then the voltage was increased to 160 V, using the Tris-glycine buffer (25 mM Tris base, 0.192 mM glycine and 0.1% SDS, pH 8.3). A molecular mass marker ladder (10 kDa, Gibco-BRL, Grand Island, NY, USA) was used. The analytical gels (11 × 15 cm) were visualized by staining with silver [17] or Coomassie Brilliant Blue (Sigma). The analysis of the differentially expressed proteins was exhaustive and only the differential spots present in at least two repetitions were considered.

2.5 Electroblotting and *N*-terminal sequencing

The gels transferred to membranes were prepared as described for the analytical gels, but a higher amount of protein (300 μg) was loaded onto the gel of the first dimension. Electroblotting onto PVDF membranes (Bio-Rad) was carried out, as previously described [18], with 10 mM CAPS, pH 11.0, in 10% v/v methanol and 0.01% SDS as transfer buffer. Electrotransfer was performed at 0.8 mA/cm^2 for 1.5 h. Membranes were stained with 0.1% w/v Coomassie Brilliant Blue (Sigma) in 40% methanol for 5 min and destained in 50% methanol. Selected spots were excised from dried membranes and *N*-terminal amino acid sequencing was performed by the NAPS Unit (University of British Columbia, Vancouver, Canada). Amino acid sequence homology searches were performed using the BLAST [19] program.

3 Results and discussion

3.1 Determination of optimal induction time and leaf extract concentration

A preliminary study was performed to determine the conditions in which a higher number of differentially expressed proteins could be obtained. Two parameters were examined: time of treatment and leaf extract concentration in the media. Firstly, the bacterium was grown in MM1 and MMC media using a fixed concentration of leaf extract (0.01 mg/mL) and samples were taken after 6, 12, 24 and 48 h. Proteins were extracted and the 2-DE profiles were compared. Five categories of proteins were observed, considering the protein profile obtained in MM1 as the baseline pattern: (a) induced proteins (synthesis *de novo*), (b); up- and (c) down-regulated proteins, (d) repressed proteins (absent) and (e) proteins which remained unchanged during the different times of treatment. Comparing both treatments (MM1 and MMC) only a small fraction of the total proteins were altered. One protein was induced in MMC after 6 h of incubation whereas after 12 h, differences in the expression of five proteins could be observed (one induced, two up-, one down-regulated and one repressed) when compared to the growth in the MM1 medium. The same differences in the protein profiles obtained after 12 h were observed after 24 and 48 h (data not shown). These results showed that 12 h of treatment was the optimal time to proceed with the detection of differential proteins.

To determine the optimal leaf extract concentration, the bacterium was grown during 12 h in MM1 and MMC using different concentrations of leaf extract (0.01, 0.1, 1.0 and 10 mg/mL). Proteins were isolated from the different treatments and prepared for 2-DE. Only differential spots present in at least two repetitions were considered. The profiles of the extracted proteins from the cultures in MMC in different concentrations were compared to that of the MM1 treatment (Fig. 1A). The analysis of the profiles revealed five differentially expressed proteins at the concentration of 0.01 mg/mL (one induced (B13), two up- (B10 and B11), one down-regulated (M1) and one repressed (M2)), 11 at 0.1 mg/mL and 1 mg/mL (three induced (A2, B6 and B7), seven up- (B2, B3, B8, B10, B11 and B12), one down-regulated (M1) and one repressed (M2)) and 13 at 10 mg/mL (two induced (B6 and B7), six up- (A2, B1, B2, B3, B8 and B11), four down-regulated (B9, B10, B12 and M1) and one repressed (M2)) (Figs. 1B, 1C, 1D and 1E, respectively). The spot M2 was present only in the MM1 medium. The concentration of 10 mg/mL yielded a higher number of differences and therefore this concentration was used in further experiments.

3.2 Identification of differentially expressed proteins

After the determination of the optimal induction time (12 h) and leaf extract concentration (10 mg/mL), at least three repetitions of each treatment (MM1, MMC, MME and MMP) were used to perform the 2-D electrophoresis. The pH of the gel varied from 4 to 7 and the visual analysis of all profiles revealed a total of approximately 350–400 proteins of molecular mass between 10 and 120 kDa (Fig. 1). Some small and faint spots could not be consistently seen in some gels causing the variation in the spot number.

It was observed that most proteins enhanced the level of expression in response to the leaf extract concentration (Fig. 1). Proteins B2, B3, B6 and B7 began to increase their expression at 0.1 mg/mL whereas B1 was intensively induced only at 10 mg/mL. Two other proteins, B11 and B12, reached the highest expression level at the leaf extract concentration of 1 mg/mL and were down-regulated at 10 mg/mL, showing a different response to the increase in leaf extract concentration from that observed for proteins B1–B3 and B6-B7. Proteins B9 and B10 remained unchanged in most treatments, but were also down-regulated in MMC 10 mg/mL. The concentration-dependent response indicates that the proteins were induced by the leaf extract and not by other factors such as growth phase or population-dependent factors since the bacterium grown in the different MMC media showed very similar optical density readings (A_{600} 1.0–1.4 after 12 h).

In order to confirm this assumption, a 2-DE map of the bacterium grown in the complex medium NYG was constructed and a higher number of spots (> 450) was visuali-

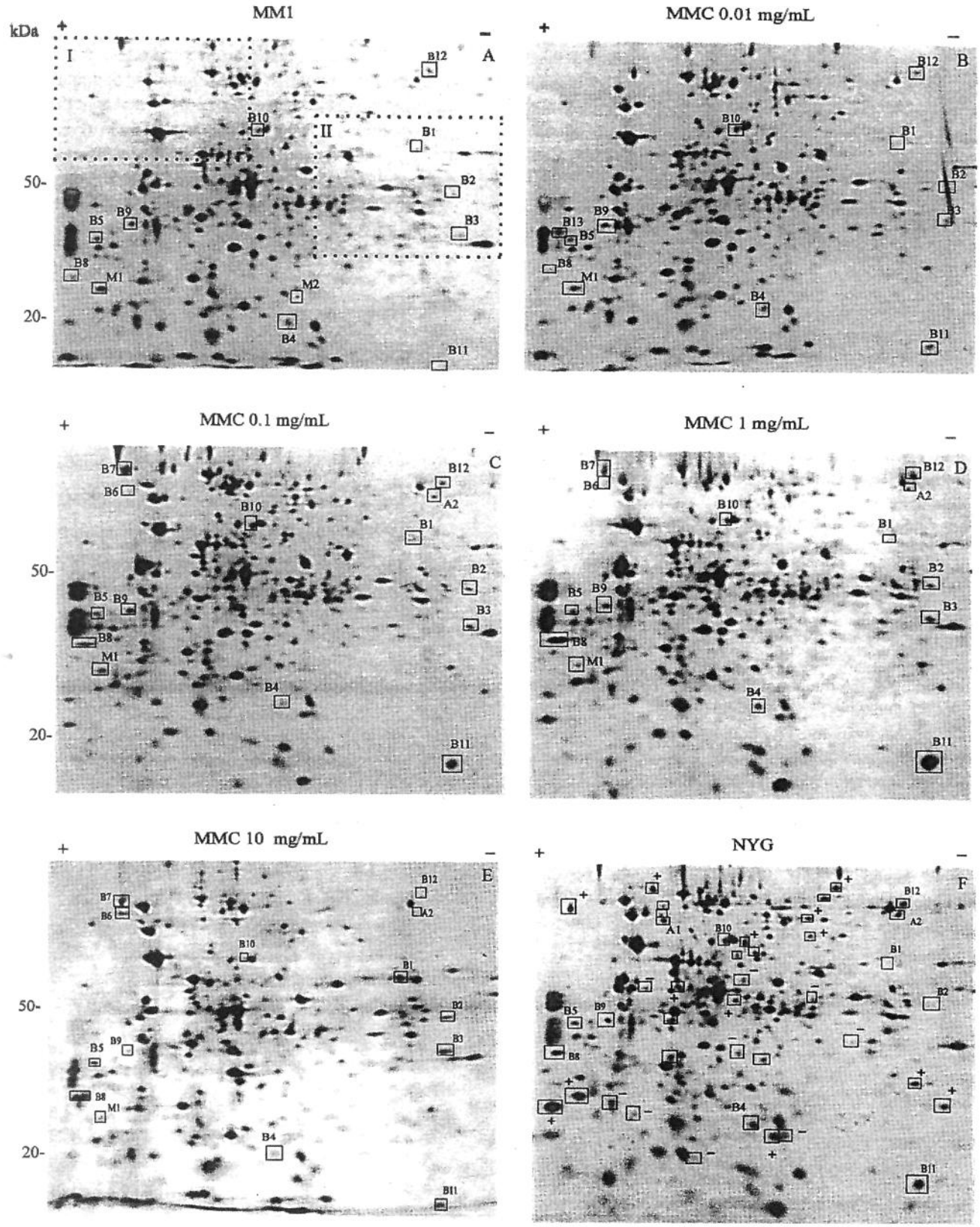

Figure 1. 2-DE protein profiles of *X. axonopodis* pv. *citri* grown in different treatments, as indicated above the gels. The symbols + and – in panel F indicate up-and down-regulated spots, respectively, when compared to MM1 (panel A). Squares with no symbols or letters indicate novel proteins.

zed (Fig. 1F). It seems that the active growth occurring in rich medium demands more proteins (enzymes) compared to the slow growing cells in MM1. The growth rate on both media was contrasted and at the sampling time used (12 h), the A_{600} on NYG medium was 1.4 whereas on MM1 it was 0.5. On the other hand, with the addition of plant leaf extract to MM1, the growth rate was regained as in the NYG medium. The comparison of the protein profile of the bacterium cultured in NYG to that obtained by growth in MM1 revealed 36 differentially expressed proteins, including 13 up-, nine down-regulated, eight induced and one repressed protein. An increased expression of the spots B8, B10 and B11 in relation to MM1 was found in NYG, as reported for the MMC media. However, spot B12 was highly expressed in the complex medium. These results indicate that these proteins may be related to the growth-phase of the bacterium. The differential expression of any other protein observed in the MMC media in relation to MM1 was not detected in the NYG protein profile. The differential spots A1 and A2 were selected for *N*-terminal sequencing. Although spot A2 was present in the MMC media in different concentrations, it was selected for sequencing due to its absence in the MM1 medium and therefore considered a differential spot from the NYG profile.

Five differential spots (B1, B2, B3, B6 and B7) were selected from the MMC 10 mg/mL profile as potential proteins for *N*-terminal sequencing. The differential expression of these proteins was also searched in the profiles of the bacterium grown in the presence of leaf extract of a nonhost (MME) and resistant host (MMP) plants. The MME treatment revealed no major differences in the panel of proteins displayed when compared to MM1 and none of the five spots selected for sequencing presented differential expression in this treatment (Fig. 2). However, in the MMP profile, protein B1 was also up-regulated with the same intensity as in MMC, whereas proteins B2, B3, B6 and B7 were observed at a lower level (Fig. 2). These proteins were repeatedly found and recognized in both citrus treatments. These results indicated that the differential proteins B1, B2, B3, B6 and B7 were specifically induced by citrus extracts (sweet orange or ponkan).

The maximum number of spots visualized in the 2-DE maps was obtained with the NYG treatment and it corresponds to 10% of the entire genome of *Xanthomonas*, considering an estimated size of 5.0×10^6 bp [20]. Attempts to increase the spot number were carried out by loading the gel with higher amounts of protein (up to 500 μg). In fact the heavy loading was disadvantageous since the more intense spots covered the close, smaller and weaker spots and many of them could not be individualized. Certainly the number of expressed proteins could be increased using a wider range of pH gradients. Another limitation in the visualization of a total proteome is the method of sample preparation, which could be inadequate to solubilize all cell proteins. Although the presumed number of spots was low considering other bacterial species analyzed such as the 1700 spots detected for *Sinorhizobium meliloti* [21], differential spots were detected and five were *N*-terminal sequenced.

3.3 *N*-terminal sequencing of differential proteins

Protein spots transferred to PVDF membranes were subjected to *N*-terminal sequencing. A total of nine spots were selected, including two differential spots from the complex medium (A1 and A2), five differential spots from the MMC medium (B1, B2, B3, B6 and B7) and two spots (B4 and B5) expressed constitutively in all treatments. Spots B2, B6, B7 and A2 could not be sequenced due to insufficient amount of protein or because their *N*-terminus was blocked. Nine amino acid residues were determined for A1, B1, B3, B4 and B5. In homology searches in the databases, only proteins showing similarity close to the *N*-terminus were considered (Table 1).

3.4 Constitutively expressed proteins (B4 and B5)

Spot B4 is a basic protein with molecular mass of 25 kDa. This protein showed *N*-terminal similarity to pseudouridine synthase 3 from *Saccharomyces cerevisae* (similarity 100% and identity 66%), an enzyme known to form pseudouridine at positions 38 and 39 in yeast tRNA.

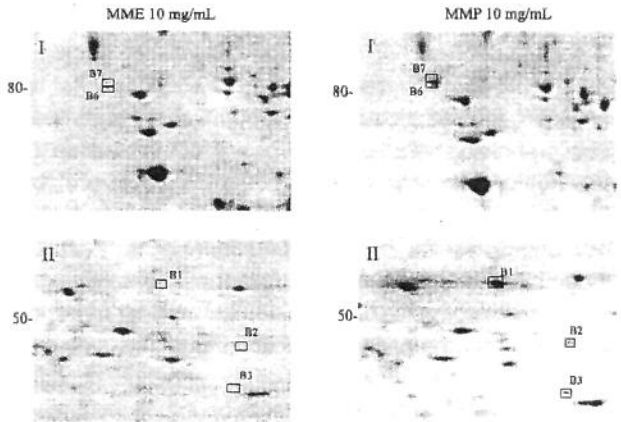

Figure 2. Gel portions I and II of MME and MMP (which correspond to the regions marked by dotted lines in Fig. 1A), as indicated, showing the differentially expressed proteins present in MMP (B1, B2, B3, B6 and B7) and absent in MME.

Table 1. Characteristics of the differentially expressed proteins sequenced and matching to other proteins

Spot	*N*-terminal sequence alignment	Mass kDa	Matching (Accession#)	Similarity/ Identity (%)
B4	E 1 SKIENANKP 43 TELENANKP : : :*******	25	pseudouridine synthase 3 (P31115)	100/66
B5	E 1 MKANDIKKG 2 MKASEMKKG ***:::***	35	elongation factor P (XF2203)	100/66
A1	E 1 TVETDKQTL 2 TLEADKQTH *:*:****	75	heat shock protein G (HtpG) (XF0978)	88/66
B1	E 1 AKVGFXAGV 9 AKVGFKAGV ***** ***	55	1,5-biphosphate carboxylase/oxygenase (Rubisco) (P05699)	88/88
B3	E 1 REIGLLNVS 4 NKIGLLNVH ***** E 1 REIGLLNVS 1 KDIQLLNVS ::* *****	40	mrkE fimbriae gene (P21649) sulfate binding protein (P02906)	66/66 88/66

The numbers before the sequences indicate the position of the amino acid.
E, experimentally determined sequence; X, undetermined amino acid
* identical amino acid; : similar amino acid

Several pseudouridine synthases have been described in *Escherichia coli*, with molecular mass of approximately 30 kDa. They act on specific uridine residues of the RNA molecules and the lack of these enzymes in bacterial cells cause difficulties in translation, slow growth rates, and inability to compete effectively with wild-type strains in mixed culture [22]. Although the function of pseudouridine synthase has been determined, very little is known about the number of these enzymes in a given cell as well as their mechanism and their RNA recognition mode.

Spot B5 represents a basic protein of approximately 35 kDa showing homology (similarity of 100% and identity of 66%) to the elongation factor P (EFP) protein of *Xylella fastidiosa* (molecular mass of 20 kDa). EFP is a protein that stimulates the peptidyltransferase activity of fully assembled 70 S prokaryotic ribosomes and enhances the synthesis of certain dipeptides initiated by *N*-formylmethionine. In the presence of EFP, 70 S ribosomes are significantly more efficient than 50 S particles in catalyzing either peptide-bond synthesis or transesterification [23]. In *E. coli*, EFP has been reported as essential for cell viability and is required for protein synthesis [24]. It is well known that this protein has a vital role for bacterial growth, however, other functions have also been attributed to this protein. Peng *et al.* [25] reported that *E. coli epf* gene complemented detergent sensitivity and virulence in an *Agrobacterium tumefaciens* mutant for the locus *chvH*, which is required for tumor formation.

3.5 Induced protein in the complex medium NYG (A1)

The spot A1 is a basic protein with molecular mass of approximately 75 kDa showing high similarity and identity (88% and 66%, respectively) to the heat shock protein G (HtpG) from *X. fastidiosa*. The HtpG is a molecular chaperon and the apparent molecular mass is approximately 72 kDa in *E.coli,* which is close to the A1 molecular mass. Although the heat shock proteins expression was originally associated to heat shock, it is known that a variety of environmental stresses may induce their expression including nutrient starvation [26], nutrient exhaustion [27], among others. The function of these induced proteins is to protect the cell against the harmful effects of altered environmental conditions. Many of the induced proteins facilitate the adaptation of metabolism to growth under altered conditions or enable the cell to adapt in order to enhance survival mechanisms [27]. In *X. axonopodis* pv. *citri*, spot A1, possibly a heat shock protein, was expressed exclusively in the complex medium. After 12 h of cultivation, the bacterium population was close to the

stationary phase and a nutritionally exhausted environment may have been established, causing the induction of the heat shock protein. Culture conditions affecting the expression of heat shock proteins have already been described by Mason *et al.* [28]. These authors showed that in *E. coli* the heat shock protein HtpG was overexpressed when the bacterium was grown in complex medium, while its expression remained unchanged in the minimal medium.

3.6 Induced proteins in the presence of citrus extracts (B1 and B3)

Unexpectedly, no major differential spots were observed comparing the treatments using leaf extracts from sensitive and tolerant citrus hosts (MMC and MMP). The similarity in the protein expression in both cases indicates that the composition of the extracts could also be very similar although some studies showed qualitative differences in amino acid, phenol and sugar contents [29, 30]. It is plausible to assume that the different composition in the leaf extract would affect the protein expression, however the correspondent alterations might be processed at a low level and thus undetectable by the 2-DE method.

Spot B1 is an acid protein of 55 kDa, present in the MMC and MMP treatments. Surprisingly, the search of this sequence in the database revealed a high homology score (similarity and identity of 88%) to the large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase (RubisCO) of several plants. RubisCO is the key enzyme of the Calvin cycle and catalyzes two competing reactions: the carboxylase and the oxygenase reactions. This enzyme can be found in photosynthetic organisms such as higher plants, algae and cyanobacteria, and in non-photosynthetic chemoautotrophic bacteria, such as *Thiobacillus denitrificans* [31]. RubisCO has also been found in nonautotrophic strains of *Beggiatoa* [32]. The enzyme showed a significant increase following exhaustion of the carbon source used (acetate). Exhaustion of organic compounds is thus postulated to derepress the genes coding for RubisCO. The finding of a RubisCO-like protein in xanthomonads is quite striking since the bacterium is an obligate aerobic and nonautotrophic. The search for the corresponding gene should be performed to confirm if it was a protein from the plant extract or if it was maintained as a vestige of ancient origin as has been suggested for *Beggiatoa* strains by Nelson *et al.* [32]. What could be the physiological role of RubisCO in nonautotrophic bacterium remains obscure. Nakamura [33] observed a high activity of RubisCO in *X. autotrophicus* grown under 40% of oxygen and the intracellular oxygen tension was partially lowered by this enzyme indicating that it may be involved in oxygen-resistance. Reactive oxygen species, including superoxides and H_2O_2, are important components of plant defense response against microbial infection and are consequence of normal aerobic metabolism. The ability of bacterial cells to detoxify and repair damage caused by reactive oxygen species plays an important role in microbial infection. Genes involved in resistance to oxygen species have been reported in *X. campestris* [34] and RubisCO may be an additional enzyme important for controlling the oxygen reactive components within the cell.

Spot B3 is an acid protein with molecular mass of approximately 40 kDa and the *N*-terminal sequence showed homology with a sulfate binding protein (SBP) of *Salmonella typhimurium* (similarity 88% and identity 66%). This protein of 35 kDa was the first periplasmic binding protein identified [35] and it is involved in sulfate transport and sulfate-binding activity. Repression of the enzyme takes place on medium containing cysteine. The SBP presents an overall structure similarity to other periplasmic binding proteins specific for L-arabinose, D-galactose/D-glucose, leucine/isoleucine/valine and leucine in spite of the low sequence homology [36]. In *E. coli* the gene encoding SBP is part of the cysteine regulon [37]. The induction of SBP in xanthomonas is likely caused by either the amino acids [29] or different sugars [38] present in citrus leaf extracts. Further studies are necessary to determine which leaf extract compound is responsible for the specific induction of SBP in the bacterium.

4 Concluding remarks

In conclusion, we report herein the use of 2-DE maps of *X. axonopodis* pv. *citri* cultured in different media to identify differentially expressed proteins. Some proteins presented a higher expression which was dependent on the leaf extract concentration used, whereas others were down-regulated. The plant leaf extract from susceptible and resistant plants showed few differences, indicating that the specific mechanisms of host recognition may be induced at a low level by the bacterium and undetectable by the method employed. All the proteins microsequenced have not been described before in xanthomonads and their association to the corresponding media or to pathogenicity are still speculative and demand further studies. The homology search in the databases did not give direct and conclusive answers since few xanthomonads genes have been described and deposited. Thus, a sound and consistent functional assignment for each protein was found to be a major problem. The sequencing of the *X. axonopodis* pv. *citri* genome is underway, and we expect to find the protein sequences determined herein,

although a few alterations in some amino acid residues is possible due to the presence of more than one protein in the gel spots. Upon completion of the genome sequencing, the search for the corresponding genes will be easily carried out although other studies will be necessary to ascertain the role played by these genes in the plant-pathogen interaction and to clarify the underlying genetic pathways.

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ARTIGO II

A SIMPLE METHOD FOR *IN VIVO* EXPRESSION STUDIES OF *Xanthomonas axonopodis* pv. *citri*

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Submetido para "Microbiology"

A simple method for *in vivo* expression studies of *Xanthomonas axonopodis* pv. *citri*

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Summary

A major problem in studying bacterial plant pathogens is to obtain the microorganism directly from the plant tissue to perform *in vivo* expression (protein or mRNA) analyses. Here we report an easy and fast protocol to isolate *Xanthomonas axonopodis* pv. *citri* directly from the host plant, in sufficient amounts to perform protein fingerprinting by 2-D gel electrophoresis as well as RNA expression assays. The protein profile obtained was very similar to that of *X. axonopodis* pv. *citri* grown in the presence of leaf extract of *Citrus sinensis*, however some differential proteins expressed *in vivo* were observed. Total RNA extraction revealed typical 16S and 23S bands in the agarose gel, and RT-PCR reactions using primers specific for genes of the bacterium confirmed the quality of the RNA preparation. Also RT-PCR reactions using plant ribosomal primers were employed and no amplification product was obtained, indicating that plant RNA is not present in the bacterium RNA sample.

INTRODUCTION

Xanthomonas axonopodis pv. *citri* is the causal agent of citrus canker, a serious disease that affects many citrus growing areas around the world. Several studies have been performed with this bacterium, most of them related to genetic diversity (Egel *et al.*, 1991; Leite *et al.*, 1994), search for specific primers (Hartung *et al.*, 1993) and pathogenicity (Swarup *et al.*, 1992; Duan *et al.*, 1999). Recently the genome sequence of this bacterium was published (da Silva *et al.*, 2002) and substantial efforts will be required to decipher the function of the genes found. Among the most interesting genes are those related to pathogenicity. One of the major problems to analyse such genes is the choice of the culture media to be used. Schulte & Bonas (1992) have developed a medium that induces the *hrp* genes, however the expression of other genes, which could be related or not to pathogenicity remains undetermined. The absence of an efficient method to obtain bacterial cells from plant tissue has limited *in vivo* expression analyses of most plant interacting bacteria. An exception is found with the symbiotic bacterium since bacteroids can be isolated directly from nodules to perform RNA isolation (Cabanes *et al.*, 2000). Generally, the amount of bacteria isolated from the plant is insufficient for expression assays, especially for protein studies. Moreover, when bacterial proteins are extracted from macerated infected tissue, one of the major drawbacks is the amount of contaminant plant components (protein, DNA or RNA) present in the samples, demanding further steps of purification.

In the present work we describe a simple method for the recovery of *X. axonopodis* pv. *citri* cells directly from infiltrated leaf tissue of the host plant *Citrus sinensis* which can

be used for extraction of proteins and RNA. Half of the amount of protein obtained from 40 leaves infiltrated with the bacterium was sufficient to perform a 2-D gel electrophoresis. Total RNA was also extracted from 20 infiltrated leaves and typical bands of 16S and 23S rRNA were obtained. RT-PCR was performed using primers specific to some genes (*mobL*, involved in plasmid mobilization, *pthA* and *clp*, both associated with pathogenicity) of the bacterium, as well as primers for plant ribosomal genes to investigate if plant RNA was present.

METHODS

Bacterial strain, culture conditions and plant inoculations

X. axonopodis pv. *citri* 306, obtained from the culture collection of plant pathogenic bacteria of IAPAR (Instituto Agronômico do Paraná, PR-Brazil), was used in this study. This strain was cultured in nutrient yeast glycerol (NYG) medium (Daniels *et al.*, 1984) with or without agar addition, at 28 °C. The host plant (*Citrus sinensis*) was grown in greenhouse during approximately 3 months. Young leaves, measuring approximately 7 cm along the main midrib, were used. The inoculum was prepared growing the bacterium overnight in NYG in order to obtain cellular mass. The cells were centrifuged, washed, resuspended in distilled water and adjusted to A_{600}=0.6. The suspension was then used to infiltrate 40 young leaves by using a syringe.

Recovery of the bacterium from inoculated leaves

Six days after the inoculation, when only light or no symptoms were visible, leaves were collected, decontaminated with alcohol and the midribs were removed. The leaves were cut into pieces using a sterile razor blade and maintained for 1 hour in sterile glass plates containing 20 ml of distilled water. The incubation during this period of time allowed the bacteria to exudate from the leaf tissue into the water. The leaves were separated from the suspension by pipeting the water, which was centrifuged to pellet the bacterial cells. The bacterium was washed with water and used for protein extraction or RNA isolation.

Protein extraction and 2-D electrophoresis

Total proteins were extracted from bacterial cells recovered from the host plant leaves and grown in the modified MM1 medium (Schulte & Bonas, 1992) containing 10 mg/ml leaf extract of *Citrus sinensis* (Mehta & Rosato, 2001). Briefly, the proteins were extracted using an extraction buffer (0.7 M sucrose, 0.5 M TrisHCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM DTT) and the same volume of phenol. The proteins were washed once with acetone 80%, dried and solubilized in 75 µl of lysis buffer (9.8 M urea, 0.2% (v/v) Nonidet P-40 (Sigma), 100 mM DTT and 2% (v/v) of a mixture of ampholytes of pH 5-7 and pH 3.5-10 (BioRad) in a ratio of 5:1). The samples were maintained at -70 °C. The first dimension electrophoresis was performed according to the method described by de Mot & Vanderleyden (1989). Gel strips of 3.6% acrylamide, 0.21% bis-acrylamide, 7.2% ampholytes pH 5-7 and 3.5-10 in the proportion of 5:1 (v/v); 2% of Nonidet P-40 and 55% of urea were prepared. Each gel strip of the first dimension was placed onto the

stacking gel of the SDS-PAGE (Laemmli, 1970). The gels (11 x 15 cm) were visualized by staining with silver (Blum *et al.*, 1987).

RNA preparation, cDNA synthesis and RT-PCR with specific primers

RNA of *X. axonopodis* pv. *citri* cells grown in the minimal medium MM1 and recovered from the host plant leaves was extracted using 750 µl extraction buffer (1 mM EDTA; 0,1 M Tris HCl; 0,1 M LiCl). The same volume of phenol/chloroform/isoamyl alcohol (25:24:1) containing SDS 1% was added and the suspension centrifuged for 3 min. The supernatant was recovered and the phenol extraction was repeated twice. RNA was precipitated by the addition of 1/20 volume of sodium acetate 40% (w/v) and 2 volumes of ethanol, resuspended in 80µl RNAse free H_2O and treated with DNAse (Gibco). Plant RNA was extracted using Trizol (Gibco) according to manufacturer's instructions. The amount and quality of RNA were verified by agarose gel electrophoresis using a denaturing agarose gel 1%, containing formaldheyde 6% and MOPS buffer (Sigma) 1X. cDNA was synthesized as described by Ausubel (1994) and RT-PCR was performed as described by Shepard & Gilmore (1999), in a final volume of 25 µl containing 2.5 µl of cDNA, 1.5 mM $MgCl_2$, 125 µM dNTP, 25 pmol of primers and 5 U de *Taq* DNA polymerase (Amersham). After 5 min of denaturation at 95°C, the amplification was followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 74°C and the bands were visualised in agarose gels 1% stained with ethidium bromide. Primers for the genes *mob*L mobD 5'-TGATCACTGATGCAATGG-3' and mobR 5'-GCGTTGTCTCACAACAAATG-3', *pthA* PTHD 5'-TGCCTGATGGATCCCATTCG-3' and PTHR 5'-

TGGCTGAGCGCAACGATGTG-3' and *clp* 0483D 5'-ATGAGCCCAGGAAATACG-3' and 0483R 5'-TACAGCACGACGGTCTTG-3' were used in the RT-PCR reactions. The expected sizes of the amplification products were 420, 600 and 680 bp, respectively. The plant ribosomal primers for the ITS (Internal Transcribed Spacer) region 92 5'-AAGGTTTCCGTAGTTGAAC-3' and 75 5'-TATGCTTAAACTCAGCGGG-3' rDNA (Desfeux & Lejeune, 1996) were also used with an annealing temperature of 55°C. These primers were designed from conserved regions of the 18S and 28S rDNA.

RESULTS AND DISCUSSION

Forty inoculated leaves were used to obtain bacterial cells for protein extraction. The total amount of protein was resuspended in 20 µl of lysis buffer and half of this amount was loaded onto the gel. The protein pattern revealed by the 2-D electrophoresis (Fig. 1a) was very similar to that obtained from the bacterium cultured in the modified minimal medium MM1 containing leaf extract of the host plant *Citrus sinensis* (Fig 1b), previously used for the protein expression analysis of *X. axonopodis* pv. *citri* (Mehta & Rosato, 2001). Several proteins used as reference of the general pattern of *X. axonopodis* pv. *citri* could be identified in the gel and are indicated with arrows in Fig. 1. It is possible that some plant or other bacterial (saprophytic or endophytic) proteins are present in the gel, however they should be in very low amounts, probably undetectable by 2-D electrophoresis. Five novel differential proteins were observed in the panel obtained from *in vivo* samples when

compared to that obtained from the bacterium grown in the presence of leaf extract (Fig 1). At this time, the origin of these proteins, if from plant or other bacteria, was not confirmed, however the differential proteins identified were absent in a 2-DE protein panel of *Citrus sinensis* leaf extract (data not shown). Moreover, macerated leaf tissue of *Citrus sinensis* infiltrated with bacterial suspension was plated onto NYG medium and only typical *X. axonopodis* pv. *citri* colonies were observed. Therefore it is likely that the new differential proteins seen in the gel belong to *X. axonopodis* pv. *citri* and are regulated *in vivo* by the host plant and therefore not previously described.

RNA extraction was carried out using 20 inoculated leaves. The presence of two sharp and bright bands representing the 16S and 23S rRNAs indicated the quality of the RNA preparation (Fig. 2) and a total of 150 µg of RNA was obtained. Contamination by RNA from other bacteria is not easy to detect since the rRNA sizes and sequences are similar within the prokaryotes. To further confirm the quality of the RNA obtained, RT-PCR reactions were performed using specific primers for the genes *pthA*, required for the development of citrus canker disease (Swarup *et al.*, 1991; 1992), *clp*, a gene that encodes the CAP-like protein, which regulates directly or indirectly genes implicated in pathogenicity (de Crecy-Lagard *et al.*, 1990), as well as the *mobL* gene, associated with plasmid mobilization and considered exclusive to *X. axonopodis* pv. *citri* in the genome comparison with *Xanthomonas campestris* pv. *campestris* (da Silva *et al.*, 2002). In all reactions the expected amplification products were obtained (Fig. 3). Contamination of the bacterium RNA preparation with plant RNA was also tested employing plant ribosomal

primers. No amplification product was observed when cDNA from *X. axonopodis* pv. *citri* cultured *in vivo* and in the minimal medium MM1 was used (Fig. 4).

In this report we show that 40 infiltrated leaves provide sufficient bacterial cells for the analysis of the protein profile *in vivo*. The bacterial cells obtained from the leaf tissues can also be used for RNA extraction, which allows the analysis of the expression of several genes. Overall, the results obtained in this study show that by using a simple, fast and inexpensive technique it is possible to obtain sufficient amounts of cells for *in vivo* expression studies. This method may also be applicable to other plant pathogenic bacteria, which colonize plant leaves.

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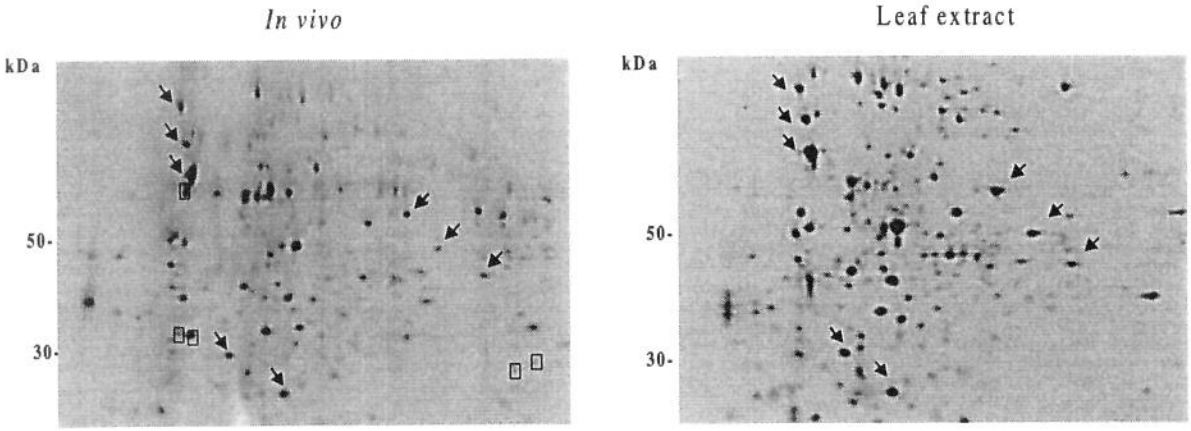

Fig.1. 2-DE protein profile of *X. axonopodis* pv. *citri* grown in the minimal medium MM1 containing leaf extract of *Citrus sinensis* (A) and recovered from leaves of the host plant *Citrus sinensis* (B). Arrows show reference proteins of the general pattern of *X. axonopodis* pv. *citri* and squares indicate differentially expressed proteins when compared to the 2-DE profile of the bacterium grown in the presence of leaf extract.

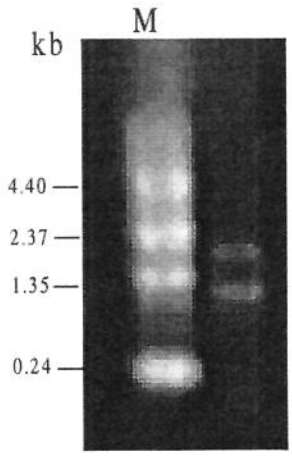

Fig. 2. 1, RNA of *X. axonopodis* pv. *citri* recovered from leaves of the host plant *Citrus sinensis*. M, molecular size marker Ladder 1 kb (Gibco).

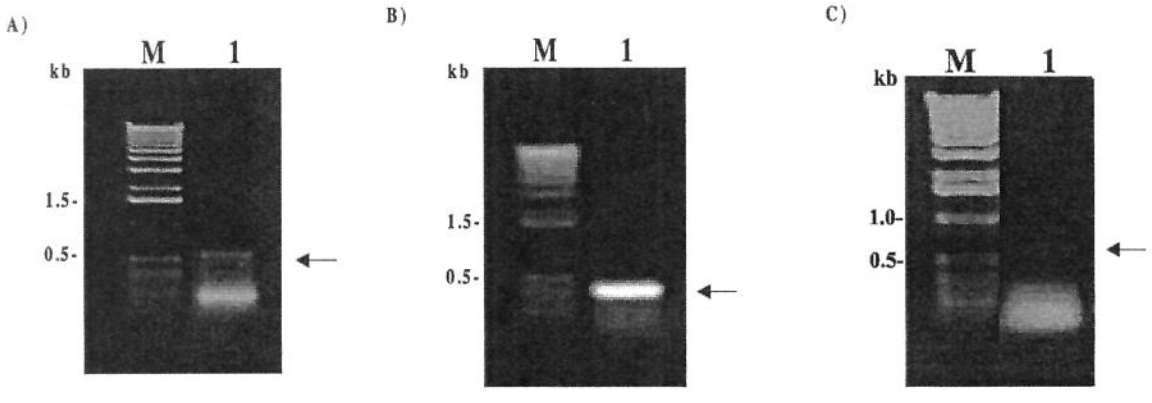

Fig. 3. RT-PCR using specific primers for the *pthA* (A), *mobL* (B) and *clp* (C) genes of *X. axonopodis* pv.*citri*. M, molecular size marker Ladder 1kb (Gibco). Arrows indicate the expected amplification products of 600 bp (A), 420 bp (B) and 680 bp (C).

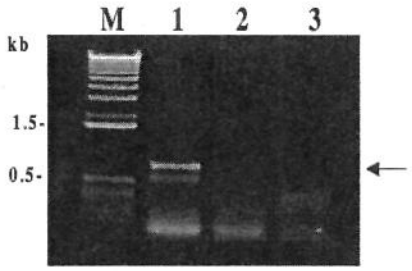

Fig. 4. RT-PCR using cDNA from *Citrus sinensis* (1) and *X. axonopodis* pv *citri* grown *in vivo* (2) and in the minimal medium MM1 (3) with primers corresponding to plant ITS (internal transcribed spacer) region. M, molecular size marker Ladder 1 kb (Gibco). Arrow indicates the expected amplification product of approximately 700 bp observed only in lane 1.

ARTIGO III

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES OF *Xanthomonas axonopodis* pv. *citri* BY REPRESENTATIONAL DIFFERENCE ANALYSIS OF cDNA

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Submetido para "Genome Research"

Identification of differentially expressed genes of *Xanthomonas axonopodis* pv. *citri* by representational difference analysis of cDNA

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ABSTRACT

Xanthomonas axonopodis pv. *citri* is a phytopathogenic bacterium responsible for citrus canker, a serious disease which causes severe losses in citriculture around the world. In this study we report the differential expression of *X. axonopodis* pv. *citri* in response to specific treatments by using cDNA RDA (Representational Difference Analysis). cDNAs from *X. axonopodis* pv. *citri* cultured in the presence of leaf extract of the host plant (*Citrus sinensis*), *in vivo*, as well as in the complex medium were hybridized against cDNA of the bacterium grown in the minimal medium. Sequencing of the difference products obtained after the second and third hybridizations revealed a total of 37 distinct genes identified by homology searches in the genome of *X. axonopodis* pv. *citri*. These genes were distributed in different functional categories, including genes that encode hypothetical proteins (12), genes involved in metabolism (13), cellular processes (6) and pathogenicity (4), and mobile genetic elements (2). Most of these genes are likely related to growth and/or acquisition of nutrients in specific treatments whereas others might be important for the bacterium pathogenicity.

INTRODUCTION

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri,* is a serious disease which causes severe losses in the production of citrus in several areas around the world (Rossetti, 1977; Stall and Seymour 1983). The symptoms include the formation of raised lesions on branches, leaves and fruits.

Several genes involved in pathogenicity have been isolated in xanthomonads, such as the *hrp* (hypersensitive response and pathogenicity), *avr* (avirulence) and *rpf* (regulation of pathogenicity factors) that influence the disease and the severity of the symptoms (Dangl, 1994). Although the major genes involved in the pathogenicity of *X. axonopodis* pv. *citri* are already known, there are very few reports on the general differential expression of this bacterium in the interaction with the host or in environmentally controlled conditions. Most studies have been limited to the analysis of the expression of specific genes (Swarup *et al.*, 1992; Duan *et al.*, 1999).

The cDNA RDA ("Representational Difference Analysis") technique has been considered efficient for differential expression studies. This method is based on the subtractive hybridization of two cDNA populations followed by enrichment of the differential products by PCR amplification (Bowler *et al.*, 1999). cDNA RDA was initially developed for eucaryotic cells and has successfully identified several differentially expressed genes in different tissues (Cooper *et al.*, 2000; Kim *et al.*, 2001). In bacteria, cDNA RDA has been rarely used and only few reports are available (Bowler *et al.*, 1999; Becker *et al.*, 2001).

In the present study we have identified differentially expressed genes of *X. axonopodis* pv. *citri* in response to different growth conditions by cDNA RDA. The

modified MM1 medium (Schulte and Bonas, 1992), described in the induction of the *hrp* genes in *X. campestris*, was used as a basal medium. cDNA from the bacterium grown in the presence of leaf extract from a susceptible host (sweet orange) as well as in the host plant leaves and complex medium was used in successive rounds of subtractive hybridizations against cDNA from the MM1 treatment.

METHODS

Bacterial strains and culture conditions

X. axonopodis pv. *citri* 306, obtained from the culture collection of plant pathogenic bacteria of IAPAR (Instituto Agronômico do Paraná, PR-Brazil), was used in this study. This strain was cultured in nutrient yeast glycerol (NYG) medium (Daniels *et al.*, 1984) with or without agar addition, at 28°C. The modified MM1 medium (Schulte and Bonas, 1992) containing 20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4 and 10 mM sucrose was used as the basal medium. For the induction experiments, the bacterium was grown in NYG medium until an A_{600} = 1.2 was reached in order to obtain cellular mass. The cells were centrifuged, resuspended in distilled water and added to the different media (MM1, MM1 + leaf extract and NYG). The initial A_{600} in all the media was of approximately 0.3.

Plant leaf extract preparation

The leaf extract was prepared by triturating 10 g of fresh leaves of sweet orange (*Citrus sinensis*) without the midribs in 100 ml of MM1 medium. The mixture was

homogenized (Politron - Superohm) and centrifuged twice at 3800 g for 20 min. The supernatant was recovered, filtered in Millipore 0.2 µm and maintained at -20°C. A final concentration of 10 mg/ml of leaf extract was used.

Inoculations of *Citrus sinensis* leaves

For the *in vivo* experiments, the bacterium was grown overnight in NYG medium, centrifuged, washed and resuspended in distilled water and adjusted to an $A_{600} = 0.6$. *Citrus sinensis* leaves were infiltrated using a seringe and bacterial cells were recovered after 6 days according to Mehta and Rosato (submitted) and used for RNA extraction.

RNA preparation and cDNA synthesis

The bacterium was grown for 12 h in the minimal medium MM1, MM1 containing leaf extract of the host plant (10 mg/ml) and in the complex medium NYG. The induction time and leaf extract concentration of the culture media were previously determined by Mehta and Rosato (2001) in the analysis of the differential expression of proteins of *X. axonopodis* pv *citri*. The bacterium was also recovered from host plant leaves 6 days after inoculation. The RNA from each treatment was extracted using 750 µl extraction buffer (1 mM EDTA; 0,1 M Tris HCl; 0,1 M LiCl). The same volume of phenol/chloroform/isoamyl alcohol (25:24:1) containing SDS 1% was added and the suspension centrifuged for 3 min. The supernatant was recovered and the phenol extraction was repeated twice. RNA was precipitated by the addition of 1/20 volume of sodium acetate 40% (w/v) and 2 volumes of ethanol, resuspended in 80µl RNAse free H_2O and treated with DNAse (Gibco). The

quality and amount of RNA were verified by agarose gel electrophoresis using a denaturing agarose gel 1%, containing formaldehyde 6% and MOPS buffer 1X. cDNA synthesis was performed using the Time Saver cDNA synthesis kit (Amersham) with some modifications as reported by Bowler *et al.* (1999).

Generation of subtractive libraries by cDNA RDA

cDNA RDA was performed essentially as described by Bowler *et al.* (1999), with some modifications. cDNA of the bacterium grown in the presence of leaf extract, *in vivo* and in the complex medium was used in independent experiments as tester. In all subtractions, cDNA of the bacterium grown in the minimal medium was used as driver1. Since rRNA is present in high amounts in bacterial RNA preparations, the 16S and 23S genes were also included as driver2. Each cDNA population was digested with *Dpn*II (New England) and adaptors were ligated. After ligation, tester and driver populations were amplified using 30 cycles of 1' at 95°C and 3' at 72°C for the generation of the representations. All representations were digested with *Dpn*II and new adaptors were ligated only to the tester fragments. Subsequently, a subtractive hybridization was performed with a tester:driver ratio of 1:100, where the ribosomal genes represented half of the amount of driver used (1:50:50 - tester:driver1:driver2). PCR reactions using diluted hybridized DNA were performed to amplify the first difference products (DP1). These products were digested with *Dpn*II, ligated to new adaptors and then used in a second subtractive hybridization using a tester:driver1:driver2 ratio of 1:400:400. The third round of subtractive hybridization was carried out following the same procedure described above using tester:driver1:driver2 ratios of 1:2500:2500 or 1:2500:5000.

DNA sequencing and analysis

The difference products obtained after the second and third subtractive hybridizations were cloned into pGEM T-easy vector (Promega) and the plasmids were purified using the Concert Rapid Plasmid Miniprep System (Gibco). At least 40 fragments from each treatment were sequenced. The sequencing reactions were performed in a total volume of 10 µl containing 800 ng of DNA, 5 pmoles of primer (M13 forward or M13 reverse), 3 µl of ABI PRISM big dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The reactions were conducted with an initial denaturation of 2 min at 95° C, followed by 25 cycles of 12 s at 95° C, 6 s at 50° C and 4 min at 60° C. The sequencing was performed in an automatic sequencer (ABI PRISM™ 377, Perkin Elmer) and the homology of the sequences was searched in the genome of *X. axonopodis* pv. *citri* deposited under the accession number AE008923, using the BLAST (Altschul *et al.*, 1990) program.

RT-PCR using specific primers

RT-PCR was performed as previously described (Shepard and Gilmore, 1999). cDNA was synthesized with random primers using the Time Saver cDNA synthesis kit (Amersham) and PCR reactions were performed in a final volume of 25 µl containing 2.0 µl of cDNA, 1.5 mM $MgCl_2$, 125 µM dNTP, 25 pmol of primer and 5 U *Taq* DNA polymerase (Pharmacia). After 5 min denaturation at 95°C, the amplification was followed by 25 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 74°C. The amplification products

were visualized in 1% agarose gels stained with ethidium bromide. The primers used were designed from the complete sequence of the genes obtained from the genome database of *X. axonopodis* pv. *citri*).

RESULTS AND DISCUSSION

cDNA RDA

The cDNA RDA technique was employed for *X. axonopodis* pv. *citri* in an attempt to discriminate genes under regulation of different conditions. The cDNAs of the bacterium recovered directly from infiltrated leaves, grown in medium containing leaf extract or in complex medium were used in 3 rounds of subtractive hybridization against cDNAs of the bacterium cultured in the minimal medium. The cDNA population of each treatment was digested with *Dpn*II, as described for *Neisseria meningitides* (Bowler *et al.*, 1999), however, most representation fragments obtained for *X. axonopodis* pv. *citri* showed smaller sizes (300-800 bp). The difference products obtained were also small and ranged between 100-300 bp (Fig. 1), however the actual sizes of the sequences representing the difference products ranged from 70-150 bp due to the presence of concatenated adaptors linked together. The identification of the differentially expressed genes, although generally based on short sequences, was performed successfully since the genome sequence of *X. axonopodis* pv. *citri* was already available (da Silva *et al.*, 2002). Another problem encountered was the high amount of ribosomal genes identified in the sequencing step (approximately 30%). In order to reduce the number of clones representing ribosomal

genes, the third subtraction in the NYG and *in vivo* conditions was performed using a higher proportion of 16S e 23S representations. A tester:driver1:driver2 proportion of 1:2500:5000 was used and a reduction in the number of 16S and 23S sequences identified was observed (approximately 15%).

According to Bowler *et al.* (1999), the second difference products represent genes, which are more abundantly expressed in a specific treatment, whereas the third difference products reveal genuine differences. Although a third round of subtractive hybridization may reduce the diversity of genes, in the present study, a higher number of fragments obtained after the third hybridization was sequenced in an attempt to identify genes exclusively expressed in the treatments analyzed.

After the subtractive hybridizations of the three treatments (MM1+leaf extract, *in vivo* and NYG) with MM1, the difference products were visualized in agarose gels in a high number as a smear (Fig 1). The difference products obtained after the second and third hybridizations were cloned and sequenced and a total of 37 genes were identified. Overall, the genes revealed belonged to five major functional categories: hypothetical (12), metabolism (13), cellular processes (6), mobile genetic elements (2) and pathogenicity (4). Out of the 11 genes identified in the leaf extract treatment, 3 encoded hypothetical proteins, 7 represented genes related to metabolism and 1 was associated to pathogenicity (Table 1). In the *in vivo* treatment, a total of 18 genes were identified, including 5 genes encoding hypothetical proteins, 7 genes associated with metabolism, 4 related to cellular processes, 1 mobile genetic element and 1 gene involved in pathogenicity (Table 1). In the NYG medium, out of the 14 genes identified, 6 represented genes encoding hypothetical proteins,

5 genes related to metabolism, 1 to cellular processes, 1 mobile genetic element and 1 gene associated to pathogenicity (Table 2).

RT-PCR using specific primers

In order to confirm the differential expression of the genes identified by cDNA RDA, specific primers for 17 genes revealed in this study were synthesized and used in RT-PCR reactions (Table 1 and Table 2). Also primers for the gene *efp* encoding the elongation factor P protein (XAC1849), which was constitutively expressed in the treatments MM1, MM1+leaf extract and NYG (Mehta and Rosato, 2001) was synthesized and used as a control. The RT-PCR reactions performed herein were not quantitative and therefore only presence or absence of bands was considered.

The differential expression was confirmed for most genes tested (Fig. 2). Only a gene encoding the hypothetical protein (XAC1749) expressed *in vivo* showed an unexpected result with an amplification product from the MM1 treatment. These results reveal a false positive proportion of approximately 6%. The constitutive gene used as a control was expressed in all media.

To investigate whether the genes identified *in vivo* were also expressed in the presence of leaf extract, primers for 5 genes (*flgE*, *yfcB*, *intS*, *nodI* and *pstK*) expressed in the *in vivo* condition were tested using cDNA from the bacterium grown in leaf extract and the amplification product was obtained in all cases (Fig. 3A). Also cDNA from the bacterium recovered from the plant leaves was used in RT-PCR reactions with the primers designed for the genes *atpD*, *nuoH* and *tlyC*, all differentially expressed in the leaf extract condition. The results revealed the amplification product for the first two genes, but not for

hemolysin (Fig. 3B). The comparison of the use of leaf extract of the host plant with *in vivo* conditions revealed that the use of the leaf extract may be considered a valid method mimicking the *in vivo* environment, since several differentially expressed genes were detected in both treatment.

Primers for the genes *atpD*, *nuoH*, *tlyC*, *yfcB*, *flgE*, *pstK*, *intS* and *nodI*, differentially expressed *in vivo* and in the presence of leaf extract were also tested using cDNA from the NYG medium and an amplification product was revealed only when primers for *nuoH* (ABC transporter) and *nodI* (NADH ubiquinone oxidoreductase, NQO8 subunit) were used (Fig. 3C). These results indicate that the other genes are not expressed in the rich medium and may be specifically induced by components present in the leaf tissue.

Differentially expressed genes

Genes with unassigned function

A high number of genes found in the present work represented genes that encode hypothetical proteins (31%). This finding is not surprising since in *E. coli*, one of the most studied bacterium, similar results have been obtained when comparing the expression in minimal and complex media (Tao *et al.*, 1999). Studies with pathogenic bacteria have emphasized mostly the major pathogenicity related genes and therefore a high number of genes with unknown function are observed. Indeed, in *X. axonopodis* pv. *citri*, hypothetical proteins represent approximately 37% of the total genome (da Silva *et al.*, 2002). In this

study, genes with unassigned function were identified in all treatments analyzed and these genes may play an important role in metabolism or in the plant-pathogen interaction since they were regulated in response to the specific treatments. Interestingly, one hypothetical protein (XAC4181) appeared several times (>10) in the sequencing step in the leaf extract and *in vivo* treatments, indicating that this gene may be highly expressed in these treatments.

Genes expressed in leaf extract, in vivo and NYG conditions

In the present study, several genes involved in metabolism and transport were expressed in all treatments analyzed. Among these differentially expressed sequences were genes that encode a peptidyl dipeptidase, which removes dipeptides from the C terminal of substrates and NADH-ubiquinone oxidoreductase, NQO12 and NQO8 subunits, which constitute a proton pump considered the first complex of energy transduction of several respiratory chains (Scheide *et al.*, 2002). A sequence between two genes that encode a hypothetical protein (XAC0289) and an oxidoreductase associated with electron transport was also obtained in all conditions. Another gene involved in the acquisition of nutrients identified in all treatments was an ABC transporter. These transporters are energy-dependent membrane proteins that translocate a variety of substrates such as biotin, sulfate, maltose, ribose, phosphate, among others, through the cellular membrane (Mishima *et al.*, 2001). ABC transporters have been considered important for phytopathogenic bacteria to grow *in planta* (Llama-Palacios *et al.*, 2002), probably due to their involvement in the transport of sugar and amino acids present in the plant tissue. Many of these genes may have been differentially expressed due to the difference in the presence of nutrients in the

media used. The minimal medium MM1, used as a control is poor in nutrients and therefore the bacterium grows slowly, when compared to the other culture conditions (Mehta and Rosato, 2001). Peptides and amino acids, for example, are essential growth factors for several bacteria and enzymes such as peptidyl dipeptidase should be important for the degradation of proteins which are more abundant in the leaf extract, NYG and probably *in vivo* treatments.

Genes involved in metabolism/transport/cellular processes

Some genes were identified in specific treatments and included genes encoding ribosomal proteins, which are also highly expressed by fast growing bacterial cells (Karlin and Mrázek, 2000). Two genes that encode ribosomal proteins L21 and L5 were differentially expressed in NYG and *in vivo*, respectively. Protein L21 ligates to the 23S rRNA in the presence of protein L20 and L5 is one of the proteins that mediates the ligation of the 5S RNA subunit to the large ribosome subunit and has an important role in the conformation of the 5S rRNA. Karlin and Mrázek (2000) report that for rapid division, many ribosomes are indispensable, augmented by abundant transcription processing factors and chaperones needed to assure properly translated, modified and folded protein products. A chaperone (GroEL) was also differentially expressed in the *in vivo* condition. These proteins contribute to conformational changes and to minimize protein damage during stress.

Other genes differentially expressed associated with metabolism were an adenine specific methylase in the *in vivo* treatment and an endonuclease precursor in the NYG condition. Both genes are related to DNA and RNA modification and play a role in the

regulation of gene expression. The adenine specific methylase recognizes a sequence of 4-8 nucleotides and modifies the nucleotide inside the sequence. This enzyme also has an important role in the DNA replication, methyl-directed mismatch repair, transposition and gene expression (Radlinska *et al.*, 2001). The endonuclease catalyzes the degradation of DNA and RNA, and in general has an effect over the stability of the RNA and therefore also on the levels of translation.

Several transport genes, involving Na and Fe, were also identified in the treatments analyzed. The solute/Na+ symporter, expressed in the presence of leaf extract and NYG, uses the free energy from the electrochemical gradients of Na+ to accumulate solutes (Jung, 2001). In *E. coli*, for example, the transport of melobiose, proline, pantothenate and glutamate is coupled to Na+. The majority of transporters from this family are involved in the acquisition of nutrients and others are associated with osmoadaptation (Jung, 2001). The membrane energetics based on Na+ has several advantages such as the increase of the versatility of the pathogen by providing an additional form of ATP synthesis, motility and solute transport. These factors increase the chances of colonizing the host cell and survival of the pathogen in the host organism (Hase *et al.*, 2001).

Another differential sequence obtained was localized between two genes, one of them associated with the transport of Fe (TonB dependent receptor) and the other was identified as a transcriptional regulator. TonB is associated with the transport of Fe, and has a crucial role in the plant-pathogen interactions (Expert *et al.*, 1996; Loper and Buyer, 1991). Fe is the limiting factor of bacterial growth *in planta* since micromolar concentrations of iron are necessary to permit bacterial growth and multiplication. The TonB system also mediates the signal transduction from the cellular surface to the

cytoplasm, as reported in *E. coli* and *Pseudomonas putida* (Harle *et al.*, 1995; Koster *et al.*, 1994). In *X. campestris*, the genes involved in the uptake of Fe are essential for the induction of a hypersensitive response (Wiggerich and Puhler, 2000).

At this stage, it is difficult to know the specific role played by these genes. It is possible that they are associated with the intense bacterial growth in the different culture conditions (presence of leaf extract, *in vivo* and NYG) when compared to the slow growing cells in the minimal medium MM1. Intense bacterial growth is also considered essential for plant colonization and therefore for invasion of plant tissue. Although the genes differentially expressed in the presence of leaf extract and *in vivo*, are directly involved in metabolism, they may also play a role in pathogenicity by regulating the level and time of the appearance of plant symptoms.

Two-component systems

The signal transduction systems have been extensively studied in Gram negative bacteria and can influence bacterium-host interactions. These systems involve a signal transduction of a sensor protein, such as histidine kinase, to a transcriptional regulator of several genes and are essential for the adaptation of bacteria to stress and environmental changes (Matsushita and Janda, 2002). Bacteria also use these systems to secrete several proteins into the extracellular environment. In the present study, genes involved in two-component systems were identified in all three treatments analyzed. In the leaf extract and *in vivo* treatments the HPr kinase/phosphatase gene was differentially expressed. Also a histidine kinase/response regulator hibrid protein was differentially expressed *in vivo*. Is it possible that components *in planta* and in the leaf extract induced the expression of these

genes allowing the bacterium to sense the new environment. A two-component regulatory protein was also identified in the NYG medium. Similar results were obtained in *Erwinia amylovora* where putative two-component response regulators were expressed in rich media (Wei *et al.*, 2000).

A protein related to the flagellum biosynthesis, the hook protein, was also differentially expressed *in vivo*. Although the flagellum is classified as related with metabolism and chemotaxis, flagellar proteins have been associated with efficient plant colonization (Nasser *et al.*, 2001). For the assembly of the flagellum, several protein subunits are exported from the cytoplasm to the outer surface of the cell by a mechanism, which is similar to the type III secretion system (Young *et al.*, 1999). This system has been well studied in phytopathogenic bacteria and several proteins involved in this mechanism share homology to proteins associated with the biosynthesis of the flagellum. In *E. coli*, the flagellar export apparatus also functions as a protein secretion system and in several bacteria it has been considered essential for bacterial viability (Young *et al.*, 1999). Moreover, it has been reported that the flagellum biosynthesis occurs in response to environmental signals and therefore the regulation of the synthesis of the flagellum can influence bacterium-host interactions independent of motility (Young *et al.*, 1999).

Mobile genetic elements

An insertion sequence (ISXac3) was differentially expressed in the NYG medium. This insertion sequence belongs to the IS3 family, which is highly representative in *X. axonopodis* pv. *citri*. Twenty one copies of ISXac3 were identified in the genome of this bacterium (da Silva *et al.*, 2002), however the expression of this gene had not been reported

before. Insertion sequences have been related to mutation, and the expression of these genes may be associated with bacterial growth. Studies in *X. oryzae* pv. *oryzae* revealed that spontaneous mutants deficient for virulence and extracellular polysaccharides accumulate in the stationary phase. Results of these studies showed that these mutations occur due to insertion sequences (IS) (Rajeshwari and Sonti, 2000). After 12 h growth in NYG, *X. axonopodis* pv. *citri* is close to the stationary phase (Mehta and Rosato, 2001), which could explain the expression of the transposon ISXac3 in this treatment. In *X. axonopodis* pv. *citri* more than 100 mobile genetic elements were identified (da Silva *et al.*, 2002), and further studies about the factors influencing the activation of these elements would be important to understand the mechanism of mutation caused by transcription events and generation of genetic diversity.

A phage-related integrase was another mobile genetic element identified in the *in vivo* condition. Integrases, besides permitting the integration of sequences in specific sites of the chromosome, may also play an indirect role in pathogenicity by changing the expression of pathogenicity related genes. In *Vibrio cholerae*, for example, the avirulence gene cluster is associated to an integrase (Kovach *et al.*, 1996). Integrases have also been associated to antibiotic resistance (Oh *et al.*, 2002) as well as integration of pathogenicity islands (Tauschek *et al.*, 2002).

Pathogenicity genes

Among the pathogenicity genes identified in the present work is the gene that encodes hemolysin, which was differentially expressed by the bacterium cultured in the presence of leaf extract. Hemolysin is a glycolipid synthesized by bacteria in the stationary

phase in conditions where there is a prevalence of carbon sources over nitrogen sources in the medium (Denisov *et al.*, 1996). Hemolysin has been considered a virulence factor in bacteria such as *Aeromonas*, due to its hemolytic properties and enterotoxic activities (Nomura, 2001). In *Pseudomonas putida*, hemolysin was considered important for the colonization of the rhizophere of several economically important plants (Espinosa-Urgel *et al.*, 2000). In phytopathogenic bacteria, the function of this enzyme is not well established. Studies with *Erwinia chrysanthemi* revealed that the flanking regions of the *hrpC* and *hrpN* genes, involved in the hypersensitive reaction in tobacco leaves, contain homologs of hemolysin (Kim *et al.*, 1998).

Another sequence identified in the *in vivo* treatment was an intergenic region between the *rpf*F and *rpf*B genes, which are involved in pathogenicity. The *rpf* ("regulation of pathogenicity factors") genes control the production of pathogenicity factors such as enzymes and extracellular polysaccharydes (Dow *et al.*, 2000). The *rpf* cluster is formed by at least 7 genes *rpf*A-G (Tang *et al.*, 1991) and studies have shown that mutations in these genes decrease the production of extracellular enzymes and reduce virulence (Barber *et al.*, 1997). The *rpf*F and *rpf*B genes are involved in the regulation of the synthesis of a diffusible extracellular factor of low molecular weight called DSF ("diffusible signal factor"), which is involved in the production of protease, endoglucanase and polygalacturonate liase (Barber *et al.*, 1997). It is assumed that DSF is a fatty-acid derivative, used by several Gram negative bacteria for intercellular signalling and regulation. This regulatory system based on a small molecule seems to be essential for pathogenicity.

Unexpectedly, a CAP-like protein ("catabolite activator protein") was differentially expressed by the bacterium grown in the complex medium. This protein regulates directly or indirectly genes implicated in pathogenicity, which are usually repressed in complex media in *Xanthomonas* (Schulte and Bonas, 1992; Wei *et al.*, 1992). de Crecy-Lagard *et al.* (1990) analyzed a mutant of *X. campestris* pv. *campestris* for the CAP-like protein and observed the differential expression of several genes including those involved in the production of xanthan gum, pigment and extracellular enzymes.

CONCLUDING REMARKS

In the present study we have reported the analysis of the differential expression of *X. axonopodis* pv. *citri* by cDNA RDA and several genes more abundantly or exclusively expressed in specific treatments were identified. The results obtained herein indicate that cDNA RDA is a rapid and efficient method for the analysis of the differential gene expression of bacteria grown in different culture conditions, and has the advantage of eliminating most of the ribosomal RNA, which is usually a drawback in several techniques such as Differential Display. In the present study, a total of 37 distinct genes regulated by specific environmental conditions were identified. Since the sequencing of the difference products were not exhaustive, it is possible that the isolation of a higher number of these products from each treatment may lead to the identification of additional genes.

The majority of the genes were classified as encoding hypothetical proteins (31%) or related to metabolism (34%). It is likely that macromolecule and energy metabolism genes are induced in the fast growing bacterial cells in the treatments analyzed, however a

few genes could be related specifically to the interaction with the citrus plant (presence of leaf extract or *in vivo*) and could be involved in pathogenicty. Among the potential genes associated with the plant-pathogen interaction are those involved in the signal transduction system and transcription regulation that could act in a cascade controlling the expression of different genes. Genes involved in cellular processes and transport such as those associated with flagellum biosynthesis and Hrp kinase/phosphatase are potential candidates for further studies related to pathogenicity. The solute Na+ symporter and TonB receptor genes seem to be regulated by specific conditions but may be more related to the acquisition of inorganic elements than directly in pathogenicity. The category of hypothetical proteins is a great challenge since they are found in high percentages in all bacterial genomes. Several of these genes are regulated by specific conditions suggesting that they can play a significant role in the bacterium life cycle.

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Table 1. Differentially expressed genes, regulated in the presence of leaf extract and *in vivo* detected by cDNA RDA

DP[a]	Homology	Gene[b]	ORF
	MM1 + leaf extract of *Citrus sinensis* against MM1		
	Hypothetical proteins		
DP3	Conserved hypothetical protein	none	XAC0744
DP3	Conserved hypothetical protein	none	XAC4181*
DP3	Conserved hypothetical protein[c]	none	XAC0289*
DP3	Conserved hypothetical protein	none	XAC0259
	Macromolecule metabolism		
DP3	Peptidyl-dipeptidase[c]	*dcp*	XAC0249
	Intermediary metabolismo		
DP3	Oxidoreductase[c]	*mocA*	XAC0288*
DP2	ATP synthase, beta chain	*atpD*	XAC3649*
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[c]	*nuoL*	XAC2693
DP3	NADH-ubiquinone oxidoreductase, NQO8 subunit[a]	*nuoH*	XAC2697*
	Cellular processes/Transport		
DP2	HPr kinase/phosphatase	*pstK*	XAC2975*
DP2	Cell division protein	*ftsY*	XAC2552
DP3	Solute:Na+ symporter	*ppa*	XAC4176
	Pathogenicity, virulence and adaptation		
DP2	Hemolysin	*tlyC*	XAC1709*
	In vivo against MM1		
	Hypothetical proteins		
DP3	Conserved hypothetical protein[c]	none	XAC0289*
DP2	Conserved hypothetical protein	none	XAC2902
DP3	Conserved hypothetical protein	none	XAC4181*
DP3	Conserved hypothetical protein	none	XAC0376
DP3	Conserved hypothetical protein	none	XAC0357
DP3	Conserved hypothetical protein	none	XAC1749*
	Macromolecule metabolism		
DP2	60 KDa chaperonin	*groEL*	XAC0542*
DP2	Adenine-specific methylase	*yfcB*	XAC2726*
DP3	Peptidyl-dipeptidase[c]	*dcp*	XAC0249
DP3	50S ribosomal protein L5	*rplE*	XAC0984*
	Intermediary metabolismo		
DP3	Oxidoreductase[c]	*mocA*	XAC0288*
DP2	Histidine kinase/response regulador hybrid protein	none	XAC0685
DP2	Transcription regulator	none	XAC1311
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[c]	*nuoL*	XAC2693
	Cellular processes/Transport		
DP2	TonB-dependent receptor	*btuB*	XAC1310
DP3	ABC transporter ATP-binding protein[a]	*nodI*	XAC1547*
DP3	Flagellar biosynthesis, hook protein	*flgE*	XAC1983*
DP3	HPr kinase/phosphatase	*pstK*	XAC2975*
	Mobile genetic elements		
DP3	Phage-related integrase	*intS*	XAC2286*
	Pathogenicity, virulence and adaptation		
DP2	Sequence between rpfF and rpfB	*rpfF/rpfB*	XAC1879/XAC1880

[a] Difference product: DP2 indicates products from the second hybridization and DP3 from the third

[b] http://cancer.lbi.ic.unicamp.br//xanthomonas/

[c] differentially expressed sequences in all treatments identified by cDNA RDA

[d] differentially expressed sequences in all treatments identified by RT-PCR

* Selected ORFs to design primers for RT-PCR reactions

Table 2. Differentially expressed genes, regulated in the complex medium NYG detected by cDNA RDA

DP[a]	Homology	Gene[b]	ORF
	NYG against MM1		
	Hypothetical proteins		
DP2	Conserved hypothetical protein	none	XAC2444
DP2	Conserved hypothetical protein	none	XAC2902
DP2	Conserved hypothetical protein	none	XAC2925
DP3	Conserved hypothetical protein	none	XAC1372
DP3	Conserved hypothetical protein[c]	none	XAC0289*
DP3	Conserved hypothetical protein	none	XAC0259
DP3	Hypothetical protein	none	XAC3025
	Macromolecule metabolism		
DP2	50S ribosomal protein L21	*rplU*	XAC1248
DP3	Peptidyl-dipeptidase[c]	*dcp*	XAC0249
DP3	Endonuclease precursor	*nucA*	XAC3769*
	Intermediary metabolismo		
DP3	Oxidoreductase[c]	*mocA*	XAC0288*
DP3	Two-component system regulatory protein	none	XAC0684
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[c]	*nuoL*	XAC2693
	Cellular processes/Transport		
DP2	Solute:Na+ symporter	*ppa*	XAC4176
	Mobile genetic elements		
DP3	ISXac 3 transposase	*ISxac3*	XAC0091*
	Pathogenicity, virulence and adaptation		
DP3	CAP-like protein	*clp*	XAC0483*

[a] Difference product: DP2 indicates products from the second hybridization and DP3 from
[b] http://cancer.lbi.ic.unicamp.br//xanthomonas/
[c] differentially expressed sequences in all treatments identified by cDNA RDA
* Selected ORFs to design primers for RT-PCR reactions

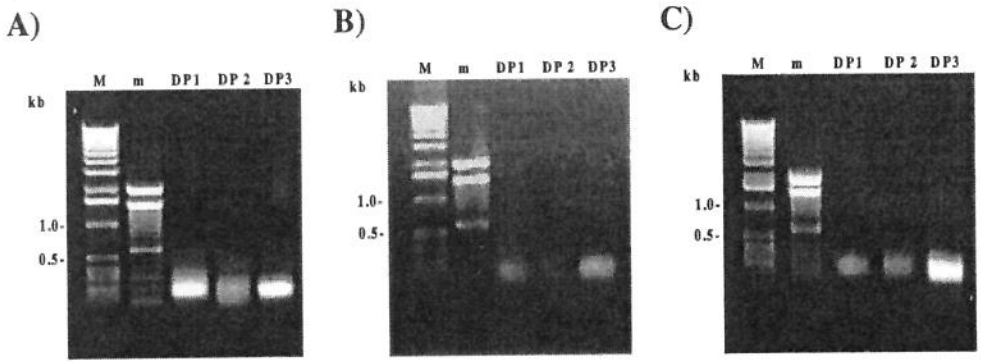

Fig. 1. Agarose gel electrophoresis of the difference products obtained after the first (DP1), second (DP2) and third (DP3) subtractive hybridization of *X. axonopodis* pv. *citri* grown in the presence of leaf extract (A), *in vivo* (B) and in the complex medium NYG (C). M and m, molecular size marker Ladder 1kb and 100bp, respectively (Gibco).

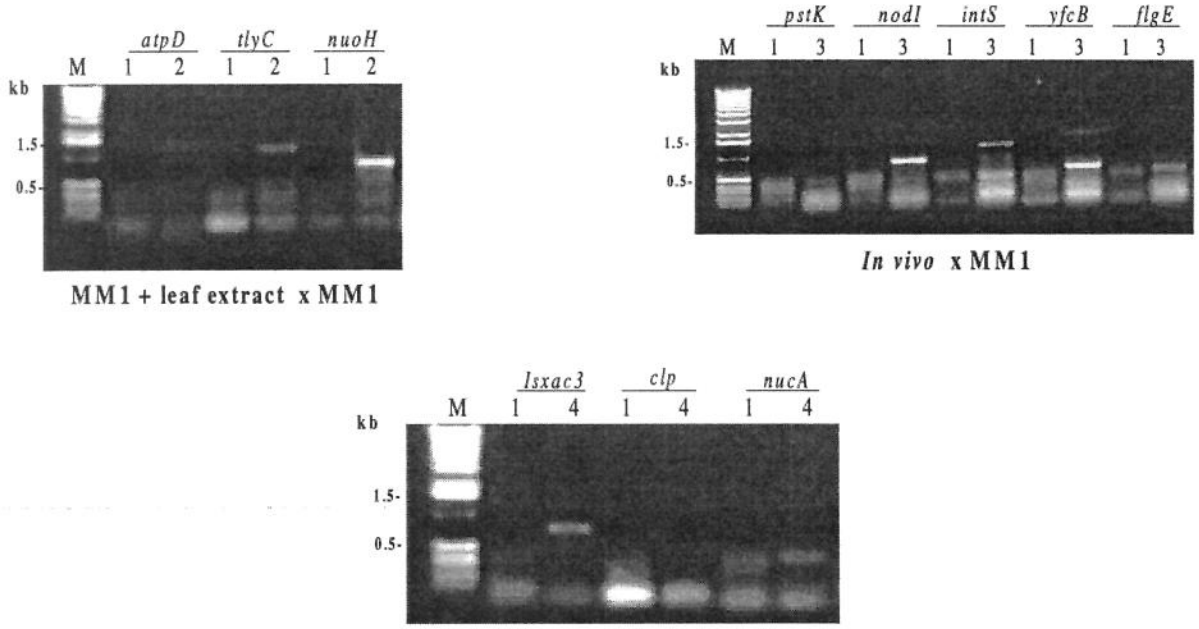

Fig.2. RT-PCR using cDNA of *X. axonopodis* pv. *citri* grown in: 1, MM1; 2, MM1 + leaf extract of *Citrus sinensis*; 3, *in vivo* and 4, NYG. The reactions were performed using primers for the genes *atpD* (1349 bp), *tlyC* (1290 bp), *nuoH* (899 bp), *pstK* (936 bp), *nodI* (814 bp), *intS* (1200 bp), *yfcB* (600 bp), *flgE* (1200 bp), *ISxac3* (762 bp), *clp* (680 bp) and *nucA* (371 bp), as indicated. M, molecular size marker Ladder 1kb (Gibco).

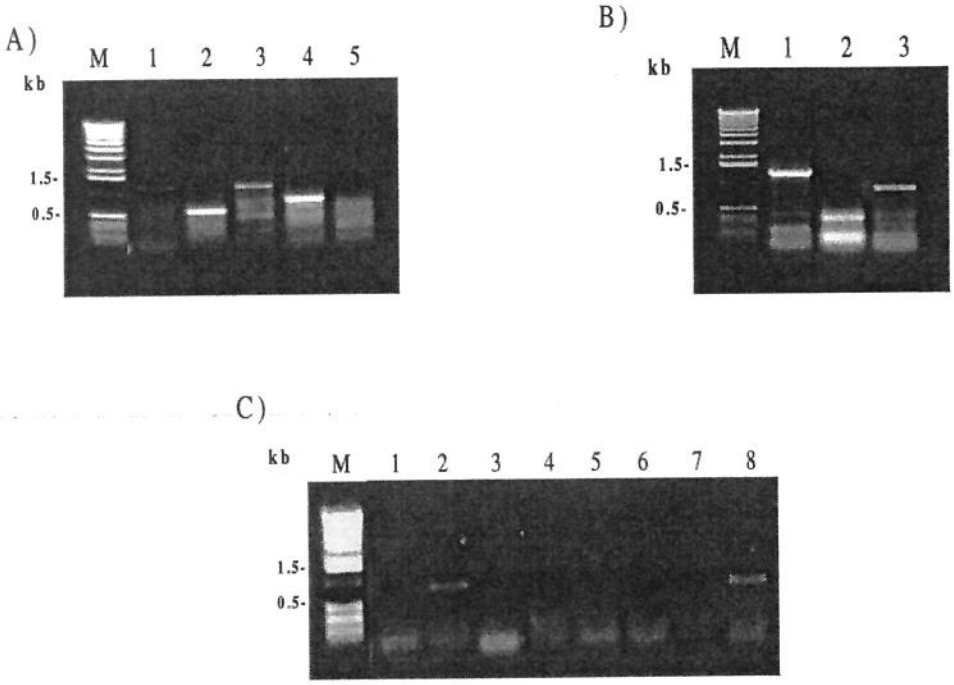

Fig.3. RT-PCR using (A) cDNA of the bacterium grown in leaf extract and primers designed for the genes *flgE* (1), *yfcB* (2), *intS* (3), *nodI* (4) and *pstK* (5), differentially expressed *in vivo*, (B) cDNA of the bacterium recovered from *Citrus sinensis* leaves and primers designed for the genes *atpD* (1), *tlyC* (2) and *nuoH* (3), differentially expressed in the presence of leaf extract, and (C) cDNA of the bacterium grown in NYG and primers designed for the genes *atpD* (1), *nuoH* (2) and *tlyC* (3), *yfcB* (4), *flgE* (5), *pstK* (6), *intS* (7) and *nodI* (8), differentially expressed in the presence of leaf extract and *in vivo*. M, molecular size marker Ladder 1kb (Gibco).

CONSIDERAÇÕES FINAIS

Neste estudo, a análise da expressão diferencial de *X. axonopodis* pv. *citri* foi analisada utilizando duas técnicas distintas: análise de proteínas através de eletroforese bidimensional e cDNA RDA ("Representational Difference Analysis").

A técnica de eletroforese bidimensional de proteínas mostrou ser um método eficiente e útil para o estudo da expressão diferencial e várias proteínas diferencialmente expressas foram observadas nos diferentes tratamentos. Entretanto, o número de proteínas que puderam ser identificadas através do seqüenciamento NH_2-terminal foi limitado devido a grande quantidade de proteína necessária e ao fato de que algumas delas tinham a região NH_2-terminal bloqueada. Recentes avanços para a otimização das tecnologias de identificação de proteínas têm contribuído significativamente para que um maior número de proteínas seja identificado, acompanhando a velocidade com que dados de seqüenciamento de genomas são gerados. A espectrometria de massa tem se revelado um importante método para a identificação de proteínas. Com a disponibilidade de seqüências do genoma do organismo, a massa dos peptídeos obtida após digestão das proteínas pode ser comparada e as proteínas podem ser identificadas. Outra vantagem da espectrometria de massa é que as proteínas podem ser excisadas diretamente dos géis de poliacrilamida, evitando assim a perda de material durante a transferência para membranas de PVDF. Embora um pequeno número de proteínas tenha sido identificado neste estudo, os resultados obtidos fornecem informações importantes a respeito da expressão diferencial de *X. axonopodis* pv. *citri* nos diferentes tratamentos.

A técnica de cDNA RDA revelou um maior número de genes diferencialmente expressos (37) nos tratamentos analisados, sendo que aproximadamente 30 fragmentos de cada tratamento foram seqüenciados. É possível que o seqüenciamento de um maior número de produtos diferenciais possa levar a identificação de um maior número de genes. cDNA RDA mostrou ser um método fácil e rápido para a identificação de vários genes diferencialmente expressos e possui várias vantagens sobre outros métodos. A principal vantagem é a subtração de genes ribossomais que constituem mais de 90% do RNA total de bactérias. Na maioria das técnicas a presença de grande quantidade de rRNA representa um problema uma vez que interferem nos resultados obtidos. Na técnica de "Differential Display" por exemplo, uma grande quantidade de genes ribossomais (30-50%) é obtida após seqüenciamento das bandas diferenciais. Em cDNA RDA, aproximadamente 30% do total de seqüências obtidas representaram genes ribossomais. Entretanto, quando a quantidade de representações dos genes 16S e 23S foi aumentada nas hibridizações subtrativas, este número caiu para 15%. É possível que um aumento na proporção de representações desses genes possa levar a uma diminuição ainda maior desta porcentagem. Outra vantagem da técnica é a facilidade da análise dos resultados uma vez que genes expressos nos dois tratamentos utilizados são eliminados e as bandas observadas nos géis de agarose representam apenas os genes diferencialmente expressos. Os resultados obtidos com cDNA RDA foram validados através da técnica de RT-PCR, que confirmou a expressão diferencial para a maioria dos genes testados, indicando que esta técnica revela informações confiáveis e consistentes e representa um método eficiente para a análise da expressão diferencial.

Neste estudo foi também descrito um método para a análise da expressão de *X. axonopodis* pv. *citri in vivo*. Análises do perfil de proteínas e RNA revelou a validade do método que contribui para a resolução de um dos principais problemas nos estudos de genes/proteínas envolvidos na patogenicidade ou interação planta-patógeno, que é a escolha do meio de cultura a ser utilizado. O RNA obtido de células recuperadas de folhas de *Citrus sinensis* infiltradas foi utilizado para cDNA RDA e vários genes diferencialmente expressos foram identificados. Esses resultados reforçam a confiabilidade e eficiência do método de recuperação de células bacterianas de tecidos infiltrados que pode ser utilizado para outras bactérias fitopatogênicas. É importante ressaltar que a expressão de genes *in vivo* não tem sido relatada até o presente momento devido às dificuldades na recuperação das bactérias diretamente da planta. Os resultados obtidos com a bactéria cultivada *in vivo* permitiram também a comparação com as condições *in vitro* (uso de extrato de citros) e revelou que a utilização de extrato pode ser considerada um método válido para mimetizar as condições *in vivo* uma vez que vários genes foram expressos em ambas as condições.

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