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Identification of seven novel virulence genes from *Xanthomonas citri* subsp. *citri* by Tn5-based random mutagenesis[§]

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To identify novel virulence genes, a mutant library of *Xanthomonas citri* subsp. *citri* 29-1 was produced using EZ-Tn5 transposon and the mutants were inoculated into susceptible grapefruit. Forty mutants with altered virulence phenotypes were identified. Nine of the mutants showed a complete loss of citrus canker induction, and the other 31 mutants resulted in attenuated canker symptoms. Southern blot analysis revealed that each of the mutants carried a single copy of Tn5. The flanking sequence was identified by plasmid rescue and 18 different ORFs were identified in the genome sequence. Of these 18 ORFs, seven had not been previously associated with the virulence of *X. citri* subsp. *citri* and were therefore confirmed by complementation analysis. Real-time PCR analysis showed that the seven genes were upregulated when the bacteria were grown in citrus plants, suggesting that the expression of these genes was essential for canker development.

***Keywords*:** *Xanthomonas citri* subsp. *citri*, mutant library, virulence genes, canker development

Introduction

Xanthomonas citri subsp. *citri* (*Xac*) is the causative agent of citrus canker, which is one of the most destructive bacterial disease in citrus (Brunings and Gabriel, 2003). This pathogen infects nearly all important citrus varieties, including grapefruit, lemons, Mexican limes and sweet oranges. Wind-driven rain is the primary cause of canker prevalence and facilitates bacterial penetration through wounds, stomata and lenticels (Stall and Civerolo, 1991; Pruvost *et al.*, 2002). Currently, the only efficient way to eliminate the disease is through the eradication of sick plants, which results in significant economic losses (Gottwald *et al.*, 2001).

Epiphytic survival and secretion systems are major mechanisms employed by *X. citri* subsp. *citri* to develop canker symptoms in host plants. The attachment of the bacteria and subsequent biofilm formation on host plant tissue are correlated with extracellular polysaccharide (EPS) biosynthesis. A *gumD* mutant defective in EPS production does not form a mature biofilm on either abiotic or biotic surface (Rigano *et al.*, 2007). Through a genome wide scale, at least 92 genes have been shown to be involved in biofilm formation, including the filamentous hemagglutinin-like protein secreted *via* the type V secretion system (Gottig *et al.*, 2009; Li and Wang, 2011). The complete *hrp* gene cluster encoding the type III secretion system (T3SS) is required for the development of disease symptoms in susceptible citrus plants as well as for the hypersensitive response (HR) in resistant plants (Alegria *et al.*, 2004; Dunger *et al.*, 2005). A T3SS-dependent *pthA* homolog encoded by the *hssB3.0* gene was found to be required for the virulence of *X. citri* subsp. *citri* KC21 on citrus grandis cultivars (Shiotani *et al.*, 2007). Other possible T3SS-related factors have been identified in the *X. citri* subsp. *citri* genome by bioinformatics analysis (da Silva *et al.*, 2002), and some of these factors have been carefully elucidated in recent studies (Dunger *et al.*, 2012; Malamud *et al.*, 2012; Sgro *et al.*, 2012).

A complete genome sequence of XAC306 from Brazil was reported ten years ago (da Silva *et al.*, 2002), and a number of virulence defective mutants have been identified in *Xac* 306 via Tn5-mediated mutagenesis screening (Laia *et al.*, 2009; Baptista *et al.*, 2010; Li and Wang, 2011). Genome-wide screening is a powerful method for mutant construction and has been used for high-throughput screening of virulence genes in several *Xanthomonas* members (Qian *et al.*, 2005; Wang *et al.*, 2008; Zou *et al.*, 2011). Four *tal* genes in a chinese strain *Xac* 29-1 correspond to 14.5, 15.5 18.5 and 23.5 tandem repeats, implying a high level of genetic diversity (Ye *et al.*, 2013). To uncover new virulence genes from *Xac* 29-1 isolated from Jiangxi Province in China, approximately 20,000 mutants were individually screened for virulence defects in grapefruit. Among 18 virulence-related genes identified to be involved in citrus canker symptom development, seven were novel genes which significantly advances our understanding of the genetic basis of this disease.

Materials and Methods

Bacteria strains, culture media, and growth conditions

All of the strains and plasmids used in this study are listed in Supplementary data Table S1. *Xac* 29-1 and its derivatives

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were grown at 28°C in NB broth or NA agar media (Zou *et al*., 2011; Ye *et al*., 2013). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium. When required, the growth media were supplemented with kanamycin (Km, 25 μg/ml) or gentamycin (Gm, 10 μg/ml).

Construction of a Tn5-tagged mutant library

An EZ-Tn5^{TM}<R6Kγori/KAN-2>TnpTransposome^{TM} Kit (Epicentre) was used to make the *Xac* 29-1 mutant library according to the manufacturer's instructions. In brief, 0.5 μl of transposon DNA was transformed into 100 μl of competent cell by electroporation using a Gene Pulser electroporation apparatus (Micropulser^{TM}, Bio-Rad) at 2.5 kV and 4 mS in 0.2 cm pre-chilled cuvettes. The recovered cells were diluted 1000-fold, spread on NA plates containing Km, and incubated at 28°C for 2 to 3 days. Individual Km-resistant colonies were numbered and transferred to fresh NA plates with Km for further investigation.

Pathogenicity and hypersensitive response (HR) assays

The wild type *Xac* 29-1 and mutant strains were grown overnight in a shaker at 28°C in NB and then suspended in sterile distilled water to a final concentration of 10^8 CFU/ml. For the pathogenicity assay, bacterial suspensions were injection infiltrated into fully expanded grapefruit leaves with a needleless syringe (*Citrus paradise* Macf. cv Duncan). Disease symptoms were scored and photographed at 15 days post-inoculation. For the hypersensitive response assay, bacteria were injected into tomato leaves, and the plant reactions were viewed at 2 days post-inoculation. Both tests were repeated three times.

EZ-Tn5 transposon copy number determination

Genomic DNA was individually extracted from *X. citri* subsp. *citri* mutants using a genomic DNA purification kit according to the manufacturer's protocols (Axygen). DNA samples were fully digested with *Pst*I, subjected to electrophoresis on a 1.0% agarose gel, and transferred to a positively charged nylon membrane (Roche). A 500-bp DNA fragment of the kanamycin resistance gene of the EZ-Tn5 transposon was amplified as a hybridization probe with primers Tn5.F and Tn5.R (Supplementary data Table S2). Probe labeling, hybridization, and detection were performed using a DIG-high prime II DNA labeling and detection starter kit following the manufacturer's instructions (Roche).

Identification of the EZ-Tn5 flanking sequence

The EZ-Tn5 flanking sequence was identified using the plasmid rescue method. Genomic DNA from each mutant was digested with *Pst*I, self-ligated with DNA ligase, and transformed into the *E. coli* strain S17-1 λpir using the heat shock method. After incubation in LB medium containing km overnight, plasmids of three separate kanamycin-resistant colonies were extracted and sequenced using the primer R6KAN-2 RP-1 (Supplementary data Table S2). The flanking sequence was analyzed by BLAST in GenBank against the *Xac* 29-1 genome sequence.

Complementation constructs

Seven entire ORFs of the mutated genes were amplified from the genomic DNA of wild type *Xac* 29-1 using specific primers (Supplementary data Table S2). To constitutively express these genes, a 502-bp DNA fragment containing the *wxacO* gene promoter was first cloned into the vector pBBR1MC-5 at the *Xho*I and *Xba*I sites after amplification with the primers *wxacO*.p.F and *wxacO*.p.R, resulting in pBBP (Supplementary data Table S1). Next, each ORF was individually inserted into the *Xba*I, *Sac*I or *Xba*I-*Sac*I sites with the corresponding ORF-specific primers (Supplementary data Table S2). The recombinant plasmids were screened with primer *wxacO*.p.F combined with each reverse primer for ORF amplification. All of the constructs were confirmed by sequencing and then transformed into the corresponding Tn5 insertion mutants via electroporation. The complemented strains were screened on NA plates containing Km and Gm.

Bacterial growth in grapefruit

Wild type *Xac* 29-1, seven mutants and the complemented strains were infiltrated into fully expanded grapefruit leaves at an OD_{600}=0.3. At 0, 2, 4, 6, 8 days after infiltration, leaf discs 1 cm in diameter were punched off from the infiltration area. The samples were rinsed twice in sterile water, surface sterilized for 15 sec in 75% ethanol, and rinsed three times in sterile water to completely remove the ethanol. After fully grinding the discs in 1 ml of sterile water, the suspension samples were diluted with 10-fold series and spread on NA plates containing the suitable antibiotic. The colonies were counted after 3 days of incubation at 28°C. The experiments were repeated three times.

Real-time PCR

Real-time polymerase chain reaction was employed to evaluate the expression levels of seven virulence genes. To obtain RNA from cells growing in NA medium, *Xac* 29-1 was cultured in 3 ml NA medium for 24 h and then subcultured (1:100) into 20 ml liquid NA medium for 36 h in a shaker (200 rpm, 28°C). The total RNA was extracted with Trizol reagent as recommended by the manufacturer (Invitrogen). To obtain RNA from cells growing in host plant, *Xac* 29-1 cells (OD_{600}=0.3) were infiltrated into fully expanded citrus leaves. At 2 days after inoculation, leaves were collected for RNA extraction. DNase I treatment and reverse transcription were performed as described previously (Zou *et al*., 2012). The primers used to evaluate gene expression are listed in Supplementary data Table S2. The PCR reaction was performed with the Applied Biosystems 7500 real-time PCR System using SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (TaKaRa). The thermal cycler conditions were run as follows: denaturation temperature at 95°C for 30 sec and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The expression level of *gyrA* was used as an internal standard (Supplementary data Table S2). The comparative threshold method was used to calculate the relative mRNA levels with respect to the corresponding transcripts in cells cultured in NB or growing in citrus plants. All RT-PCRs were performed in triplicate.

Results

EZ-Tn5 mediated genome-wide mutagenesis

To uncover novel virulence genes in *Xac* 29-1, a mutant library of 20,000 mutants was produced using the EZ-Tn5 mutagenesis kit. One-hundred microliters of competent cells were transformed with 0.5 μl of transposon DNA by electroporation. To obtain a random insertion ratio, we spread 40 plates for each electroporation, and 2000 Km-resistance colonies were isolated. After ten transformations, approximately 20,000 colonies were obtained. The probability of finding one transposon insertion for a certain gene was calculated from the formula $P = 1 - (1 - X/G)^n$, where P is the probability of finding one transposon insertion within a given gene, X is the length of the gene (1,000 bp), G is the length of the genome (5.15×10^6 bp in *Xac* 29-1), and n is the number of transposon inserts present in the population (Krysan *et al.*, 1999). The probability of finding one transposon insertion for a certain gene with 20,000 mutants was 98.0% (Laia *et al.*, 2009).

Virulence-deficient mutant screening

The mutants were infiltrated into susceptible citrus grapefruit for pathogenicity assay and into a non-host tomato to evaluate the capacity to elicit a hypersensitive response. The pathogenic phenotype was tested and scored at 15 days after

Table 1. **Virulence-related genes of *Xanthomonas citri* subsp. *citri* 29-1 identified this study**

Mutant	Gene ID	Function of gene product	Insertion site	Virulence Phenotype in Grapefruit	HR in Tomato
Mxac17-72	XAC29_00040	Energy transducer TonB protein	(-44/672)	lost	normal
Mxac 92-31	XAC29_00040	Energy transducer TonB protein	(9/672)	lost	normal
Mxac 179-53	XAC29_00045	Biopolymer transport ExbB protein	(-79/762)	weakened	normal
Mxac 165-69	XAC29_00045	Biopolymer transport ExbB protein	(-84/762)	weakened	normal
Mxac 46-27	XAC29_00045	Biopolymer transport ExbB protein	(-26/762)	weakened	normal
Mxac 92-72	XAC29_00050	Biopolymer transport ExbD1 protein	(10/423)	lost	normal
Mxac 45-32	XAC29_00050	biopolymer transport ExbD1 protein	(-44/423)	weakened	normal
Mxac 14-73	XAC29_01060	Nitrogen regulatory protein P-II(GlnB)	(-135/345)	weakened	normal
Mxac 7-73	XAC29_01170	Putative secreted protein	(610/753)	lost	normal
Mxac 21-36	XAC29_03475	Two-component system sensor protein	(1654/1860)	weakened	normal
Mxac 8-20	XAC29_06185	Putative membrane protein	(324/1167)	lost	normal
Mxac 39-87	XAC29_06185	Putative membrane protein	(70/1167)	weakened	normal
Mxac 33-10	XAC29_06185	Putative membrane protein	(301/1167)	weakened	normal
Mxac 51-74	XAC29_06795	Outer membrane protein	(122/342)	lost	normal
Mxac 111-54	XAC29_06795	Outer membrane protein	(40/342)	lost	normal
Mxac 97-43	XAC29_07675	Chaperone protein DnaJ	(32/1128)	weakened	normal
Mxac 80-68	XAC29_07675	Chaperone protein DnaJ	(623/1128)	weakened	normal
Mxac 110-45	XAC29_07675	Chaperone protein DnaJ	(34/1128)	weakened	normal
Mxac 121-23	XAC29_07810	Putative GntR-family transcriptional regulator	(225/363)	lost	normal
Mxac 27-7	XAC29_07810	Putative GntR-family transcriptional regulator	(207/363)	lost	normal
Mxac 181-45	XAC29_09685	Serine/threonine protein kinase	(411/519)	weakened	normal
Mxac 1-30	XAC29_09950	Response regulator	(237/384)	weakened	lost
Mxac 39-5	XAC29_09950	Response regulator	(339/384)	weakened	lost
Mxac 58-23	XAC29_09950	Response regulator	(165/384)	weakened	lost
Mxac 10-72	XAC29_09950	Response regulator	(124/384)	weakened	lost
Mxac 80-21	XAC29_10340	Probable phosphoenolpyruvate synthase protein (*ppsA*)	(2331/2379)	weakened	normal
Mxac 13-83	XAC29_10340	Probable phosphoenolpyruvate synthase protein (*ppsA*)	(1745/2379)	weakened	normal
Mxac 112-9	XAC29_10340	Probable phosphoenolpyruvate synthase protein (*ppsA*)	(1321/2379)	weakened	normal
Mxac 6-15	XAC29_11940	Putative major facilitator superfamily transmembrane transporter	(182/1251)	weakened	normal
Mxac 156-66	XAC29_11950	Conserved hypothetical protein	(495/669)	weakened	normal
Mxac 44-35	XAC29_13795	Probable triosephosphate isomerase (*tpiA*)	(509/756)	weakened	normal
Mxac 155-15	XAC29_22454	PthA4	(2282/3492)	weakened	normal
Mxac 51-14	XAC29_22454	PthA4	(762/3492)	weakened	normal
Mxac 87-52	XAC29_22454	PthA4	(1461/3492)	weakened	normal
Mxac 191-41	XAC29_22454	PthA4	(196/3492)	weakened	normal
Mxac 173-4	XAC29_22454	PthA4	(668/3492)	weakened	normal
Mxac 126-80	XAC29_22454	PthA4	(1789/3492)	weakened	normal
Mxac 4-69	XAC29_22454	PthA4	(954/3492)	weakened	normal
Mxac 140-68	XAC29_22454	PthA4	(284/3492)	weakened	normal
Mxac 56-20	XAC29_17990	General secretion pathway protein D	(74/2292)	weakened	normal

inoculation. The 2000 mutants isolated from each round of electroporation were infiltrated into citrus leaves at the same time. This was followed by the next electroporation transformation to obtain the next set of 2000 mutants while waiting to view and record the virulence phenotype of the infiltrated mutants. After the 10 rounds of pathogenic assays, 40 mutants were identified as showing a phenotypic alteration in citrus. Nine mutants completely lost the pathogenicity, and the remaining 31 mutants showed reduced virulence in citrus plants. Four mutants were impaired in their ability to induce a hypersensitive response in tomatoes compared to the wild type cells (Table 1).

The Tn5 insertion copy number was validated using Southern blot analysis (Fig. 1) and showed that each of the 40 mutants contained a single-copy insertion, which was consistent with the low rate (less than 1%) of multi-copy transposon insertions that was described by the manufacturer of the EZ-Tn5 insertion kit. This allowed us to identify the flanking sequence in each mutant using the plasmid rescue cloning procedure. Among the 40 mutants, there were 34 with an insertion in the coding region and 6 with an insertion in intergenic regions, as shown by sequencing and blast analyses of the flanking fragments (Table 1). Three intergenic region insertions occurred in the promoter region of the gene *exbB* that encodes a biopolymer transport protein. The other three intergenic insertions were found in *tonB*, *exbD1*, and *glnB*; the last two of these genes also had one insertion each within their coding sequences. The gene *pthA4* was the sole gene identified from the plasmid and was mutated 8 times. The other 17 genes were located on the chromosome. In total, 18 different ORFs were hit in the genome sequence, and some ORFs had multiple insertions. These genes were classified into several functional groups, including the type II secretion system, type III secretion effectors, extracellular polysaccharide biosynthesis, transportation, regulation and general metabolic pathways (Table 1).

Complementation analysis of seven virulence defective mutants

The contribution of the genes *tpiA*, *ppsA*, *glnB*, *dnaJ*, XAC29_09950, XAC29_03475 and XAC_11940 to pathogenicity has not been previously reported in *X. citri* subsp. *citri*, and they were confirmed by a complementation assay in this study. The mutants produced extremely weak canker symptoms in citrus plants (Fig. 2). The growth of a response regulator mutant (XAC29_09950) was almost similar with wild type, showing slight effect on bacterial growth. The growth of the other six mutant strains was significantly decreased in citrus plants. All of the mutants were partially restored to wild type levels of virulence at 15 days after infiltration, and similar results were observed in their growth in *planta* (Fig. 2).

Gene expression

To evaluate the correlation of the mutated genes with canker development, the expression levels of the seven genes *in planta* were analyzed by real-time PCR. Total RNA were isolated from bacteria cultured in NB media and bacteria grown in citrus plants. When compared with expression levels of the genes in the NB medium, the expression levels of the seven genes were all remarkably induced in *planta* (Fig. 3). The most significantly induced genes were *glnB* and *dnaJ*, which were enhanced by approximately 60-fold and 30-fold, respectively.

Discussion

In the present study, a mutant library of *Xac* 29-1 was obtained by EZ-Tn5 transposon mutagenesis and the mutants were inoculated into citrus plants to identify virulence-related genes. The flanking sequences of 9 mutants that caused no symptoms in host corresponded to 6 genes, and those of

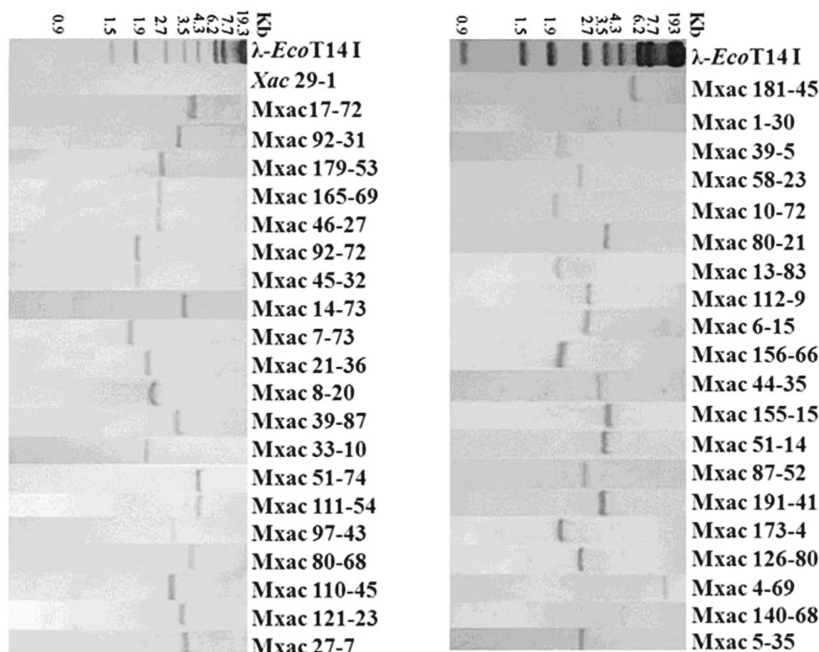

Fig. 1. Southern blot analysis of Tn5 insertion in mutants. gDNA was digested with *Pst*I and hybridized against a 500-bp Tn5 transposon DNA fragment amplified with the primers Tn5.F and Tn5.R.

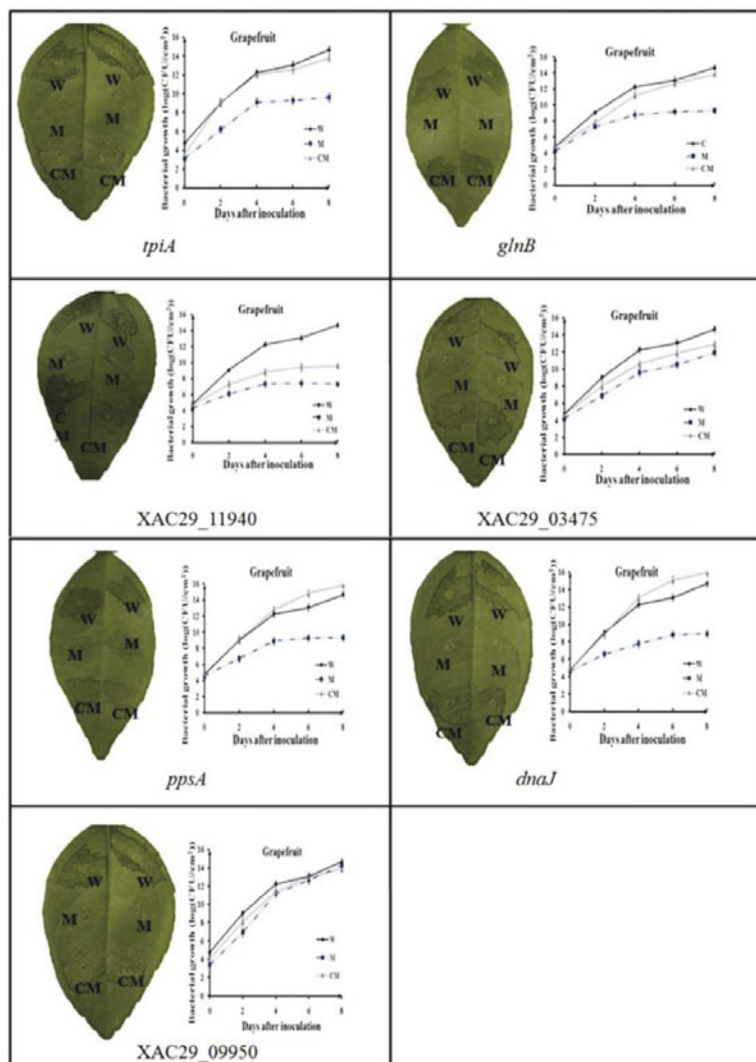

Fig. 2. The pathogenicity of *Xanthomonas citri* subsp. *citri* mutants in citrus plants. The bacteria were inoculated into intercellular spaces of citrus leaves at a cell concentration of 10^8 CFU/ml. Symptoms were observed at 15 days post inoculation. Bacterial growth was monitored over a period of 8 days. Values represent the mean of three samples from three different plants, and the experiment was repeated three times. W, Wild-type; M, Mutant; CM, Complemented mutant.

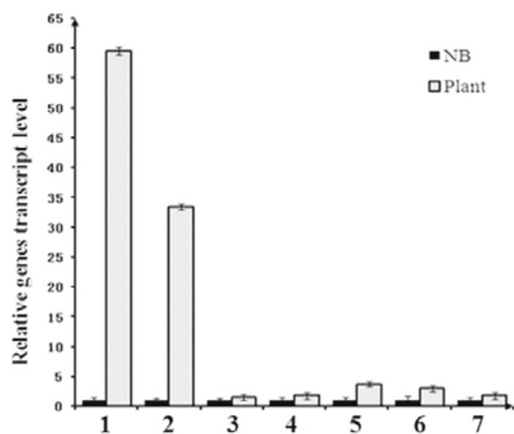

Fig. 3. Evaluation of the expression levels of seven gene candidates by real-time PCR. RNA was isolated from cultures of the wild type *Xac* 29-1 grown in NB medium (black) and the citrus leaves injected with wild type 29-1 (grey). 1, *glnB*; 2, *dnaJ*; 3, XAC29_09950; 4, XAC29_03475; 5, XAC29_11940; 6, *tpiA*; 7, *ppsA*. The relative mRNAs levels were calculated with respect to the level of *gyrA*. The data presented are the means of triplicate measurements from a representative experiment.

31 mutants that caused weak symptom corresponded to 12 genes. These genes were classified as type III secretion effectors or components of the type II secretion system, extracellular polysaccharide biosynthesis pathway, transportation, regulation or general metabolic pathways. Seven mutants *glnB*, *dnaJ*, *tpiA*, *ppsA*, XAC29_03475, XAC29_09950, and XAC_11940 were confirmed by complementation analysis. Their expression was upregulated when grown in citrus plants.

The ability to move proteins out of the cytoplasm and across membranes is a key aspect of the physiology and pathogenicity of Gram-negative bacteria. The T3SS encoded by *hrp* gene cluster is responsible for delivering a number of HR elicitors or pathogenic factors into plant cells (Galan and Collmer, 1999). The T3SS effector PthA is responsible for modulating host transcription to promote citrus canker (de Souza *et al.*, 2012). *Xac* 29-1 has four copies of PthA encoded on two plasmids. However, the repeat number of each copy of PthA is different from the previous sequenced strain Xac 306 from Brazil (Ye *et al.*, 2013) even though they have the same genetic loci in the genome. In *Xac* 29-1, the PthA

with 23.5 repeats functions as the major determinant of bacterial virulence. Eight mutants with diverse insertion sites within the *pthA* ORF showed a phenotype of reduced virulence in citrus plants (Table 1). This is different from the phenotype of PthA in *Xac* 306, which causes a complete loss of pathogenicity, and no visible hypertrophy symptoms at the inoculated area (AI-Saadi *et al*., 2007) which may be caused by genetic diversity of PthA in *Xac* 29-1.

The DnaK-DnaJ-GrpE (KJE) chaperone system functions in the regulation of protein homeostasis in bacteria by facilitating the folding of nascent polypeptides and the re-folding of damaged proteins (Diamant and Goloubinoff, 1998). DnaJ is thought to bind first and then recruit DnaK through its J domain (Perales-Calvo *et al*., 2010). Stimulation of ATP turnover by the combined action of DnaJ and interactions with the substrate would then enable tight binding. In line with its key role in protein folding, KJE has been considered an essential building block for the minimal bacterial genome and to be common to all bacteria. The KJE system is present in almost all sequenced bacterial genomes. A rigorous survey of 1,233 complete bacterial genomes reported that the entire KJE system is absent in *Desulfurobacterium thermolithotrophum* and *Thermovibrio ammonificans*, two thermophilic bacteria isolated independently from deep-sea hydrothermal vents in the Atlantic and Pacific Oceans, respectively (Warnecke, 2012). In *Xac* 306, DnaK, DnaJ, and GrpE are encoded by XAC1521, XAC1522, and XAC1523 (da Silva *et al*., 2002). The identities of XAC1521 and XAC-1522 were reported by a previous work (Yan and Wang, 2012). In our work, DnaJ was reduced in pathogenicity and could be complemented. These results demonstrated that the KJE system is involved in canker development.

The utilization of carbon and nitrogen resources is essential for growth followed by successful colonization of host plants by pathogenic bacteria. We identified three novel virulence-associated genes involved in central metabolism. Triosephosphate isomerase (TPI) catalyzes the isomerization of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in the fifth step of the glycolytic pathway (Knowles, 1991; Cui and Karplus, 2001). Phosphoenolpyruvate synthetase (*ppsA*) catalyzes the conversion of pyruvate and ATP to phosphoenolpyruvate (PEP), AMP, and phosphate and is thought to function in gluconeogenesis. The synthesis of PEP from pyruvate is catalyzed by *ppsA* (Tang *et al*., 2005). Although the role of *ppsA* in bacterial virulence has been reported in previous studies, it has not been reported in *X. citri* subsp. *citri* (Wang *et al*., 2008; Yang *et al*., 2008). Both *tpiA* and *ppsA* are involved in the glycolytic pathway (Knowles, 1991; Cui and Karplus, 2001). Nitrogen is an important material for synthesizing proteins, nucleic acids and other nitrogen compounds in bacteria. The PII protein encoded by *glnB* senses α-ketoglutarate levels within the cell as an indicator of carbon status and controls NtrB activity in response to the level of α-ketoglutarate (Yurgel *et al*., 2010). In ammonia-limited conditions, GlnB is uridylylated rapidly by a bifunctional, uridylyltransferase/uridylyl-removing enzyme encoded by *glnD*, resulting in an increase in glutamine synthetase (GS) and the activation of the two component regulator NtrB/NtrC (Atkinson and Ninfa, 1998). This indicated that there existed a complex carbon and nitrogen metabolic event within *X. citri* subsp. *citri* during canker development.

The major facilitator superfamily (MFS) was required for *X. citri* subsp. *citri* to live in citrus tissue. Mutation in XAC29_11940 which encodes a MFS reduced bacterial growth and virulence remarkably. The communication between bacterial cells and the environment includes the export of waste compounds and secondary metabolites involved in infection or competition (Mitchell, 1967). The MFS proteins transport a wide range of structurally unrelated compounds such as sugars, Krebs-cycle metabolites, vitamins, amino acids, iron-siderophores, ions, drugs and endogenous toxins (Pao *et al*., 1998; Del Sorbo *et al*., 2000). Further studies are needed to identify the exact substrate for XAC_11940.

Two-component systems are structured around two conserved proteins: a histidine protein kinase and a response regulator protein. The transmembrane kinase sensor is autophosphorylated at a conserved histidine residue and then transfers to the response regulator, resulting in activation of the response regulator and generation of the output response of the signaling pathway (Stock *et al*., 2000). In this work, we reported a two component sensor XAC29_03475 and a response regulator XAC29_09950. Mutation in XAC29_09950 had no distinct effect on bacterial growth in citrus, suggesting that it was involved in bacterial virulence directly. Added a previous report about ColR/ColS (Yan and Wang, 2011), we propose that two-component systems play crucial roles in multiple cellular functions in *X. citri* subsp. *citri.*

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