High-Throughput Screening and Analysis of Genes of *Xanthomonas citri* subsp. *citri* Involved in Citrus Canker Symptom Development

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Citrus canker is caused by *Xanthomonas citri* subsp. *citri* and is one of the most devastating diseases on citrus plants. To investigate the virulence mechanism of this pathogen, a mutant library of strain 306 containing approximately 22,000 mutants was screened for virulence-deficient mutants in grapefruit (*Citrus paradise*). Eighty-two genes were identified that contribute to citrus canker symptom development caused by *X. citri* subsp. *citri*. Among the 82 identified genes, 23 genes were classified as essential genes, as mutation of these genes caused severe reduction of bacterial growth in M9 medium. The remaining 59 genes were classified as putative virulence-related genes that include 32 previously reported virulence-related genes and 27 novel genes. The 32 known virulence-related genes include genes that are involved in the type III secretion system (T3SS) and T3SS effectors, the quorum-sensing system, extracellular polysaccharide and lipopolysaccharide synthesis, and general metabolic pathways. The contribution to virulence by nine genes (*pthA4*, *trpG*, *trpC*, *purD*, *hrpM*, *peh-1*, XAC1230, XAC1548, and XAC3049) was confirmed by complementation assays. We further validated the mutated genes and their phenotypes by analyzing the EZ-Tn5 insertion copy number using Southern blot analysis. In conclusion, we have significantly advanced our understanding of the putative genetic determinants of the virulence mechanism of *X. citri* subsp. *citri* by identifying 59 putative virulence-related genes, including 27 novel genes.

Plant pathogenic bacteria of the genus *Xanthomonas* cause a variety of diseases in economically important crop plants worldwide (Subramoni et al. 2006). *X. citri* subsp. *citri* (syn. *X. axonopodis* pv. *citri*, *X. campestris* pv. *citri*, or *X. citri*) is the causative agent of citrus bacterial canker disease, which is distributed in many tropical and subtropical citrus growing areas and has a significant impact on citrus markets and trade worldwide (Civerolo 1984; Graham et al. 2004). Citrus-producing areas without citrus canker consider *X. citri* subsp. *citri* a quarantine pest and subject it to strict international regulation. *X. citri* subsp. *citri* is spread primarily by rain splash; it enters its hosts through openings such as stomata and wounds and forms distinctive raised, necrotic lesions surrounded by oily, water-

soaked margins and yellow chlorotic rings on leaves, stems, and fruits (Brunings and Gabriel 2003; Stall and Seymour 1983). Eventually, the epidermis ruptures, and massive numbers of pathogen cells emerge to the plant surface, where the bacteria are readily available for rain splash and repeated infection cycles (Duan et al. 1999). Severe invasion by the pathogen leads to defoliation, dieback, and fruit-drop, and the infected fruit is less valuable or completely unmarketable (Gottwald et al. 2002). However, no efficient, environmentally friendly treatment has been developed to control the disease once the pathogen has been established (Graham et al. 2004).

Intensive studies of the molecular virulence mechanism of the citrus canker pathogen have been reported (Brunings and Gabriel 2003; Moreira et al. 2010). PthA, the type III secretion system (T3SS) effector, is a canker pathogenicity determinant and is widely present in the *Xanthomonas* spp. that causes citrus canker disease (Cubero and Graham 2002; Swarup et al. 1992). The exogenous insertion of the *pthA* gene confers the ability to elicit canker disease in citrus plants to *X. axonopodis* pv. *citrumelo*, which causes citrus bacterial spot disease without eliciting the erumpent lesions (Swarup et al. 1991). Transient expression of *pthA* in host plant cells was sufficient to induce citrus canker symptoms including hypertrophy, hyperplasia, and cell death (Duan et al. 1999). PthA is transported via the T3SS, which comprises the products of the *hrp* gene cluster. The transcriptional expression of *hrp* genes is induced in planta and is regulated by the HrpG and HrpX regulators (Alegría et al. 2004; Guo et al. 2011; Wengelnik and Bonas 1996; Wengelnik et al. 1996), and mutations in the *hrpG* or *hrpX* genes in *X. citri* subsp. *citri* resulted in a loss of pathogenicity in citrus (Guo et al. 2011; Laia et al. 2009). In addition to the *pthA* and *hrp* genes, other virulence-related genes are required for *X. citri* subsp. *citri* to cause disease (Brunings and Gabriel 2003). For example, *opsX* has been reported to play roles in the production of lipopolysaccharides (LPS) and extracellular polysaccharides (EPS), growth, and virulence in planta (Kingsley et al. 1993). The *pgi* mutant was unable to grow on minimal medium containing fructose or glycerol as the sole carbon source and was unable to multiply in lemons (*Citrus limon*) (Tung and Kuo 1999). The diffusible signaling factor (DSF)-mediated *rpf* quorum-sensing system was reported to positively regulate genes that encode EPS, extracellular enzymes such as proteases, endoglucanases, and other virulence genes in lemons (Siciliano et al. 2006). The *gum* gene cluster is responsible for the biosynthesis of EPS in many xanthomonads, and a mutant of *gumB* showed defective EPS production and biofilm formation and led to reduced disease symptoms in lemons (Rigano et al. 2007). A knockout of the *galU* gene

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caused a reduction in EPS levels, an inability to grow in planta, and no symptoms in grapefruit (Guo et al. 2010). Furthermore, mutations in the LPS biosynthesis genes *wxaO* and *rfbC* resulted in deficiency in motility, stress resistance, and virulence in grapefruit (Li and Wang 2011). Additionally, the ColR/ColS two-component regulatory system (TCRS) was

reported to play multiple roles in LPS and catalase production, biofilm formation, stress resistance, and transcription of *hrpD6* and *hpaF*, and the knockout of either *colR* or *colS* resulted in a loss of pathogenicity in grapefruit (Yan and Wang 2011).

Although significant progress has been made in identifying important virulence genes that are involved in canker disease

Table 1. Virulence-related and essential growth-related genes identified in this work and the phenotypes of the mutants in minimal medium and host plants.

Gene ID ^a	Gene ^b	Function of gene product ^c	Insertion site ^d	Growth in planta ^e	Growth on M9 plate ^f
Secretion systems					
XAC3534	<i>xpsD</i> §++	General secretion pathway protein D	4178715	+++	++
XAC3535#	<i>xpsN</i> §+	General secretion pathway protein N	4181542	+++	++
XAC3536#	<i>xpsM</i> §+	General secretion pathway protein M	4181884	++	++
XAC3542	<i>xpsG</i> §+	General secretion pathway protein G	4185890	+++	++
XAC3543	<i>xpsF</i> §+	General secretion pathway protein F	4187142	++	++
XAC3544	<i>xpsE</i> §+	General secretion pathway protein E	4188184	+++	++
XAC0396	<i>hpaB</i> §+	T3SS HpaB protein	470195	++	++
XAC0405	<i>hrcV</i> §+	T3SS HrcV protein	477286	+	++
XAC0412	<i>hrcN</i> §+	T3SS HrcN protein	481742	+	++
T3SS effector					
XACb0065*	<i>pthA4</i> §++	T3SS effector of AvrBs3/PthA family	58482	+++	++
Metabolic pathway					
<u>XAC0033</u>	<i>gluB</i>	Glutamate synthase alpha subunit	41666	+++	–
<u>XAC0476</u>	<i>trpE</i> §+	Anthranilate synthase component I	567293	+++	–
<u>XAC0478</u> *	<i>trpG</i> §+	Anthranilate synthase component II	569312	++++	+
<u>XAC0481</u> *	<i>trpC</i>	Indole-3-glycerol phosphate synthase	571902	+++	–
<u>XAC2717</u>	<i>trpB</i> §	Tryptophan synthase beta chain	3177648	+++	–
XAC2496	NA	Glutamate dehydrogenase	2912244	++++	++
<u>XAC2725</u>	<i>aroC</i> §	Chorismate synthase	3186589	+	–
<u>XAC3010</u> #	<i>aroK</i> §+	Shikimate kinase	3520694	++++	–
<u>XAC3458</u>	<i>leuC</i> §+	3-isopropylmalate dehydratase large subunit	4080401	++++	+
XAC3494	<i>aspC</i>	Aspartate aminotransferase	4132553	++	++
<u>XAC1835</u>	<i>hisI</i> §+	Phosphoribosyl-AMP cyclohydrolase	2130611	++++	+
XAC1808	NA	Aldehyde dehydrogenase	2081819	++++	++
<u>XAC1139</u>	<i>acnA</i>	Aconitate hydratase 1	1291310	++++	+
XAC1788	<i>pgi</i> §++	Glucose-6-phosphate isomerase	2057186	+	++
XAC1840	NA	Sugar aldolase	2135693	+++	++
XAC3890	<i>putA</i>	Bifunctional PutA protein	4574489	NSD	++
XAC1247#	<i>uvrA</i>	Excinuclease ABC subunit A	1425150	++	++
<u>XAC0511</u> *	<i>purD</i>	Phosphoribosylamine-glycine ligase	600972	+++	–
<u>XAC0513</u>	<i>purH</i> §+	Bifunctional purine biosynthesis protein	603947	++++	–
<u>XAC1032</u>	<i>purF</i> §	Amidophosphoribosyltransferase	1190150	+	–
<u>XAC3549</u>	<i>purL</i>	Phosphoribosylformylglycinamide synthetase	4207480	+++	–
<u>XAC0012</u>	<i>pdxJ</i> §+	Pyridoxal phosphate biosynthetic protein	13736	++++	–
<u>XAC3068</u>	<i>bioA</i> §	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	3589299	NSD	+
<u>XAC0387</u>	<i>bioF</i> §	8-amino-7-oxononanoate synthase	458172	+++	–
XAC1099	<i>moaD</i>	Molybdopterin-converting factor chain 1	1251717	NSD	++
<u>XAC2778</u>	<i>nadD</i>	Nicotinate-nucleotide adenyltransferase	3257107	++	–
<u>XAC3220</u>	<i>nadE</i> §	NH3-dependent NAD synthetase	3793982	++	–
XAC0618*	<i>hrpM</i> §++	Periplasmic glucan biosynthesis protein	733719	++	++
XAC0014	<i>cls</i> §++	Cardiolipin synthetase	15609	NSD	++
XAC2292#	<i>galU</i> §++	UTP-glucose-1-phosphate uridylyltransferase	2682811	+++	++
XAC3602	<i>metB</i> §+	Cystathionine gamma-lyase-like protein	4273998	++++	++
EPS and LPS production					
XAC2576	<i>gumK</i> §+	GumK protein	3035219	+++	++
XAC2581#	<i>gumF</i> §+	GumF protein	3041792	+++	++

(continued on next page)

^a #: Transposon insertion may have a polar effect. *: Mutants that have been complemented to the virulence in planta by a corresponding gene cloned from wild type. Essential physiological genes are underlined. The mutants inserted by EZ-Tn5 in the intergenic region are also listed.

^b NA: not available. §: Genes that have been reported previously to be virulence-related in the pathogenic bacteria; §+: Genes that have been reported to be virulence-related in members of genus *Xanthomonas*; §++: Genes that have been reported to be virulence-related in *Xanthomonas citri* subsp. *citri*. Genes common with the Laia and associates (2009) appear in bold font.

^c Function of the putative corresponding protein or the putative domain of the hypothetical protein.

^d The insertion site of EZ-Tn5 in the chromosome or plasmid of pXAC64 and pXAC33 of strain *X. citri* subsp. *citri* 306. The insertion site in one representative mutant was shown here if the same gene was inserted by EZ-Tn5 in more than one mutant.

^e Mutants and wild-type were inoculated into the intercellular spaces of citrus leaves at a cell concentration of 10⁶ CFU/ml. The leaves were sampled at 4 days postinoculation, and the bacterial cell density was determined by serial dilution on nutrient agar plates containing corresponding antibiotics. Symbols indicates the value calculated by (cell density of wild-type)/(cell density of mutant): + = ≥1,000; ++ = ≥100 and <1,000; +++ = ≥10 and <100; ++++ = <10 and significantly lower than wild type. NSD = no significant difference between wild-type and mutant. Significant difference: *P* < 0.05, analyzed by Student's two-sample *t* test.

^f Growth of mutants on M9 minimal media plates. ++ = grow properly similar with wild-type; + = grow poorly; – = not grown.

development, the comprehensive understanding of the virulence mechanism of *X. citri* subsp. *citri* is lacking and remains to be elucidated. A large number of virulence-related genes were identified by sequencing the genomes of respective pathogens (da Silva et al. 2002; Moreira et al. 2010) as well as transcriptome analyses of critical virulence regulatory genes, such as *hrpX* and *hrpG* (Guo et al. 2011). However, their roles in

virulence have not been experimentally determined. Tn5-mediated mutagenesis screening is an efficient method for creating mutants and has been used for high-throughput screening of potential virulence-related genes in several *Xanthomonas* members (Laia et al. 2009; Qian et al. 2005; Rott et al. 2011; J. Wang et al. 2008). The genome of *X. citri* subsp. *citri* 306 has been sequenced and is a good model system for investigating

Table 1. (continued from previous page)

Gene ID ^a	Gene ^b	Function of gene product ^c	Insertion site ^d	Growth in planta ^e	Growth on M9 plate ^f
EPS and LPS production (continued)					
XAC3598	<i>rfbC</i> §++	Truncated O-antigen biosynthesis protein	4268484	++++	++
XAC3599	NA	Hypothetical protein	4270078	+++	++
XAC3600	<i>wzt</i> §++	ABC transporter ATP-binding protein	4271693	+++	++
XAC3601	<i>wzm</i> §+	ABC transporter permease	4273662	++++	++
Regulatory element					
XAC1548*	NA	Transcriptional regulator GntR family	1789466	+	++
XAC1878	<i>rpfC</i> §++	RpfC protein	2180203	NSD	++
XAC2358	<i>dkaA</i> §	Regulator of rRNA expression	2753209	++	++
XAC2670	NA	TCRC LytT family, sensor histidine kinase.	3128487	++	++
Transport protein					
XAC0010	<i>exbD1</i> §+	Biopolymer transport ExbD1 protein	12925	+	++
XAC0642#	<i>rmrB</i>	MFS transporter	756373	NSD	++
XAC2293	NA	Epimerase	2684644	+++	++
XAC2334	<i>cydC</i> §	ABC transporter ATP-binding protein	2728289	NSD	++
<u>XAC2468</u>	<i>corA</i> §+	Magnesium and cobalt transport protein	2872360	++++	+
XAC4217	<i>tatB</i> §	Sec-independent protein translocase	4976723	NSD	++
XAC4216	<i>tatC</i> §+	Sec-independent protein translocase	4976057	++	++
XAC4340	<i>yrbE</i> §	Toluene tolerance protein	5135801	NSD	++
Protein fate					
XAC0019	<i>ompP1</i> §	Outer membrane protease	20681	++++	++
XAC1585	NA§+	Peptidyl-prolyl cis-trans isomerase	1831534	++	++
XAC2001	<i>clpA</i> §+	ATP-dependent Clp protease subunit	2337204	++++	++
Plasmid mobilization and stabilization					
XAC1507	<i>mobL</i> §	Plasmid mobilization protein	1739717	+++	++
XACa0028	<i>pemI</i>	Plasmid stable inheritance protein I	18713	++	++
Extracellular enzyme					
XAC0661*	<i>peh-1</i> §++	Endopolygalacturonase	784188	NSD	++
Adhesion					
XAC1812#	<i>hmsF</i>	Polysaccharide deacetylase	2085866	++++	++
Hypothetical gene					
XAC0007#	NA	Tetratricopeptide repeat (TPR); signal peptide	10317	+++	++
XAC0013	NA	Signal peptide	14688	+++	++
XAC0464	NA	No known domain	554061	NSD	++
<u>XAC1142</u>	NA	PRPF domain	1293608	++++	–
<u>XAC1230*</u>	NA	Polyketide cyclase; Signal peptide	1408918	++	–
XAC1347	NA	Signal peptide	1554134	NSD	++
XAC1434	NA	Domain present in carbohydrate binding proteins and sugar hydrolyses (CASH)	1654334	+	++
XAC1496	NA	Signal peptide	1728074	+	++
XAC2307	NA	F5/8 type C domain; Signal peptide	2697403	NSD	++
<u>XAC2357#</u>	NA	Five transmembrane domains	2753636	++	+
XAC2673	NA	No known domain	3134049	++++	++
XAC2903	NA	Phage integrase domain	3409390	+++	++
XAC2976	NA	P-loop ATPase protein family	3477864	++++	++
XAC3049*	NA	Signal peptide	3567127	+	++
XAC3576	NA	Transmembrane domain	4242586	NSD	++
XAC3795	NA	Glyphos-transf domain	4464472	++	++
Intergenic region					
XAC0007-0008	XAC0007- <i>tonB</i>	10913	+++	++	
XAC0008-0009	<i>tonB-exbB</i>	11731	+	–	
XAC0122-0123	<i>tldD</i> -XAC0123	144933	+++	++	
XAC0333-0334	<i>metR-sflA</i>	399501	++++	+	
XAC1521-1522	<i>grpE-dnaK</i>	1755217	+	+	
XAC1988-1989	<i>flgA-flgM</i>	2321007	NSD	++	
XAC1990-1991	XAC1990-1991	2321849	++++	++	
XAC2576-2577	<i>gumK-gumJ</i>	3036089	+++	++	
XAC3273-3274	XAC3273- <i>cheY</i>	3855976	++++	–	
XAC3575-3576	Etf-QO-XAC3576	4240787	+++	++	
XAC3578-3579	<i>ipsI-xanA</i>	4244075	NSD	++	
XAC3597-3598	XAC3597- <i>rfbC</i>	4267725	+++	++	
XAC3599-3600	XAC3599- <i>wzt</i>	4270688	+++	++	
XACb0012-0013	XACb0012-0013	12393	++++	++	

pathogen-host interactions (Brunings and Gabriel 2003; da Silva et al. 2002). A mutant library of strain 306 was constructed previously in our group (Guo et al. 2010). In the present study, approximately 22,000 mutants were individually screened for virulence deficiency in grapefruit (*Citrus paradise* Macf. cv. Duncan). In total, 59 putative virulence-related genes, including 27 novel genes, were identified to be involved in citrus canker symptom development, thereby significantly advancing our understanding of the genetic basis underlying the virulence mechanism of citrus canker pathogen.

RESULTS

High-throughput screening of virulence-deficient mutants of *X. citri* subsp. *citri*.

A mutant library of *X. citri* subsp. *citri* 306 was previously constructed using the EZ-Tn5 mutagenesis kit (Guo et al. 2011). To understand the genetic basis of the pathogen's virulence mechanism, a total of 22,000 mutants were individually subjected to a two-time pathogenicity screening protocol using susceptible grapefruit (*C. paradise* Macf. cv. Duncan). At each screening, the mutants were inoculated into the intercellular spaces of the citrus leaves with two different cell suspensions (10^6 or 10^8 CFU/ml). Four independent replicates were performed for each mutant at each bacterial concentration, and the experiment was repeated three times. Thus, the pathogenicity assay was performed with at least 24 independent replicates for each mutant. A total of 217 mutants that elicited either no symptoms or reduced symptoms in grapefruit leaves were collected and were subjected to a Southern blot assay to determine the copy number of EZ-Tn5 (Supplementary Fig. S1).

Only one of the mutants was shown to contain two copies of EZ-Tn5 and was excluded from further investigation. The 216 virulence-deficient mutants (containing one single copy of the transposon) were investigated further.

To determine the insertion site of the transposon, the 216 mutants were subjected to a rescue cloning procedure to clone the EZ-Tn5 flanking sequences. The 216 mutants included mutations in 97 protein-coding sequences and intergenic regions, as shown by sequencing and blast analyses of the flanking fragments. In addition to 14 intergenic regions, one gene (*rplJ*) encoding a ribosomal protein and 82 genes that were involved in citrus canker symptom development caused by *X. citri* subsp. *citri* were identified in this work. Only these 82 genes (excluding the intergenic regions and ribosomal encoding gene) were further investigated in this study (Table 1; Supplementary Table S1). Among the 82 genes, 80 were mapped to the chromosome, and each of the other two was mapped to megaplasmsids pXAC64 and pXAC33 of *X. citri* subsp. *citri* 306 (Fig. 1). Based on the analysis of protein function, the 82 genes were classified into 11 diverse categories (Table 1). Among the identified genes, 32 genes were previously characterized virulence factors, including 22 genes in the genus *Xanthomonas* and nine genes in *X. citri* subsp. *citri* (Table 1). In addition to the 32 previously characterized virulence factors in the pathogenic bacteria, 27 novel genes, including 13 hypothetical genes, were identified in this study as being potentially involved in the pathogenicity of *X. citri* subsp. *citri*.

To test whether EZ-Tn5 insertion has a polar effect on downstream genes in operons, a polarity analysis was performed for the mutated genes in a putative operon. As listed in Table 1, the insertion of EZ-Tn5 into 10 genes (*xpsN*, *xpsM*, *aroK*, *uvrA*,

galU, *gumF*, *rmrB*, *hmsF*, XAC0007, and XAC2357) may have a polar effect on downstream genes within the same operon. For example, both *xpsN* and *xpsM* are located in the *xps* operon, and the insertion of EZ-Tn5 in either *xpsN* or *xpsM* may have a potential polar effect on the downstream gene *xpsD*, which encodes the channel-forming protein in type II secretion system (T2SS) machinery. Moreover, *gumF* is located in the *gum* operon, and the insertion of EZ-Tn5 in *gumF* may have a polar effect on the downstream *gum* genes that are involved in EPS biosynthesis. Those genes were included for further analysis, since one operon usually contains functionally related genes.

The mutant phenotypes in planta and in vitro.

Compared with the typical canker disease symptoms caused by the wild-type strain, diverse symptoms were observed in grapefruit leaves following inoculation of the identified virulence-deficient mutants (Table 2). Similar canker symptoms could be induced by inoculating the wild-type strain at concentrations of either 10^6 or 10^8 CFU/ml. The disease progression with an inoculation of 10^6 CFU/ml was delayed by approximately 7 days as compared with the higher inoculation concentration of 10^8 CFU/ml (data not shown). In the following analysis, the symptoms were categorized on the basis of the symptoms that were caused by the mutants that were inocu-

lated at a concentration of 10^6 CFU/ml. The symptoms were observed at 4 to 30 days postinoculation (dpi). As summarized in Table 2, the diverse symptoms were classified into five categories. Category I contained mutants that caused no visible symptoms on the grapefruit leaves. Mutated genes in this category included three T3SS genes (*hpaB*, *hrcV*, and *hrcN*), four genes of the metabolism pathways (*pgi*, *nadD*, *hrpM*, and *galU*), two genes encoding regulators (*dksA* and XAC1548), one gene encoding a transporter (*exbD1*), and two hypothetical genes (XAC1230 and XAC3049). Category II mutants caused clearly evident chlorosis but no visible canker lesions on the grapefruit leaves. Mutated genes in this category included the T3SS effector gene *pthA4* and the hypothetical gene XAC1496. Category III mutants caused a reduction in water soaking and caused thicker lesions than the wild-type strain. Mutated genes in this category contained *gumK* and *gumF* of the EPS biosynthesis cluster. Category IV mutants caused delayed canker lesion symptoms on the grapefruit leaves. The mutants caused water-soaking and lesion symptoms on citrus leaves, but the development of these symptoms was markedly delayed compared with simultaneously inoculated wild-type strain. Mutated genes in this category included six genes in the T2SS (*xpsD*, *xpsM*, *xpsN*, *xpsE*, *xpsF*, and *xpsG*), five genes of metabolism pathways (*gltB*, *trpG*, *trpC*, *leuC*, and XAC1840), two genes encoding transporters (*tatB* and *tatC*), one gene of

Table 2. Classification of identified genes based on the symptoms in host plants caused by respective mutants

Category ^a	Representative symptoms ^b	Mutated genes
I, No symptoms		<i>hpaB</i> , <i>hrcV</i> , <i>hrcN</i> , <i>pgi</i> , <i>nadD</i> , <i>hrpM</i> , <i>galU</i> , <i>dksA</i> , <i>exbD1</i> , XAC1230, XAC1548, XAC3049
II, Chlorosis only		<i>pthA4</i> , XAC1496
III, Reduced water soaking and thicker lesion		<i>gumK</i> , <i>gumF</i>
IV, Delayed symptoms		<i>xpsD</i> , <i>xpsN</i> , <i>xpsM</i> , <i>xpsG</i> , <i>xpsF</i> , <i>xpsE</i> , <i>gltB</i> , <i>trpG</i> , <i>trpC</i> , <i>leuC</i> , <i>tatB</i> , <i>tatC</i> , <i>clpA</i> , <i>pehI</i> , XAC1142, XAC1840
V, Fewer symptoms		<i>aroC</i> , <i>aroK</i> , <i>trpE</i> , <i>trpB</i> , <i>aspC</i> , <i>hisI</i> , <i>acnA</i> , <i>putA</i> , <i>uvrA</i> , <i>purD</i> , <i>purH</i> , <i>purF</i> , <i>purL</i> , <i>pdxJ</i> , <i>bioA</i> , <i>bioF</i> , <i>moaD</i> , <i>nadE</i> , <i>cls</i> , <i>wzt</i> , <i>wzm</i> , <i>metB</i> , <i>rfbC</i> , <i>rpfC</i> , <i>ompP1</i> , <i>rmrB</i> , <i>cydC</i> , <i>corA</i> , <i>yrbE</i> , <i>mobL</i> , <i>pemI</i> , <i>hmsF</i> , XAC0007, XAC0013, XAC0464, XAC1347, XAC1434, XAC1585, XAC1808, XAC2293, XAC2307, XAC2357, XAC2496, XAC2670, XAC2673, XAC2903, XAC2976, XAC3576, XAC3599, XAC3795

^a Mutants and wild type were inoculated into the intercellular spaces of citrus leaves at a cell concentration of 10^6 CFU/ml. Symptoms were observed from 4 to 30 days postinoculation and symptoms caused by different mutants were classified into five categories.

^b Only the symptom caused by one representative mutant was present in each category. W = wild-type; M = mutant. The representative mutants used in each category were as follows: category I, the mutant of *hrcV*; II, the mutant of *pthA4*; III, the mutant of *gumK*; IV, the mutant of *xpsD*; and V, the mutant of *aroK*.

protein fate (*clpA*), one gene encoding an extracellular enzyme (*peh-1*), and one hypothetical gene (XAC1142). Category V mutants caused citrus canker lesions, but these symptoms were much milder than those of the wild-type strain. Mutated genes in this category contained 22 genes of metabolism pathways (*aroC*, *aroK*, *trpE*, *trpB*, *aspC*, *hisI*, *acnA*, *putA*, *uvrA*, *purD*, *purH*, *purF*, *purL*, *pdxJ*, *bioA*, *bioF*, *moaD*, *nadE*, *cls*, *metB*, XAC1808, and XAC2496), four genes in the LPS biosynthesis gene cluster (*wzt*, *wzm*, *rfbC*, and XAC3599), two genes encoding regulators (*rpfC* and XAC2670), five genes encoding transporters (*rmrB*, *cydC*, *corA*, *yrbE*, and XAC2293), two genes of protein fate (*ompP1* and XAC1585), two genes of plasmid stabilization and mobilization (*mobL* and *pemI*), one gene of adhesion (*hmsF*), and 12 hypothetical genes (XAC0007, XAC0013, XAC0464, XAC1347, XAC1434, XAC2307, XAC2357, XAC2673, XAC2903, XAC2976, XAC3576, and XAC3795).

To understand how various genes contribute to virulence, growth of the mutants in planta was investigated. Each of the mutants was inoculated into the intercellular space of grapefruit leaves, using a 10^6 CFU/ml cell suspension. The inoculated leaves were sampled at 0, 2, and 4 dpi, and the bacterial cell density was measured using serial dilutions on nutrient agar (NA) plates. No significant difference in bacterial density was observed at 0 dpi, ensuring that any differences among the strains at later stages would not be due to different initial concentrations. Only the results at 4 dpi are presented here. As shown in Table 1, compared with the wild-type strain, mutations in nine genes caused more than a 1,000-fold reduction in cell density inside the grapefruit leaves; mutations in 17 genes caused 100- to 1,000-fold reductions of cell density inside the leaves; mutations in 23 genes caused 10- to 100-fold reductions of cell density inside the leaves; mutations in 19 genes caused less than 10-fold reductions.

In addition to the phenotypes that were observed in planta, the phenotypes of these mutants were also assayed in vitro by measuring growth on minimal medium plates and observing colony morphology on NA plates. Mutants of nine genes (*galU*, *gumK*, *gumF*, *rfbC*, *wzt*, *wzm*, *metB*, *rpfC*, and XAC2293) formed nonmucoid colonies on the NA plates, and mutants of six genes (*gltB*, *trpE*, *aroC*, *aroK*, XAC2357, and XAC1142) formed white colonies on the NA plates. Additionally, mutants of 16 genes showed no growth and mutants of seven genes grew poorly on M9 minimal medium plates (Table 1), indicating that these 23 genes are required for the proper bacterial growth in vitro. Thus, these 23 genes were classified as essential genes and the other 59 genes were classified as putative virulence-related genes in this work (Table 1).

General metabolism factors.

The category of the general metabolism pathway contains the largest number of genes (31 in total) identified in this study (Table 1). As shown in Figure 2, 26 of these genes were sorted into 12 different pathways on the basis of their analysis, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. These 12 pathways are involved in the metabolism of many essential compounds and include vitamin B6 metabolism; glycolysis and gluconeogenesis; the citrate cycle; alanine, aspartate, and glutamate metabolism; nicotinate and nicotinamide metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; glycerophospholipid metabolism; biotin metabolism; histidine metabolism; purine metabolism; cysteine and methionine metabolism; and valine, leucine, and isoleucine metabolism. Of the 31 metabolism-related genes, 19 were classified as essential genes, since the individual mutation of these 19 genes led to auxotrophic or poor growth in minimal medium and the other 12 genes were classified as putative virulence-related genes (Table

1; Fig. 2). Knockout of four genes (*pgi*, *nadD*, *hrpM*, and *galU*) caused no symptoms (symptom category I) on grapefruit leaves. Knockout of five genes (*gltB*, *trpG*, *trpC*, *leuC*, and XAC1840) caused delayed symptoms (symptom category IV) on grapefruit leaves. Mutants of the other 21 genes caused milder symptoms (symptom category V) on grapefruit leaves. The cell densities of the mutants were significantly lower than those of the wild-type strain in planta, with the exceptions of mutants in *putA*, *bioA*, *moaD*, and *cls*, which were not significantly different from wild type at 4 dpi. The virulence of four mutants (*trpG*, *trpC*, *purD*, and *hrpM*) was restored to the wild-type level by complementation using the intact corresponding genes (Table 1).

Mutants in T2SS caused delayed symptom development in host plants.

In a wide variety of pathogenic bacteria, T2SS mediates the transportation of toxins and hydrolytic enzymes from the periplasmic space, across the outer membrane to the extracellular surroundings (Pugsley 1993). In our screening, six T2SS-related genes (*xpsD*, *xpsM*, *xpsN*, *xpsE*, *xpsF*, and *xpsG*) were identified to potentially contribute to the virulence of the canker pathogen (Table 1; Fig. 3). Mutations in these six *xps* genes caused delayed symptoms (symptom category IV) in host plants. Compared with wild-type, all six *xps* mutants showed fewer symptoms at 8 dpi and caused more water soaking in the citrus leaves at 15 dpi. In addition, the canker lesion symptom was observed with the mutants but appeared approximately one week later than with wild type (Fig. 3D). The cell densities of the *xps* mutants in the intercellular space of the leaves were reduced compared with wild type (Table 1). A variety of degrading enzymes are secreted via the T2SS in *Xanthomonas* spp., including protease, amylase, pectate lyase, cellulase, and xylanase (Baptista et al. 2010; Furutani et al. 2004; Lee et al. 2004; Szczesny et al. 2010). Consistently, the extracellular activities of protease and amylase of all six *xps* mutants were much lower than those of the wild-type strain (Fig. 3B and C).

T3SS and *pthA4* are essential for the virulence of *X. citri* subsp. *citri*.

T3SS plays important roles in the hypersensitive response and pathogenicity of gram-negative bacterial pathogens. Three genes of the T3SS (*hpaB*, *hrcV*, and *hrcN*) were identified in our screening (Table 1). All three mutants caused no visible symptoms (symptom category I) on grapefruit leaves, and the cell densities of the mutants were significantly reduced in planta, which was more than 100-fold lower than that of the wild type. One T3SS effector gene, *pthA4* (XACb0065), was also identified in this work. Surprisingly, 50 separate mutants were identified in the *pthA4* gene (Fig. 4A), which accounts for 23.1% of all identified virulence-deficient mutants, and all of these *pthA4* mutants caused similar phenotypes in planta (data not shown). Following inoculation, the *pthA4* mutants caused only chlorosis on the citrus leaves (symptom category II) (Fig. 4B). Although the *pthA4* mutant proliferated in the intercellular spaces of the grapefruit leaves, the cell density of the *pthA* mutant was 20-fold lower than that of the wild type at 4 dpi (Table 2; Fig. 4C). To confirm the role of *pthA4* in pathogenicity, the intact wild-type *pthA4* gene was cloned and was transformed into five randomly chosen *pthA4* mutants. All of the complemented strains regained the ability to cause citrus canker symptoms and proliferate in the intercellular spaces of citrus leaves at levels that were similar to the wild-type strain (Fig. 4B and C).

Plasmid mobilization and stabilization factors.

The *mobL* and *pemI* genes, which encode putative proteins for plasmid mobilization and stabilization, were identified in

this work (Table 1). The *mobL* (XAC1507) gene encodes a putative plasmid conjugative transfer protein that belongs to the MobL/MobA family. Mutants of *mobL* developed fewer symptoms (symptom category V) in citrus leaves, and the bacterial density inside the leaves was 38-fold lower than that of the wild-type strain at 4 dpi. The other gene, *pemI*, is located on megaplasmid pXAC33. Interestingly, gene *pemI* (XACa0028) and downstream gene *pemK* (XACa0027) potentially comprise a toxin-antitoxin (TA) system in *X. citri* subsp. *citri* (da Silva et al. 2002). Generally, a TA system

consists of a stable toxin and an unstable antitoxin that interferes with the toxin's lethal action (Hayes 2003). In the present study, a mutation of the putative antitoxin coding gene *pemI* caused a dramatic reduction in cell density (212-fold lower than that of wild type) and virulence in host plants (symptom category V) (Table 2). The *mobL* and *pemI* genes were classified into the virulence-related genes category rather than the essential genes category because mutants of the respective genes still grew in vitro and in planta (Table 1).

Fig. 2. Schematic metabolism pathways of genes involved in general metabolism. In this work, 26 genes were identified and were sorted into 12 metabolism pathways according to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Different pathways are defined using black rectangle. Genes shown in red indicate that mutants of the respective genes are auxotrophic on M9 minimal medium plate. Arrows represent the direction of the pathway. Arrow lines with dashes mean multiple steps were involved in the reactions. Lines without arrowheads mean the same compound in different pathways.

Regulatory factors.

Although a large number of regulatory genes have been uncovered by whole-genome sequencing, only a few have been investigated for their roles in the virulence mechanism of *Xanthomonas* species (Büttner and Bonas 2010; Qian et al. 2008). In the present study, four regulatory genes were found to potentially contribute to the virulence of *X. citri* subsp. *citri* (Table 1). In addition to the previously reported quorum-sensing sensor kinase gene *rpjC* (Siciliano et al. 2006), we found that a mutation of *dksA*, which encodes an expression regulator of ribosomal RNA, abolished pathogenicity in grapefruit. DksA has been reported to play a role in regulating the virulence of *Escherichia coli* (Paul et al. 2004), *Salmonella typhimurium* (Webb et al. 1999), *Shigella flexneri* (Mogull et al. 2001), and *Pseudomonas aeruginosa* (Jude et al. 2003). However, the biological role of DksA has not been characterized in *Xanthomonas* spp. Additionally, the XAC1548 gene encodes a putative GntR family regulator that was named after the *Bacillus subtilis* repressor of the gluconate operon (Haydon and Guest 1991). Mutation in XAC1548 resulted in a loss of pathogenicity in the host plant (symptom category I) (Table 2; Fig. 5A). A growth assay revealed that the bacterial population of the mutant of XAC1548 was significantly lower than that of the wild type inside the intercellular spaces of host leaves (Fig. 5B). For complementation, the gene of XAC1548 was cloned from the wild type and was transformed into the mutant. As we ex-

pected, the complementary strain caused similar symptoms to those of the wild type and the bacterial population was also restored to the wild-type level in the host plant (Fig. 5). Furthermore, XAC2670 encodes a putative histidine kinase sensor of the TCRS and has a high similarity (81% in amino acid sequence) to AlgZ (also called FimS) in *P. aeruginosa*. The sensor AlgZ, possibly by interacting with the cognate regulator AlgR, has been shown to be a repressor of alginate biosynthesis (Yu et al. 1997) and an activator of type IV pili-mediated twitching motility (Whitchurch et al. 1996, 2002) in *P. aeruginosa*. Mutations in XAC2670 caused a notable deficiency of virulence (symptom category V) and a significant reduction of growth in host plants (Tables 1 and 2).

EPS and LPS biosynthesis genes.

EPS has long been recognized as an important virulence factor for many pathogenic bacteria (Leigh and Coplin 1992). Interestingly, recent reports revealed that the *gum* genes of *X. citri* subsp. *citri* are required for virulence in *C. limon* (Rigano et al. 2007) but are dispensable for bacterial growth and disease formation in *C. sinensis* (Dunger et al. 2007), suggesting that the contribution of EPS to virulence may depend on the host plant and environmental conditions (Büttner and Bonas 2010). In our screening, two *gum* genes (*gumF* and *gumK*) were identified as being involved in the virulence of *X. citri* subsp. *citri* in grapefruit (*C. paradise* Macf. cv. Duncan). Both

Fig. 3. The type II secretion system (T2SS) was involved in virulence in planta and the enzyme activity of protease and amylase in *Xanthomonas citri* subsp. *citri*. **A**, Arrows indicate gene location and direction in the T2SS gene cluster. Position and direction of flag indicate the insertion site of the transposon and direction of the kanamycin-resistance gene of EZ-Tn5, respectively. The red arrow represents the *xpsD* mutant used for assays of protease, amylase, and pathogenicity in this work. **B**, Protease and **C**, amylase activity assays. Bacterial cells were inoculated on skim-milk plates and starch plates at a concentration of 10^8 CFU/ml. The results were photographed at 2 days postinoculation (dpi) for amylase activity and 3 dpi for protease activity. **D**, Pathogenicity assay for the *xpsD* mutant. The wild-type (W) and mutant (M) were inoculated into the leaves of grapefruit (*Citrus paradise* Macf. cv. Duncan) at a concentration of 10^6 CFU/ml. Results were photographed at different timepoints as shown.

gum mutants showed reduced water soaking in citrus leaves at an early stage after inoculation (data not shown), and the cell densities of the *gum* mutants were significantly reduced in grapefruit leaves as compared with those of the wild-type strain (Table 1). Interestingly, the surfaces of the leaves that were inoculated with the *gum* mutants formed canker lesions that were much thicker than those formed by the wild-type strain (symptom category IV) (Table 2).

In comparison to the extensively studied virulence function of EPS, our understanding of the role of LPS in virulence is rather limited with respect to plant-pathogen interactions. In the pre-

sent study, four genes that are located in the LPS biosynthesis cluster were identified (Table 1). Among these four genes, the roles of *wzt*, *wzm*, and *rfbC* in LPS biosynthesis and bacterial virulence have been reported previously (Cuthbertson et al. 2007; Laia et al. 2009; Li and Wang 2011; Wang et al. 2010). The fourth gene, XAC3599, encodes a small protein (65 amino acids) of unknown function and is absent from the *lps* cluster of other sequenced xanthomonads (Moreira et al. 2010), indicating that this gene is specific to the species *X. citri* subsp. *citri*. Mutations in XAC3599 caused fewer symptoms (category V) (Table 2) and significantly reduced cell density in host plants (Table 1).

Fig. 4. The *pthA4* gene was required for the pathogenicity of *Xanthomonas citri* subsp. *citri*. **A**, A total of 50 separate *pthA* mutants with EZ-Tn5 insertions in different positions were identified. Arrow indicates the open reading frame of *pthA4* gene. Putative domains of the encoded protein are shown. LZ = leucine zipper repeats, NLS = nuclear localization signals, AAD = acid activation domain. **B**, Pathogenicity assay. Bacterial cells were inoculated into the leaves of grapefruit (*Citrus paradise* Macf. cv Duncan) at a concentration of 10^6 CFU/ml. Results were photographed at 14 days after inoculation. All the *pthA4* mutants showed similar symptoms in citrus plant. The symptoms caused by five randomly chosen mutants could be complemented to the wild type by the complementary plasmid p53-*pthA4*. Only the results of one mutant are shown. W = *X. citri* subsp. *citri* 306/pUFRO53, wild type containing empty vector; M = 320E11/pUFRO53, *pthA4* mutant containing empty vector; C = 320E11/p53-*pthA4*, mutant containing the complementary vector. **C**, Growth assay in planta. Bacterial cells were inoculated into grapefruit leaves at a concentration of 10^6 CFU/ml. Bacterial cells from the inoculated leaves were recovered at different timepoints, and the value represents means of three replicates. Means $\pm$ standard deviation are plotted.

Transporters.

In addition to the *wzm/wzt* ATP-binding cassette (ABC) transporter system, eight genes (*exbD1*, *rmrB*, *cydC*, *corA*, *tatB*, *tatC*, *yrbE*, and XAC2293) that encode diverse transporters were identified in our screening (Table 1). The *exbD1* gene is located in the TonB-dependent iron uptake cluster *tonB-exbB-exbD1-exbD2* (Wiggerich and Puhler 2000). Knockout of *exbD1* resulted in a loss of pathogenicity (symptom category I) in host plants, and the cell density inside the grapefruit leaves was more than 1,000-fold lower than that of wild type at 4 dpi. Furthermore, mutations in *tatB* and *tatC* of the *tatABC* operon, which encodes the twin arginine translocation pathway, caused delayed symptoms (category IV) in grapefruit leaves. Compared with the wild-type pathogen, the cell density of the *tatC* mutant was more than 100-fold decreased, whereas no significant difference in the cell density of the *tatB* mutant was detected inside the grapefruit leaves at 4 dpi (Table 2). Additionally, mutations in *rmrB*, XAC2293, *cydC*, *corA*, and *yrbE* caused fewer symptoms (category V) in host plants. Mutations in XAC2293 and *corA* caused a significant reduction in cell density, whereas mutations in *rmrB*, *cydC*, and *yrbE* had no significant effect on bacterial growth in grapefruit leaves at 4 dpi (Table 2).

Hypothetical genes.

As shown in Table 1, 16 hypothetical genes were identified in this study. Based on the bacterial growth assay in M9 medium, three genes (XAC1142, XAC1230, and XAC2357) were classified into the essential genes category and the other 13 genes were classified as putative virulence-related genes (Table 1). Mutations in 12 of these genes resulted in the formation of fewer symptoms (category V), and mutations in XAC1142 resulted in delayed symptom development (category IV) in grapefruit leaves after inoculation of the respective mutants.

Notably, mutant of XAC3049 caused no visible symptoms (category I) in host plant. The role of XAC3049 in pathogenicity was confirmed by complementation, using the XAC3049 gene cloned from the wild-type strain (X. F. Hu and N. Wang unpublished data). The mutant of XAC1496 formed only chlorosis (category II) in host plants, which was similar to the symptoms caused by the mutant of *pthA4* (Table 2), indicating that this gene potentially played a critical role in the virulence of *X. citri* subsp. *citri*. XAC1230 was inserted by EZ-Tn5 in four separate mutants (Supplementary Fig. S2). Interestingly, these four mutants displayed varied phenotypes in planta. The mutants M1, M2, and M3 caused no symptoms in citrus leaves, and M4 caused only reduced symptoms. Similarly, M1, M2, and M3 were auxotrophic and M4 grew, albeit poorly, on minimal medium. To further confirm the phenotype of the XAC1230 mutation, the intact wild-type XAC1230 gene was cloned and was transformed into the four mutants. All of the complemented mutants rescued the virulence in planta and grew at wild-type rates on minimal medium. These results suggest that the XAC1230 gene underlies the phenotypic changes in pathogenicity and auxotrophy in *X. citri* subsp. *citri*.

DISCUSSION

Random mutagenesis using the Tn5-based strategy has been widely used to identify the genetic determinants that underlie the virulence mechanisms of various pathogenic bacteria. To investigate the genetic basis of the virulence mechanism of the citrus canker pathogen *X. citri* subsp. *citri*, an EZ-Tn5 mutant library containing approximately 22,000 mutants was screened for virulence-deficient mutants in the grapefruit host plant. These 22,000 mutants provided approximately fourfold excess coverage of the predicted 4,418 open reading frames (ORF) in the genome of *X. citri* subsp. *citri* (da Silva et al. 2002). All of

Fig. 5. The XAC1548 gene was required for the pathogenicity of *Xanthomonas citri* subsp. *citri*. **A**, Pathogenicity assay. Bacterial cells were inoculated into the leaves of grapefruit (*Citrus paradise* Macf. cv Duncan) at a concentration of 10⁶ CFU/ml. Results were photographed at 12 days after inoculation. W = *X. citri* subsp. *citri* 306/pUFR053, wild-type containing empty vector; M = 414H4/pUFR053, mutant of XAC1548 containing empty vector; C = 414H4/p53-1548, mutant containing the complementary vector. **B**, Growth assay in planta. Bacterial cells were inoculated into grapefruit leaves at a concentration of 10⁶ CFU/ml. Bacterial cells from the inoculated leaves were recovered at different timepoints, and the value represents means of three replicates. Means $\pm$ standard deviation are plotted.

the virulence-deficient mutants were verified with two cell concentrations of 10^6 and 10^8 CFU/ml. An obvious insertion bias of EZ-Tn5 was noted in this study. For example, 50 separate *pthA4* and 14 separate *xpsD* mutants were identified (Figs. 3 and 4). However, despite this bias in transposon insertions, 82 genes and 14 intergenic regions were identified as being involved in the virulence of *X. citri* subsp. *citri*, accounting for 2.22% of all genes in the complete genome. Among the 82 genes, 32 are known to be virulence-related in pathogenic bacteria and include genes encoding T2SS, T3SS, T3SS effectors, the quorum-sensing system, EPS or LPS, and general metabolic pathways. The identification of many previously identified virulence-related genes further validates the pathogenicity screening approach used in this work. A similar mutagenesis approach was recently employed to identify virulence-related genes in *X. citri* subsp. *citri* (Laia et al. 2009). In total, 3,300 mutants were screened using a pathogenicity assay in the host plant of Rangpur limes (*C. limonia*), and 35 genes were identified as being potentially involved in the virulence of this pathogen (Laia et al. 2009). Notably, the *pthA* mutation was not identified in their screen. Moreover, only three genes (*cls*, *wzt*, and *hrpM*) reported by Laia and associates (2009) overlapped with our results. This small degree of overlap between the two studies might be explained by two different citrus cultivars that were used for the pathogenicity assays; *C. paradise* Macf. cv. Duncan was used in this study, whereas *C. limonia* was used in the previous study (Laia et al. 2009). Alternatively, another important reason could be the genome coverage of the mutant libraries of both works. In all, 3,300 mutants and 22,000 mutants were screened by Laia and associates (2009) and in this work, respectively. The probability of finding one transposon insert for a certain gene were calculated for both libraries based on the calculated formula $P = 1 - (1 - x/g)^n$, where P is the probability of finding one transposon insert within a given gene, x is the length of the gene (1,000 bp), g is the length of the genome (5.27×10^6 bp in *X. citri* subsp. *citri*), and n is the number of transposon inserts present in the population (Krysan et al. 1999). The probability of finding one transposon insert for a certain gene with 22,000 mutants is 98.5%, whereas it is 46.5% when 3,300 mutants are screened for *X. citri* subsp. *citri* (Laia et al. 2009). In the present study, the contribution to pathogenesis of nine genes, including four essential genes (*trpG*, *trpC*, *purD*, and XAC1230) and five virulence-related genes (*pthA4*, *hrpM*, *peh-1*, XAC1548, and XAC3049), was confirmed by complementation with the respective wild-type gene (Table 1). We also validated the mutated genes and their phenotypes by analyzing the EZ-Tn5 insertion copy number using Southern blot analysis. Only one of 217 mutants contained two EZ-Tn5 insertions, and the rest contained one single-copy insertion, which is consistent with the low rate (less than 1%) of multiple-copy transposon insertions that is described by the manufacturer of the EZ-Tn5 insertion kit that we used (Epicentre, Madison, WI, U.S.A.). Importantly, in addition to the 32 known virulence-related genes, 27 novel genes were identified in this work, which provides new insights into the genetic basis of *X. citri* subsp. *citri* pathogenicity.

To analyze the roles of different virulence factors, the identified virulence-related genes (excluding the essential genes) were discussed according to the diverse symptoms caused by the corresponding mutants as follows. On the basis of the changes exerted on the citrus leaves, the symptoms caused by the various mutants were classified into five categories. The mutated genes were assigned to their respective symptom categories (Table 2). Mutants in category I showed no pathogenicity in host plants, as no visible canker symptoms formed on the citrus leaves. The loss of pathogenicity of the T3SS mutants (*hpaB*, *hrcV*, and *hrcN*) was primarily due to a failure of effector translocation

into the host cells. HpaB functions as a T3SS chaperone in *X. campestris* pv. *vesicatoria* and promotes the secretion of multiple effectors, including AvrBs3, and prevents the delivery of noneffectors into the plant cells (Büttner et al. 2004). The HrcV and HrcN proteins are conserved among animal and plant pathogenic bacteria and are part of the core secretion apparatus of T3SS (Lorenz and Buttner 2009).

Mutants of the *pthA4* and XAC1496 genes caused only chlorosis in the host plant (classified as category II) (Table 2). After inoculation of the *pthA4* mutant, the citrus leaves remained flat and no visible hypertrophy symptoms were observed at the inoculated area (Fig. 4B). This observation is consistent with previous reports that PthA was responsible for hyperplasia and hypertrophy of the citrus canker lesions (Duan et al. 1999; Swarup et al. 1991). Although a mutation of *pthA4* caused significantly reduced bacterial growth, the mutant still multiplied within the intercellular spaces of the citrus leaves (Fig. 4C). Interestingly, although the cell density of the *pthA4* mutant was 20-fold lower than that of the wild type, it was at least fivefold higher than that of the T3SS mutants (*hpaB*, *hrcV*, and *hrcN*) inside the grapefruit leaves (Table 1). Combined with the observation that all three T3SS mutants (*hpaB*, *hrcV*, and *hrcN*) caused no visible symptoms on the citrus leaves, it is likely that the chlorosis symptom of the *pthA4* mutants was caused by other effectors of T3SS. Notably, *X. citri* subsp. *citri* 306 contains four *pthA* homologs named *pthA1*, *pthA2*, *pthA3*, and *pthA4* on the two plasmids pXAC33 and pXAC64 (da Silva et al. 2002). Unlike the high mutation frequency of the *pthA4* gene (in which 50 mutations were identified), no *pthA1*, *pthA2*, or *pthA3* mutant was identified by our screen, implying a minor or dispensable role of these three *pthA* homologs in the virulence of the pathogen.

Mutations in *gumK* and *gumF* caused decreased water-soaking and thicker canker-lesion symptoms and were, therefore, assigned to category III (Table 2). Water soaking is one of the first macroscopic symptoms caused by many phytopathogens of xanthomonads such as *X. axonopodis* pv. *manihotis* (Kemp et al. 2004) and *X. campestris* pv. *citrumelo* (Swarup et al. 1991). The decreased water soaking caused by the *gum* mutations is consistent with a previous report in which an injection of bacterial EPS extract caused water soaking in the corresponding plant (El-Banoby and Rudolph 1979). In addition to mediating water soaking in the intercellular spaces, others roles for EPS have been suggested in epiphytic fitness and intercellular multiplication during the bacterial invasion of plants, including protecting the bacteria from desiccation, enhancing attachment to surfaces, reducing contact with environmental toxic compounds, and concentrating minerals and nutrients (Denny 1995). In the present study, growth of the *gum* mutants was significantly reduced relative to wild type (Table 1), which is consistent with a previous report that a mutation in *gumB* significantly reduced the growth of *X. citri* subsp. *citri* in *C. limon* leaves (Rigano et al. 2007).

Mutants that showed delayed symptom development in grapefruit leaves were classified into category IV (Table 2). For example, the T2SS gene mutants caused water soaking at an early stage after inoculation in the grapefruit leaves and the water soaking was followed by canker lesions that were similar to wild type. However, the development of canker symptoms was delayed by approximately one week relative to wild type (Fig. 3D). This result is consistent with the previous report that mutation of *xpsD* affected cellulose degradation and virulence of *X. citri* subsp. *citri* (Baptista et al. 2010). T2SS is important for bacterial pathogenesis in both plants and animals (Cianciotto 2005; Johnson et al. 2006). A variety of hydrolytic enzymes are secreted via T2SS in *Xanthomonas* spp., including protease, amylase, pectate lyase, cellulase, and xylanase

(Baptista et al. 2010; Furutani et al. 2004; Lee et al. 2004; Szczesny et al. 2010). Consistently, the extracellular activities of protease and amylase in the *xps* mutants (*xpsD*, *xpsM*, *xpsN*, *xpsE*, *xpsF* and *xpsG*) were much lower than that of the wild-type pathogen (Fig. 3B and C). The extracellular enzymes that are secreted via T2SS are known to contribute to the degradation of the plant cell wall, which is usually a major obstacle to bacterial invasion (McNeil et al. 1984). Citrus canker symptoms are associated with the leaf epidermis, which is composed of cell wall. Thus, T2SS and its substrates might contribute to canker symptom development by degrading the epidermal cell wall. Consequently, loss-of-function mutations in T2SS genes will delay the development of symptoms in grapefruit leaves. In addition, substrates of T2SS have been suggested to facilitate the assembly of extracellular T3SS appendages (Szczesny et al. 2010), which transport virulence effectors into host cells to suppress the plant's immune system and thereby promote disease progression.

Mutants in category V caused water-soaking and canker-lesion symptoms on the citrus leaves, but these symptoms were much milder as compared with wild type. The mechanisms of virulence deficiency in this category might be more complicated, as diverse functional genes were assigned to this category. Some mutated genes are involved in the synthesis of known virulence factors. For example, mutants of *wzt*, *wzm*, *rfbC*, and XAC3599 also showed less symptoms in citrus leaves (Table 2). These four genes are located in a gene cluster that underlies the production of LPS, which is the major component of bacterial outer membranes and is considered an important virulence factor of many plant bacterial pathogens, including *Xanthomonas* spp. (Berry et al. 2009; Dow et al. 1995; Drigues et al. 1985; Li and Wang 2011; Titarenko et al. 1997). Wzt and Wzm constitute an ABC transporter system for the exportation of the O-antigen portion of LPS across the inner membrane of *E. coli* (Cuthbertson et al. 2007). RfbC contains two separate glycosyl transferase family 2 domains, which are involved in O-antigen biosynthesis in a wide variety of bacteria (Li and Wang 2011; P. G. Wang et al. 2008). Interestingly, XAC3599 is located in the same *lps* gene cluster, yet its function remains unknown. Mutations in four transporter genes (*cydC*, *corA*, *rmrB*, and XAC2293) also caused fewer symptoms in grapefruit leaves. The *cydC* gene encodes an ATP-binding protein in the CydDC ABC transport system and is involved in the virulence of the animal pathogen of *Shigella flexneri* in a mouse model (Way et al. 1999). In *E. coli*, CydDC is required for proper membrane assembly of the cytochrome *bd* complex (Pittman et al. 2002), which is one of the two principal membrane-bound terminal respiratory oxidases and is required for resistance to a variety of environmental stresses (Jünemann 1997). The *cydC* gene might play a similar role in the respiratory pathway of *X. citri* subsp. *citri* and, in turn, contribute to bacterial growth in planta. The product of the *corA* gene forms a Mg²⁺ transportation channel that is the primary source of intracellular Mg²⁺ in *Salmonella typhimurium* (Lunin et al. 2006), and a strain lacking *corA* exhibited attenuated virulence upon injection in mice (Papp-Wallace et al. 2008). Consistent with our results, a virulence-deficient *corA* mutant was identified in a mutant library of *X. albilineans* in a sugarcane model (Rott et al. 2011). The role of *corA* in the virulence of *Xanthomonas* spp. has not yet been characterized and needs further study. The protein encoded by *rmrB* contains 13 putative transmembrane domains and belongs to the major facilitator superfamily of efflux pump proteins. *rmrB* (XAC0642) and the downstream gene XAC0641 (which encode a multidrug-resistance efflux pump) form a putative transcriptional unit, since the two ORF overlap with four nucleotides (da Silva et al. 2002). The RmrB protein has been proposed to prevent accumulation of toxic plant com-

pounds inside bacterial cells, and an *rmrB* mutant in *Rhizobium etli* increased sensitivity to plant metabolites, including salicylic acid, phytoalexins, and flavonoids (González-Pasayo and Martínez-Romero 2000). RmrB likely plays similar roles in *X. citri* subsp. *citri*. In addition to the mutants described above, mutants of 12 hypothetical genes also showed deficient virulence (symptom category V) in host plants, and those genes will be discussed later.

Bacteria need to coordinate their limited genetic resources and their diverse virulence factors to survive harsh environments, including the intercellular spaces in host plants. In our screen, four regulatory genes (*rpfC*, *dksA*, XAC1548, and XAC2670) were identified as being potentially involved in the virulence regulation of *X. citri* subsp. *citri* (Table 1).

The DSF-mediated *rpf* quorum-sensing system was well-studied in *X. campestris* pv. *campestris* and regulates the synthesis of extracellular enzymes, EPS production, and biofilm formation and is also required for full virulence in host plants (He and Zhang 2008). A set of target proteins were found to directly interact with the quorum-sensing regulator RpfG of *X. citri* subsp. *citri* (Andrade et al. 2006), including HmsF. Insertion of EZ-Tn5 in *hmsF* has a potential polar effect on the expression of downstream genes of the *hmsHFRS* operon (Table 1). The *hmsF* gene encodes a putative polysaccharide deacetylase, and the products of the *hms* operon are required for the biofilm formation of *Yersinia pestis* (Forman et al. 2006). It is likely that the reduced virulence and cell density of the *hmsF* mutant in planta resulted from its effect on biofilm formation through its direct interaction with RpfG, since RpfG is the regulator for the DSF signaling pathway.

The putative protein that is encoded by XAC2670 shares high similarity (81% in amino acid sequence) to the TCRS sensor AlgZ (also called FimS) in *P. aeruginosa*. This sensor might interact with the cognate regulator AlgR and has been reported to be both a repressor of alginate biosynthesis (Yu et al. 1997) and an activator of type IV pili-mediated twitching motility (Whitchurch et al. 1996, 2002). Interestingly, XAC2670 is located adjacent to a gene cluster (XAC2664 to XAC2669) that is responsible for type IV pili assembly, which suggests a potential role for XAC2670 in the regulation of type IV pili, which are principal structures for host colonization and adhesion in many pathogenic bacteria (Craig et al. 2004). Mutation of *pilC* or *pilB* has been shown to cause significant reduction of virulence in *X. campestris* pv. *campestris* (Qian et al. 2005).

XAC1548 encodes a putative GntR family regulator that is named after the *B. subtilis* repressor of the gluconate operon (Haydon and Guest 1991). Regulators of this family share a conserved N-terminal DNA binding domain, but one or both of the C-terminal effector binding and oligomerization domains is quite diverse and heterogeneous (Rigali et al. 2002). As a consequence of this heterogeneity, the GntR regulators have been further classified into six subfamilies, FadR, HutC, MocR, YtrA, AraR, and PlmA (Lee et al. 2003; Rigali et al. 2004). Homolog analysis showed that the protein encoded by XAC1548 belongs to the YtrA subfamily, which contains a reduced C-terminal domain. The *ytrA* gene, which is a part of a self-regulated operon, has been experimentally explored in *B. subtilis*. This operon consists of genes encoding an ABC transporter system that is involved in acetoin utilization (Yoshida et al. 2000). Interestingly, XAC1548 is the first gene in a putative operon that contains *nodI* (XAC1547) and the three hypothetical genes XAC1546, XAC1545, and XAC1544. The *nodI* gene encodes a putative ABC transporter ATP-binding protein, and knockout of a homolog *nodI* in *R. leguminosarum* resulted in a "nodulation-delayed" phenotype in pea host plants (Evans and Downie 1986). Moreover, XAC1546 en-

codes a putative ABC transporter system permease protein. It is likely that XAC1548 contributes to virulence by regulating the ABC transporter system that is encoded by XAC1544, XAC1545, XAC1546, and XAC1547. Further characterization of XAC1548 and the ABC transporter system is needed to fully understand their roles in the virulence of *X. citri* subsp. *citri*.

Importantly, we identified 13 hypothetical genes that were classified into putative virulence-related genes in the present study. Most of these hypothetical genes have no significant homology with known virulence genes. Thus, the identification of these novel virulence genes will be important for increasing our understanding of the virulence mechanism of *X. citri* subsp. *citri* and other foliar bacterial pathogens, even though further characterization of those hypothetical genes is necessary. For example, the putative protein encoded by the hypothetical gene XAC1142 contains a PrpF domain. PrpF was recently characterized as a non-PLP (pyridoxal-5-phosphate) dependent isomerase that is involved in the catabolism of propionate (Garvey et al. 2007). Propionate is a short-chain fatty acid that is commonly used as a food preservative because of its broad negative effects on cellular functions (Eklund 1980). Interestingly, this bacteria-toxic compound is present in citrus plants (Moshonas and Shaw 1983). XAC1142 might play a similar role in protecting the pathogen against the toxicity of propionate, which is consistent with our observed reduction of cell density of the XAC1142 mutant in planta (Table 1).

In conclusion, we have significantly advanced the understanding of the putative genetic determinants of the virulence mechanism of *X. citri* subsp. *citri* by identifying 59 putative virulence-related genes, including 27 novel genes. Further characterization of the putative virulence-related genes is needed. Characterization of the roles of these genes in mediating citrus canker disease progression will facilitate the design of new strategies for controlling citrus canker and other foliar bacterial diseases.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids.

The wild-type strain of *X. citri* subsp. *citri* 306 was provided by the Division of Plant Industry of the Florida Department of Agriculture and Consumer Services, Gainesville, FL, U.S.A. The *X. citri* subsp. *citri* strains were cultured at 28°C in nutrient broth (Difco, Detroit), on NA (Difco) plates, or on M9 minimal medium plates (Sambrook and Russell 2001). The *E. coli* strain was cultured at 37°C in Luria-Bertani (LB) medium. When required, the growth media were supplemented with ampicillin (50 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹), gentamycin (5 µg ml⁻¹), kanamycin (50 µg ml⁻¹), and rifampicin (50 µg ml⁻¹).

Screening of the mutant library of the *X. citri* subsp. *citri* in grapefruit.

The mutant library of *X. citri* subsp. *citri* was constructed previously (Guo et al. 2010). A total of approximately 22,000 independent mutants were subjected to the pathogenicity test for screening for virulence-deficient mutants. The EZ-Tn5 mutants were stored at -80°C and were cultured on NA plates containing kanamycin. The pathogenicity assays of the mutants were performed as previously described (Yan and Wang 2011). Briefly, fully expanded, immature grapefruit (*C. paradise* Macf. cv. Duncan) leaves were prepared in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. The *X. citri* subsp. *citri* strains were cultured for 2 days on NA plates at 28°C and

were resuspended in sterile tap water. For each mutant, two bacterial suspensions (10⁶ and 10⁸ CFU/ml) were injected into the intercellular spaces of the leaves with a needleless syringe. For each concentration, the cell suspension was inoculated into two independent leaves with two inoculation sites on each leaf. The wild-type pathogen was inoculated into the same leaves at the same cell suspension concentration. All of the tests were repeated three times with similar results. Similar canker symptoms could be induced by inoculating the wild-type pathogen at concentrations of either 10⁶ or 10⁸ CFU/ml. The disease progression with an inoculation of 10⁶ CFU/ml was delayed by approximately 7 days compared with the higher inoculation concentration of 10⁸ CFU/ml (data not shown). Only the symptoms induced by the pathogen with an inoculation of 10⁶ CFU/ml were present.

Growth assays in planta.

The *X. citri* subsp. *citri* strains were cultured on NA plates for 2 days at 28°C. The bacterial cells were resuspended in sterile tap water at a concentration of 10⁶ CFU/ml and were infiltrated into the grapefruit leaves with a needleless syringe. To evaluate the growth of wild-type *X. citri* subsp. *citri* strain 306 and its derivatives in the intercellular spaces of the plants, leaf disks (with a leaf area of 1 cm²) from inoculated leaves (four leaves for each sample) were excised with a cork borer and were then ground in 1 ml of sterile tap water. The samples were serially diluted and were plated on NA plates with appropriate antibiotics. The bacterial colonies were counted following incubation at 28°C for 3 days. The in planta growth was measured in quadruplicate, and the assays were repeated three times with similar results.

Southern blot analysis.

Genomic DNA from the mutant strains was extracted using a genomic DNA purification kit (Promega, Madison, WI, U.S.A.), and 3 µg of genomic DNA was digested with *EcoRI* overnight at 37°C and was subjected to electrophoresis on a 0.7% agarose gel. The DNA was transferred to a positively charged nylon membrane (Roche, Indianapolis, IN, U.S.A.), using a standard protocol. Probe generation, hybridization, and chemiluminescence detection were performed using the Digoxigenin-High Prime II DNA labeling and detection starter kit according to the manufacturer's instructions (Roche, Indianapolis, IN, U.S.A.).

Identification of the EZ-Tn5 insertion sites of the *X. citri* subsp. *citri* mutants.

Rescue cloning was performed to determine the insertion sites of the EZ-Tn5 transposon in the mutants according to the manufacturer's instructions (Epicentre). In brief, 1 µg of genomic DNA of the mutants of interest was digested with *PstI* or *EcoRI* overnight at 37°C. The digested DNA was purified, self-ligated, and transformed into the *E. coli* strain EC100D by electroporation. The transformed cells were spread onto LB plates containing kanamycin and were incubated overnight at 37°C. At least three separate kanamycin-resistant colonies were subjected to plasmid extraction for each mutant. The plasmid was sequenced using the primer R6KAN-2 RP-1 (Epicentre). BLAST analyses and alignment with the genomic sequence of *X. citri* subsp. *citri* 306 were performed to determine the insertion site of the EZ-Tn5 transposon.

For the identified genes located in potential operons, polarity analyses were conducted to determine if the insertion of EZ-Tn5 caused polar effect on the downstream genes in the operons, following the instructions of the kit (Epicentre). Briefly, the insertion of EZ-Tn5 has a potential polar effect on the expression of one or more of the downstream genes, if the

direction of the kanamycin-resistance gene of EZ-Tn5 is different from that of the mutated gene. The insertion of EZ-Tn5 has no polar effect on the expression of one or more downstream genes if their directions are same.

Construction of complementary plasmids.

The polymerase chain reaction (PCR) primers used for the amplification of target genes are listed in Supplementary Table S2. To construct the complementary plasmid for *pthA4*, the megaplasmids of *X. citri* subsp. *citri* 306 were extracted and digested with *KpnI* and *XhoI* and were then ligated into the pBluescript II SK(+) plasmid (Invitrogen, Carlsbad, CA, U.S.A.). The resulting plasmids were sequenced to select the plasmid that contained the 16-kb *KpnI-XhoI* fragment harboring the *pthA4* gene. This plasmid was named pBS-A4. Using the primers pthA4F and pthA4R and pBS-A4 as the template, a PCR fragment of 3,896 bp containing the *pthA4* gene was amplified and inserted into the T-vector of pCR2.1 (Invitrogen). The resulting plasmid (p2.1-*pthA4*) was digested with *EcoRI* and *HindIII* and was ligated with pUFR053 (El Yacoubi et al. 2007) to yield the complementary plasmid p53-*pthA4*. To generate the complementary plasmid of p53-0478, the intact *trpG* gene was amplified using the primers 0478F and 0478R and the genomic DNA of *X. citri* subsp. *citri* 306 as the template. The PCR product was digested with *SacI* and *HindIII* and was ligated into pUFR053 to yield the plasmid p53-0478. The complementary plasmids p53-0481, p53-0511, p53-0618, p53-0661, p53-1548, and p53-1230 were constructed using a similar approach with their respective primers. The complementary plasmids and the empty vector pUFR053 were transformed into the corresponding mutants.

Auxotrophic, protease, and amylase assays.

M9 medium was used to test whether the *X. citri* subsp. *citri* mutants were auxotrophic on minimal medium. In brief, the mutants and the wild type were initially cultured on NA plates for 2 days, and then, the bacteria were restreaked on M9 medium plates and were cultured at 28°C. The growth of the bacteria was measured over the following 5 days after inoculation. For each assay, three replicates were used.

Bacterial protease activity was assayed on skim-milk plates. The bacterial cells of wild type and *xps* mutant strains were collected from NA plates and were resuspended in sterile water at a concentration of 10^8 CFU/ml, and then, 2 µl of the cell suspension was inoculated on a skim-milk plate and was cultured at 28°C. The protease activity of wild type and mutant strains was observed and photographed 3 dpi.

For the amylase assay, the bacterial cells of wild type and *xps* mutant strains were collected from NA plates and were resuspended in sterile water at a concentration of 10^8 CFU/ml, and 2 µl of the cell suspension was then inoculated on a NA plate containing 1% (wt/vol) starch and was cultured at 28°C. The plates were stained using Gram's iodine solution (containing 0.5 and 1 g of potassium iodide in 100 ml of water) at 2 dpi, and the results were photographed after staining.

Public software used for the analyses performed in this work.

The National Center for Biotechnology Information database was used to determine the insertion site of EZ-Tn5 in all mutants obtained in this work. The J. Craig Venter Institute database was used to analyze the functional categories of all of the genes that were identified in this work. The KEGG database was used to analyze the general metabolic pathways of the genes of interest that were identified in this work. The SMART database was used to analyze the one or more functional domains of the genes that were identified in this work.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- J. Craig Venter Institute website: www.jcvi.org
 KEGG:Kyoto Encyclopedia of Genes and Genomes website: www.genome.jp/kegg
 The National Center for Biotechnology Information website: www.ncbi.nlm.nih.gov
 European Molecular Biology Laboratory SMART database: smart.embl-heidelberg.de