Arch Microbiol (2014) 196:729–738
DOI 10.1007/s00203-014-1012-0

ORIGINAL PAPER

The *galU* gene of *Xanthomonas campestris* pv. *campestris* is involved in bacterial attachment, cell motility, polysaccharide synthesis, virulence, and tolerance to various stresses

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Received: 19 February 2014 / Revised: 11 June 2014 / Accepted: 4 July 2014 / Published online: 18 July 2014

Abstract Uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase (GalU; EC 2.7.7.9) is an enzyme that catalyzes the formation of uridine diphosphate (UDP)-glucose from UTP and glucose-1-phosphate. GalU is involved in virulence in a number of animal-pathogenic bacteria since its product, UDP-glucose, is indispensable for the biosynthesis of virulence factors such as lipopolysaccharide and exopolysaccharide. However, its function in *Xanthomonas campestris* pv. *campestris*, the phytopathogen that causes black rot in cruciferous plants, is unclear. Here, we characterized a *galU* mutant of *X. campestris* pv. *campestris* and showed that the *X. campestris* pv. *campestris galU* mutant resulted in a reduction in virulence on the host cabbage. We also demonstrated that *galU* is involved in bacterial attachment, cell motility, and polysaccharide synthesis. Furthermore, the *galU* mutant showed increased sensitivity to various stress conditions including copper sulfate, hydrogen peroxide, and sodium dodecyl sulfate. In addition, mutation of *galU* impairs the expression of the flagellin gene *fliC* as well as the attachment-related genes *xadA*, *fhaC*, and *yapH*. In conclusion, our results indicate involvement of *galU* in the virulence factor production and pathogenicity in *X. campestris* pv. *campestris*, and a role for *galU* in stress tolerance of this crucifer pathogen.

Communicated by Erko Stackebrandt.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-014-1012-0) contains supplementary material, which is available to authorized users.

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Keywords Motility · Pathogenicity · Polysaccharide · *Xanthomonas*

Introduction

Uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase (GalU; EC 2.7.7.9), which is also called uridine diphosphate (UDP)-glucose pyrophosphorylase, is an enzyme responsible for the production of UDP-glucose from UTP and glucose-1-phosphate. UDP-glucose is required to synthesize different surface structures, exopolysaccharide (EPS), and lipopolysaccharide (LPS) (Jiang et al. 2010). Since GalU is involved in producing glycosyl donors for EPS and LPS biosynthesis, the enzyme is essential for virulence in many bacterial pathogens, such as *Actinobacillus pleuropneumoniae* (Rioux et al. 1999), *Aeromonas hydrophila* (Vilches et al. 2007), *Klebsiella pneumonia* (Chang et al. 1996), *Proteus mirabilis* (Jiang et al. 2010), *Pseudomonas aeruginosa* (Priebe et al. 2004), *Pseudomonas syringae* (Deng et al. 2010), and *Vibrio cholerae* (Nesper et al. 2001).

Xanthomonas is a large genus of Gram-negative bacteria that cause disease in hundreds of plant hosts, including many economically important crops (Ryan et al. 2011). *Xanthomonas* species produce a characteristic EPS, xanthan, which leads to the mucoid appearance of the bacterial colonies (Buttner and Bonas 2010). A putative GalU encoding gene was found in the complete genome sequence of several *Xanthomonas* species, such as *X. albilineans* (Pieretti et al. 2009), *X. axonopodis* pv. *citri* (synonyms, *X. citri* pv. *citri*, and *X. citri* subsp. *citri*) (da Silva et al. 2002), *X. axonopodis* pv. *citrumelo* (Jalan et al. 2011), *X. campestris* pv. *campestris* (da Silva et al. 2002; Qian et al. 2005; Vorholter et al. 2008), *X. campestris* pv. *raphani*

(Bogdanove et al. 2011), and *X. campestris* pv. *vesicatoria* (Thieme et al. 2005), *X. oryzae* pv. *oryzae* (Lee et al. 2005; Salzberg et al. 2008), and *X. oryzae* pv. *oryzicola* (Bogdanove et al. 2011). To date, only three reports were found in the literature regarding *Xanthomonas* GalU. First, it was indicated that the gene encoding *X. campestris* pv. *campestris* GalU could restore a non-mucoid mutant isolated from *X. campestris* pv. *campestris* by Tn*5* mutagenesis to mucoid phenotype (Wei et al. 1996). Second, the GalU encoding sequence in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* was cloned and overexpressed in *Escherichia coli*, and the recombinant GalU proteins were purified and characterized (Bosco et al. 2009). Third, the role of the *galU* gene in the virulence of *X. axonopodis* pv. *citri* was evaluated, and it was found that the *galU* gene is required for EPS production and pathogenicity in *X. axonopodis* pv. *citri* (Guo et al. 2010).

X. campestris pv. *campestris* is the causative agent of black rot in crucifers, a disease that causes tremendous agricultural losses (Williams 1980). The virulence of *X. campestris* pv. *campestris* toward plants depends on a number of factors, including the ability to produce EPS and LPS, secrete several extracellular enzymes (such as cellulase, and mannanase), and cell motility (Dow and Daniels 1994; Dow et al. 1995; Chan and Goodwin 1999; Dow et al. 2003; McCarthy et al. 2008; Buttner and Bonas 2010). Although a previous study indicated that inactivation of *galU* by transposon mutagenesis revealed a non-mucoid phenotype and the cloned *galU* gene responsible for the mucoid phenotype restoration (Wei et al. 1996), no other biological functions were examined. The aim of the current work was to further characterize *galU* and to gain insights into its additional biological functions in *X. campestris* pv. *campestris*. The presented data indicated that GalU plays a role in bacterial attachment, cell motility, EPS and LPS synthesis, pathogenicity, and stress tolerance.

Materials and methods

Bacterial strains, plasmids, media, and culture conditions

E. coli DH5α (Hanahan 1983) served as the host for DNA cloning. *X. campestris* pv. *campestris* strain Xcc17 was a virulent wild-type strain isolated in Taiwan (Yang and Tseng 1988). The *wzt* mutant was Xcc17-derived mutant with EZ-Tn*5* inserted in *wzt* gene collected in our laboratory. The putative function of *wzt* gene product is the ATP-binding component of an ABC-transporter that specifically exports surface polysaccharides (Vorholter et al. 2001). Luria-Bertani (LB) medium (Miller 1972) was the general-purpose medium for cultivating *E. coli* and *X. campestris* pv. *campestris* at 37 and 28 °C, respectively. The compositions of XOLN and XVM2 media are given elsewhere (Fu and Tseng 1990; Wengelnik et al. 1996). The following antibiotics were added when necessary: ampicillin (50 μg/ml), gentamycin (15 μg/ml), and tetracycline (15 μg/ml). Liquid cultures were shaken at 220 rpm. Solid media contained 1.5 % agar.

DNA techniques

Enzymes were purchased from Promega and Roche. Standard protocols have been described elsewhere (Sambrook et al. 1989). Polymerase chain reaction (PCR) was carried out as previously described (Hsiao et al. 2005). DNA sequences were determined by Mission Biotech Co., Ltd. (Taipei, Taiwan). Transformation of *E. coli* was performed by the standard method (Sambrook et al. 1989) and that of *X. campestris* pv. *campestris* by electroporation (Wang and Tseng 1992).

Construction of *galU* mutant

A *galU* mutant was constructed by insertional mutagenesis of Xcc17 and designated as SXG17. For the insertion, the 438-bp fragment internal to the Xcc17 *galU* gene was PCR amplified using primers MTF (5′-CGAGCGCGCAGGCAAGCTCG-3′) and MTR (5′-GCGCCCGTGGACTCCAGGTACT-3′) and cloned into pUC19G (Yen et al. 2002). The resultant plasmid, pUCgalU, was electroporated into Xcc17 allowing for homologous recombination through the identical regions in the chromosome and the plasmid by a single crossover. Insertion of pUCgalU into *galU* was confirmed by PCR.

Complementation of *galU* mutant

The 982-bp *Pst*I-*Xba*I fragment encompassing the upstream 87-bp fragment plus the entire coding region of the Xcc17 *galU* was PCR amplified using primers CMF (5′-CTGCAGCCGGATTTTGCGGCTGCT-3′; *Pst*I site underlined) and CMR (5′-TCTAGACTCAGCCGCGTGCGTCGG-3′; *Xba*I site underlined) and cloned into the *Pst*I-*Xba*I sites of the broad-host-range vector pRK415 (Keen et al. 1988), generating pRKgalU. This construct contained the *galU* gene in the downstream and orientated in the same direction as the *lac* promoter. For complementation of *galU* mutant, plasmid pRKgalU was electroporated into the mutant SXG17.

Pathogenicity test

The virulence of *X. campestris* pv. *campestris* in cabbage was estimated after bacteria were introduced into the leaves by the leaf-clipping method (Hsiao et al. 2011). Lesion lengths were measured 14 days post-inoculation. Three

independent experiments with six replicates each were carried out.

EPS production assay and LPS analysis

The levels of EPS were measured as described previously (Hsiao et al. 2011) with a minor modification. Briefly, bacterial cells from an overnight culture were diluted 50-fold into fresh LB broth and grown at 28 °C for 24 h. The EPS in the supernatant was precipitated by addition of two volumes of ethanol (95 %), and the mixtures were kept at −20 °C for 1 h. The precipitated EPS was centrifuged at 12,000 *g* for 10 min and dried at 65 °C in an oven overnight before determination of dry weights. LPS was isolated according to a previously described method (Nesper et al. 2000) with minor modifications. Bacteria were grown overnight in LB medium at 28 °C. Five-milliliter overnight cultures were centrifuged at 12,000 *g* at 4 °C for 2 min. The collected cells were washed in 1 ml of TNE (10 mM Tris–HCl, pH 8, 10 mM NaCl, 10 mM EDTA) and resuspended in 540 μl of TNEX (TNE–1 % Triton X-100). Fifteen microliter of lysozyme (20 mg/ml) was added, and the mixture was incubated for 20 min at 37 °C. Following lysozyme digestion, the mixture was treated with 60 μl protease K (10 mg/ml) overnight at 55 °C to obtain the LPS samples. The isolated LPS was resolved by Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Lesse et al. 1990) and visualized by silver stain following the manufacturer's instructions (Bio-Rad). Standard LPS from *Salmonella enterica* serovar Typhimurium was purchased from Sigma.

Plate assay for extracellular enzyme activity

Assays of the activities of two extracellular enzymes (cellulase and mannanase) were performed on agar plates containing the appropriate substrate according to published methods (Hsiao et al. 2011) with minor modifications. Briefly, 3 μl of overnight culture ($OD_{550} = 1$) was deposited onto the surface of LB plates containing carboxymethyl cellulose (0.5 %) or locust bean gum (0.2 %). After 2 days of incubation, enzyme activity was measured as described previously (Hsiao et al. 2011).

Motility and attachment assay

To test the motility, 3 μl of overnight culture ($OD_{550} = 1$) was deposited onto the surface of XOLN plates (0.3 % agar). The diameter of the migration ring was measured after 2 days of incubation at 28 °C. Bacterial adhesion under static conditions was quantified by a crystal violet incorporation assay with polystyrene plates according to a previously described method (Hsiao et al. 2012). Briefly, overnight culture of each strain was diluted with XOLN medium containing 2 % glucose. Then, 150 μl of this diluted culture ($OD_{550} = 0.1$) was pipetted into the wells of polystyrene 96-well flat-bottom microtiter plates (Nunc), which were then allowed to stand for 24 h at 28 °C. Cells that attached to the surface of the wells were measured by removing the medium, rinsing the wells with 180 μl distilled water (three times), and staining attached bacteria with 180 μl 0.1 % (w/v) crystal violet at room temperature for 5 min. Then, free dye was removed, and the wells were rinsed three times with distilled water. The dye incorporated in attached cells was solubilized in 200 μl ethanol (95 %) and the OD_{595} was determined.

Stress tolerance assays

Strains were grown in XVM2 media ($OD_{550} = 0.35$) in the presence or absence of one type of stress condition, and growth of each strain was evaluated by measuring OD_{550} after growth for 24 h at 28 °C with shaking at 200 rpm. The stress conditions were as follows: for copper sulfate treatment, 0.05 mM $CuSO_4$ was added; for oxidative stress, 0.003 % H_2O_2 was added; for high osmolarity, 0.3 M NaCl was added; for SDS stress, 0.01 % SDS was added.

Promoter activity assay

Reporter constructs containing the upstream region of flagellar gene *fliC* and attachment-related genes *xadA*, *fhaC*, and *yapH* were obtained as previously described (Hsiao et al. 2012; Liu et al. 2013). Briefly, the upstream regions of these genes were PCR amplified and cloned into the upstream of the promoter-less *lacZ* gene in the promoter-probing vector pFY13–9 (Lee et al. 2001), giving pFYfliC, pFYxadA, pFYfhaC, and pFYyapH. These reporter constructs were electroporated separately into *X. campestris* pv. *campestris* wild-type Xcc17 and *galU* mutant SXG17. *X. campestris* pv. *campestris* strains harboring these constructs were grown overnight and inoculated into fresh media to obtain an initial OD_{550} of 0.35, after which growth was allowed to continue. Samples were taken in triplicate at 6 h, and the β-galactosidase activity was assayed as previously described, with the enzyme activity expressed in Miller units (Miller 1972).

Sequence alignment

Multiple sequence alignment was generated by use of the CLUSTALX package, with the default protein weight matrix (standard point accepted mutation series).

Statistical analysis

Values are the means of three technical replicates per experiment, and each experiment was performed at least

three times. A nonparametric Kruskal–Wallis test followed by a Tukey HSD test was used to determine the statistical significance of differences between means. All statistical analyses were performed using the SPSS statistical software program (version 17.0; SPSS, Inc., Chicago, IL).

Results and discussion

Characteristics of the *X. campestris* pv. *campestris galU* gene

To date, complete genomic sequences of three *X. campestris* pv. *campestris* strains (ATCC33913, 8004, and B100) have been deposited in a public database (da Silva et al. 2002; Qian et al. 2005; Vorholter et al. 2008). A survey of the genome sequence data of the *X. campestris* pv. *campestris* revealed that one *galU* gene is annotated in the fully sequenced genomes of these three different *X. campestris* pv. *campestris* strains. The open reading frame number of *galU* gene in strains ATCC33913, 8004, and B100 is XCC2188, XC_1930, and xccb100_1992, respectively (Table S1) (da Silva et al. 2002; Qian et al. 2005; Vorholter et al. 2008). There is also a homologue, XC861, in the genome sequence of strain Xcc17 (a draft genome) (http://xcc.life.nthu.edu.tw). The Xcc17 GalU shared 99.7, 99.7, and 100 % identity with homologues from ATCC33913, 8004, and B100, respectively. A database search revealed that GalU is highly conserved in several sequenced *Xanthomonas* species, such as *X. axonopodis*, *X. campestris*, and *X. oryzae,* with >80 % amino acid identity (Table S1).

From database searches, the deduced amino acid sequence of *X. campestris* pv. *campestris* GalU was found to be, among structurally characterized proteins, the most similar to GalU from *Sphingomonas elodea* (PDB code 2UX8) (Aragao et al. 2007), with 53 % identity and 68 % similarity. In addition, it also shared over 40 % identity with several crystalized GalU proteins from other bacteria, such as *Helicobacter pylori* (PDB code 3JUJ) (Kim et al. 2010), *E. coli* (PDB code 2E3D) (Thoden and Holden 2007b), and *Corynebacterium glutamicum* (PDB code 2PA4) (Thoden and Holden 2007a). Multiple sequence alignment revealed that several residues involved in enzyme activity described by crystallographic or mutagenetic data are well conserved in *X. campestris* pv. *campestris* GalU (Figure S1). When compared with the crystallographic data from *S. elodea* GalU, it is revealed that: (1) Asp135, Gly174, Glu193, and Val206 form hydrogen bonds with glucose-1-phosphate; (2) Ala13, Gly14, Gln 106, Gly111, and Asp 134 form hydrogen bonds with nucleotide; and (3) Leu112, Leu132, Tyr210, and Leu233 constitute a hydrophobic cap to the base of the sugar ring at the catalytic cavity. Catalytic residue Arg15 (numbering as in the *H. pylori* GalU) has been confirmed to be essential for enzyme activity by mutagenetic analysis (Kim et al. 2010). It is also conserved in the sequence of GalU proteins aligned and is situated at Arg18 in *X. campestris* pv. *campestris* GalU (Figure S1).

The *galU* gene is essential for full virulence of *X. campestris* pv. *campestris*

Although bioinformatics analysis revealed several orthologous *galU* genes in *Xanthomonas* species, only the *galU* genes from *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* have been biochemically characterized to encode a functional UTP-glucose-1-phosphate uridylyltransferase (Bosco et al. 2009). Recently, the *galU* gene from *X. axonopodis* pv. *citri* has been functionally evaluated (Guo et al. 2010). In *X. campestris* pv. *campestris*, it is only known that a recombinant clone, pEK135, has an insert of 3.0-kb *Kpn*I-*Eco*RI fragment harboring a complete *galU* gene and was able to restore the non-mucoid mutant G76E (Wei et al. 1996). To explore the physiological role of *galU* in *X. campestris* pv. *campestris*, a *galU* mutant was constructed by mutagenizing the wild-type strain Xcc17 using homologous recombination (see Materials and methods section for details). The obtained mutants were designated as SXG17. A complemented strain, named SXG17(pRKgalU), was simultaneously created by introducing the *galU*-expression plasmid pRKgalU into the mutant strain SXG17.

In many animal-pathogenic bacteria, such as *A. pleuropneumoniae* (Rioux et al. 1999), *A. hydrophila* (Vilches et al. 2007), *K. pneumonia* (Chang et al. 1996), *P. mirabilis* (Jiang et al. 2010), *P. aeruginosa* (Priebe et al. 2004), and *V. cholerae* (Nesper et al. 2001), mutation in *galU* caused attenuated virulence. Comparatively little is known regarding the role of the *galU* gene in the virulence of plant-pathogenic bacteria. Only *galU* genes from *P. syringae* (a necrotizing plant pathogen) and *X. axonopodis* pv. *citri* (a causal agent of citrus canker) have been reported to play a role in pathogenicity (Deng et al. 2010; Guo et al. 2010). Nothing is known about the role of *galU* in *X. campestris* pv. *campestris* virulence. To investigate the association between *galU* and pathogenicity of *X. campestris* pv. *campestris*, the virulence of the mutant was tested on host plant cabbage by the leaf-clipping method (Hsiao et al. 2011). Fourteen days post-inoculation, no obvious black rot symptoms were observed on the leaves inoculated with *galU* mutant SXG17, while typical "V"-shape black rot symptoms were observed on leaves inoculated with wild-type Xcc17 (Fig. 1). Introduction of the cloned gene into the *galU* mutant restored virulence to wild-type level (Fig. 1). The mean lesion lengths were 1.90 ± 0.13 and 1.94 ± 0.17 cm, respectively, for Xcc17 (wild type)

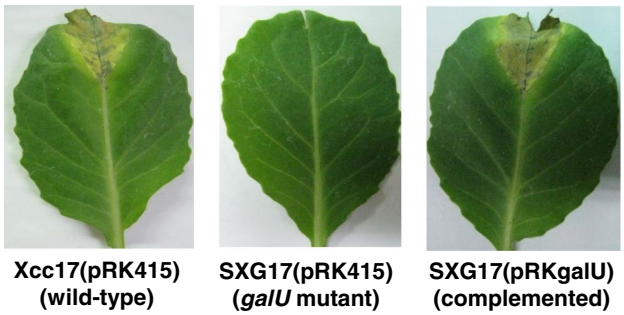

Fig. 1 Effect of mutation of *galU* on virulence of *X. campestris* pv. *campestris* to cabbage. *X. campestris* pv. *campestris* wild type carrying empty broad-host-range vector pRK415 and the complemented *galU* mutant strains caused *black rot* symptoms when the host plant cabbage was inoculated, while the *galU* mutant evoked no obvious disease symptoms. Photographs were taken on day 14 post-inoculation

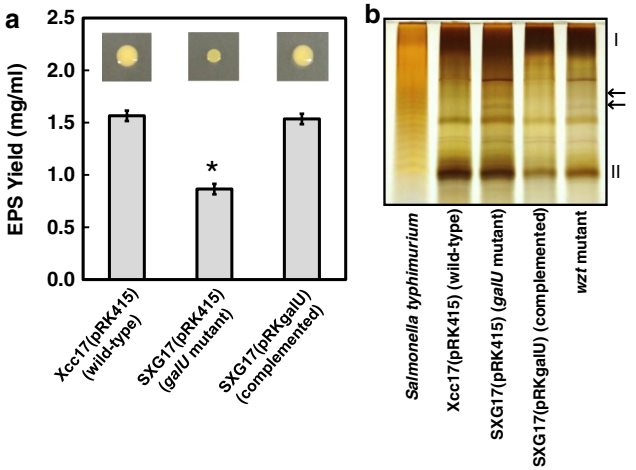

Fig. 2 Effect of mutation of *galU* on polysaccharide synthesis. **a** EPS production in LB medium by wild-type Xcc17 and its derivatives. Values presented are the means ± standard deviations from three repeats. *Asterisk* indicates significance at $P < 0.001$. **b** Analysis of LPS extracted from *X. campestris* wild-type and mutant strains. The LPS produced by wild-type Xcc17 and its derivatives were extracted, subjected to Tricine SDS-PAGE analysis, and visualized by silver staining. LPS of *Salmonella enterica* serovar Typhimurium was used as a standard. Bands I and II represent O-antigen containing LPS and core oligosaccharide, respectively. The *arrows* denote new bands in the mutants. The experiments were repeated three times with similar results, and the results of only one experiment are presented

and SXG17(pRKgalU) (complemented) at 14 days after inoculation. The mean lesion lengths caused by the complemented strain and the wild-type strain were not significantly different ($P = 0.673$). These results demonstrated that *galU* is required for virulence of *X. campestris* pv. *campestris*. *X. campestris* pv. *campestris* is a vascular pathogen mainly gains entry into plants via hydathodes at the leaf margins or through wounds. Leaf-clipping method introduces the bacteria directly into the vascular system. Whether different inoculation techniques possess different lesion lengths remain to be evaluated.

The lack of pathogenicity of the *galU* mutant might result from its inability to grow in *planta*. It has been reported that the growth of *galU* mutant of *X. axonopodis* pv. *citri* was significantly reduced in grapefruit leaves (Guo et al. 2010) and mutation in *galU* of *P. syringae* reduces bacterial multiplication in susceptible bean plants (Deng et al. 2010). Whether similar situation exists in *X. campestris* pv. *campestris galU* mutant remains to be determined.

The *galU* gene is involved in polysaccharide biosynthesis in *X. campestris* pv. *campestris*

In *Xanthomonas* species, it is indicated that successful infection and bacterial multiplication in the host tissue depend on a number of virulence factors, such as EPS, LPS, and extracellular degradative enzymes (Buttner and Bonas 2010; Ryan et al. 2011). The results showing the *galU* gene is essential for pathogenicity suggested that the ability of SXG17 to produce these virulence factors might have been impaired.

Compared to the wild type, the colony of SXG17 showed a much drier surface, indicating that the EPS synthesis was decreased (Fig. 2a). After EPS quantification, the result clearly showed that the EPS productivity was about 1.57 mg/ml for Xcc17 and 0.87 mg/ml for SXG17 grown in LB media (Fig. 2a) ($P < 0.001$). Complementation of SXG17 with plasmid to express full-length GalU restored the mucoid phenotype to that of the wild type and restored the EPS production to a near-wild-type level (1.54 mg/ml) (Fig. 2a) ($P = 0.847$). Similar situations are observed in *X. axonopodis* pv. *citri*, in which the *galU* gene is involved in EPS biosynthesis and the *galU* mutant is less viscous (Guo et al. 2010). The *galU* mutant of *K. pneumonia* also revealed a nonmucoid colony morphology (Chang et al. 1996).

The effect of a *galU* mutation on the production of extracellular degradative enzymes was evaluated by a substrate-supplementary plate assay. The diameters of the colonies formed by different cells on the same plate were similar. No significant differences in the activities of extracellular enzymes, including cellulase and mannanase were observed between Xcc17 and SXG17 (data not shown). In *X. axonopodis* pv. *citri*, three genes encoding cell wall degrading enzymes (*XAC0028*, *pelB*, and *XAC0165*) were significantly downregulated at the transcriptional level in *galU* mutant compared to the wild-type strain (Guo et al. 2010). The encoding enzyme products are cellulase for *XAC0028*, pectate lyase II for *pelB*, and arabinosidase for *XAC0165* (da Silva et al. 2002). In *X. campestris* pv. *campestris* genome, such homologues are present with gene

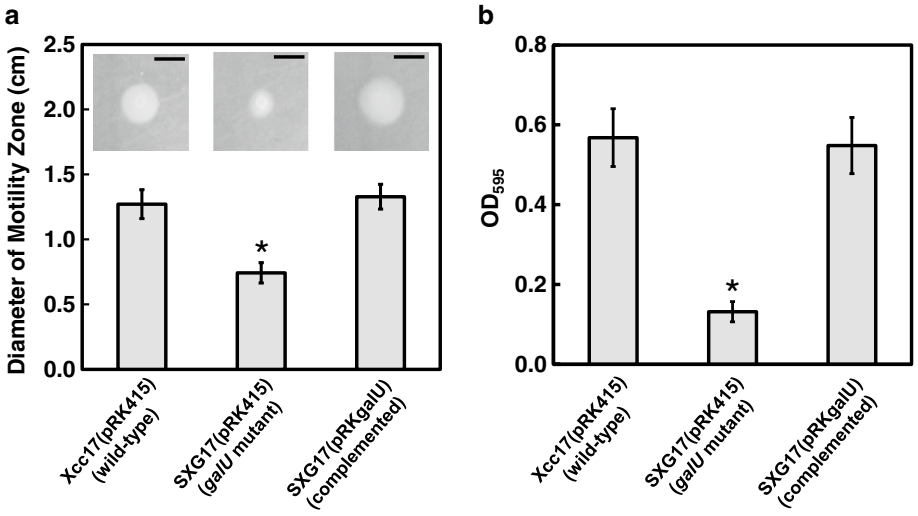

Fig. 3 Effect of mutation of *galU* on cell motility (**a**) and bacteria attachment (**b**). **a** The cell motility was evaluated using XOLN medium supplemented with 0.3 % agar plate. *Scale bars* = 1 cm. Similar results were obtained at least three times. The diameter of the motility zone of each strain was measured after 2 days of incubation at 28 °C. Values presented are the means ± standard deviations from three repeats. *Asterisk* indicates significance at $P < 0.001$. **b** Cells were grown in XOLN medium supplemented with glucose in 96-well polystyrene microtiter plates and incubated at 28 °C for 24 h. After crystal violet staining, attached cells were quantified by solubilizing the dye in ethanol and measuring the absorbance at 595 nm. Values presented are the means ± standard deviations from three repeats. *Asterisk* indicates significance at $P < 0.001$

numbers XCC0026, XCC2815, and XCC0149 in strain ATCC33913 (da Silva et al. 2002). The involvement in virulence of these genes is still unknown. Whether a *galU* mutation has any impact on the expression of these genes requires further study.

To investigate the role of *X. campestris* pv. *campestris galU* gene in LPS production, LPS was isolated, analyzed by Tricine SDS-PAGE followed by silver staining, and was compared with LPS from *X. campestris* pv. *campestris* wild-type Xcc17, as well as with LPS from a *wzt* mutant in which the biosynthesis of LPS was impaired (Vorholter et al. 2001). As shown in Fig. 2b, (1) the LPS of the *galU* mutant migrated similarly to that of *wzt* mutant and showed an altered LPS pattern compared with Xcc17; (2) the LPS production of the complemented strain was similar to that of the wild-type strain. These results indicated that *galU* is involved in LPS synthesis of *X. campestris* pv. *campestris*. In previous studies, disruption of the *galU* gene of several bacteria affected the LPS profile compared with that of the wild type, such as *A. pleuropneumoniae* (Rioux et al. 1999), *A. hydrophila* (Vilches et al. 2007), *E. coli* (Genevaux et al. 1999), *K. pneumonia* (Chang et al. 1996), *P. mirabilis* (Jiang et al. 2010), *P. aeruginosa* (Priebe et al. 2004), *P. syringae* (Deng et al. 2010), and *V. cholerae* (Nesper et al. 2001). However, this is not the case in *X. axonopodis* pv. *citri*, in which the LPS pattern of the *galU* mutant strain was indistinguishable from that of the wild-type strain (Guo et al. 2010), suggesting that the function of GalU may differ between these two *Xanthomonas* species.

Cell motility is reduced after *galU* mutation

The *galU* mutation also drastically affected the cell motility of several bacteria, such as *E. coli* (Komeda et al. 1977; Genevaux et al. 1999), *P. mirabilis* (Jiang et al. 2010), and *P. syringae* (Deng et al. 2010). To test whether a mutation in *galU* has any effect on the cell motility of *X. campestris* pv. *campestris*, the *galU* mutant was evaluated for the mobile ability on 0.3 % agar plate. The results showed that the motility zone diameters of SXG17(pRK415) exhibited a significant decrease (0.74 cm) when compared with Xcc17(pRK415) (1.27 cm) (Fig. 3a) ($P < 0.001$). In SXG17 with cloned *galU*, SXG17(pRKgalU), wild-type-level motility (1.33 cm) was restored (Fig. 3a). These results indicated that the GalU of *X. campestris* pv. *campestris* is implicated in bacterial motility.

Mutation of *galU* affects the attachment of *X. campestris* pv. *campestris* to polystyrene

The *galU* gene is involved in bacterial attachment in several bacteria, such as *E. coli* (Genevaux et al. 1999), *Haemophilus parasuis* (Zou et al. 2013), *P. syringae* (Deng et al. 2010), *V. cholerae* (Nesper et al. 2001), and *X. axonopodis* pv. *citri* (Guo et al. 2010). To test the impact of *galU* mutation in *X. campestris* pv. *campestris* adhesion, bacterial attachment was examined in minimal XOLN medium plus 2 % glucose and analyzed by crystal violet staining. The *galU* mutant SXG17(pRK415) exhibited

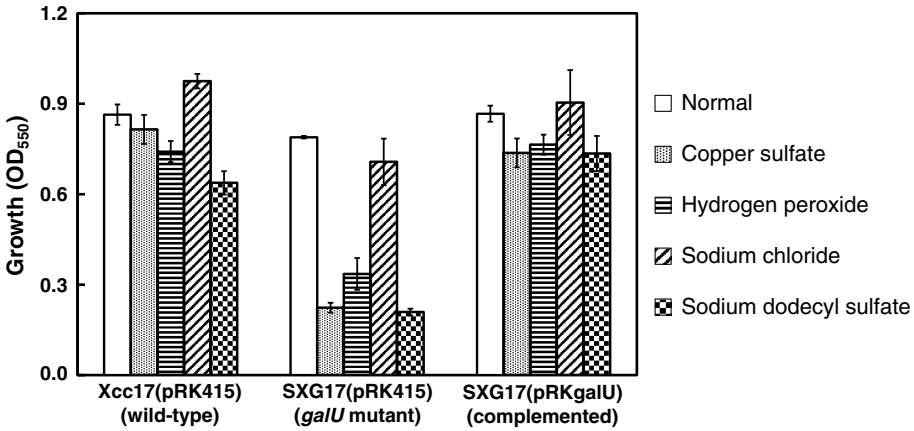

Fig. 4 Effect of mutation of *galU* on stress tolerance. Cells were grown in XVM2 medium with or without different stress conditions. The cell density was measured at OD_{550} after 24 h. Values presented are the means ± standard deviations from three repeats

significant reduction in bacterial attachment on a polystyrene surface compared with that of the wild type, where the level of adherence was approximately 75 % of the wild-type level (Fig. 3b) ($P < 0.001$). The complementary strain SXG17(pRKgalU) was restored to levels similar to those of the wild-type strain (Fig. 3b). These findings indicated that the *galU* gene is involved in bacterial attachment in *X. campestris* pv. *campestris*.

The *galU* gene is involved in the tolerance of *X. campestris* pv. *campestris* to various stresses

Experimental evidence suggests that EPS not only acts as a virulence factor but also suppresses basal plant defense responses and protect bacteria against environmental stress (Buttner and Bonas 2010). Furthermore, mutations in LPS gene clusters render the bacteria more susceptible against harsh environmental conditions (Buttner and Bonas 2010). The observations that mutation of *galU* resulted in reduced EPS synthesis and altered LPS pattern suggested that this gene might play a role in environmental stress tolerance. To verify whether *galU* plays a role in environmental stress tolerance of *X. campestris* pv. *campestris*, the growth of wild-type Xcc17(pRK415), *galU* mutant SXG17(pRK415), and complementary strain SXG17(pRKgalU) under different stress conditions was evaluated. As shown in Fig. 4, the *galU* mutant revealed a remarkable growth reduction with 30.0, 49.6, 79.5, and 35.9 % of growth retained in XVM2 supplemented with $CuSO_4$, H_2O_2, NaCl, and SDS, respectively, compared to wild type under the same condition. These phenotypic changes were restored in the complemented strain (Fig. 4). These data indicated that the *galU* mutant was more sensitive to these stresses than the wild type. The involvement of *galU* in stress tolerance has been studied in *P. mirabilis* (Jiang et al. 2010), *P. syringae* (Deng et al. 2010), and *V. cholerae* (Nesper et al. 2001). The *P. mirabilis* and *V. cholerae galU* mutant is more sensitive to SDS compared to the wild-type strain (Nesper et al. 2001; Jiang et al. 2010). The *P. syringae galU* mutant is more sensitive to H_2O_2 than the wild type (Deng et al. 2010).

Mutation of *galU* in *X. campestris* pv. *campestris* changes the transcriptional expression of certain motility and attachment-related genes

Reduction in cell motility and attachment could result from downregulation of genes related to these phenotypes. In *X. campestris* pv. *campestris*, flagellin gene *fliC* is essential for motility and flagellar biogenesis (Lee et al. 2003). The adhesion ability of *X. campestris* pv. *campestris* to surfaces has not been previously studied. In other *Xanthomonas* species, mutations in genes encoding XadA, YapH, and FhaC show altered adherence abilities (Darsonval et al. 2009; Das et al. 2009; Gottig et al. 2009). In *X. campestris* pv. *campestris* genomes, such homologues are present. To test the involvement of *galU* in the expression of these genes in Xcc17, a reporter assay was performed to analyze their expression in SXG17 using Xcc17 for comparison. The results showed that the promoter activity of *fliC*, *xadA*, *fhaC*, and *yapH* in SXG17 was decreased to 74, 29, 91, and 52 %, respectively, of that in Xcc17 (Table 1). These results suggested that the transcription level of the genes encoding flagellin (*fliC*), the outer membrane adhesin (*xadA*), the outer membrane hemolysin activator protein (*fhaC*), and the YapH protein (*yapH*) was reduced after *galU* mutation.

Although the *galU* gene has a role in cell motility in several bacteria, the effect of *galU* mutation on flagellin synthesis is different (Komeda et al. 1977; Deng et al. 2010; Jiang et al. 2010). In *E. coli*, it is indicated that the *galU* mutant is nonmotile and has much reduced amounts of flagellin and flagellin-specific mRNA (Komeda et al. 1977). In *P. mirabilis*, the *galU* mutant is defective in swarming motility and synthesized lower level of flagellin than did the wild-type strain, and synthesized a smaller amount of mRNA of *flhDC*, which is a master regulator controlling

Table 1 Promoter activities of *fliC*, *xadA*, *fhaC*, and *yapH* in Xcc17 and SXG17

Gene (ID)[a]	Predicted product	Mean β-galactosidase activity (Miller units) ± SD[b]	
		Xcc17 (wild type)	SXG17 (*galU* mutant)
fliC (XCC1941)	Flagellin	81.18 ± 2.00 (100)	60.26 ± 2.40 (74)
xadA (XCC0658)	Outer membrane adhesin	66.49 ± 1.95 (100)	19.32 ± 1.99 (29)
fhaC (XCC1793)	Outer membrane hemolysin activator protein	420.59 ± 8.02 (100)	382.65 ± 14.43 (91)
yapH (XCC2024)	YapH protein	440.34 ± 20.50 (100)	227.03 ± 42.25 (52)

[a] The gene ID is based on strain ATCC33913 and listed in parentheses

[b] Values in parentheses are percent activity relative to that in Xcc17

the expression of flagellum genes (Jiang et al. 2010). However, the *P. syringae galU* mutant has marked reduction in motility, whereas the amount of flagellins in the total flagellin fractions of both wild-type and mutant strains was equal (Deng et al. 2010). The observation showing *fliC* expression is reduced after *galU* mutation in this study is similar to the case of *E. coli*. In addition to *fliC*, the genes *rpoN2*, *fleQ*, *fliA*, *flhA*, *flhB* and *flgM* are essential for motility and normal flagellar biogenesis in *X. campestris* pv. *campestris* (Liu et al. 2013). Whether GalU has a role in FliC protein synthesis and in the expression of other genes involved in flagellar synthesis remains to be evaluated.

While *galU* mutants in several bacteria reduces bacteria attachment (Genevaux et al. 1999; Deng et al. 2010; Guo et al. 2010), no information is available regarding the role of *galU* in the expression of attachment-related genes. Here, the expression of three potential attachment-related genes was reduced in the *X. campestris* pv. *campestris galU* mutant. Although the involvement of virulence and adhesion in *X. campestris* pv. *campestris* is not yet recognized, these results extend our insight into the physiological role of GalU.

Conclusion

To date, only two reports have documented *galU* in *X. campestris* pv. *campestris*. One described the role of *galU* in EPS synthesis and revealed that a DNA fragment carrying *galU* gene can restore the non-mucoid phenotype (Wei et al. 1996), and the other described the coding product of *galU* and demonstrated that the coding product possesses UTP-glucose-1-phosphate uridylyltransferase activity (Bosco et al. 2009). In this study, we characterized *galU* in strain Xcc17 of *X. campestris* pv. *campestris* to gain more insights into the physiological role of the *X. campestris* pv. *campestris* GalU. To this end, we set out to construct a *galU* mutant and subjected it to phenotypic evaluation. There were several important findings: (1) deletion of *galU* has an impact on pathogenicity; (2) GalU is involved in polysaccharide production, including EPS and LPS; (3) GalU is required for cell motility and bacterial attachment; (4) GalU is implicated in environmental stress tolerance; (5) GalU has a role in the expression of flagellin gene *fliC* as well as the attachment-related genes *xadA*, *fhaC*, and *yapH*. The findings presented here showing GalU plays an essential role in *X. campestris* pv. *campestris* pathogenicity, in virulence determinant production and environmental response suggest that GalU is a potential target for antibacterial agent screening and may help to develop new strategies for the control of the black rot disease.

Acknowledgments This work was supported by the National Science Council of Taiwan Grant No. NSC 101-2313-B-166-001-MY3 to Yi-Min Hsiao, and the Central Taiwan University of Science and Technology Grant No. CTU100-PC-010 to Chao-Tsai Liao.

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