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Functional characterization and transcriptional analysis of *galE* gene encoding a UDP-galactose 4-epimerase in *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

The Gram-negative plant pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) is the causative agent of black rot in crucifers, a disease that causes tremendous agricultural loss. In this study, the Xcc *galE* gene was characterized. Sequence and mutational analysis demonstrated that the Xcc *galE* encodes a UDP-galactose 4-epimerase (EC 5.1.3.2), which catalyzes the interconversion of UDP-galactose and UDP-glucose. Alanine substitution of the putative catalytic residues (Ser124, Tyr147, and Lys151) of GalE caused loss of epimerase activity. Further study showed that the Xcc *galE* mutant had reduced biofilm formation ability. Furthermore, reporter assays revealed that *galE* transcription exhibits a distinct expression profile under different culture conditions, is subject to catabolite repression, and is positively regulated by Clp and RpfF. In addition, the *galE* transcription initiation site was mapped. This is the first time that UDP-galactose 4-epimerase has been characterized in the crucifer pathogen Xcc.

1. Introduction

UDP-galactose 4-epimerase (GalE; EC 5.1.3.2), which is also called UDP-glucose 4-epimerase, is an enzyme responsible for interconversion of UDP-galactose and UDP-glucose. UDP-galactose generally serves as the donor molecule for the biosynthesis of galactosyl residues in glycoproteins and complex lipopolysaccharides (Chung et al. 2012). GalE is also an important virulence factor in a number of bacterial pathogens such as *Erwinia amylovora* (Metzger et al. 1994), *Vibrio cholera* (Nesper et al. 2001), *Porphyromonas gingivalis* (Nakao et al. 2006), and *Aeromonas hydrophila* (Agarwal et al. 2007).

Xanthomonas campestris pv. *campestris* (Xcc) is a plant-pathogenic bacterium that causes black rot in crucifers (Williams 1980). The virulence of Xcc toward plants depends on a number of factors, including the ability to secrete several extracellular enzymes (such as protease, cellulase, and mannanase), produce exopolysaccharide, and cell motility (Chan and Goodwin 1999; Dow et al. 2003; Dow and Daniels 1994; McCarthy et al. 2008). Expression of these virulence determinants is upregulated by the cAMP receptor protein-like protein (Clp) and RpfF, an enoyl-CoA hydratase homolog required for synthesis of diffusible signal factor (Barber et al. 1997; He et al. 2006, 2007; Slater et al. 2000).

One potential *galE* gene has been annotated in the fully sequenced genomes of Xcc strains ATCC33913, 8004, and B100 (da Silva et al. 2002; Qian et al. 2005; Vorholter et al. 2008) as well as in strain Xc17, in which the genome sequence is almost complete (a draft genome) (http://xcc.life.nthu.edu.tw). The biological functions of these putative *galE* genes have not been studied. In view of the findings that GalE is an important virulence factor in diverse bacteria, it is suggested that GalE might play a similar physiological role in Xcc. To address this possibility, the objective of this study was to characterize the putative *galE* gene in Xcc.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Luria–Bertani (LB) broth and LB agar (Miller 1972) were the general-purpose media for cultivating *Escherichia coli* and Xcc at 37 and 28 °C, respectively. XOLN was the basal salt medium containing 0.625 g/l tryptone and 0.625 g/l yeast extract (Fu and

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Table 1
Bacterial strains and plasmids used in this study.

	Description	Reference or source
E. coli strain		
DH5α	F⁻ *endA1 hsdR17* (r_K^-) *supE44 thi-1* λ⁻ *recA1 deoR gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*DM15	Hanahan (1983)
X. campestris pv. campestris strain		
Xc17	Virulent wild type strain isolated in Taiwan, Ap^R	Yang and Tseng (1988)
AU56E	Xc17-derived mutant with Tn*5*(pfm)CmKm inserted in *clp* gene, AP^R, Cm^R, Km^R	Tseng et al. (1999)
RM17F	Xc17-derived mutant with a Gm^R cartridge inserted in *rpfF* gene, AP^R, Gm^R	Hsiao et al. (2008)
SC17	Xc17-derived mutant with EZ-Tn*5* inserted in *galE* gene, AP^R, Km^R	This study
Plasmids		
yT&A	PCR cloning vector, Ap^R	Yeastern
pTgalE	A 1149 bp RCR amplified fragment from *galE* (+202/+1350 relative to TIS) and cloned into yT&A	This study
pTgalES123A	pTgalE derivative carrying an S → A mutation at position 123 of GalE	This study
pTgalEY147A	pTgalE derivative carrying a Y → A mutation at position 147 of GalE	This study
pTgalEK151A	pTgalE derivative carrying a K → A mutation at position 151 of GalE	This study
pET30b	Expression vector, Km^R	Novagen
pETgalE	The 1149 bp *Nde*I-*Xho*I fragment of pTgalE cloned into the *Nde*I and *Xho*I sites of pET30b	This study
pETgalES123A	The 1149 bp *Nde*I-*Xho*I fragment of pTgalES123A cloned into the *Nde*I and *Xho*I sites of pET30b	This study
pETgalEY147A	The 1149 bp *Nde*I-*Xho*I fragment of pTgalEY147A cloned into the *Nde*I and *Xho*I sites of pET30b	This study
pETgalEK151A	The 1149 bp *Nde*I-*Xho*I fragment of pTgalEK151A cloned into the *Nde*I and *Xho*I sites of pET30b	This study
pUC19G	Gm^R cartridge from pUCGM ligated with the blunt-ended *Ava*II-*Ssp*I large fragment from pUC19	Yen et al. (2002)
pUCgalE	The 1149 bp *Eco*RI-*Bam*HI fragment of pTgalE cloned into the *Eco*RI and *Bam*HI sites of pUC19G	This study
pUCgalEK	pUCgalE derivative with Km^R inserted in the internal region of *galE* gene	This study
pRK415	Broad-host-range vector, RK2 *ori*, Tc^R	Keen et al. (1988)
pRKgalE	A 1369 bp RCR amplified fragment from *galE* (+14/+1382 relative to TIS) and cloned into pRK415	This study
pFY13-9	Promoter-probing vector derived from pRK415, using *lacZ* as the reporter, Tc^R	Lee et al. (2001)
pFYgalE	The 335-bp fragment, −113/+222 relative to *galE* TIS, cloned into the *Pst*I/*Xba*I sites of pFY13-9	This study

Ap^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; Gm^R, gentamycin-resistant; Km^R, kanamycin-resistant; Tc^R, tetracycline-resistant; TIS, transcription initiation site.

Tseng 1990). Glycerol, glucose, or galactose was supplemented (2%) as required. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), gentamycin (15 μg/ml), and tetracycline (15 μg/ml) were added as required.

2.2. Recombinant DNA techniques

Enzymes were purchased from Promega (Madison, WI, USA) and Roche (Indianapolis, IN, USA). Standard protocols have been described elsewhere (Sambrook et al. 1989). The polymerase chain reaction (PCR) was carried out as described previously (Hsiao et al. 2005) using the primers listed in Table 2. DNA sequences were determined by Mission Biotech Co., Ltd. (Taipei, Taiwan). Transformation of *E. coli* was performed using a standard method (Sambrook et al. 1989) and that of Xcc was performed by electroporation (Wang and Tseng 1992).

2.3. Production of recombinant GalE protein and site-directed mutagenesis

The 1149-bp fragment containing the entire coding sequence of the Xc17 *galE* gene was PCR-amplified using the primer pair *490NdeI*/*1638XhoI* and ligated into the yT&A cloning vector (Yeastern) to produce pTgalE. After sequence confirmation, the fragment was excised from pTgalE and cloned into the pET30b expression vector (Novagen, Gibbstown, NJ, USA), giving pETgalE. pETgalE was used as a template for recombinant GalE protein production to express the cloned *galE* with the S30 T7 high-yield protein expression system from Promega, following the manufacturer's instructions. The reaction was incubated at 37 °C with shaking (200 rpm) for 1 h. GalE activity of the synthesized protein was evaluated. For a negative control, pET30b was used to clarify the protein expression background on the GalE activity analysis.

Alanine substitution mutations were produced in GalE using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. The mutations were constructed in the highly conserved active site residues of GalE (S123A, Y147A, or K151), using pTgalE as a template and the primers listed in Table 2. After verifying the DNA sequence, the mutated *galE* was cloned into pET30b to give pETgalES123A, pETgalEY147A, and pETgalEK151A. The constructs were then separately used to produce mutated GalE with a S30 T7 high-yield protein expression system and to evaluate GalE enzyme activity.

2.4. galE mutant construction and complementation

Procedures for constructing *galE* mutant were as follows and illustrated in Fig. 1A. The 1149-bp PCR-amplified *galE*-coding region from pTgalE was excised and cloned into pUC19G (Yen et al. 2002), yielding pUCgalE. The EZ-Tn5™ <KAN-2> Transposon (Km^R, 1221 bp) was randomly inserted into pUCgalE using the EZ-Tn5™ <KAN-2> insertion kit according to the manufacturer's instructions (Epicentre Biotechnologies, Madison, WI, USA). One plasmid, pUCgalEK, with the transposon inserted into the *galE*-coding sequence at 422 bp from the start codon was selected for further use. This plasmid was electroporated into Xc17, allowing for double crossover, and transformants were selected on LB medium containing kanamycin (transposon selection marker). Integration of the transposon into the chromosome by homologous recombination was confirmed by PCR. Briefly, genomic DNA isolated from kanamycin resistant strains was used as template, and Km^R cartridge inserted *galE* region was amplified by primer pair *490NdeI*/*1638XhoI*. In parallel, PCR amplification using Xc17 as template was used for comparison. As shown in Fig. 1B, the *galE* mutant showed an amplified product of size 2370 bp (lane 2) and for the Xc17 the amplified product was 1149 bp (lane 1). The confirmed *galE* mutant strain was named SC17.

For construction of the SC17 complementation plasmid, the 1369-bp fragment encompassing the upstream 178-bp fragment plus the entire coding region of the Xc17 *galE* was PCR-amplified using primer pair *302XbaI*/*1670BamHI* and cloned into the broad-host-range vector pRK415 (Keen et al. 1988), generating pRKgalE. Plasmid pRKgalE was electroporated into the mutant SC17 to complement the *galE* mutant.