HrpG and HrpX Play Global Roles in Coordinating Different Virulence Traits of *Xanthomonas axonopodis* pv. *citri*

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Xanthomonas axonopodis pv. *citri* is the causal agent of citrus canker, which is one of the most serious diseases of citrus. To understand the virulence mechanisms of *X. axonopodis* pv. *citri*, we designed and conducted genome-wide microarray analyses to characterize the HrpG and HrpX regulons, which are critical for the pathogenicity of *X. axonopodis* pv. *citri*. Our analyses revealed that 232 and 181 genes belonged to the HrpG and HrpX regulons, respectively. In total, 123 genes were overlapped in the two regulons at any of the three selected timepoints representing three growth stages of *X. axonopodis* pv. *citri* in XVM2 medium. Our results showed that HrpG and HrpX regulated all 24 type III secretion system genes, 23 type III secretion system effector genes, and 29 type II secretion system substrate genes. Our data revealed that *X. axonopodis* pv. *citri* regulates multiple cellular activities responding to the host environment, such as amino acid biosynthesis; oxidative phosphorylation; pentose-phosphate pathway; transport of sugar, iron, and potassium; and phenolic catabolism, through HrpX and HrpG. We found that 124 and 90 unknown genes were controlled by HrpG and HrpX, respectively. Our results suggest that HrpG and HrpX interplay with a global signaling network and co-ordinate the expression of multiple virulence factors for modification and adaption of host environment during *X. axonopodis* pv. *citri* infection.

The genus *Xanthomonas* is an important group of gram-negative plant-pathogenic bacteria that infect approximately 124 monocotyledonous and 268 dicotyledonous plants (Chan and Goodwin 1999; Leyns et al. 1984). Among the diseases caused by members of the genus *Xanthomonas*, citrus canker is one of the most serious diseases of most commercial citrus cultivars resulting in significant losses worldwide. Citrus-producing areas without citrus canker consider the citrus canker pathogen a quarantine pest due to the potential threat to citrus production. Thus, citrus canker has a significant impact on national and international citrus markets and trade (Graham et al. 2004). This devastating disease is caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. citri*, *X. campestris* pv. *citri*, or *X. citri* subsp. *citri*) (Cubero and Graham 2002; Schaad et al.

2006; Vauterin et al. 1995). The Asiatic form or A type of citrus canker is the most virulent and affects the widest range of hosts, including *Citrus* spp. and many closely related Rutaceae plants. Citrus canker disease is characterized by formation of necrotic raised lesions on leaves, stems, and fruit. On heavily infected trees, citrus canker causes defoliation, twig dieback, general tree decline, blemished fruit, and premature fruit drop. *X. axonopodis* pv. *citri* infects citrus from rain-splashed inocula directly through stomata (especially wind-blown rain) or by wounds, and grows in the intercellular spaces of the spongy mesophyll (Graham et al. 2004). The genus *Xanthomonas* has become an important model organism for studying plant-microbe interaction and for understanding bacterial pathogenicity and virulence mechanisms (Büttner and Bonas 2010).

In *Xanthomonas* spp., the type III secretion system (T3SS) encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes is one critical pathogenicity factor. The expression of *hrp* genes is controlled by two regulators: HrpG and HrpX. HrpG, as an OmpR family regulator, activates the expression of *hrcC* and *hrpX* in *X. campestris* pv. *vesicatoria* (Wengelnik et al. 1996b) or only *hrpX* in *X. campestris* pv. *campestris* (Huang et al. 2009). HrpX, an AraC-type transcriptional activator, controls the expression of other *hrp* genes along with some effector genes (Astua-Monge et al. 2000; Wengelnik and Bonas 1996). The HrpG regulon has been extended beyond the *hrp* genes using cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (Noël et al. 2001). Thirty HrpG-induced and five HrpG-repressed genes were found, including the *hrp* gene cluster, effector genes, and genes encoding protease. Many genes in the HrpX regulon in xanthomonads possess a consensus sequence motif, called the plant-inducible promoter (PIP) box (TTTCG-N15-TTTCG), in the promoter regions (Fenselau and Bonas 1995). Comparative genomic analysis revealed that 12 and 15 candidates for HrpX regulons existed in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*, respectively, which did not include the genes in *hrp* clusters (da Silva et al. 2002). However, genes with an imperfect PIP box and genes without a PIP box have been found to be controlled in an HrpX-dependent manner (Noël et al. 2001).

Recent studies showed that some type II secretion system (T2SS) substrate genes also belong to the HrpG or HrpX regulon (Furutani et al. 2004; Wang et al. 2008; Yamazaki et al. 2008). T2SS substrates, including proteases, lipases, and cell-wall-degrading enzymes (CWDE), might contribute to bacterial infection by degradation of the plant cell wall. Co-regulation of T3SS and T2SS by HrpG and HrpX helps bacteria

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overcome plant defenses and acquire nutrients for growth in planta.

Although a few studies have been performed to identify genes in the HrpG or HrpX regulon, no comprehensive study has been done in genus *Xanthomonas*. In this study, we designed a genome-wide *X. axonopodis* pv. *citri* oligonucleotide microarray for the transcriptome analysis of the HrpX and HrpG regulons. Based on the microarray results, we expanded the knowledge about the HrpX and HrpG regulons with genes related to more functions such as chemotaxis and flagellar biosynthesis and transport, and a large set of unknown genes in addition to T3SS, effector, and T2SS substrate genes. We also found the cross-talk between HrpG regulatory cascade and quorum sensing (QS).

RESULT

Generation of the *hrpG* mutant.

The *hrpG* insertion mutant was generated by double cross-over homologous recombination in this study. The insertion of the kanamycin (Km) cassette in *hrpG* was confirmed by polymerase chain reaction (PCR) with three pairs of primers targeting different locations of inside and flanking regions of *hrpG*. The *hrpG* insertion mutant lost pathogenicity in grapefruit and showed no symptoms (Supplementary Fig. S1). The complementation plasmid containing the intact *hrpG* gene restored its pathogenicity in planta.

Microarray analyses and validation of microarray data.

It has been reported that, in *Xanthomonas* spp., the expression of *hrp* genes, including the regulators *hrpG* and *hrpX*, was repressed in nutrient-rich media but strongly induced in planta and in nutrient-deficient medium XVM2, which is known to mimic the intercellular space of plant cells (Wengelnik et al. 1996a). Therefore, XVM2 medium was selected to explore the HrpG and HrpX regulons of *X. axonopodis* pv. *citri*. The growth curves of both *hrpG* and *hrpX* mutants in XVM2 medium were similar to that of the wild-type strain (Supplementary Fig. S2).

In order to characterize the HrpG and HrpX regulons, microarray analyses were conducted to compare gene expression of the *hrpG* or *hrpX* mutants, respectively, with that of the wild-type strain 306 at different growth stages. The three timepoints used in this time-course study, A (optical density at 600 nm [OD₆₀₀] = 0.25), B (OD₆₀₀ = 0.4), and C (OD₆₀₀ = 0.5), represent three growth stages (two in exponential stage and one in early stationary stage) of *X. axonopodis* pv. *citri* in XVM2 medium. In this study, false discovery rate (FDR) = 0.05 and absolute value of log₂-fold change = 0.585 (equivalent to fold change of 1.5) were used as the cut-off value. For the *hrpG* mutant, 99, 28, and 174 genes showed significant expression changes at timepoints A, B, and C, respectively (Fig. 1). Similarly, 53, 63, and 159 genes were differentially expressed in the *hrpX* mutant at the three corresponding timepoints (Fig. 1). The greatest number of differentially expressed genes, and the greatest magnitude of changes, occurred at timepoint C in both mutants (Fig. 1). Overall, 232 genes belonged to the HrpG regulon, while 181 genes belonged to the HrpX regulon (Supplementary Table S1). HrpG and HrpX not only activated gene expression but also repressed gene expression. For the *hrpG* mutant, 29, 9, and 95 genes were underexpressed and 70, 19, and 79 genes were overexpressed compared with the wild-type strain at timepoints A, B, and C, respectively. Similarly, 42, 58, and 104 genes were underexpressed and 11, 5, and 55 genes were overexpressed in the *hrpX* mutant at the three corresponding timepoints (Fig. 1).

To validate the microarray data, two-step quantitative reverse-transcription (QRT)-PCR was used to verify the microarray data. Eight genes were chosen from among the genes belonging to both the HrpG and HrpX regulons to compare the data obtained from the two methods (Supplementary Table S2), which showed underexpression at timepoint C in both the *hrpG* and *hrpX* mutants in microarray analysis. The resulting transcriptional ratio from QRT-PCR analysis was log₂ transformed and compared with the average log₂ ratio values obtained by microarray analysis (Fig. 2). Although the amplitude of fold changes between the two techniques is different for different genes, the general trend of gene expression is consistent.

Fig. 1. Diagram displaying the numbers of differentially expressed genes resulting from the comparison of the *hrpG* mutant versus the wild-type strain and the *hrpX* mutant versus wild-type strain at selected timepoints. Genes with the absolute value of log₂-fold change >0.585 and false discovery rate <0.05 were selected as significantly differentially expressed genes. Black columns represent overexpressed genes in mutants and white columns represent underexpressed genes in either the *hrpX* or *hrpG* mutant. Numbers adjacent to the columns represent the numbers of genes in each condition.

Functional classification of differentially regulated genes.

The 232 genes belonging to the HrpG regulon could be assigned to 18 functional categories according to the annotation from the J. Craig Venter Institute (JCVI) role categories, including amino acid biosynthesis; biosynthesis of cofactors, prosthetic groups, and carriers; cell envelope; cellular processes; central intermediary metabolism; DNA metabolism; energy metabolism; fatty acid and phospholipid metabolism; mobile and extrachromosomal element functions; protein fate; protein synthesis; regulatory functions; transcription; transport and binding proteins; hypothetical proteins; unknown function; unclassified, and not in JCVI (which means that the gene is not assigned to any JCVI categories) (Fig. 3). The 181 genes of the HrpX regulon were assigned into the same 17 functional categories as HrpG, except mobile and extrachromosomal element functions. Approximately 53.4% (124 of 232) of genes of the HrpG regulon and 49.7% (90 of 181) genes of the HrpX regulon were in the categories of hypothetical protein, unclassified, unknown function, and not in JCVI.

Clustering analysis.

Clustering analysis was performed to group genes with a similar expression pattern. Genes with similar time-dependent regulation patterns were assigned to the specific clusters. This enables the characterization of clearer and more meaningful expression patterns from a large array of differentially regulated

genes. Accordingly, five major gene clusters were assigned (Fig. 4).

Cluster I consisted of 104 genes showing underexpression in the *hrpX* mutants at timepoint C. Of these, 73 were also underexpressed in the *hrpG* mutant at timepoint C. The genes in this cluster consist of genes encoding T3SS and T3SS effectors, T2SS substrates, and genes involved in amino acid biosynthesis, regulation pathway, and energy metabolism.

Cluster II was upregulated in both *hrpG* and *hrpX* mutants at timepoint C. This cluster is composed of 31 genes, including those related to amino acid biosynthesis, DNA metabolism, and thiamine biosynthesis, and 23 hypothetical genes.

Cluster III was upregulated only in the *hrpG* mutant at timepoint C but not in the *hrpX* mutant at any stage. It comprises 44 genes encoding two-component regulation systems, chemotaxis and bacterial motility, amino acid biosynthesis, and sugar and starch metabolism.

Cluster IV was downregulated at any of the three timepoints of the *hrpG* mutant but not in the *hrpX* mutant. It contains 44 genes, including those related to energy metabolism, cell envelope, substrate transport and binding, and regulation.

Cluster V was upregulated in the *hrpX* mutant only at timepoint C but not in the *hrpG* mutant. It includes 19 genes, consisting of those responsible for histidine biosynthesis, energy metabolism, and ABC transporter.

Genes not in clusters included 48 genes showing altered expression at least at one timepoint in either *hrpG* or *hrpX* mu-

Fig. 2. Comparison of gene expression by quantitative reverse-transcription polymerase chain reaction (QRT-PCR) and microarray. The log₂-fold change of each gene was derived from the comparison of either *hrpX* or *hrpG* mutant versus wild-type at timepoint C. Two genes, 16S rRNA and *gyrA*, were used as endogenous controls in QRT-PCR, both resulting in similar results. Only the QRT-PCR results with 16S rRNA as endogenous control are shown. Values of log₂-fold change were means of four replicates. Experiments were repeated three times with similar results and only one result was presented. Error bars indicate standard deviation. White bars represent the values from microarray and black bars represent the values from QRT-PCR.

tant. They encode proteins responsible for energy metabolism, regulation, substrate transportation, and pathogenicity factors (PthA1-4).

T3SS and T3SS effectors.

The whole *hrp* gene cluster, which contains 24 genes (XAC0393 to XAC0417), was downregulated in both *hrpG* and *hrpX* mutants. The magnitude of downregulation of *hrp* genes in the *hrpX* mutant was greater than that in the *hrpG* mutant when compared at the same growth stage in XVM2. For instance, at timepoint C, the expression of *hrp* genes was downregulated 0.94 to 2.18 log₂-fold (1.92- to 4.53-fold) in the *hrpG* mutant, whereas it was downregulated 1.23 to 5.54 log₂-fold (2.35- to 46.53-fold) in the *hrpX* mutant. The expression of most putative and known T3SS effector genes was under HrpG and HrpX regulation. Among 24 putative and known T3SS effectors found in *X. axonopodis* pv. *citri* genome (Moreira et al. 2010b), 23 effectors belong to the HrpG or HrpX regulon (Supplementary Table S3). Except for the *pthA1-4* and *avrXacE2* genes, the remaining 18 effector genes were downregulated in both mutants and grouped into cluster I. The *pthA1-4* genes showed altered expression (overexpressed) at timepoint A and B in the *hrpG* mutant while the *avrXacE2* genes was overexpressed at timepoint A in the *hrpG* mutant. HrpX did not regulate the expression of *pthA* and *avrXacE2* genes. In addition, one putative effector gene, XAC2990, was identified in this study. The protein sequence of this gene shares 45% identity to the putative T3SS effector RCFBP_mp20163 from *Ralstonia solanacearum* (Remenant et al. 2010). The products of both genes have a lipase domain that hydrolyzes ester linkages of triglycerides. It was reported that lipase acted as a virulence factor and played important roles in disease development caused by *Fusarium graminearum* (Feng et al. 2005) and *Pseudomonas aeruginosa* (Reimann et al. 1997). Two genes, XAC3225 and XACb0007, sharing 99% identity with each other and encoding transglycosylase, belong to the HrpX regulon because their expression was downregulated in the *hrpX* mutant at timepoint C. It was reported that XAC3225 shares high identity with *hopAII* of

P. syringae pv. *tomato* DC3000, which is a T3SS helper protein contributing to effector translocation (Moreira et al. 2010b; Oh et al. 2007). Mutation of XAC3225 reduced the disease symptoms on citrus (Moreira et al. 2010b).

T2SS substrates.

Eight genes (Supplementary Table S4), including *virK* (XAC0435), XAC0552, *phe-1* (XAC0661), *xcp* (XAC0795), XAC0817, *pglA* (XAC2374), XAC2831, and XAC2835, which have been confirmed to encode T2SS substrate experimentally, showed underexpression in both mutants except for *pglA* (XAC2374), which was underexpressed only in the *hrpX* mutant at timepoint C. Two of them, *pglA* (XAC2374) and *phe-1* (XAC0661), encode CWDE. In addition, 21 genes were predicted as putative T2SS substrates in this study based on the clustering with the known T2SS substrates, homology to known secreted proteins, as well as signal peptide prediction. All 21 genes encoding putative T2SS substrates were upregulated by HrpX, and 12 of them were also upregulated by HrpG. In addition, XAC2654 encoding a plant natriuretic peptide-like protein in *X. axonopodis* pv. *citri* (XacPNP) was underexpressed in both *hrpG* and *hrpX* mutants. Especially at timepoint C in the *hrpX* mutant, its expression was more than five log₂-fold lower than that in the wild-type strain.

Signal transduction and regulation.

Several genes belonging to the two-component system showed changed expression in this study (Table 1). Two genes encoding sensor kinases, XAC1488 and XAC2192, and two genes encoding response regulators, *rpjG* (XAC1877) and XAC2897, were upregulated in the *hrpG* mutant at timepoint C. Two genes (XAC1939 and XAC1940) encoding GGDEF family proteins showed an expression pattern similar to *rpjG*, which break down an important bacterial intracellular secondary messenger, 3,5-cyclic diguanylic acid (c-di-GMP). Gene *regS* (XAC1798) encoding a two-component system sensor protein showed overexpression in the *hrpG* mutant at timepoint C. Gene *stkXac1* (XAC1171) encoding a serine/thre-

Fig. 3. Distribution of genes of the HrpG and HrpX regulons that were assigned into various J. Craig Venter Institute functional categories. White and black bars represent genes in the HrpX and HrpG regulon, respectively. * indicates biosynthesis of cofactors, prosthetic groups, and carriers.

onine kinase showed underexpression in both mutants at timepoint C. Gene *phoC* (XAC4369) encoding phosphatase precursor was underexpressed in the *hrpX* mutant at timepoint C.

A few genes encoding transcriptional regulators were also differentially expressed in the study (Table 1). The expression of five genes (*rpoE* (XAC1682), XAC1555, XAC2166, XAC3445, and XAC4272) encoding one sigma factor RpoE and four transcriptional regulators was changed in the *hrpX* mutant. The expression of three transcriptional regulatory genes—XAC0917, *phoU* (XAC1573), and *flgM* (XAC1989)—was changed only in the *hrpG* mutant. Two genes encoding a transcription regulator, *pcaQ* (XAC0880) and XAC1455, showed altered expression in both *hrpX* and *hrpG* mutants.

Chemotaxis and bacterial motility.

A number of genes involved in motility and chemotaxis were regulated at transcription level in the *hrpG* mutant. The *fliJ* gene (XAC1950), one of the flagellar genes whose product responds to chemotactic stimuli, was upregulated in the *hrpG* mutant at timepoint A. Two genes, *motB* (XAC1908), encoding flagellar motor protein D, and *fliO* (XAC1945), encoding flagellar protein for flagellum apparatus, were underexpressed in the *hrpG* mutant at timepoint B. At timepoint C, eight genes were overexpressed in the *hrpG* mutant, including chemotaxis genes *cheA* (XAC2865), encoding chemotaxis histidine protein kinase, *cheW* (XAC1906), encoding a coupling protein, and *cheY* (XAC1904), encoding chemotaxis response regulator; and genes in early flagellar biosynthesis that are responsible

Fig. 4. Hierarchical clustering of genes in the HrpG and HrpX regulons based on similar time-dependent expression patterns. Clustering analysis was performed with Cluster 3.0 using complete linking with uncentered correlation distance. Columns represent individual timepoints and rows represent individual genes. Red bars indicate overexpression in mutants and green bars indicate underexpression in mutants. The color scale portrays $\log_2$ -fold change differences between the mutants and wild-type strains. Genes with the absolute values of $\log_2$ -fold change < 0.585 ($|\text{fold change}| < 1.5$) were displayed in black. Five major clusters were assigned as I, II, III, IV, and V.

for flagellum basal body assembly, such as *flgF* (XAC1982), encoding flagellar basal body rod protein, *fliF* (XAC1954), encoding flagellar M-ring protein, *fliI* (XAC1951), encoding flagellum-specific ATP synthase, and *fliG* (XAC1953) encoding flagellar motor switch protein. Gene *flgM* (XAC1989), encoding an anti- σ -28 factor that is a negative regulator of flagellin synthesis, was overexpressed in the *hrpG* mutant at timepoint C.

To test whether the expression changes of flagella genes we found from the microarray analysis led to motility changes in the *hrpG* mutant, both swarming and swimming assays were performed on XVM2 plates containing either 0.7 or 0.3% agar. The *hrpG* mutant did not show different swimming ability on XVM2 supplemented with 0.3% agar in the incubation period (data not shown). Higher swarming ability of the *hrpG* mutant was observed after 13 days of inoculation on XVM2 containing 0.7% agar (Fig. 5). This result is consistent with the microarray analysis of the *hrpG* mutant showing that most of the differentially expressed genes related to flagellar biosynthesis were changed at the early stationary stage.

Amino acid biosynthesis.

Sixteen genes related to amino acid biosynthesis showed altered expression in this study. Eight genes (XAC1828 to XAC1835) involved in histidine biosynthesis were upregulated only in the *hrpX* mutant at timepoint C. Two genes, *ilvC* (XAC3451) and *ilvG* (XAC3452), involved in valine, leucine, and isoleucine biosynthesis showed an expression pattern similar to that of histidine biosynthesis genes. Three genes—*metE* (XAC0336), involved in cysteine and methionine metabolism; *dapA* (XAC1760), involved in lysine biosynthesis; and *asnB* (XAC1433), involved in alanine, aspartate, and glutamate me-

tabolism—showed expression changes in the *hrpG* mutant. Two genes involved in phenylalanine, tyrosine, and tryptophan biosynthesis—*tyrA* (XAC1525) and *pheA* (XAC3647)—showed expression changes in both *hrpX* and *hrpG* mutants. PheA is a chorismate mutase, which is a key enzyme in the

Fig. 5. Swarming assay on XVM2 medium containing 0.7% agar. Black columns represent wild-type strain, gray columns represent the *hrpG* mutant, and white columns indicate the *hrpX* mutant. dpi = days postinoculation. Experiments were performed in quadruplicate and repeated three times with similar results; only one representative result was presented.

Table 1. Regulatory genes under the control of HrpG and HrpX

JCVI ^b	Locus tag	Gene	Log ₂ ratio of fold change ^a						Product description
			hrpGA/WA	hrpGB/WB	hrpGC/WC	hrpXA/WA	hrpXB/WB	hrpXC/WC	
Both									
R	XAC1171	<i>stkXacI</i>	0.984	NS	−1.031	NS	−0.778	−2.025	Serine/threonine kinase
R	XAC0880	<i>pcaQ</i>	0.675	NS	−0.854	NS	−0.883	−1.999	Transcriptional regulator
R	XAC1455	...	0.638	NS	NS	NS	NS	−1.195	MarR family transcriptional regulator
HrpG									
T	XAC1320	...	NS	NS	−0.716	NS	NS	NS	Regulatory protein
U	XAC1939	...	NS	NS	0.652	NS	NS	NS	GGDEF family protein
R	XAC3771	...	NS	NS	0.675	NS	NS	NS	Hypothetical protein; pfam03466-LysR substrate binding domain ^c
R	XAC2192	...	NS	NS	0.759	NS	NS	NS	Two-component system sensor protein
R	XAC1877	<i>rpfG</i>	NS	NS	0.780	NS	NS	NS	Response regulator
U	XAC1940	...	NS	NS	0.817	NS	NS	NS	GGDEF family protein
R	XAC1488	...	NS	NS	0.951	NS	NS	NS	Sensor histidine kinase
R	XAC1989	<i>flgM</i>	NS	NS	0.995	NS	NS	NS	Flagellar protein
R	XAC2897	...	NS	NS	1.128	NS	NS	NS	Response regulator
R	XAC1573	<i>phoU</i>	NS	NS	1.139	NS	NS	NS	Phosphate regulon transcriptional regulator
R	XAC1798	<i>regS</i>	NS	0.601	NS	NS	NS	NS	Two-component system sensor protein
R	XAC0917	...	−0.734	NS	NS	NS	NS	NS	Transcriptional regulator
HrpX									
R	XAC2166	...	NS	NS	NS	NS	NS	−1.021	Transcriptional regulator
R	XAC2163	...	NS	NS	NS	NS	NS	−0.742	Hypothetical protein; pfam07969-amidohydrolase family ^c
R	XAC3445	...	NS	NS	NS	2.221	2.879	3.457	Transcriptional regulator
R	XAC3446	...	NS	NS	NS	−0.708	NS	NS	Hypothetical protein; pfam00486-transcriptional regulatory protein, C terminal ^c
R	XAC4272	...	NS	NS	NS	−0.617	NS	NS	LacI family transcription regulator
R	XAC1555	...	NS	NS	NS	−0.653	NS	NS	Transcriptional regulator
T	XAC1682	<i>rpoE</i>	NS	NS	NS	NS	NS	−0.822	RNA polymerase σ-E factor

^a Fold change was derived from mutants versus wild type at timepoints A, B, and C. NS = not significantly differentially expressed ($|\log_2\text{-fold change}| < 0.585$ or false discovery rate > 0.05).

^b J. Craig Venter Institute (JCVI) functional categories for HrpG and HrpX regulon (Both) or HrpG and HrpX regulons individually. Categories: R = regulatory functions, T = transcription, and U = unknown function.

^c Pfam analysis of the hypothetical proteins.

shikimate pathway related to aromatic amino acid synthesis. It was reported that chorismate mutase was either translocated to the periplasm or secreted in some pathogenic bacteria (Calhoun et al. 2001; Sasso et al. 2005; Xia et al. 1993). The chorismate mutase found in *X. oryzae* pv. *oryzae* XKK.12 is involved in virulence on its host rice (Degrassi et al. 2010).

General metabolism and transport.

Many genes involved in general metabolism are regulated by HrpG and HrpX, including those involved in energy metabolism, fatty acid and phospholipid metabolism, and sugar transport. Three genes—*GNL* (XAC0548), *fruK* (XAC2502), and *kdgK* (XAC0143)—involved in the pentose phosphate pathway were differentially expressed in the *hrpG* mutant. Three genes encoding cytochrome C oxidase, which is the complex IV in oxidative phosphorylation, were underexpressed in both mutants at timepoint A. The *atpB* (XAC3655) gene in oxidative phosphorylation was overexpressed only in the *hrpX* mutant at timepoint C. Three genes in fatty acid and phospholipid metabolism—*accD* (XAC0264), *fadE9* (XAC1313), and *paaF* (XAC1314)—were overexpressed in the *hrpG* mutant at timepoint C. Gene *paaF* also showed overexpression in the *hrpX* mutant at timepoint A.

Thirteen genes encoding transport and binding proteins were differentially expressed in either *hrpG* or *hrpX* mutant. Four genes encoding TonB-dependent receptors (XAC3050, XAC3489, XAC3444, and XAC1143) showed differential expression. These proteins are assembled in the outer membrane of gram-negative bacteria and are mainly known in the transport of iron-siderophore complexes and vitamin B12 into the periplasm (Postle and Kadner 2003). In recent studies, novel substrates of TonB-dependent receptors were revealed, such as nickel, zinc, maltodextrin, and sucrose (Blanvillain et al. 2007; Neugebauer et al. 2005; Schauer et al. 2007; Stork et al. 2010). XAC3489 shares 93% identity at the amino acid sequence with XCC3358, which plays a major role in pathogenicity. The XCC3358 insertion mutant of *X. campestris* pv. *campestris* showed delayed symptom development compared with the wild-type strain (Blanvillain et al. 2007). A few genes encoding transporters were differentially expressed in either the *hrpG* or *hrpX* mutant, such as sugar transporters (e.g., *fucP* [XAC1556], *suc1* [XAC3488], and *fruA* [XAC2503]), K⁺ transporter *kdpC* (XAC0758), ABC transporter components (XAC 0827 to XAC0828), and MFS transporter (XAC1705).

DISCUSSION

DNA microarray has been widely used to study the transcriptional responses of many organisms to genetic and environmental perturbations (Dharmadi and Gonzalez 2004; Ye et al. 2001). In this study, we developed the first whole-genome DNA microarray for *X. axonopodis* pv. *citri*. In previous studies, two gene-array platforms for expression analysis have been designed and have played important roles in characterization of the pathogenicity and virulence of *X. axonopodis* pv. *citri* (Astua-Monge et al. 2005; Moreira et al. 2010a). However, both previous gene arrays represented part of the genome. The first *X. axonopodis* pv. *citri* gene array is a macroarray that only contains 279 *X. axonopodis* pv. *citri* genes associated with pathogenicity and virulence (Astua-Monge et al. 2005), while the second *X. axonopodis* pv. *citri* gene array only consists of 2,365 open reading frames (ORF), which correspond to 52.7% of the annotated ORF of the *X. axonopodis* pv. *citri* genome (Moreira et al. 2010a). The microarray developed in our study represents all 4,427 annotated protein-coding ORF. In addition, we used an 8-by-15-K format so that eight microarrays could be printed on a single slide, which will significantly

reduce the variation when multiple slides are used (Tseng et al. 2001). The reliability of this array has been proven with the following evidence: i) gene expression of all selected genes in the microarray result was validated in QRT-PCR (Fig. 2); ii) T3SS and T3SS effector genes that have been shown to be regulated by HrpX and HrpG (Wengelnik and Bonas 1996; Wengelnik et al. 1996b, 1999) were identified in our study (Fig. 4); and iii) T2SS substrate genes that have been shown to be controlled by HrpX and HrpG were confirmed using this microarray. Thus, our microarray provides a robust and comprehensive tool for transcriptome analysis of *X. axonopodis* pv. *citri*.

HrpX and HrpG play important roles in coordinating different categories of genes. First, they regulate pathogenicity and virulence genes to overcome the plant defense responses and survive in the intercellular spaces. The most well-known HrpG- and HrpX-dependent virulence factors were distributed in cluster I (Fig. 4), including T3SS (*hrp* genes), T3SS effectors, T2SS substrates, and XacPNP. The major roles of T2SS substrates include degradation of the plant cell wall, cytotoxicity, adherence, spread, and transmission (Cianciotto 2005), which might facilitate the assembly of extracellular appendages of secretion systems such as T3SS and, therefore, promote pathogenesis. T3SS translocates the T3SS effector proteins into plant cells that either suppress the host defense or interfere with host cellular processes. For instance, some T3SS effectors act as cysteine proteases in plants such as XopD, a member of the YopJ/AvrRxv family (Grant et al. 2006; Noël et al. 2002). Members of the AvrBs3/PthA family of transcriptional factors directly interact with the host transcription to modulate the host gene expression (Schornack et al. 2006). Our microarray data showed that *pthA1-4* genes were not controlled by HrpX. However, the expression of *pthA1-4* genes was induced in the *hrpG* mutant at timepoint A and B. *X. axonopodis* pv. *citri* employs XacPNP to mimic host PNP and to systemically regulate plant homeostasis, resulting in a suppressed host defense responses and better survival environment for bacteria (Gottig et al. 2009). Importantly, a significant induction of *hrpG* expression was observed when *Xanthomonas* spp. enter the plant apoplast (Zhang et al. 2009). Thus, HrpG coordinates multiple virulence factors in the *X. axonopodis* pv. *citri* infection process. Second, in addition to manipulation of plant defenses, *X. axonopodis* pv. *citri* has to adapt its metabolism to the intercellular spaces, which are nutrient poor (Alfano and Collmer 1996) and laden with toxic substances (either preformed or induced) as part of the host defense responses (Osborn 1996; Segura et al. 1999). Multiple intracellular metabolic activities, such as amino acid biosynthesis; oxidative phosphorylation; pentose-phosphate pathway; transport of sugar, iron, and potassium; and the phenolic compounds catabolism pathway are regulated through HrpX and HrpG to help the bacteria to overcome environmental stresses. *X. axonopodis* pv. *citri* activates the gene expression of sugar transporters through HrpX and HrpG in order to uptake more sugars as a carbon and energy source, particularly sucrose, glucose, and fructose, which are the most common sugars in the plant apoplast (Joosten et al. 1990; Rico et al. 2009). The activation of a phenolic catabolism pathway could convert toxic plant chemicals to less toxic compounds or even carbon sources. Third, we also identified a large set of unknown genes controlled by HrpG and HrpX. In all, 53.4% (124 of 232) of genes in the HrpG regulon and 49.7% (90 of 181) of genes in the HrpX regulon encode unknown proteins. Their roles are unknown in *X. axonopodis* pv. *citri* infection. Nonetheless, the list might provide targets for further study. Among the unknown proteins, some seem to play important roles in *X. axonopodis* pv. *citri* virulence. For example, one putative

effector gene, XAC2990, was predicted in this study. The protein sequence of this gene shares 45% identity with the putative T3SS effector RCFBP_mp20163 from *R. solanacearum* (Remenant et al. 2010).

The regulation of HrpX and HrpG on multiple cellular activities is through both direct and indirect controls (Fig. 6). For the T3SS and T3SS effector genes, five T3SS genes and eight T3SS effector genes have been shown to contain PIP boxes in their promoter regions (da Silva et al. 2002). HrpX was shown to interact with the PIP box (Koebnik et al. 2006). For other genes controlled by HrpX and HrpG but without PIP box, one putative explanation is that HrpX and HrpG control multiple regulatory genes (Table 1; Fig. 6), which, in turn, regulate those genes. In total, HrpX and HrpG manipulate the gene expression of 21 regulatory genes and one sigma factor gene (Table 1). Among the regulatory genes controlled by HrpG, *rpfG*

encodes a response regulator (discussed below) in the diffusible signal factor (DSF)-mediated QS system (Barber et al. 1997; Tang et al. 1991). RpfG was reported to regulate genes involved in synthesis of extracellular enzymes and exopolysaccharide (EPS) and biofilm formation (Slater et al. 2000). The regulation of flagellar genes might be related to the regulation of *flgM* and XAC0917 by HrpG. Gene *flgM* (XAC1989) over-expressed in the *hrpG* mutant at the early stationary stage, which encodes an anti- σ -28 factor that is involved in temporal regulation of flagellar biosynthesis (Yang et al. 2009). XAC0917 is a TetR family member. It was reported that FhrR, a TetR family member, positively regulates expression of the genes encoding flagellar and ribosomal protein genes and negatively regulates expression of *hrp* genes under the control of Clp in *X. campestris* pv. *campestris* (He et al. 2007). The *phoU* gene encodes a transcriptional regulator, which partici-

Fig. 6. Schematic model of HrpG- and HrpX-related regulation cascades of *Xanthomonas* genus. The environmental or plant signals activate the unknown sensor protein which relays the signal to the master regulator HrpG. The activated HrpG is involved in four major aspects of regulatory cascades: 1) Inducing the transcription of HrpX, which in turn activates the expression of a large set of virulence genes such as T3SS, T3SS effector, and T2SS substrate genes. HrpX represses the transcription of genes involved in histidine biosynthesis at the same time. During this process, HrpX also regulates the transcription of some genes involved in signal transduction and regulation such as transcriptional factors, as well as transport which may result in change of nutrient uptake and energy metabolism. 2) HrpG represses the transcription of genes involved in chemotaxis and flagellar biosynthesis in order to repress bacterial motility at the early stationary stage of growth. 3) HrpG also controls the expression of genes related to signal transduction and regulation, transport and general metabolism. 4) HrpG cross-talks with DSF-mediated quorum sensing pathway via repressing the transcription of RpfG and two GGDEF proteins at the early stationary stage of growth. Those proteins contribute the concentration of the central messenger cyclic-di-GMP. The high levels of c-di-GMP promote biofilm formation, while low levels promote motility and transcription of virulence factors. One important target of c-di-GMP is Clp which is a transcriptional activator. C-di-GMP binds to the Clp to prevent it from DNA binding and the expression of genes encoding extracellular enzymes, and genes involved in T3SS, and EPS biosynthesis. Two transcriptional factors, FhrR and Zur are under the control of Clp and they have regulatory effect on expression of the *hrp* genes. Blue lines indicate both positive and negative regulations occurring in different genes of the listed functional categories. + and – represent positive and negative transcriptional regulation, respectively.

pates in phosphate transport and also negatively controls the expression of phosphate ABC transporter genes (Muda et al. 1992). The *stkXacI* (XAC1171) gene encodes a serine/threonine kinase, which plays important roles in a multitude of cellular processes, including division, proliferation, apoptosis, and differentiation (Manning et al. 2002). The HrpX and HrpG regulons also manipulate the gene expression of multiple regulators, including *pcaQ* (XAC0880), XAC1455, XAC1555, XAC4272, XAC3445, and XAC2166, which have not been studied extensively in *X. axonopodis* pv. *citri*. However, their homologs have been studied in other bacteria. For example, the homolog of PcaQ (XAC0880), a LysR-family transcription regulator, activated the expression of the *pcaDCHGB* operon, which encodes enzymes involved in phenolic compounds catabolism in *Agrobacterium tumefaciens* A348, (Parke 1996). In agreement with the regulation of PcaQ, two genes encoding subunits of protocatechuate 4,5-dioxygenase, *pcaH* (XAC0878) and *ligA* (XAC0879), were underexpressed in both *hrpX* and *hrpG* mutants. Protocatechuate 4,5-dioxygenase is the first enzyme in the protocatechuate branch of the β -ketoadipate pathway to use aromatic compounds as carbon sources (Parke 1996). XAC1455 shares 85% identity of amino acid sequence with XC_2827, which belongs to the MarR family transcriptional regulator in *X. campestris* pv. *campestris* 8004. The product of XC_2827, designated as HpaR, is involved in hypersensitive response, pathogenicity, and extracellular protease production in *X. campestris* pv. *campestris* and was positively regulated by HrpG and HrpX (Wei et al. 2007). Thus, it seems that HrpX and HrpG control multiple cellular activities through multiple regulatory genes.

Our data suggest that a cross-talk exists between HrpG and the QS system in *X. axonopodis* pv. *citri*. The *rpfG* gene (XAC1877) was upregulated at the early stationary stage in the *hrpG* mutant. Two GGDEF genes showed expression patterns similar to *rpfG*, which break down an important bacterial intracellular secondary messenger, c-di-GMP. RpfG is the response regulator in the DSF-mediated QS system, which mediates virulence, biofilm formation, and many cellular activities in *Xanthomonas* spp. (Barber et al. 1997; Tang et al. 1991). RpfG contains an HY-GYP domain, which interacts with GGDEF proteins to mediate signal transduction to c-di-GMP. The cross-talk between DSF-mediated QS and the HrpG regulon was also found in genome-scale analysis of the DSF regulon in *X. campestris* pv. *campestris* (He et al. 2006, 2007). In addition, for the *hrpG* mutant, 99, 28, and 174 genes showed significant expression changes at timepoints A, B, and C, respectively (Fig. 3). Similarly, 53, 63, and 159 genes were differentially expressed in the *hrpX* mutant at the three corresponding timepoints (Fig. 3). The temporal changes of gene regulation might be related to QS. QS is likely to be involved in controlling the timing and level of gene expression in the HrpG regulon. Bacteria might monitor cell density and host stimuli by timing the expression of virulence factors such as T3SS. The messenger c-di-GMP might be the molecule that links QS and the HrpG regulatory cascade. RpfG and GGDEF proteins control the concentration of the central messenger, c-di-GMP. The high levels of c-di-GMP promote biofilm formation, whereas low levels promote motility and transcription of virulence factors (Römling et al. 2005; Simm et al. 2004; Tischler and Camilli 2004). One important target of c-di-GMP is the cAMP receptor protein-like protein (Clp), which is a transcriptional activator. C-di-GMP binds to the Clp to prevent DNA binding and the expression of genes encoding extracellular enzymes and genes involved in T3SS and EPS biosynthesis (He et al. 2007). Two transcriptional factors, FhrR and Zur, were identified in the transcriptomic analysis of Clp regulons of *X. campestris* pv. *campestris* (He et al. 2007). FhrR controls the

expression of genes encoding flagellar, T3SS, and ribosomal proteins, while Zur regulates genes involved in iron uptake, multidrug resistance, and detoxification (Huang et al. 2009). Therefore, it is possible that bacteria temporally regulate the cellular functions to adapt to different stages of growth or infection through multiple signaling cascades, including the QS system.

Comparison of the HrpX and HrpG regulons at different timepoints gives us an insight into the dynamic expression of common genes shared by both regulons. In all, 33 genes were shared by the two regulons at timepoint A and 9 genes at timepoint B, whereas 104 common genes were found at timepoint C (Supplementary Table S5). In general, 123 genes were overlapped in the two regulons at any of the three timepoints, which corresponds to 68% of the HrpX regulon (123 of 181), and 53% of the HrpG regulon (123 of 232). The most common genes were grouped into cluster I, which contains genes encoding T3SS, T3SS effectors, and T2SS substrates. Interestingly, 58 genes in the HrpX regulon are HrpG independent, while 109 genes in the HrpG regulon are HrpX independent. As to the HrpG-independent genes in the HrpX regulon, further analysis revealed that 23 of them showed subtle expression changes (the absolute value of $\log_2$ -fold change < 0.585), with statistical significance FDR < 0.05 in the *hrpG* mutant in at least one of the three timepoints (particularly 17 of the 23 genes are in cluster I). The expression of genes in cluster I responded to *hrpX* mutation more rapidly and dramatically than to HrpG mutation based the $\log_2$ -fold value (Fig. 4). Thus, those 23 genes might also belong to the HrpG regulon but have weaker responses to HrpG. For the remaining 35 HrpG-independent genes in the HrpX regulon, one possibility is that the *hrpX* gene is regulated by other regulators besides HrpG. For instance, it was reported that Zur in *X. campestris* pv. *campestris* 8004 positively controls *hrp* gene expressions via HrpX (Huang et al. 2009). The HrpX-independent genes in the HrpG regulon were as we expected because HrpG is upstream of HrpX in the regulatory cascade. These genes in the HrpG regulon include 10 regulatory genes and 11 genes related to chemotaxis and flagellar biosynthesis. Those regulatory genes contribute to controlling the large set of genes in the HrpG regulon and also indicate that HrpG is a master regulator in the pathogenesis process of *X. axonopodis* pv. *citri*. In addition, we confirmed the repression effect of HrpG on bacterial motility due to the altered expression of genes involved in chemotaxis and flagellar biosynthesis by testing the motility of the *hrpG* mutant (Fig. 5).

This study is the first exhaustive genome-wide analysis of HrpG and HrpX regulons in the genus *Xanthomonas*. We identified a large set of HrpX- or HrpG-dependent unknown or hypothetical proteins that might be candidate virulence genes, particularly multiple ORF in cluster I. This study also provides us a global view of regulation cascades that control the expression of multiple virulence genes, including T3SS, effector, and T2SS substrate genes, during infection. Further microarray analysis of the mutants of the regulatory genes identified in this study will help us explore the regulation circuits of HrpG and HrpX regulons.

MATERIALS AND METHODS

Strains and growth conditions.

All of the strains used in this study are listed in Supplementary Table S6. *X. axonopodis* pv. *citri* wild-type strain 306 was provided by the Division of Plant Industry of the Florida Department of Agriculture and Consumer Services, Gainesville, U.S.A. The *X. axonopodis* pv. *citri* *hrpG* mutant was generated in this study as described below and the *hrpX* mutant was gen-

erated and confirmed as described in the previous study (Figueiredo et al. 2011). Wild-type strain 306 and mutant strains were grown in nutrient broth (NB), on nutrient agar (NA), or in nutrient yeast glycerol (NYG) medium (Daniels et al. 1984) at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. Antibiotics were used at the following concentrations: rifamycin (Rif), 50 µg/ml; Km, 50 µg/ml; ampicillin (Ap), 50 µg/ml; spectinomycin (Sp), 50 µg/ml; gentamicin (Gm), 5 µg/ml; and chloramphenicol (Cm), 35 µg/ml.

Generation of the *hrpG* mutant.

The 1,180-bp Km-resistance gene *kanR* gene was isolated from pBSL15 plasmid by *EcoRI* digestion and ligated with similarly digested pGEM-T easy vector. The plasmid was named pUSS01-1 when the *kanR* and *lacZ* were in the same transcription direction, and the plasmid was named pUSS01-2 when they were not in the same transcription direction. The pUSS01-2 was chosen for further manipulation. The 693-bp upstream region of *hrpG* (86 bp at 5' of *hrpG*) was amplified using genomic DNA of *X. axonopodis* pv. *citri* 306 as template and primers AChrpG-Nde1F3 and AChrpG-NSi1R2, then cleaved with *NdeI*-*NsiI*. The *NdeI*-*NsiI* fragment was ligated into similarly digested pUSS01-2. The resulting construct was named phrpGDV1-3', which contains the upstream sequence of *hrpG* downstream of the Km cassette. The 834-bp fragment containing a 436-bp region downstream of and 398 bp of coding region of *hrpG* was obtained by *Apal*-*SphI* digestion of the 1,200-bp fragment amplified using genomic DNA of strain 306 as template and primers AChrpG-ApalF2 and AChrpG-R1. Then, the 834-bp fragment was cloned into phrpGDV1-3' and located upstream of the Km cassette, which resulted in the construct phrpGDV1 carrying a mutated *hrpG* fragment consisting of the Km cassette flanked by *hrpG* upstream and downstream fragments. The *hrpG* disruption fragment was cleaved out with *Bam*HI from phrpGDV1 and ligated with *Bam*HI-cut pOK1. The resulting recombination construct, named phrpGDV2, was transformed into *E. coli* DH5αλPIR (Huguet et al. 1998) and subsequently transformed into strain 306. Transconjugants were selected on NYG medium supplemented with Rif and Sp. Positive colonies were replicated on both NA plates supplemented with 5% (wt/vol) sucrose, Sp, and Rif and NA with only Rif. The sucrose-sensitive colonies were selected from NA Rif plate and dilution-plated on NA containing Rif, Km, and 5% sucrose to select for resolution of the construct by a second cross-over event. The resulting *hrpG* mutant was confirmed by PCR analysis with three pairs of primers: AChrpG-Kpn1F4 and AChrpG-Kpn1R4, AChrpG-ApalF2 and AChrpG-SphR3, and AChrpG-F1 and AChrpG-NSi1R2.

Complementation of the *hrpG* mutant.

The entire *hrpG* gene, with a 738-bp upstream sequence and 399-bp downstream sequence, was amplified from genomic DNA of *X. axonopodis* pv. *citri* wild-type strain 306 using PCR with primers AChrpG-Kpn1F4 and AChrpG-Kpn1R4, which contain a *KpnI* restriction site. The resulting 1,929-bp fragment was ligated to PCR 2.1-TOPO following the manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.), resulting in phrpG. From phrpG, a *KpnI* fragment containing the *hrpG* gene was isolated and cloned into similarly digested pUFR053 that was treated with calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA, U.S.A.) (El Yacoubi et al. 2007), resulting in phrpGC. The plasmid phrpGC was transferred into the *hrpG* mutant by triparental mating with helper *E. coli* strain containing pRK2013 (Swarup et al. 1991). The transconjugants were selected on NA with Rif and Gm. The presence of phrpGC was verified using PCR.

Pathogenicity assay.

Pathogenicity assays were conducted in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. Assays were performed using fully expanded, immature leaves of Duncan grapefruit (*Citrus paradisi* Macfadyen). *X. axonopodis* pv. *citri* wild-type and mutant strains used in this assay were grown with shaking overnight at 28°C in NB, centrifuged down, and suspended in sterile tap water, and the concentrations were adjusted to 10⁸ CFU/ml. For the pathogenicity assays, bacterial solutions of both 10⁸ and 10⁵ CFU/ml were infiltrated into leaves with needleless syringes (Rybak et al. 2009; Vilorio et al. 2004). The test was repeated three times with similar results. Disease symptoms were photographed at 5, 10, and 12 days postinoculation.

RNA extraction.

Single bacterial colonies were picked and grown in 5 ml of NB at 28°C for 24 h with shaking, then transferred into 50 ml of NB for overnight incubation. The bacterial cultures in middle exponential stage were centrifuged down, washed with XVM2 medium once, and inoculated in XVM2 medium with an initial concentration at OD₆₀₀ = 0.03. Bacteria were grown in XVM2 medium with shaking at 200 rpm at 28°C and samples of culture were collected at three different timepoints: OD₆₀₀ at 0.25, 0.4, and 0.5 (marked as A, B, and C, respectively) according to the growth curve in XVM2. Four biological replicates were used for each strain per timepoint. RNA was stabilized immediately by mixing bacterial culture with two volumes of RNeasy Protect Bacteria reagent (Qiagen, Valencia, CA, U.S.A.) and incubated at room temperature for 5 min. Bacterial cells were centrifuged at 5000 × g for 10 min and cell pellets were used for RNA extraction.

Cell pellets were treated with lysozyme and RNA extractions were performed using a RiboPure bacteria kit (Ambion, Austin, TX, U.S.A.). Contaminated genomic DNA was removed from RNA by treatment with a TURBO DNA-free kit (Ambion). RNA quantity was initially determined on a ND-8000 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.).

X. axonopodis pv. *citri* oligonucleotide microarray design.

Based on the annotated genome sequence of *X. axonopodis* pv. *citri* 306 (da Silva et al. 2002), 8-by-15-K DNA microarray chips covering the whole genome of *X. axonopodis* pv. *citri* 306 were designed using Agilent's eArray custom design tool and produced by Agilent Technologies. Each of 4,427 protein-coding genes has three unique optimized 60-mer oligonucleotide probes in microarray. Agilent's standard controls were printed on the array as well, allowing for measurement of proper hybridization conditions.

Microarray hybridization.

Microarray analysis using the Agilent microarray platform was performed at Interdisciplinary Center for Biotechnology Research Microarray Core Facility, University of Florida. Labeled cDNA was generated using a Fairplay III microarray labeling kit (Agilent Technologies). Total RNA input (5 µg) was used to generate labeled cDNA according to the manufacturer's protocol. Briefly, cDNA was synthesized from 5 µg of the total RNA with AffinityScript HC and random primer and then modified cDNA was labeled with either cy3 or cy5; labeled cDNA was purified following the manufacturer's instructions. The microarray analysis was performed using the Agilent 8-by-15-K *X. axonopodis* pv. *citri* genome array. Four independent biological replicates were performed for three time-

point comparisons with dye-swap design. A total of 300 ng of labeled cDNA per sample was used for the hybridization. A dye swap was performed to remove any bias from the labeling dyes. Hybridization was performed using a Gene Expression Hybridization Kit (Agilent Technologies) according to user's manual and was done at hybridization oven for 17.5 h at 65°C. The arrays were washed according to the manufacturer's recommended protocols. Briefly, arrays were washed with gene expression wash buffer 1, containing 0.005% Triton X-102, for 1 min at room temperature and were then washed with 37°C-warmed gene expression wash buffer 2, containing 0.005% Triton X-102, for 1 min and were dried by the Agilent Stabilization and Dyeing Solution. The arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C; Agilent Technologies). The data were extracted from a scanned image using Feature Extraction 10.1.1.1 software (Agilent Technologies).

Microarray data analysis and statistical methods.

The raw data were imported into R environment and statistical tests were performed using BioConductor statistical software, which is an open source and open development software project for analysis of microarray and other high-throughput data based primarily on the R programming language (Gentleman et al. 2004). Data preprocessing and normalization were performed using the Linear Models for Microarray Data (LIMMA) package (Smyth 2004). Raw mean signal intensities from all microarray spots were background corrected and normalized using within-array lowess approach. Log₂-transformed values were used for statistical analysis. Histograms, box plots, and pairwise scatter plots were generated to examine data quality and comparability. A linear modeling approach and the empirical Bayes statistics as implemented in the LIMMA package (Smyth 2004) were then employed for differential expression analysis. The *P* values were adjusted using the Benjamini and Hochberg method, designated as FDR (Benjamini and Hochberg 1995). Differentially expressed genes were ranked based on FDR, and genes with FDR < 0.05 and a minimum absolute value of log₂-fold-change > 0.585 (equivalent to 1.5-fold) were considered to be significantly differentially expressed. If the gene had three probes and only one was filtered, the gene was removed from further analysis. The log₂-fold change values of the differentially expressed genes were averaged from the values of the two or three probes of the corresponding genes. Annotations for the differentially expressed genes were extracted from the Integrated Microbial Genome database and the JCVI database and manually verified. Hierarchical clustering analysis was performed with Cluster 3.0 using complete linking with uncentered correlation distance (de Hoon et al. 2004). The resulting dendrograms were visualized using Java Treeview software (Saldanha 2004).

All primary data from transcriptome experiments as well as experimental protocols used are available from Gene Expression Omnibus datasets, the National Center of Biotechnology Information (accession number GSE24016).

QRT-PCR.

To verify the microarray result, QRT-PCR assays were conducted using the same set of RNA for microarray analysis. Aliquot RNA samples (1 µg) used for microarray were reverse transcribed using a RETROscript kit with random hexamer primers (Ambion) for two-step QRT-PCR. Gene-specific primers were designed to generate products 100 to 250 bp in length from sequences on *X. axonopodis* pv. *citri* genome using DNASTAR software (DNASTAR, Madison, WI, U.S.A.). Real-time PCR was performed for all four biological replicates

obtained at timepoint C on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) using a QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's instructions. The 16S rRNA and *gyrA* were used as endogenous controls. The relative fold change of gene expression was calculated by using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The values of fold change were log₂ transformed to compare with values generated from microarray analysis.

Motility assays.

The media for motility assays was XVM2 containing 0.3% agar for swimming and 0.7% agar for swarming. Bacteria were grown in NB overnight with shaking at 200 rpm, and then centrifuged down, washed, and diluted to OD₆₀₀ = 0.3 in sterile water. Suspension (1 µl) was spotted on the center of each swimming or swarming plate and incubated at room temperature. The assay was repeated three times independently in quadruplicate.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BioConductor website: www.bioconductor.org
 Gene Expression Omnibus (GEO) database: www.ncbi.nlm.nih.gov/geo
 The Integrated Microbial Genome (IMG) *Xanthomonas axonopodis* pv. *citri* 306 genome database:
img.jgi.doe.gov/cgi-bin/pub/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=637000342
 The J. Craig Venter Institute (JCVI) *Xanthomonas axonopodis* pv. *citri* 306 genome database:
cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=ntxa01
 Pfam website: pfam.janelia.org
 Phobius website: <http://phobius.sbc.su.se>