

# Requirement of the *galU* Gene for Polysaccharide Production by and Pathogenicity and Growth *In Planta* of *Xanthomonas citri* subsp. *citri*<sup>∇</sup>

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*Xanthomonas citri* subsp. *citri* is the causal agent of citrus canker, which has a significant impact on citrus production. In this study, we characterized the *galU* gene of *X. citri* subsp. *citri*. Two *galU* mutants (F6 and D12) were identified in an *X. citri* subsp. *citri* EZ-Tn5 <R6Kγori/KAN-2> Tnp transposon library. Rescue cloning, sequence analysis, and Southern blot analysis indicated that both of these mutants had a single copy of the EZ-Tn5 transposon inserted in *galU* in the chromosome. Further study showed that *galU* was required for biosynthesis of extracellular polysaccharides (EPS; xanthan gum) and capsular polysaccharide (CPS) and biofilm formation. Mutation of *galU* resulted in a loss of pathogenicity for grapefruit. The loss of pathogenicity of a *galU* mutant resulted from its inability to grow *in planta* rather than from the effect on virulence genes. Quantitative reverse transcription-PCR assays indicated that mutation of *galU* did not impair the expression of key virulence genes, such as *pthA* of *X. citri* subsp. *citri*. Although D12 had a growth rate similar to that of the wild-type strain in nutrient broth, no D12 population became established in the intercellular spaces of citrus leaves. Coinoculation of a *galU* mutant with the wild-type strain did not promote growth of the *galU* mutant *in planta*. Defects in EPS and CPS production, pathogenicity, and growth *in planta* of the *galU* mutant were complemented to the wild-type level using plasmid pCGU2.1 containing an intact *galU* gene. These data indicate that the *galU* gene contributes to *X. citri* subsp. *citri* growth in intercellular spaces and is involved in EPS and CPS synthesis and biofilm formation.

Citrus canker is a serious disease of most commercial citrus cultivars in subtropical citrus-producing areas of the world (12, 18). Citrus-producing areas without citrus canker consider the disease a quarantine pest due to the potential threat to citrus production. Thus, citrus canker has a significant impact on national and international citrus markets and trade (18). Citrus canker is a disease that is characterized by formation of necrotic raised lesions on leaves, stems, and fruits. On severely affected trees, citrus canker causes defoliation, twig dieback, general tree decline, blemished fruit, and premature fruit drop (19). Citrus canker is caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri* (synonyms, *Xanthomonas citri*, *Xanthomonas campestris* pv. *citri*, and *Xanthomonas axonopodis* pv. *citri*) strain A (10, 44, 48). This bacterium is dispersed via windblown rain and enters the plant directly through stomata or through wounds, and then it grows in the intercellular spaces of the spongy mesophyll (18). The intercellular spaces are nutrient poor (1) and contain toxic substances (either preformed or induced) as part of the host defense response (35, 45). Bacteria have evolved complicated mechanisms to overcome the plant defense and acquire nutrients to colonize susceptible hosts.

Genome sequencing of *X. citri* subsp. *citri* has greatly in-

creased our understanding of the interaction between citrus and *X. citri* subsp. *citri*. *X. citri* subsp. *citri* contains 4,313 genes, including 2,710 genes with assigned functions, 1,603 genes without known functions, and 54 RNA genes (13). About 6% of the *X. citri* subsp. *citri* genes are involved in pathogenicity, virulence, and adaptation. The major genes involved in pathogenicity and virulence are genes encoding secretion systems, including type III secretion system genes, effector genes, genes encoding cell wall-degrading enzymes, toxins, bacterial adhesins, and surface structural elements, and *rpf* (regulation of pathogenicity factors) genes, which are related to cell-cell signaling (13). Even though the genome of *X. citri* subsp. *citri* has been sequenced and 62.83% of the predicted open reading frames have been assigned functions, functional studies are necessary to experimentally characterize the genes related to *X. citri* subsp. *citri* pathogenesis and host adaptation. Transposon mutagenesis has been widely used for this purpose (23, 41).

GalU is a UTP-glucose-1-phosphate uridylyltransferase (synonym, UDP-glucose pyrophosphorylase), and it catalyzes the formation of UDP-glucose from glucose 1-phosphate and UTP. UDP-glucose is involved in synthesis of glucosylated surface structures as a substrate for glucosyltransferase, and it is a glycosyl donor in the enzymatic biosynthesis of carbohydrates (9, 46). Mutations in the *galU* gene have led to reduced virulence of a number of bacterial pathogens, including *Escherichia coli* (21, 28), *Klebsiella pneumoniae* (7), *Shigella flexneri* (43), *Actinobacillus pleuropneumoniae* (38), *Vibrio cholerae* (34), *Pseudomonas aeruginosa* (37), and *Mesophilic aeromonas* (49). The reduced virulence of *galU* mutants has been associated mainly with changes in lipopolysaccharides (LPS), capsu-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
Mach1 T1R	F <sup>-</sup> $\phi$ 80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>hsdR</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) Δ <i>recA1398</i> <i>endA1</i> <i>tonA</i>	Invitrogen
TransforMax EC100D <i>pir</i> <sup>+</sup>	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ( <i>ara-leu</i> )7697 <i>galU</i> <i>galk</i> λ <sup>-</sup> <i>rpsL</i> <i>nupG</i> <i>pir</i> <sup>+</sup> (DHFR)	Epicentre
HB101	F <sup>-</sup> <i>supE44</i> <i>hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacY1</i> <i>galk2</i> <i>rpsL20</i> <i>xyl-5</i> <i>mtl-1</i> <i>leuB6</i> <i>thi</i>	4
<i>X. citri</i> subsp. <i>citri</i> strains		
306	Rif <sup>r</sup> , causes citrus canker on citrus	40
D12	<i>galU</i> ::Tn5 derivative of strain 306, Rif <sup>r</sup> Kn <sup>r</sup>	This study
F6	<i>galU</i> ::Tn5 derivative of strain 306, Rif <sup>r</sup> Kn <sup>r</sup>	This study
CD	D12 containing pCGU2.1, Kn <sup>r</sup> Cm <sup>r</sup> Gm <sup>r</sup>	This study
CF	F6 containing pCGU2.1, Kn <sup>r</sup> Cm <sup>r</sup> Gm <sup>r</sup>	This study
Plasmids		
pCR2.1	PUC ori f1 ori <i>lacZ</i> α <sup>+</sup> Kn <sup>r</sup> Ap <sup>r</sup>	Invitrogen
pRK2013	ColE1 Kn <sup>r</sup> Tra <sup>+</sup> , conjugation helper plasmid	14
pUFR053	IncW Mob <sup>+</sup> <i>mob</i> (P) <i>lacZ</i> α <sup>+</sup> Par <sup>+</sup> Cm <sup>r</sup> Kn <sup>r</sup> , shuttle vector	15
pCGU1.1	<i>galU</i> gene from <i>X. citri</i> subsp. <i>citri</i> 306 cloned in pCR2.1	This study
pCGU2.1	<i>galU</i> gene on BamHI fragment from pCGU1.1 cloned in pUFR053	This study

lar polysaccharides (CPS), or exopolysaccharides (EPS). *GalU* has also been reported to be involved in adhesion of *E. coli* (17). However, the role of the *galU* gene in the virulence of *X. citri* subsp. *citri* and other plant-pathogenic bacteria has not been studied. In this study, we characterized the *galU* gene of *X. citri* subsp. *citri*. This was part of our effort to characterize critical genes involved in the virulence of *X. citri* subsp. *citri*. To our knowledge, it is the first report of an analysis of *galU* for plant-pathogenic bacteria.

#### MATERIALS AND METHODS

**Bacterial strains and growth media.** All of the strains used in this study are listed in Table 1. *X. citri* subsp. *citri* wild-type strain 306 (rifamycin resistant) (40) and mutant strains were grown in nutrient broth (NB), on nutrient agar (NA), or in NYG medium (11) at 28°C. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used at the following concentrations: rifamycin (Rif), 50 μg/ml; kanamycin (Kn), 50 μg/ml; ampicillin (Ap), 50 μg/ml; gentamicin (Gm), 5 μg/ml; and chloramphenicol (Cm), 35 μg/ml.

**Construction of *X. citri* subsp. *citri* mutant library.** An EZ-Tn5 <R6Kγori/KAN-2> Tnp Transposome kit (Epicentre, Madison, WI) was used to make the *X. citri* subsp. *citri* mutant library by following the manufacturer's instructions. The cells recovered were diluted 500- to 1,000-fold, spread on NA plates containing Rif and Kn, and incubated at 28°C for 2 to 3 days. Mutants were kept at -80°C in 20% glycerol until they were used. The mutant library was screened by performing pathogenicity assays (see below) with a susceptible host, cultivar Duncan grapefruit (*Citrus paradisi* Macf. cv. Duncan). Mutants that caused no symptoms or reduced symptoms were selected for further study.

**Rescue cloning of two nonmucoid mutants.** Two nonmucoid mutants, D12 and F6 (Table 1), were chosen from the mutant library for further analysis based on their nonpathogenic phenotypes in pathogenicity assays. To identify the insertion site in mutants D12 and F6, the rescue cloning method was used by following the manufacturer's instructions (Epicentre, Madison, WI). Briefly, genomic DNA (1 μg) of the D12 and F6 transformants were digested overnight with BamHI and SphI, end repaired (made blunt ended) using T4 DNA polymerase (New England Biolabs, Ipswich, MA), and 5' phosphorylated so that they self-ligate. The digested DNA was purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and allowed to self-ligate in the presence of T4 DNA ligase in a 10-μl mixture for 4 h at 16°C. The ligation mixture was electroporated into electrocompetent *E. coli* TransforMax EC100D *pir*<sup>+</sup> (Epicentre, Madison, WI). Cells were immediately transferred to a 17- by 100-mm round-bottom polypropylene tube which contained 1 ml SOC (super optimal broth with catabolite repression) (20), and the preparation was gently mixed by pipetting. The cells were incubated for 1 h at 37°C with gentle shaking and then plated on LB agar containing Kn. A plasmid was isolated from Kn-resistant colonies, purified,

and sequenced with primers R6KAN-2 RP-1 and KAN-2 FP-1 (Table 2). Sequencing was performed at the Interdisciplinary Center for Biotechnology Research sequencing facility at the University of Florida. Since the genome sequence of *X. citri* subsp. *citri* strain 306 has been published (13), transposon insertion sites in the F6 and D12 transformants were identified by aligning the corresponding *X. citri* subsp. *citri* 306 and mutated loci.

**Nucleic acid isolation and PCR.** Genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI) by using the protocol for isolating genomic DNA from bacteria. After DNA precipitation, the pellet was dried in a Vacufuge (Eppendorf, Westbury, NY) for 5 min and dissolved in the DNA rehydration solution supplied with the kit. Bacterial plasmid DNA was isolated using the Wizard miniprep DNA purification system (Promega, Madison, WI). The concentration and purity of DNA were determined using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA).

All conventional PCRs were performed with a Bio-Rad DNAEngine Peltier thermal cycler (Bio-Rad, Hercules, CA). Amplification of the DNA was performed using 50-μl (total volume) mixtures with *Taq* DNA polymerase (Promega, Madison, WI). The PCR conditions were 95°C for 5 min, followed by 40 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 52°C, and 1 to 3 min of extension (depending on the length of the amplicons) at 72°C.

**Southern blot analysis.** For Southern blot hybridization, genomic DNA samples were purified (after isolation using a Wizard genomic DNA purification kit) using phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol) and a standard molecular biology protocol (42). The DNA was precipitated, washed with 70% (vol/vol) ethanol, and resuspended in RNase- and DNase-free water. Genomic DNA (3 μg per sample) was digested with BglIII, subjected to electrophoresis on a 0.9% agarose gel, and transferred to

TABLE 2. Primers used in this study

Primer	Sequence (5' → 3') <sup>a</sup>
<i>galU</i> -F1	AGACAGTGCCTGCGAAAGAAATGCTGC
<i>galU</i> -R1	AACAGCGATCAGGCAACAATCAGC
Kan2-F1	CGAGGCCGCGATTAAATTCACAACA
Kan2-R1	AGGCAGTTCATAGGATGGCAAGA
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC
R6KAN-2 RP-1	CTACCCTGTGGAACACCTACATCT
CGU-F	AATGATGGATCCCTGCCAAAGCCT TGACGC
CGU-R	AACAGAGGATCCAACATCACCACG CCCAAC

<sup>a</sup> Underlined nucleotides are not exact matches and were introduced to add a BamHI restriction enzyme site.

a positively charged nylon membrane (Roche, Indianapolis, IN) using standard procedures (42). Probe generation, hybridization, and detection of chemiluminescence were performed using a digoxigenin (DIG)-High Prime II DNA labeling and detection starter kit as recommended by the manufacturer (Roche, Indianapolis, IN).

**Complementation of the *galU* mutants.** The entire *galU* gene with 624 bp of upstream sequence and 388 bp of downstream sequence was amplified from genomic DNA of *X. citri* subsp. *citri* wild-type strain 306 by a PCR performed with primers CGU-F and CGU-R, which contained a BamHI restriction site (Table 2). The resulting 1.9-kb fragment was ligated to PCR 2.1-TOPO using the manufacturer's protocol (Invitrogen, Carlsbad, CA), resulting in pCGU1.1. A BamHI fragment containing the *galU* gene was isolated from pCGU1.1 and cloned into similarly digested pUFR053 that was treated with calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA) (15), resulting in pCGU2.1 (Table 1). Plasmid pCGU2.1 was transferred into *galU* mutants D12 and F6 (*galU::Tn5*) by triparental mating with an *E. coli* helper strain containing pRK2013 (47). Transconjugants were selected on NA with Rif and Gm. The presence of pCGU2.1 was verified using PCR. Complementation assays were conducted to examine EPS and CPS production, the mucoid phenotype on NA medium, pathogenicity, and growth of the *galU* mutants *in planta* using plasmid pCGU2.1 containing an intact *galU* gene. Empty vector pUFR053 without the *galU* gene was used as a control.

**Quantitative determination of EPS production.** To measure the quantity of EPS in culture supernatants, bacterial cells were grown in NB supplemented with 2% D-glucose at 28°C for 24 h with shaking at 200 rpm. Then 10-ml portions of the cultures were removed, and the cells were removed by centrifugation (5,000 × g for 20 min). Three volumes of 99% ethyl alcohol was added to the supernatants. The precipitated EPS was pelleted by centrifugation, dried, and weighed (51). Three independent replicates were used for each strain. The test was performed three times independently; only the results of one test are shown below.

**Capsule assays.** Bacteria were grown at 28°C on NA with appropriate antibiotics. Samples were prepared by mixing and spreading a loop of bacteria with 1 drop of distilled water on a pre-cleaned slide and air dried. Each sample was stained with 1% crystal violet and washed with 20% copper sulfate supplied with a capsule-staining kit (Eng Scientific Inc., Clifton, NJ) by following the manufacturer's instructions. The samples were photographed using a light microscope (Leica DMLB2; Leica Microsystems Wetzlar GmbH, Germany) with an oil immersion lens at a magnification of ×1,000.

**LPS assays.** Bacteria were grown overnight at 28°C in NB, M9 glucose medium (8), or XVM2 medium (52). Ten-milliliter cultures were harvested at the exponential phase of growth and pelleted. LPS was isolated and treated with proteinase K (33), and then it was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, LPS samples were mixed with an equal volume of Laemmli sample buffer (pH 6.8) containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, and 0.01% bromophenol blue. The mixtures were boiled for 5 min, and 20-μl samples were loaded on precast Ready Gel Tris-HCl polyacrylamide gels (86 mm by 68 mm by 1.0 mm) containing 4 and 15% acrylamide in the stacking and separating gels, respectively (Bio-Rad Laboratories, Inc., Hercules, CA). Electrophoresis was performed at 12 mA for the stacking gels and at 25 mA for the separating gels until the bromophenol blue was 1 cm above the bottom of the gel. Immediately after electrophoresis each gel was stained using a silver stain kit (Bio-Rad Laboratories, Inc., Hercules, CA).

**Biofilm assays.** Biofilm formation in borosilicate glass tubes was quantified as described previously (36). Bacteria were grown in NB with shaking to the mid-exponential growth phase and then diluted 1:10 in fresh NB containing appropriate antibiotics. One milliliter of a diluted bacterial suspension was placed in each borosilicate glass tube (Fisher Scientific, Pittsburgh, PA) and incubated without shaking for 48 h at 28°C. The culture medium was poured out, and attached bacterial cells were gently washed three times with distilled water, incubated at 60°C for 20 min, and then stained with 1.5 ml 0.1% crystal violet for 45 min. The unbound crystal violet was poured out, and the wells were washed with water. The crystal violet-stained cells were solubilized in 1.5 ml of ethanol-acetone (80:20, vol/vol). Biofilm formation was quantified by measuring the absorbance at 590 nm using an Agilent 8453 UV-visible spectrophotometer. Ten replicates were used for quantitative measurement.

**Pathogenicity assays on plants.** Pathogenicity assays were conducted in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL. Assays were performed using fully expanded, immature leaves of cv. Duncan grapefruit. The *X. citri* subsp. *citri* wild-type and mutant strains used in this assay were grown with shaking at 28°C overnight in NB and were suspended in sterile tap water, and the concentration was adjusted to 10<sup>8</sup> CFU/ml. For the pathogenicity assays, bacterial suspensions containing both 10<sup>8</sup> and

10<sup>5</sup> CFU/ml were injection infiltrated into leaves with a needleless syringe (40, 50). The test was repeated three times, and similar results were obtained in all tests. Disease symptoms were photographed 5, 10, and 12 days postinoculation (DPI).

**Bacterial growth assays *in planta*.** For assays of bacterial growth in the intercellular spaces of citrus leaves, the concentration of the starting inoculum used was 10<sup>6</sup> CFU/ml, and whole leaves were inoculated by infiltration of the abaxial leaf surface using a needleless syringe as described above. For coinoculation, wild-type strain 306 and the D12 mutant were mixed together (1:1) before inoculation. Briefly, bacterial cell counts were determined for four biological replicates at each sampling time point (0, 1, 2, 3, 4, 6, 8, or 10 DPI). Leaf disks from inoculated leaves were excised with a cork borer (leaf area, 1 cm<sup>2</sup>) and then ground in 1 ml sterile tap water. The samples were serially diluted and plated on NA plates with appropriate antibiotics. The colonies were counted 48 h after plating. The *in planta* growth assays were repeated three times independently with four replicates each time, but the results of only one experiment are described here.

**RNA extraction and quantitative reverse transcription-PCR (QRT-PCR).** Bacteria were grown in XVM2 medium at 28°C with shaking at 200 rpm, and 1-ml samples of *X. citri* subsp. *citri* wild-type strain and D12 mutant cultures were collected at 13 h after inoculation. RNA was stabilized immediately by mixing it with 2 volumes of RNAlater bacterial reagent (Qiagen, Valencia, CA) and was incubated at room temperature for 5 min. Bacterial cells were centrifuged at 5,000 × g for 10 min, and the cell pellets were stored at -80°C prior to RNA extraction.

Cell pellets were treated with lysozyme, and RNA extraction was performed using an RNeasy minikit (Qiagen, Valencia, CA). Contaminated genomic DNA was removed from RNA by treatment with a TURBO DNA-free kit (Ambion, Austin, TX). The concentration of RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and adjusted to 50 ng/μl for QRT-PCR.

Fifteen target genes were chosen for an expression study with 16S rRNA as the endogenous control, and the primers shown in Table 3 were designed using sequences of the *X. citri* subsp. *citri* genome and DNASTAR software (DNASTAR, Inc., Madison, WI). A one-step QRT-PCR was performed with a 7500 Fast real-time PCR system (Applied Biosystems, Inc., Foster City, CA) using a QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA). The total volume of the one-step QRT-PCR mixture was 25 μl, and this mixture contained 2× QuantiTect SYBR green RT-PCR master mixture (12.5 μl), 10 μM gene-specific primers (1.25 μl), QuantiTect RT mixture (0.5 μl), and 50 ng of RNA template (1 μl). The reaction mixture was incubated at 50°C for 30 min and at 95°C for 15 min and then was subjected to 40 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 30 s. A melting curve analysis was performed to verify the specificity and identity of the QRT-PCR products. Three biological replicates were used for each strain. QRT-PCR was repeated once with another three independent biological replicates. A total of six independent biological replicates were used for each strain.

The amount of fluorescence was plotted as a function of the PCR cycle using the 7500 Fast system SDS software, and the threshold cycle ( $C_T$ ) values for each gene were determined. The  $\Delta C_T$  values for each target gene were calculated by subtracting the  $C_T$  values for 16S rRNA (the endogenous control) from the  $C_T$  values for the target gene. The  $\Delta C_T$  values for each gene for the D12 and wild-type strains were subjected to a simple *t* test (SAS), and the  $\Delta\Delta C_T$  values for target genes for D12 were obtained using the wild-type as a calibrator as described previously (53). The relative expression levels of target genes in XVM2 medium were obtained by using the  $2^{-\Delta\Delta C_T}$  method, which calculated the fold change of the transcript levels of the target genes in D12 relative to the wild-type level.

## RESULTS

**Generation of *galU* mutants of *X. citri* subsp. *citri*.** Two nonmucoid *X. citri* subsp. *citri* mutants, F6 and D12, were selected from the *X. citri* subsp. *citri* EZ-Tn5 library. Compared to wild-type strain 306 colonies, colonies of F6 and D12 on NA plates were smaller and less viscous. However, the growth rates of the mutants in NB broth were indistinguishable from that of the wild-type strain (data not shown).

The sites of transposon insertion in the F6 and D12 mutants were determined by rescue cloning. Sequencing results indicated that EZ-Tn5 was inserted between nucleotides 235 and



TABLE 3. Genes and corresponding primers used for QRT-PCR analysis

Gene	Product		Primer <sup>b</sup>	
	Function <sup>a</sup>	Component or protein	Direction <sup>c</sup>	Sequence (5' → 3')
16S rRNA		Ribosome component	F	CGCTTTCGTGCCTCAGTGTCTAGTGTGG
			R	GGCGTAAAGCGTGCCTAGGTGGTGGTT
<i>hprG</i>	TTSS	TTSS regulator	F	GCCTTTCGAATTCGCACGAGTTACACG
			R	CACACGCCGGGCTGGAAAAGA
<i>hprX</i>	TTSS	TTSS regulator	F	AGCGATCTCTGGCTTGCTCTAC
			R	ATACGCATCTTCGGCCTCTTCTCTGA
<i>hrcV</i>	TTSS	TTSS component	F	GCGTTTGC GGCGTGTCTCATCT
			R	CAATCTGGTGGTAGGCCTGGTCGTTTTCTT
<i>pthA</i>	TTSS	TTSS effector	F	TGGCGTCGGCAAACAGTGGTC
			R	TGCTCCGGGGTCAGGTTCAAG
<i>avrBs2</i>	TTSS	Avirulence protein	F	CGCGCAAATCACGACAAGGACTACTAC
			R	CGGGCCAGCGTGC GGTTTTTC
<i>avrXacE1</i>	TTSS	Avirulence protein	F	TCGCGCTGGGCCGGAACATAACC
			R	GCGTCCGCGGCGATAACTCTTG
<i>XAC0028</i>	CWDE	Cellulase	F	CGCTCCACGCTGCTTTTCAT
			R	ATTCCGGCACCGGACAGATTG
<i>XAC0160</i>	CWDE	Xylanase	F	CATGGCCTGGCGGTCCTTGTGTCT
			R	GCGGATCCGGCTGGCTTGTTTC
<i>XAC0165</i>	CWDE	Xylosidase	F	AGGGCGGGGCGTTGCTGTTCTAC
			R	TCTTGCCGTCGGGACTGCTGTGA
<i>peh-1</i>	CWDE	Endopolygalacturonase	F	AGTGGCAACGCGTTTCTGACC
			R	CGCTGCGTTGTTGCCCTTGAC
<i>celD</i>	CWDE	Glucan 1,4-beta-glucosidase	F	GATTTCCGGCCGAGCGTCTGGA
			R	GGATGCCGGCCTGGTTCTTCA
<i>pglA</i>	CWDE	Polygalacturonase	F	CAGCGCCGACGTCACCTTGTA
			R	GTAGCCGGGACGCGAATAGACC
<i>pelB</i>	CWDE	Pectate lyase II	F	GAACTTCGGCGTGC GTGTGGTG
			R	GTGAGCGAGGCGAAGATGGTGTGGTGC
<i>gumB</i>	EPS synthesis	Polysaccharide exporter	F	CTGACCGAAATCGAGAAGGGCACCAATC
			R	GCGCCACACCATACAGAGGAGTCAGTTC
<i>kpsF</i>	CPS synthesis	Arabinose 5-phosphate isomerase	F	GCTTACC GCCGACTTC
			R	CGCTTGCGGCTCATTTCATC

<sup>a</sup> TTSS, type III secretion system; CWDE, cell wall-degrading enzyme; EPS, extracellular polysaccharides; CPS, capsular polysaccharide.

<sup>b</sup> The primers were derived from the sequences of corresponding genes.

<sup>c</sup> F, forward; R, reverse.

236 in D12 and between nucleotides 665 to 666 in F6 downstream of the translation start site (Fig. 1A). Insertion of EZ-Tn5 into the *galU* gene was confirmed by PCR analysis. PCR with gene-specific primers *galU*-F1 and *galU*-R1 targeted amplification of the interior region of *galU* (Table 2). This resulted in a 0.84-kb amplicon when *X. citri* subsp. *citri* 306 genomic DNA was the template but in approximately 2.8-kb amplicons when F6 and D12 were the templates due to insertion of the 1.938-kb EZ-Tn5 transposon (Fig. 1B). In addition, the F6 and D12 transformants were confirmed to have a single copy of EZ-Tn5 using Southern blot analysis (Fig. 1C). Southern blot analysis showed that a DIG-labeled 675-bp Kan2 DNA fragment hybridized to a 7.3-kb band of D12 and to a 2.8-kb band of F6. The difference in the sizes of the D12 and F6 bands that hybridized resulted from differences in the locations of the transposon insertion sites and their relative distances from the restriction site of BglII, which was used for digestion of the genomic DNA, in the *galU* gene. For D12, EZ-Tn5 (1.938 kb) was inserted into a 5.535-kb BglII fragment containing the 5' end of the *galU* gene. For F6, EZ-Tn5 (1.938 kb) was inserted into a 0.9-kb BglII fragment containing the 3' end of the *galU* gene. Consequently, a 7.3-kb band and a 2.8-kb band were hybridized for D12 and F6, respectively. This confirmed that a single copy of EZ-Tn5 was inserted into the

genomes of F6 and D12 (Fig. 1C). No hybridization to the genomic DNA of *X. citri* subsp. *citri* wild-type strain 306 was observed when the Kan2 probe was used.

***galU* gene involvement in polysaccharide biosynthesis.** In order to study the role of the *galU* gene in polysaccharide biosynthesis, the major polysaccharides of *X. citri* subsp. *citri*, EPS, CPS, and LPS, were investigated. The results for only one *galU* mutant, D12, are described here since there were no differences between the results for *galU* mutants D12 and F6. There was a significant difference in EPS production between the *X. citri* subsp. *citri* wild-type strain (1.325 mg/ml) and D12 (0.012 mg/ml). Complementation with plasmid pCGU2.1 containing the entire *galU* gene restored EPS production in D12 to the wild-type level (1.725 mg/ml). The empty vector pUFR053 did not affect EPS production (0.0175 mg/ml). The D12 and wild-type strains were stained using a capsule-staining kit and observed with a microscope. The *X. citri* subsp. *citri* wild-type strain was covered with capsule, while there appeared to be no capsule in the *galU* mutant (Fig. 2A). Complementation with plasmid pCGU2.1 restored CPS production in D12 to the wild-type level. The empty vector pUFR053 did not complement CPS production in D12 (Fig. 2A). After proteinase K treatment, the LPS pattern of the *galU* mutant strain was indistinguishable from that of the wild-type strain when these

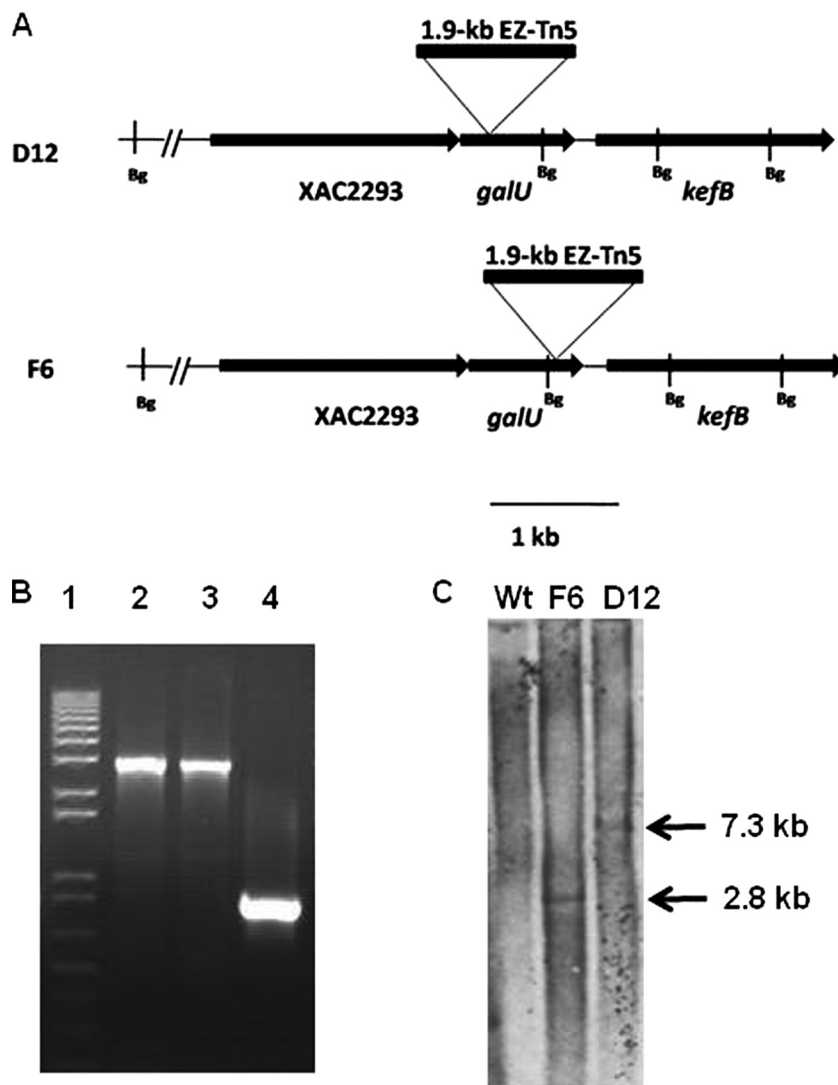


FIG. 1. Sequence analysis of EZ-Tn5 insertion in the *galU* mutants. (A) Genomic location of *galU* on the *X. citri* subsp. *citri* chromosome and transposon insertion sites in the *galU* mutants. *kefB* encodes a transport protein, *galU* encodes a UTP-glucose-1-phosphate uridylyltransferase, and *XCC2293* encodes a dehydratase protein. Bg, BglIII restriction site. (B) PCR analysis confirming insertion of EZ-Tn5 into the *galU* gene: agarose gel electrophoresis of DNA amplified using primers *galU*-F1 and *galU*-R1 targeting the interior region of the *galU* gene from the *X. citri* subsp. *citri* wild-type 306, D12, and F6 strains. Lane 1, Invitrogen 1 Kb Plus DNA size marker; lane 2, D12, lane 3, F6, lane 4, *X. citri* subsp. *citri* 306. (C) Southern blot of DNA of *X. citri* subsp. *citri* wild-type strain 306 and *galU* mutants F6 and D12 digested with BglIII. The membrane was probed with a 675-bp *kan-2* gene fragment that was amplified using PCR with primers Kan-F1 and Kan-R1. Wt, wild type.

strains were grown in NB, M9 glucose medium, or XVM2 medium (data not shown). The mucoid phenotype of strain D12 colonies was restored to that of the wild-type colonies on NA by complementation with plasmid pCGU2.1 but not by complementation with the empty vector pUFR053 (data not shown).

***galU* gene involvement in biofilm formation.** Because of the significant effect of the *galU* gene on polysaccharide production, the *galU* gene was hypothesized to be involved in biofilm formation. As shown in Fig. 2B, less biofilm was observed for the *galU* mutant than for the wild-type strain. Ten replicates were used for quantitative measurement of biofilm. The absorbance of the crystal violet in the biofilm-staining assay for the wild-type strain (optical density at 590 nm [OD<sub>590</sub>], 0.811 ±

0.083) was 12.5 times greater than that for *galU* mutant strain D12 (OD<sub>590</sub>, 0.065 ± 0.011). Biofilm formation by the D12 mutant strain was restored by complementation with plasmid pCGU2.1 containing the intact *galU* gene (OD<sub>590</sub>, 1.753 ± 0.063) but not by complementation with the empty vector pUFR053 lacking the *galU* gene (OD<sub>590</sub>, 0.040 ± 0.013).

**Pathogenicity assays.** Pathogenicity assays indicated that neither F6 nor D12 elicited a reaction on grapefruit, while the wild-type strain caused necrotic raised lesions typical of citrus canker on leaves when an inoculum containing a high concentration of bacteria (10<sup>8</sup> CFU/ml) was used (Fig. 3A). Similar results were obtained for an inoculum containing a lower concentration of bacteria (10<sup>5</sup> CFU/ml) (data not shown). Complementation with plasmid pCGU2.1 contain-

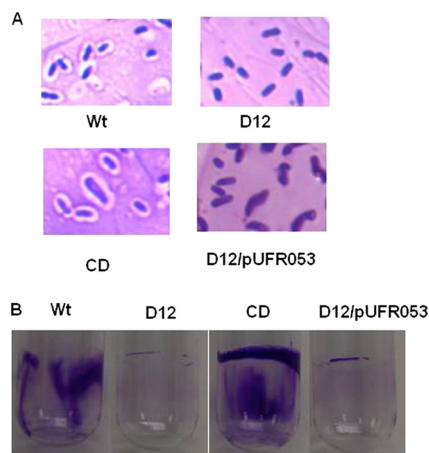


FIG. 2. Effects of *galU* on the capsular polysaccharide and biofilm formation. (A) Capsule-stained *X. citri* subsp. *citri* strains observed with a light microscope (magnification,  $\times 1,000$ ). (B) Biofilm formation by *X. citri* subsp. *citri* strains as determined using crystal violet staining. Wt, *X. citri* subsp. *citri* wild-type strain 306; D12, *galU* mutant; CD, strain D12 complemented with pCGU2.1; D12/pUFR053, D12 complemented with empty vector pUFR053 without the *galU* gene.

ing the entire *galU* gene restored the pathogenicity of the D12 and F6 mutants to the wild-type level and resulted in symptoms similar to those caused by the wild type on grapefruit (Fig. 3A). The empty vector pUFR053 without the *galU* gene did not complement the pathogenicity of either F6 and

D12, and no symptoms were observed with the two mutants on grapefruit leaves (Fig. 3A).

**Effects of mutation of the *galU* gene on expression of key virulence genes.** Loss of pathogenicity could result from down-regulation of key virulence genes. In order to test whether mutation of the *galU* gene affected the expression of key virulence genes, QRT-PCR assays were conducted. XVM2 medium has been reported to mimic the environment of plant intercellular spaces, and most virulence factors of *Xanthomonas* spp. can be induced in this medium (2, 52). Therefore, we grew D12 and the wild-type strain in XVM2 medium, removed samples at the exponential phase, and compared the levels of transcription of 15 genes using QRT-PCR. The 15 target genes included genes encoding enzymes involved in EPS and CPS biosynthesis and virulence factors, such as the type III secretion system (TTSS), effector proteins, and cell wall-degrading enzymes (CWDEs).

For QRT-PCR, the  $\Delta C_T$  and  $\Delta\Delta C_T$  values are appropriate subjects for statistical analysis (53). A *t* test was performed for the  $\Delta C_T$  values of the D12 and wild-type strains, and the  $\Delta\Delta C_T$  values of target genes for D12 were obtained using the wild type as a calibrator. Table 4 shows that five genes, including three CWDE genes (*XAC0160*, *celD*, and *pglA*), a CPS biosynthesis gene (*kpsF*), and a TTSS effector gene (*pthA*), were not expressed differently in the D12 and wild-type strains when they were grown in XVM2 medium. However, the expression of seven genes was significantly upregulated in D12 compared to the wild-type strain; these genes were two avirulence genes

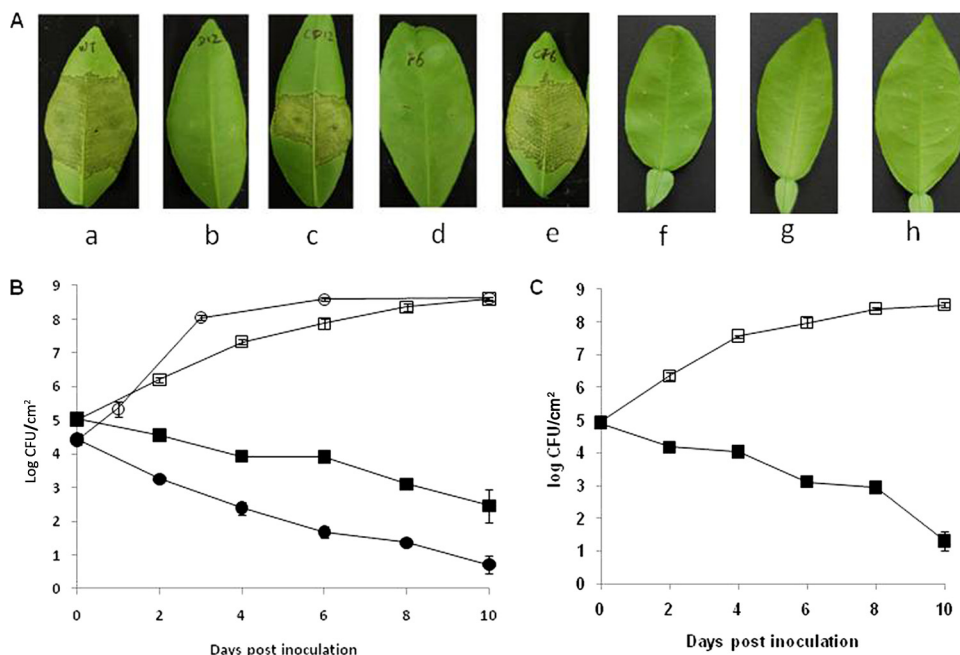


FIG. 3. Pathogenicity assays and growth of *X. citri* subsp. *citri* strains *in planta*. All strains were infiltrated into leaves with needleless syringes. (A) Host responses of cv. Duncan grapefruit inoculated with *X. citri* subsp. *citri* wild-type strain 306 (a), *galU* mutant D12 (b), CD (complemented mutant D12) (c), *galU* mutant F6 (d), CF (complemented mutant F6) (e), D12 with empty vector pUFR053 without the *galU* gene (f), F6 with pUFR053 without the *galU* gene (g), and water control (h). (B) Growth of *X. citri* subsp. *citri* wild-type strain 306, *galU* mutant D12, CD (complemented mutant D12), and D12 with empty vector pUFR053 in grapefruit leaves. (C) Growth of coinoculated *X. citri* subsp. *citri* wild-type strain 306 and *galU* mutant D12 in grapefruit leaves.  $\square$ , wild-type strain 306;  $\blacksquare$ , D12;  $\circ$ , CD (complemented mutant D12);  $\bullet$ , D12 with pUFR053. The *in planta* growth assays were repeated three times independently with four replicates each time, but the results of only one experiment are shown. The error bars indicate the standard errors of the means.

TABLE 4. Comparison of expression of key virulence genes in the wild type and the *galU* mutant grown in XVM2 medium using QRT-PCR

Gene	Mean $\Delta\Delta C_T^a$	SD	95% Confidence interval	<i>P</i>	Change (fold) <sup>b</sup>
<i>hrpG</i>	-1.6804	0.5275	-2.3590, -1.0018	0.0003 <sup>c</sup>	3.2052
<i>hrpX</i>	-2.1666	0.5206	-2.8363, -1.4968	<0.0001 <sup>c</sup>	4.4896
<i>hrcV</i>	-2.0417	0.4961	-2.6799, -1.4035	<0.0001 <sup>c</sup>	4.1173
<i>pthA</i>	-0.2077	0.3894	-0.7086, 0.2933	0.3774	1.1548
<i>avrBs2</i>	-2.1722	0.6046	-2.9499, -1.3944	<0.0001 <sup>c</sup>	4.5071
<i>avrXacE1</i>	-2.4682	1.0118	-3.7699, -1.1665	0.0018 <sup>c</sup>	5.5335
<i>XAC0028</i>	2.2138	0.7257	1.2802, 3.1474	0.0004 <sup>c</sup>	0.2156
<i>XAC0160</i>	0.5413	1.3596	-1.2078, 1.2903	0.5062	0.6872
<i>XAC0165</i>	1.2379	0.3362	0.8054, 1.6705	<0.0001 <sup>c</sup>	0.4240
<i>peh-1</i>	-2.8464	0.9095	-4.0164, -1.6764	0.0003 <sup>c</sup>	7.1920
<i>celD</i>	0.4748	1.0723	-0.9047, 1.8542	0.4609	0.7196
<i>pglA</i>	-0.5460	0.8444	-1.6322, 0.5402	0.2889	1.4600
<i>pelB</i>	1.2397	0.2500	0.9182, 1.5613	<0.0001 <sup>c</sup>	0.4235
<i>gumB</i>	-0.9043	0.5975	-1.6730, -0.1356	0.0255 <sup>c</sup>	1.8716
<i>kpsF</i>	-0.3704	0.9612	-1.6069, 0.8661	0.5196	1.2927

<sup>a</sup> The mean  $\Delta\Delta C_T$  was determined using six biological replicates.

<sup>b</sup> The change in expression in D12 was calculated using  $2^{-\Delta\Delta C_T}$ .

<sup>c</sup> Values are significantly different when *P* is <0.05.

(*avrXacE1* and *avrBs2*), one TTSS component gene (*hrcV*), two TTSS regulator genes (*hrpG* and *hrpX*), one CWDE gene (*peh-1*), and one EPS synthesis gene (*gumB*). Three genes encoding CWDEs (*XAC0028*, *pelB*, and *XAC0165*) were significantly downregulated at the transcription level in D12 compared to the wild-type strain.

**In planta growth of the *galU* mutant.** Since mutation of the *galU* gene did not impair most key virulence genes of *X. citri* subsp. *citri*, the loss of pathogenicity of the *galU* mutant was hypothesized to be due to the lack of growth *in planta*. To test this hypothesis, the wild-type and D12 mutant strains were tested to determine their growth in grapefruit leaves. Although there were no differences between the wild type and the *galU* mutants in the ability to grow in NB (data not shown), the growth of *galU* mutant D12 was significantly reduced *in planta* compared to the growth of the wild-type strain. The concentration of the wild-type strain *in planta* was  $4 \times 10^8$  CFU/cm<sup>2</sup> at 10 DPI, compared to  $6 \times 10^2$  CFU/cm<sup>2</sup> for *galU* mutant D12 (Fig. 3B). Strain D12 with complementation plasmid pCGU2.1 containing the entire *galU* gene grew to the same concentration as the wild type *in planta* (Fig. 3B). Addition of the empty vector pUFR053 without the *galU* gene did not restore the growth of D12 *in planta*.

To determine whether the *X. citri* subsp. *citri* wild-type strain affected the growth of the *galU* mutant *in planta*, the wild-type and D12 strains were coinoculated into grapefruit leaves as described above. Coinoculation of the wild-type strain and the D12 strain did not affect the growth of D12 *in planta* compared to the growth of D12 alone (Fig. 3C).

## DISCUSSION

Multiple genes, including *galETKM*, *galU*, and *galR*, are involved in formation of the sugar nucleotide precursors UDP-galactose and UDP-glucose for polysaccharide synthesis. The *galETKM* genes normally form an operon, while the *galU* and *galR* genes are not clustered in the genome (17). Knockout of these genes affects the outer membrane properties of bacteria and the virulence of *Azospirillum brasilense* (25), *Klebsiella pneumoniae* (7), *Streptococcus pneumoniae* (32), and *E. coli*

O157:H7 (21) in hosts. In this study, a *galU* mutant of *X. citri* subsp. *citri* was characterized to determine the effects of a mutation on the synthesis of major polysaccharides, pathogenicity, and growth in intercellular spaces. Apparently, the *galU* gene in *X. citri* subsp. *citri* is involved in EPS and CPS production. This is consistent with the critical role of the UTP-glucose-1-phosphate uridylyltransferase, which is responsible for synthesis of UDP-glucose from glucose 1-phosphate and UTP and for galactose and glucose interconversion through the Leloir pathway and plays a pivotal role in carbohydrate metabolism in different organisms (16). The phenotype of the mutant apparently resulted from mutation of the *galU* gene rather than from malfunction of downstream genes. The *galU* gene is the last gene of the operon containing *XAC2295* encoding one hypothetical protein, *XAC2294* encoding a lipopolysaccharide core biosynthesis protein, *XAC2293* encoding a dehydratase, and *galU* (13). The intergenic distance between the *galU* gene and the downstream gene *kefB* is 174 bp. The *galU* gene and the downstream *kefB* gene were predicted to belong to different operons based on operon prediction using SOFTBERRY (Softberry, Inc.). Thus, transposon mutation of the *galU* gene should not affect the function of the *kefB* gene (13). In addition, defects of the *galU* mutant in EPS production, the mucoid phenotype, pathogenicity, and growth *in planta* were complemented so that the levels were the same as the wild-type levels when plasmid pCGU2.1 containing an intact *galU* gene was used but not when the pUFR053 vector without the *galU* gene was used.

Mutation of the *galU* gene blocks EPS and CPS biosynthesis in *X. citri* subsp. *citri*. Both EPS and CPS are important components of the bacterial outer surface. Capsular polysaccharides are linked to the cell surface, while EPS molecules appear to be released onto the cell surface with no visible means of attachment and form an amorphous layer on the outer surface (39). The EPS produced by xanthomonads, xanthan, consists of repeating pentasaccharide units with the structure mannose-( $\beta$ 1,4)-glucuronic acid-( $\beta$ 1,2)-mannose-( $\alpha$ 1,3)-cellobiose (24). Three sugar nucleotides, UDP-glucose, UDP-glucuronic acid, and GDP-mannose, are required precursors for EPS synthesis.



UDP-glucose is the substrate for UDP-glucuronic acid synthesis and also affects the intercellular concentration of UDP-galactose (3). Mutation of the *galU* gene eliminated synthesis of one of the major precursors, UDP-glucose, and may also affect UDP-glucuronic acid for EPS synthesis in *X. citri* subsp. *citri*. Therefore, EPS production is deficient in the *galU* mutant of *X. citri* subsp. *citri*. Likewise, a deficiency of the major sugar nucleotide precursor, UDP-glucose, and possibly UDP-galactose and UDP-glucuronic acid in the *galU* mutant also accounts for the defect in CPS synthesis in the *galU* mutant. Previous studies also indicated that GalU is involved in EPS and CPS synthesis (3, 29, 31, 32).

The lack of pathogenicity of the *galU* mutant results from its inability to grow *in planta* rather than from an effect on the virulence genes. In order to determine why the *galU* mutant was not pathogenic, we first investigated the effect of the *galU* gene on the expression of genes encoding EPS and CPS biosynthesis proteins and key virulence factors, such as the type III secretion system (TTSS), effector proteins, and cell wall-degrading enzymes (CWDEs). The TTSS effector PthA is the pathogenicity determinant of *X. citri* subsp. *citri* that is required to cause canker symptoms on hosts (5). The level of expression of the *pthA* gene was the same in the *galU* mutant as it was in the wild-type strain. Overall, none of the virulence genes tested in this study showed reduced expression in the *galU* mutant, except for three CWDE genes whose involvement in *X. citri* subsp. *citri* virulence is still not known. Growth assays indicated that the *galU* mutant grew poorly in the intercellular environment (Fig. 3). This was probably due to a defect in synthesis of EPS and CPS, which have been shown to act as a barrier under stress conditions (39). Capsular polysaccharides have been reported to be essential for growth of *Erwinia amylovora* *in planta* (6). Capsule-like structures around *X. citri* subsp. *citri* were observed in infected Mexican lime and Yuzu leaves by transmission electron microscopy (30). Interestingly, coinoculation of the wild-type strain and the D12 strain did not rescue the growth of D12 *in planta*. *E. amylovora* exopolysaccharide amylovoran mutants can be rescued by wild-type strains, which presumably envelope the mutants in a biofilm (26, 27). Apparently, the mechanism by which the growth of the *galU* mutant was impaired *in planta* was different from the mechanism by which the growth of the amylovoran mutants of *E. amylovora* was impaired and remains to be explored.

Interestingly, GalU represents a potential target for screening antimicrobial compounds for control of citrus canker disease. Since GalU is required for *X. citri* subsp. *citri* growth *in planta*, antimicrobial compounds that inhibit GalU activity could potentially render *X. citri* subsp. *citri* virtually avirulent. In addition, prokaryotic UTP-glucose-1-phosphate uridylyl-transferases appear to be completely unrelated to their eukaryotic counterparts and have totally different structures even though they have almost identical catalytic properties (22). This interesting finding suggests that putative antimicrobial inhibitors of GalU might not be harmful for humans and the citrus hosts.

In summary, our data provide insights into the roles of the *galU* gene in EPS and CPS production, as well as biofilm formation, in *X. citri* subsp. *citri*. Our results also suggest that the *galU* gene plays a pivotal role in *X. citri* subsp. *citri* growth in the intercellular spaces of citrus leaves.

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## REFERENCES

- Alfano, J. R., and A. Collmer. 1996. Bacterial pathogens in plants: life up against the wall. *Plant Cell* 8:1683–1698.
- Astua-Monge, G., J. Freitas-Astua, G. Bacocina, J. Roncoletta, S. Carvalho, and M. Machado. 2005. Expression profiling of virulence and pathogenicity genes of *Xanthomonas axonopodis* pv. *citri*. *J. Bacteriol.* 187:1201–1205.
- Boels, I., A. Ramos, M. Kleerebezem, and W. de Vos. 2001. Functional analysis of the *Lactococcus lactis galU* and *galE* genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis. *Appl. Environ. Microbiol.* 67:3033–3040.
- Boyer, H., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459–472.
- Brunings, A. M., and D. W. Gabriel. 2003. *Xanthomonas citri*: breaking the surface. *Mol. Plant Pathol.* 4:141–157.
- Bugert, P., and K. Geider. 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Mol. Microbiol.* 15:917–933.
- Chang, H. Y., J. H. Lee, W. L. Deng, T. F. Fu, and H. L. Peng. 1996. Virulence and outer membrane properties of a *galU* mutant of *Klebsiella pneumoniae* CG43. *Microb. Pathog.* 20:255–261.
- Clowes, R. C., and W. Hayes. 1968. Experiments in microbial genetics. Blackwell Scientific Publications, Oxford, United Kingdom.
- Csonka, L. N., and W. Epstein. 1996. Osmoregulation, p. 1210–1223. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
- Cubero, J., and J. H. Graham. 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl. Environ. Microbiol.* 68:1257–1264.
- Daniels, M., C. Barber, P. Turner, M. Sawczyk, R. Byrde, and A. Fielding. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. *EMBO J.* 3:3323–3328.
- Das, A. K. 2003. Citrus canker—a review. *J. Appl. Hortic.* 5:52–60.
- da Silva, A. C., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B. Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, L. M. Alves, A. M. do Amaral, M. C. Bertolini, L. E. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chamberg, L. P. Ciapina, R. M. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. Ferreira, R. C. Ferreira, M. I. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, E. G. Lemos, M. V. Lemos, E. C. Locali, M. A. Machado, A. M. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, A. Rossi, J. A. Sena, C. Silva, R. F. de Souza, L. A. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417:459–463.
- Ditta, G., S. Stanfield, D. Corbin, and D. Helinski. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. U. S. A.* 77:7347–7351.
- El Yacoubi, B., A. Brunings, Q. Yuan, S. Shankar, and D. Gabriel. 2007. In planta horizontal transfer of a major pathogenicity effector gene. *Appl. Environ. Microbiol.* 73:1612–1621.
- Frey, P. A. 1996. The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J.* 10:461–470.
- Genevaux, P., P. Bauda, M. DuBow, and B. Oudega. 1999. Identification of Tn10 insertions in the *rfaG*, *rfaP*, and *galU* genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. *Arch. Microbiol.* 172:1–8.
- Gottwald, T. R., J. H. Graham, and T. S. Schubert. 2002. Citrus canker: the pathogen and its impact. *Plant Health Prog.* doi:10.1094/PHP-2002-0812-01-RV.
- Graham, J. H., T. R. Gottwald, J. Cubero, and D. S. Achor. 2004. *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. *Mol. Plant Pathol.* 5:1–15.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557–580.
- Ho, T. D., and M. K. Waldor. 2007. Enterohemorrhagic *Escherichia coli*



- O157:H7 *gal* mutants are sensitive to bacteriophage P1 and defective in intestinal colonization. *Infect. Immun.* **75**:1661–1666.
22. Hossain, S., K. Tanizawa, Y. Kazuta, and T. Fukui. 1994. Overproduction and characterization of recombinant UDP-glucose pyrophosphorylase from *Escherichia coli* K-12. *J. Biochem.* **115**:965–972.
  23. Jacobs, M., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenther, D. Bovee, M. Olson, and C. Manoil. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A.* **100**:14339–14344.
  24. Jansson, P., L. Kenne, and B. Lindberg. 1975. Structure of extracellular polysaccharide from *Xanthomonas campestris*. *Carbohydr. Res.* **45**:275–282.
  25. Jofre, E., A. Lagares, and G. Mori. 2004. Disruption of dTDP-rhamnose biosynthesis modifies lipopolysaccharide core, exopolysaccharide production, and root colonization in *Azospirillum brasilense*. *FEMS Microbiol. Lett.* **231**:267–275.
  26. Koczan, J., M. McGrath, G. W. Sundin, and Y. Zhao. 2008. Biofilm formation in *Erwinia amylovora*: implications in pathogenicity, p. 67–71. In K. B. Johnson and V. O. Stockwell (ed.), *ISHS Acta Horticulturæ 793. XI International Workshop on Fire Blight*. ISHS, Leuven, Belgium.
  27. Koczan, J., M. McGrath, Y. Zhao, and G. W. Sundin. 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity *Phytopathology* **99**:1237–1244.
  28. Komeda, Y., T. Icho, and T. Iino. 1977. Effects of *galU* mutation on flagellar formation in *Escherichia coli*. *J. Bacteriol.* **129**:908–915.
  29. Lai, Y., H. Peng, and H. Chang. 2001. Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. *Infect. Immun.* **69**:7140–7145.
  30. Lee, I., K. Kim, J. Hyun, Y. Lee, and E. Park. 2009. Comparative ultrastructure of nonwounded Mexican lime and Yuzu leaves infected with the citrus canker bacterium *Xanthomonas citri* pv. *citri*. *Microsc. Res. Tech.* **72**:507–516.
  31. Mollerach, M., and E. Garcia. 2000. The *galU* gene of *Streptococcus pneumoniae* that codes for a UDP-glucose pyrophosphorylase is highly polymorphic and suitable for molecular typing and phylogenetic studies. *Gene* **260**:77–86.
  32. Mollerach, M., R. Lopez, and E. Garcia. 1998. Characterization of the *galU* gene of *Streptococcus pneumoniae* encoding a uridine diphosphoglucose pyrophosphorylase: a gene essential for capsular polysaccharide biosynthesis. *J. Exp. Med.* **188**:2047–2056.
  33. Nesper, J., D. Kapfhammer, K. Klose, H. Merkert, and J. Reidl. 2000. Characterization of *Vibrio cholerae* O1 antigen as the bacteriophage K139 receptor and identification of IS1004 insertions aborting O1 antigen biosynthesis. *J. Bacteriol.* **182**:5097–5104.
  34. Nesper, J., C. Lauriano, K. Klose, D. Kapfhammer, A. Kraiss, and J. Reidl. 2001. Characterization of *Vibrio cholerae* O1 El tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect. Immun.* **69**:435–445.
  35. Osbourn, A. E. 1996. Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **8**:1821–1831.
  36. Pratt, L., and R. Kolter. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* **30**:285–293.
  37. Priebe, G., C. Dean, T. Zaidi, G. Meluleni, F. Coleman, Y. Coutinho, M. Noto, T. Urban, G. Pier, and J. Goldberg. 2004. The *galU* gene of *Pseudomonas aeruginosa* is required for corneal infection and efficient systemic spread following pneumonia but not for infection confined to the lung. *Infect. Immun.* **72**:4224–4232.
  38. Rioux, S., C. Galarneau, J. Harel, J. Frey, J. Nicolet, M. Kobisch, J. Dubreuil, and M. Jacques. 1999. Isolation and characterization of mini-Tn10 lipopolysaccharide mutants of *Actinobacillus pleuropneumoniae* serotype 1. *Can. J. Microbiol.* **45**:1017–1026.
  39. Roberts, I. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol.* **50**:285–315.
  40. Rybak, M., G. V. Minsavage, R. E. Stall, and J. B. Jones. 2009. Identification of *Xanthomonas citri* ssp. *citri* host specificity genes in a heterologous expression host. *Mol. Plant Pathol.* **10**:249–262.
  41. Salama, N. R., and C. Manoil. 2006. Seeking completeness in bacterial mutant hunts. *Curr. Opin. Microbiol.* **9**:307–311.
  42. Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
  43. Sandlin, R., K. Lampel, S. Keasler, M. Goldberg, A. Stolzer, and A. Maurilli. 1995. Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. *Infect. Immun.* **63**:229–237.
  44. Schaad, N., E. Postnikova, G. Lacy, A. Sechler, I. Agarkova, P. Stromberg, V. Stromberg, and A. Vidaver. 2006. Emended classification of xanthomonad pathogens on citrus. *Syst. Appl. Microbiol.* **29**:690–695.
  45. Segura, A., M. Moreno, F. Madueno, A. Molina, and F. Garcia-Olmedo. 1999. Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant-Microbe Interact.* **12**:16–23.
  46. Stimson, E., M. Virji, K. Makepeace, A. Dell, H. Morris, G. Payne, J. Saunders, M. Jennings, S. Barker, and M. Panico. 1995. Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol. Microbiol.* **17**:1201–1214.
  47. Swarup, S., R. De Feyter, R. H. Bransky, and D. W. Gabriel. 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *Xanthomonas campestris* to elicit canker-like lesions on citrus. *Phytopathology* **81**:802–809.
  48. Vauterin, L., B. Hoste, K. Kersters, and J. Swings. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* **45**:472–489.
  49. Vilches, S., R. Canals, M. Wilhelms, M. T. Salo, Y. A. Knirel, E. Vinogradov, S. Merino, and J. M. Tomas. 2007. Mesophilic *Aeromonas* UDP-glucose pyrophosphorylase (GalU) mutants show two types of lipopolysaccharide structures and reduced virulence. *Microbiology* **153**:2393–2404.
  50. Vitoria, Z., D. L. Drouillard, J. H. Graham, and J. W. Grosser. 2004. Screening triploid hybrids of 'Lakeland' limequat for resistance to citrus canker. *Plant Dis.* **88**:1056–1060.
  51. Vojnov, A., A. Zorreguieta, J. Dow, M. Daniels, and M. Dankert. 1998. Evidence for a role for the *gumB* and *gumC* gene products in the formation of xanthan from its pentasaccharide repeating unit by *Xanthomonas campestris*. *Microbiology* **144**:1487–1493.
  52. Wengelnik, K., C. Marie, M. Russel, and U. Bonas. 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. *J. Bacteriol.* **178**:1061–1069.
  53. Yuan, J., A. Reed, F. Chen, and C. J. Stewart. 2006. Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**:85.