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Expression Profiling of Virulence and Pathogenicity Genes of *Xanthomonas axonopodis* pv. citri

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DNA macroarrays of 279 genes of *Xanthomonas axonopodis* pv. citri potentially associated with pathogenicity and virulence were used to compare the transcriptional alterations of this bacterium in response to two synthetic media. Data analysis indicated that 31 genes were up-regulated by synthetic medium XVM2, while only 7 genes were repressed. The results suggest that XVM2 could be used as an in vitro system to identify candidate genes involved in pathogenesis of *X. axonopodis* pv. citri.

Citrus canker, caused by *Xanthomonas axonopodis* Starr & Garces emend. Vauterin et al. pv. citri (Hasse) Dye [syn. *Xanthomonas campestris* pv. citri (Hasse)], is one of the most devastating citrus diseases in the world (44).

Within the genus *Xanthomonas*, several genes have been found associated with pathogenicity and virulence. Of these genes, the *avr* (avirulence), *rpf* (named for regulation of pathogenicity factors), and *hrp* (named for hypersensitive response and pathogenicity) genes are perhaps the most widely studied elements. The *avr* genes encode a known group of effector proteins responsible for controlling the ability of bacteria to elicit the hypersensitive reaction in resistant hosts (26) and may also participate in pathogenicity or virulence in compatible interactions (38, 47). The *rpf* operon is thought to control the production of important pathogenicity factors, such as proteases, endoglucanases, polygalacturonate ligases, and extracellular polysaccharides (5, 17). Finally, the *hrp* genes are thought to encode proteins involved in the type III secretion system, responsible for delivering effector proteins inside the host plant cells (8, 9, 20, 21).

Even though pathogenicity and virulence of *Xanthomonas axonopodis* pv. citri have been traditionally associated with the activity of a single avirulence-like gene known as *pthA* (14, 22, 45, 46), little is known about other gene products involved in these processes. In this respect, transcription profiling under natural conditions may be a good alternative to identify all the elements involved in pathogenicity and virulence of this microbial pathogen. However, leaf spot pathogens, such as *X. axonopodis* pv. citri, do not reach high population levels in infected tissues and do not yield enough material (either bacterial cells or RNA) to conduct gene expression studies. Therefore, in an attempt to develop an alternative system for gene expression studies, an in vitro system was evaluated in order to determine whether it could be used to model pathogen responses to host tissues under controlled conditions. The two media selected were NB (nutrient broth), commonly used for growing this bacterium, and XVM2, suspected to mimic the environment of the plant intercellular spaces (1, 41, 51, 55).

Construction of DNA macroarrays. To study the expression profile of *X. axonopodis* pv. citri, 279 candidate genes, associated with pathogenicity or virulence by sequence similarity, were selected from the genome of this bacterium (13). Specific primers were designed to amplify either the entire open reading frame or a fragment of it. PCR amplifications were performed in two rounds using 96-well plates and a Mastercycler Eppendorf (Eppendorf). The quality of amplimers was analyzed by agarose gel electrophoresis.

Prior to the construction of the DNA macroarray, dried and purified PCR products were resuspended in a 50% dimethyl sulfoxide solution, adjusting the concentration of each product to 100 ng/μl. PCR products were spotted onto positively charged nylon membranes (Genetix) using a Q-Bot robot (Genetix) in a 3×3 arrangement, where each spot was replicated eight times.

Transcription measurements. In order to examine the gene expression profiles under in vitro conditions, a single cell culture of *X. axonopodis* pv. citri strain 306 (kindly provided by Rui P. Leite, IAPAR, Brazil) was grown in NB and the *hrp*-inducing medium XVM2 (41) at 28°C with shaking at 100 rpm. The NB and XVM2 cultures were grown at 28°C for 12 and 20 h, respectively, until they reached an optical density at 600 nm of 0.3. Bacterial cells were harvested by centrifugation at 4°C and then stored at −80°C for further manipulations. These experiments were repeated three times using different single cell cultures.

Total RNA from *X. axonopodis* pv. citri cells was extracted using the RNeasy kit (QIAGEN) according to the recommendations of the manufacturer. In order to specifically label the cDNA of the selected genes, a set of 41 octamers was designed using the genome-directed primer approach described by Talaat and coworkers (48). cDNA was labeled with [^{33}P]dCTP during reverse transcription. Labeled cDNA was hybridized to the membranes using standard procedures. After hybridization and washes, membranes were dried by blotting, wrapped in a plastic film, and exposed to a general-purpose screen (Amersham Biosciences) for 96 h.

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Images were captured using a STORM phosphorimager (Amersham Biosciences). Signal strength and local background were quantified using the ImaGene software (BioDiscovery). Spots with signal/background ratios of less than two were flagged and excluded from the analysis. Local background was subtracted, and the data of each array were normalized by the total signal strength method as implemented in the GeneSight software (BioDiscovery).

Differentially expressed genes were identified by three methods, fold change (TF), significance analysis of microarrays (SAM), and analysis of variance (ANOVA). For the TF method, the normalized signal intensity for each spot of the treatment condition (T) was divided by that of the control condition (C) to calculate the ratio ($R = T/C$). Final ratios were calculated as the average of all individual hybridizations. Genes with R of ≥ 2 or ≤ 0.5 (twofold) were considered significantly up- and down-regulated, respectively.

The SAM method identifies significant relationships between gene expression and the response variable using a significance limit Δ (delta), a false discovery rate, and an induction or repression threshold defined by the user. The SAM analysis was performed using a Microsoft Excel add-in as described by Tusher et al. (52).

ANOVA was performed using the software package GeneANOVA (16). We used a completely randomized design in which the number of spot replicates per sample, the number of experiments performed, and genes and culture media were considered treatments. Only genes with P values of $\leq 1\%$ were considered significantly induced or repressed (http://citest.centrodecitricultura.br/gamo/JB00701-04/).

The experiments were performed under the conditions described above. After three independent biological replications of each treatment, results indicated that most *X. axonopodis* pv. citri genes (87%) exhibited similar levels of expression in the culture media, while only 13% of the genes were differentially expressed. Few differentially expressed genes were found when expression profiles were compared in *Escherichia coli* growing in rich and minimal media (50).

The low number of differentially expressed genes may be due to several possible causes. First of all, the induction of some genes could depend on one or more host-specific conditions that may not be provided by the media. Also, some of the genes may just be constitutively expressed and may not respond to alterations in the environment. Finally, it is important to consider the fact that some of the individual expression levels observed in this study may be errors caused by technical problems, such as annotation errors, cross-hybridization, misapplied DNA spots on the arrays, or scatter in the data. Therefore, the data presented here must be taken as a general trend that could be used as a guide for further research.

Comparison of the three methods of analysis indicated that SAM identified the largest number of differentially expressed genes, 32, while ANOVA and TF recognized approximately the same number, 25 and 27, respectively (Table 1). When the identities of the genes recognized by each method were compared, SAM and TF exhibited 82% agreement, while ANOVA showed an identical low level of agreement with SAM and TF (61%). Since none of the methods used in this study was better than the others, we decided to use a combination of all genes identified by the three methods, resulting in 37 differentially expressed genes.

Gene expression profile in XVM2 medium. Most differentially expressed genes (Table 1) included known pathogenicity or virulence factors, such as avirulence proteins, cell wall-degrading enzymes, proteins involved in adhesion to surfaces, peptidases, proteins associated with secretion, nucleases, putative extracellular polysaccharide (EPS) and lipopolysaccharide (LPS) synthesis proteins, and other proteins associated with oxidative stress responses and synthesis of osmoregulated periplasmic glucans (OPGs).

The ability of XVM2 medium to induce the expression of *avr* genes has been reported for other xanthomonads (1). From the eight *avr* gene candidates annotated in the genome of *X. axonopodis* pv. citri (13), only *avrXacE1*, *avrXacE2*, and XAC0076 (an *avrBs2*-like gene) were found to be induced by XVM2 medium. The four members of the *pthA* family and *avrXacE3* exhibited constitutive expression in both culture media. Other *avr* genes from *Xanthomonas* spp., including the *pthA*-like gene *avrBs3*, have been found to be constitutively expressed under different conditions including growth on XVM2 medium (11, 25, 36). Our results support the hypothesis of Ciesiolka et al. (11) who proposed that there might be at least two regulation systems that control the expression of *avr* genes in xanthomonads.

When *X. axonopodis* pv. citri was grown in XVM2 medium, eight *hrp* genes were found to be up-regulated. This result was expected, since it is already known that the expression of *hrp* genes is induced by XVM2 in *Xanthomonas campestris* pv. vesicatoria (41, 55). Interestingly, all induced *hrp* genes are similar to either structural components of the type III core apparatus or proteins required for secretion in *X. campestris* pv. vesicatoria (40). In contrast, the protein encoded by *hrpB1*, repressed in XVM2, is known to be located in the cytoplasm of the bacterial cell in *X. campestris* pv. vesicatoria (40).

When *X. axonopodis* pv. citri was grown in XVM2 medium, the *rpfG* and *rpfC* genes, four genes potentially involved in the synthesis of EPS or LPS, and one encoding a putative cell wall-degrading enzyme (CWDE) were found to be down-regulated. These results could be expected, since RpfG, RpfH, and RpfC are known to be part of a signal transduction system that indirectly induces the synthesis of EPS and extracellular enzymes in other xanthomonads (42).

However, at least three other genes encoding putative CWDEs seemed to be up-regulated by XVM2 medium (XAC0028, XAC0029, and XAC2373), suggesting that an alternative signaling system may also exist to regulate the expression of CWDEs in *X. axonopodis* pv. citri. On the other hand, it is known that EPS or LPS biosynthesis in *Ralstonia solanacearum* and *Xanthomonas campestris* pv. campestris is activated only at late stages of infection (23, 53). This late induction would allow adherence of bacterial colonies to cell surfaces, promoting the establishment of biofilms and the proper functioning of the type III secretion system (15). Our results may reflect the fact that a richer medium (NB) may induce the activation of the EPS or LPS biosynthesis earlier than a minimal medium like XVM2.

Five genes putatively associated with oxidative stress responses were found to be induced by XVM2 medium (Table 1). This defense mechanism consists of the rapid generation of

TABLE 1. Up- and down-regulated genes from *X. axonopodis* pv. citri in the XVM2 medium as determined by three different methods

Gene group and protein	Gene tag	gi no.	FC[a]	No. of differentially expressed genes[b] by:		
				SAM	TF	ANOVA
Up-regulated genes						
Avirulence protein (*avrBs2*-like)	XAC0076	gi21240850	3.98	x	x	x
Avirulence protein (*avrXacE1*)	XAC0286	gi21241060	3.45	x	ND	ND
Avirulence protein (*avrXacE2*)	XAC3224	gi21243950	2.79	x	ND	x
Beta-lactamase (*bla*)	XAC3057	gi21243784	2.38	x	ND	ND
Catalase/peroxidase (*katG*)	XAC1301	gi21242054	3.90	x	x	x
Cellulase (*egl*) endo-1,4-beta-glucanase	XAC0028	gi21240802	4.99	x	x	x
Cellulase (*egl*) endo-1,4-beta-glucanase	XAC0029	gi21240803	5.68	x	x	x
Filamentous hemagglutinin (*fhaB*)	XAC1815	gi21242559	4.58	x	x	x
General stress protein	XAC2369	gi21243103	4.39	x	x	x
Glutathione peroxidase (*btuE*)	XAC1549	gi21242300	5.30	x	x	x
Glutathione peroxidase-like protein (*gpo*)	XAC1457	gi21242209	7.08	x	x	x
Hemagglutinin/hemolysin-related protein	XAC1816	gi21242560	6.53	x	x	ND
HrcJ protein (*hrcJ*)	XAC0409	gi21241182	(1.06)	ND	ND	x
HrcR protein (*hrcR*)	XAC0402	gi21241175	4.48	x	x	x
HrcU protein (*hrcU*)	XAC0406	gi21241179	2.76	x	ND	ND
HrcV protein (*hrcV*)	XAC0405	gi21241178	7.89	x	x	ND
HrpB2 protein (*hrpB2*)	XAC0408	gi21241181	4.42	x	x	x
HrpB4 protein (*hrpB4*)	XAC0410	gi21241183	6.85	x	x	x
HrpB5 protein (*hrpB5*)	XAC0411	gi21241184	(2.00)	ND	x	ND
Methicillin resistance protein (*mecI*)	XAC0069	gi21240843	4.82	x	x	x
Nuclease (*nucH*)	XAC0447	gi21241220	6.99	x	x	x
Pectate lyase (degenerated) (*pel*)	XAC2373	gi21243107	7.98	x	x	x
Periplasmic glucan biosynthesis protein (*mdoG*)	XAC4284	gi21245001	13.61	x	x	ND
Superoxide dismutase (*sodC2*)	XAC0210	gi21240984	7.93	x	x	ND
Superoxide dismutase-like protein (*yojM*)	XAC0209	gi21240983	10.73	x	x	x
Thiophene and furan oxidation protein (*thdF*)	XAC4370	gi21245082	2.92	x	x	ND
Trehalose-6-phosphate synthase (*ostA*)	XAC3211	gi21243937	2.42	x	ND	x
VirB11 protein (*virB11*), chromosomal[c]	XAC2618	gi21243349	4.56	x	x	x
VirK protein (*virK*), chromosomal	XAC0435	gi21241208	2.17	x	ND	ND
YapH protein (*yapH*)	XAC4113	gi21244830	(1.28)	ND	ND	x
Down-regulated genes						
Cellulase (*egl2*)	XAC2522	gi21243255	0.18	x	x	ND
GumD protein (*gumD*)	XAC2583	gi21243315	(0.96)	ND	ND	x
HrpB1 protein (*hrpB1*)	XAC0407	gi21241180	0.17	x	x	x
Periplasmic glucan biosynthesis protein (*hrpM*)	XAC0618	gi21241389	0.19	x	x	x
Phosphoglucomutase/phosphomannomutase (*xanA*)	XAC3579	gi21244304	0.15	x	x	ND
RpfG protein (*rpfG*)	XAC1877	gi21242621	(0.79)	ND	ND	x
RpfC protein (*rpfC*)	XAC1878	gi21242622	0.16	x	x	x

[a] FC, average fold change as calculated by SAM. The results represent the mean of the three biological replications. Values in parentheses were calculated by using raw data and not SAM estimations.
[b] x, a differentially expressed gene; ND, not considered a diffentially expressed gene.

highly reactive oxygen species (ROS) that can kill the invading bacterium (27). Bacterial pathogens may protect themselves by degrading ROS using catalases, peroxidases, and superoxide dismutases, by detoxifying these compounds, or by maintaining reduced environments accomplished by glutathione-associated enzymes (24, 27). The expression of stress-associated genes in minimal medium has also been reported for *E. coli* as a result of its need to synthesize all its building blocks from a single carbon and energy source (50).

OPGs have been shown to play an important role in bacterium-plant interactions involving both symbionts and pathogens (6, 7, 12, 19, 28, 29, 30, 31, 35, 37, 49). Two genes similar to those involved in the biosynthesis of OPGs were found to be differentially expressed in XVM2 medium: an *mdoG*-like gene (XAC4284) appeared to be up-regulated, while an *mdoH*-like gene (XAC0618) was down-regulated. In *E. coli*, *mdoG* and *mdoH* are located side by side, forming an operon, while in *X. axonopodis* pv. citri, these two genes are located in different areas of the genome. Therefore, it is possible to speculate that this structural difference may be associated with the different regulatory strategies observed for these two species.

At least four genes encoding surface proteins seemed to be up-regulated by XVM2 medium. Of these genes, the filamentous hemagglutinin gene (XAC1815) and a hemagglutinin-related protein gene (XAC1816) may be involved in attachment to host tissues. As already shown for several bacterial plant pathogens, attachment of bacterial cells to the plant cell wall is required for successful infection (39, 43).

One of the interesting genes induced by XVM2 medium is an *ostA*-like gene (XAC3211). This gene codes for the enzyme trehalose-6-phosphate synthase involved in the synthesis of trehalose, a typical microbial sugar known to accumulate in a variety of symbiotic or pathogenic interactions of microorganisms with plants, as well as in response to salt stress (10, 33). Trehalose-producing organisms could affect the carbon metabolism of infected plants by converting the products of photo-

TABLE 2. Occurrences (sites) of the conserved motif in the promoter regions of all genes induced by XVM2 medium

Gene	Start[a]	*P* value[b]	Sequence[c]
hrcJ	147	1.14e-05	CGCCGAGCAT**ATCGATGC**CTTCAGCATG
btuE	173	1.14e-05	GACGCCATCA**ATCGATGC**ACAACGCCGC
katG	145	3.18e-05	CGCCTCGGTT**GTCGATGC**TAGCGCCTGC
gpo	77	3.18e-05	CATGGTACGA**GTCGATGC**AAGCGGAGCA
bla	214	4.51e-05	CATCAATAGG**ATCAATGC**GATGCCCATT
egl	91	4.51e-05	ATCGGTCTGA**ATCAATGC**ATCAGCCGTG
ostA	280	6.46e-05	TGATCATCGC**AGCGATGC**GCTTGTCGCG
hrpB5	269	6.46e-05	AGACGGTGCG**AGCGATGC**ATTCCTGGCC
hrpB4	126	6.46e-05	CGGAGCGGAG**AGCGATGC**GCAGTTCACC
yapH	199	7.69e-05	AGCCGGGGGC**GTCGATGT**GGTATTCAAG
yojM	214	8.88e-05	GTCGCCCACG**ATCGTTGC**CCTCACCGCG
pel	203	9.97e-05	CTGACTTGGG**AACGATGC**CAGAAAGATC
virB11	199	9.97e-05	GTTGCTGGGT**AACGATGC**CGGAGCCCAC
hrcU	34	1.50e-04	TCCTTCCGCG**ATCGCTGC**GCCCCCGATT
virK	70	1.50e-04	GCGGCGCGAG**GTCAATGC**GCGCCTGTAG
nucH	71	1.50e-04	GCTCGATGAA**ATCGCTGC**GGAGGGTGGC
mdoG	7	2.44e-04	AGTGGC**GTCGTTGC**ATGCGCATCG
hrpB2	151	3.46e-04	CGAAGAAGAC**GCCGATGC**CGACGCGGTG
hrcR	78	3.46e-04	TCACTAACCT**GCCGATGC**CGATGGTCTG
fhaB	96	3.88e-04	CGCCGGCCGC**ATCGATGG**CACCAGCGCC
mecI	173	4.16e-04	TCCAGGGGCG**GGCGATGT**GTCCAGGCTG
avrBs2	141	4.16e-04	CGTCATGCGC**GGCGATGT**GCGCGCCAAG
sodC2	43	5.17e-04	CCAGCCCAGC**GGCAATGC**CGGTGCGCGC
avrXacE1	58	5.17e-04	TTTGGCAAGC**AGCGCTGC**GAACGTTGTT
egl	89	5.31e-04	TATGAGCTCG**TTCGATGT**ACGACAGCAA
hrcV	34	6.36e-04	CTCCATCGCC**GTCGCTGT**GGTCGCGACC
thdF	110	6.88e-04	TGAGTCGCCT**ACCGTTGC**GACAATCGCG

[a] Start position of the conserved sequence within the 300 bases extracted. The start codon of each gene would be at position 301.

[b] The *P* value gives the probability of a random string (generated from the background letter frequencies) having the same match score or higher.

[c] The conserved motif is shown in bold type.

synthesis into trehalose and diverting carbon away from plant metabolism or by releasing trehalose into the plant in order to affect plant metabolism by interfering with the plant's sugar-sensing system (32, 34, 54). The role of trehalose in citrus canker development is still unknown, but induction of its biosynthetic machinery in a plant-like environment suggests that this sugar may be involved at some point in pathogenesis of *X. axonopodis* pv. citri.

Conserved upstream sequences. Regulation of genes induced by XVM2 medium has been suggested to be associated with the presence of a conserved plant-inducible promoter sequence motif known as the PIP box ($TTCGCN_{15}TTCGC$, where N is any nucleotide) upstream of the putative start codon of these genes (18). Even though this conserved sequence has already been found upstream of a large number of pathogenicity or virulence genes, such as *hrpB* pathogenicity operon, *avrXv3*, *avrXv4*, and *avrRxv*, all from *X. campestris* pv. vesicatoria (1, 2, 11, 18), its functionality has been questioned (11).

To determine conserved DNA sequence motifs potentially associated with the promoter region of genes induced by XVM2 medium, PERL script was used to construct a database of upstream sequences by extracting 300 nucleotides upstream of the start codon of each gene identified in the genome of *X. axonopodis* pv. citri. The sequences upstream of the regions of the XVM2-induced genes (300 bp) were used as a training set for the program MEME version 3.0.10 with a minimum significance E value of 10^{-3} (3). Analysis of the data indicated the presence of an 8-bp conserved motif (ATCGATGC; E value = 1.6×10^{-4}) in the promoter region of all genes induced by XVM2 medium (Table 2). This conserved sequence is probably the binding site of a transcription activator protein. The conserved motif was used to scan across the promoter regions of those genes encoding hypothetical proteins in the *X. axonopodis* pv. citri genome using the MAST version 3.0.10 software with a *P* value of $<1 \times 10^{-4}$ (4). The global search indicated the presence of this motif in the promoter regions of 125 genes encoding proteins with unknown functions. Research is under way to identify the correlation between the presence of this conserved motif and the roles of these unknown proteins in pathogenicity or virulence of this bacterial pathogen.

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