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Mini-Tn7 vectors as genetic tools for gene cloning at a single copy number in an industrially important and phytopathogenic bacteria, *Xanthomonas* spp

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Abstract

Transposon mini-Tn*7* vectors insert into the chromosome of several Gram-negative bacteria in a site-specific manner. Here, we demonstrated the application of mini-Tn*7* as single copy site-specific integration vector system for *Xanthomonas campestris* pv. *campestris*. The transposition of the mini-Tn*7* into the bacterial genome was detected at a Tn7 attachment (*att*Tn*7*) site located downstream of *glmS1*. Furthermore, using a newly constructed vector pBBR1FLP2 containing the FLP recombinase for site-specific excision of the sequence between the FLP recognition target (FRT) sites, and a *sacB* counter selection marker, an unmarked mini-Tn*7* insertion mutant was created. Mini-Tn*7* insertion did not affect bacterial virulence on the tested plant. The mini-Tn7 and FLP-*FRT* systems also work well in *X. oryzae* pv. *oryzae*.

Keywords

Single copy number gene cloning; mini-Tn7; unmarked mutation; *Xanthomonas*; virulence

Introduction

Transposons are mobile genetic elements that can transpose within the same, or other, genomes. Transposon Tn*7* and its derivatives have been shown to exert a high frequency of transposition into specific target sites on the chromosome of several bacteria (Arciszewska *et al.*, 1989). Integration of the transposable element requires the proteins encoded by Tn*7* genes (*tnsABCDE*) and can occur using two different mechanisms (Waddell & Craig, 1989). TnsABC + TnsD promote transposition into a specific Tn7 attachment site, *att*Tn7, with a high frequency, while TnsABC + TnsE promote insertion into non-*att*Tn7 sites (Peters & Craig, 2001). Generally, *att*Tn7 is located immediately downstream of *glmS*, a gene encoding glucosamine-fructose-6-phosphate aminotransferase, an enzyme that catalyzes the formation of glucosamine 6-phosphate. Most bacteria possess a single *glmS* gene and therefore a single *att*Tn7 site. However, multiple *glmS* genes and *att*Tn7 sites have been

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reported in some bacteria including *Burkholderia* spp. (Choi *et al.*, 2006; Choi *et al.*, 2008). Moreover, non-*glmS*-linked *att*Tn7 sites have also been identified in *Proteus mirabilis* (Choi & Schweizer, 2006). Mini-Tn7 vectors have been developed and proven to be useful for integration of a cloned gene and genetic analysis of several Gram-negative bacteria (Choi & Schweizer, 2006; Choi & Schweizer, 2006; Choi *et al.*, 2006; Choi *et al.*, 2005; Peters & Craig, 2001; Waddell & Craig, 1989). These mini-Tn7-based vectors are a valuable genetic tool for examining gene complementation and expression analysis at either the transcriptional or translational level.

Xanthomonas spp. are Gram-negative aerobic bacteria that are soil and plant pathogens that cause destructive diseases in many economically important crops, including rice, citrus, cabbage, cauliflower and radish. *Xanthomonas campestris* is also the producer of industrially important Xanthan gum. The development of novel genetic tools to study these bacterial phytopathogens and industrially important bacteria could lead to a better understanding of the underlying pathophysiology, improve the treatment strategies and improve production of an industrial material Xanthan gum. However, one of the important problems for molecular characterization of genes in *Xanthomonas* is lacking of vectors for specific integration of genes into bacterial chromosome either for single copy gene complementation or in vivo promoter analysis. In this communication, we demonstrate the successful application of the mini-Tn*7* vector system for integration of genes in *X. campestris* as well as *X. oryzae*. Although these bacterial species contain two *glmS* genes, insertion of the transposon was only detected at the *glmS1*-associated *att*Tn7 site, and this insertion did not affect bacterial virulence. A new vector containing FLP recombinase that facilitates excision of FRT sites a *sacB* a counter section marker are useful for making unmarked mutation in *Xanthomonas*.

Materials and methods

Bacterial strain and culture conditions

Xanthomonas strains were grown in Silva-Buddenhagen (SB) medium (Chauvatcharin *et al.*, 2005) at 28°C with continuous shaking. Bacteria containing genetic elements expressing *sacB* were cultivated in modified SB in which glucose was used instead of sucrose; *sacB*-deficient, sucrose-resistant clones were then selected on SB medium supplemented with 5% sucrose. Antibiotic concentrations used were 5 μg^{-1} ml gentamicin (Gm) and 300 μg^{-1} ml carbenicillin (Cb).

Plasmids

pUC18-mini-Tn7T-Gm, pUC18-mini-Tn7T-Gm-lacZ and the pTNS2 helper plasmid have been described previously (Choi & Schweizer, 2006; Choi *et al.*, 2006; Choi *et al.*, 2005). The FLP-mediated excision plasmid, pBBR1-FLP2 was constructed by cloning the 5-kb *Acc*65I-*Sph*I (blunt) fragment of pFLP2 (Hoang *et al.*, 1998), which contained the cI_{857}-*FLP-sacB* genes, into pBBR1MCS-4 (Kovach *et al.*, 1995) digested with *Acc*65I and *Sma*I. The complete sequence of pBBR1-FLP2 was deposited to the GenBank databases under the accession number FJ797950.

pTn7T-P_{ahpC}::lacZ was constructed by PCR amplification of 270-bp *ahpC* (*xcc0834*) promoter fragment using two specific primers (BT2305: 5’TTGCCGTTGTGGTACGCG3’ and BT2306: 5’AGCCTCAGACATGCGGCA3’) designed from the Xcc genome sequence (da Silva *et al.*, 2002). The PCR product was cloned into pDrive cloning vector (Qaigen, Germany) prior to the subcloning of the *Xho*I and *Acc*65I fragment into pUC18-mini-Tn7T-Gm-lacZ digested with the same restriction enzymes to generate pTn7T-P_{ahpC}::lacZ.

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Molecular genetic techniques

Molecular genetic techniques including genomic and plasmid DNA preparations, polymerase chain reaction (PCR), restriction endonuclease digestion, DNA ligation, transformation in *Escherichia coli*, bacterial crude lysate preparation, gel electrophoresis and Southern blotting analysis were performed using standard protocols (Sambrook *et al.*, 1989). Transformation of *X. campestris* pv. *phaseoli* (Xcc) was performed by electroporation, as previously described (Mongkolsuk *et al.*, 1996). DNA sequences were determined on an ABI 310 automated DNA sequencer (Applied Biosystems, USA).

Virulence test of *X. campestris* pv. *campestris*

The virulence of Xcc was determined on Chinese radish (*Raphanus sativus*), a compatible host plant, using leaf-clipping methods (Dow *et al.*, 2003) with some modifications. Overnight cultures of *Xanthomonas* strains in AB minimal medium (Chilton *et al.*, 1974) supplemented with 0.1 % (w/v) casamino acid were diluted to an optical density at 600 nm (OD_{600}) of 1.0 in fresh AB medium. Three leaves per plant were inoculated by leaf clipping, and five leaves were inoculated for each bacterial strain. The lesion lengths were measured at 14 days post-inoculation. Experiments were done in triplicate. The difference between strains was analyzed by *t*-test, and *P*-values less than 0.05 were considered to be significantly different.

β-galactosidase assay

Crude bacterial lysates were prepared and protein assays were performed as previously described (Panmanee *et al.*, 2002). The total protein concentration in the cleared lysate was determined using dye-binding method (Bio-Rad, USA). β-galactosidase assays were performed as previously described and expressed as international units defined as the amount of enzyme capable of releasing 1 μmol *p*-nitrophenol generated at 25°C per min (Panmanee *et al.*, 2002).

Results and Discussion

Insertion of Mini-Tn7 vectors into the *Xanthomonas* chromosome

In order to examine the utility of Tn*7*-based genetic tools vectors for the study of *Xanthomonas campestris* pv. *campestris* (Xcc), we tested whether a mini-Tn7 vector could be used in Xcc. pUC18-mini-Tn*7*T-Gm and pTNS2 (the helper plasmid encoding the TnsABCD site-specific transposition pathway) were transformed into wild-type Xcc. The transformants were selected on SB plates containing 5 μg^{-1} ml gentamicin. The analysis of the annotated Xcc genomic sequence using the BLASTP algorithm (Altschul *et al.*, 1997) and *Pseudomonas aeruginosa glmS* as the query sequence revealed that Xcc contains two putative glucosamine-fructose-6-phosphate aminotransferase genes, *glmS1* (*xcc0569*) and *glmS2* (*xcc3411*). Therefore, the insertion of the mini-Tn*7* was verified using PCR with *glmS1*- or *glmS2*-specific forward primers and a reverse primer that located in the mini-Tn*7* (Fig. 1a). The results demonstrate that the mini-Tn7-Gm was inserted downstream of *glmS1* in all 37 transformants that were analyzed in this study. Fig. 1b shows the PCR products of 398 bp and 235 bp amplified from a representative transformant using *glmS1*Up (5’ACGACCGCCTGCTGGAAAA3’) and Tn*7*R (5’-CACAGCATAACTGGACTGATT3’) primers, and *glmS1*Down (5’-ACGGGATGGCTGCGGCTT3’) and Tn*7*L (5’-ATTTGCTTACGACGCTACACC3’) primers, respectively. Amplification with *glmS1*Up and Tn*7*L or *glmS1*Down and Tn*7*R primer pairs resulted in no PCR products (Fig. 1b). The transposition of mini-Tn*7* was orientation-specific with Tn*7*R located immediately downstream of and facing *glmS1* (Fig. 1a). Site- and orientation-specific transposition is a common feature of Tn*7* insertions into other bacterial chromosomes (Choi & Schweizer,

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2006; Choi & Schweizer, 2006; Choi *et al.*, 2006; Choi *et al.*, 2005; Choi *et al.*, 2008; Waddell & Craig, 1989). Possible insertions of mini-Tn*7* at the *glmS2* site were assessed using PCR amplification with *glmS2*Up (5'GATGGCGACCTGCCGCTG3') and *glmS2*Down (5'GCATCGTCGGCCGCGACA3') (Fig. 1a). Amplification from all strains resulted in a 283 bp PCR product that indicates that no insertion of mini-Tn7 occurred at the *glmS2* site (Fig. 1b). Based on the DNA sequence analysis of the PCR products from five reactions, the insertion site of mini-Tn7 was located 25 nucleotides downstream of the *glmS1* stop codon (Fig. 1c). The analysis of the 3' *glmS1* nucleotide sequence identified a putative *att*Tn7 site that was very similar to the *att*Tn7 sites identified in *E. coli* (Waddell & Craig, 1989) and *P. aeruginosa* (Choi & Schweizer, 2006) (Fig. 1c). No putative *att*Tn7 site could be identified at *glmS2* (Fig. 1c). These results support the experimental findings that mini-Tn*7* insertions seemed to be confined to the *glmS1*-linked *att*Tn*7* site with no insertions observed at *glmS2*.

The excision of the Gm^r marker

pUC18-mini-Tn7T-Gm, a mini-Tn7 vector, contains two FLP recombinase target (*FRT*) sites flanking *aacC1* (Gm^r) (Fig. 1a). These *FRT* sites allow for flipase (FLP) mediated excision of *aacC1*. To assess whether an unmarked mini-Tn*7* insertion could be generated in Xcc, pFLP2 (Hoang *et al.*, 1998) was introduced into three representative Xcc transformants (Xcc::mini-Tn*7*T-Gm). The pFLP2 plasmid encodes the *Saccharomyces cerevisiae* FLP recombinase enzyme whose expression is driven by a λ promoter under the control of temperature sensitive λ repressor (cI_{857}). After several attempts to transform pFLP2 into Xcc::mini-Tn*7*T-Gm, no transformants could be obtained, suggesting that the plasmid may not be able to efficiently replicate and be maintained in Xcc. Hence, a *cI_{857}-FLP-sacB* containing fragment of pFLP2 was sub-cloned on a *Acc*65I-*Sph*I (blunt) fragment into the broad-host range pBBR1MCS-4 plasmid which contains a carbenicillin resistance (Cb^r) gene. The resulting expression vector pBBR1-FLP2 (Fig. 2) is capable of expressing cloned genes and replicating in *Xanthomonas*, yielding pBBR1-FLP2. This plasmid was subsequently electroporated into Xcc::mini-Tn*7*T-Gm and Cb^r transformants were selected. Excision of the Gm resistance cassette resulted in Cb^r and Gm^s transformants, and the excision event was confirmed by PCR analysis. PCR analyses using Xcc::mini-Tn*7*T-Gm genomic DNA and primers *glmS1*Up and *glmS1*Down generated a PCR product of 2,270 bp (Fig. 3a and b). Excision of *aacC1* by FLP-mediated recombination should result in a PCR product of 1,312 bp when amplification is performed using *glmS1*Up and *glmS1*Down primers and DNA from Cb^r and Gm^s colonies. As expected, 1,312 bp PCR products were detected (Fig. 3b). Hence, pBBR1-FLP2 could be efficiently used to mediate FLP-FRT recombination in order to generate an unmarked Tn*7* insertion in *Xanthomonas*. Moreover, the advantage of using pBBR1MCS and its derivative plasmids is that they can stably replicate at a moderate copy number in a number of Gram-negative bacteria, including α- and γ-proteobacteria (Khan *et al.*, 2008;Kovach *et al.*, 1995;Vattanaviboon *et al.*, 2007). Also, these plasmids can be mobilized through the IncP1 conjugative transfer system into many Gram-negative bacteria (Kovach *et al.*, 1995). The removal pBBR1-FLP2 plasmid was cured from Xcc::mini-Tn*7*T was by growing the strain on SB medium containing 5% sucrose. pBBR1-FLP2 contains a counter-selection gene, *sacB*, which encodes *Bacillus subtilis* levansucrase (Gay *et al.*, 1985). The presence of sucrose in the medium causes cell death in Gram-negative bacteria due to production of a toxic high-molecular-weight fructose polymer. Cells which survived and grew on the sucrose-supplemented medium were selected for further characterization and were shown to be Cb^s indicating plasmid loss. The pBBR1-FLP2-cured clones could be obtained after a single passage of cells through SB broth supplemented with sucrose. Therefore, the presence of the *sacB* counter-selection marker in pBBR1-FLP2 allows for the simple and rapid elimination of the plasmid from the Xcc host.

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Insertion of mini-Tn7 elements did not affect Xcc virulence

Xcc is a causative agent of black rot disease in cruciferous crops. The usefulness of the mini-Tn*7* system as a genetic tool in *Xanthomonas* and in the investigation of plant--microbe interactions depends on the premise that mini-Tn*7* insertion should not affect the virulence of the bacteria on the compatible host plant. Wild-type Xcc and Xcc::mini-Tn*7*T strains were inoculated into Chinese radish (*Raphanus sativus*) leaves by the leaf clipping method (Dow *et al.*, 2003). The results demonstrate that the lesion length in both Xcc::mini-Tn*7*T and the isogenic wild-type strains was not significantly different (P-value > 0.05). This suggests that the transposition of the mini-Tn*7* into the *glmS1* site has no effect on the virulence of Xcc on the tested host plant (Fig. 4). Thus, the mini-Tn*7* offers a potential shuttle vector system for the site-specific chromosomal integration for single copy complementation experiments, as well as gene expression analysis in *X. campestris* pv. *campestris* using reporter gene constructs.

Gene expression analysis in Xcc using mini-Tn7 vector

We have characterized *ahpC*, a gene encoding alkyl hydroperoxide reductase, in *X. campestris* pv. *phaseoli* (Loprasert *et al.*, 2000; Mongkolsuk *et al.*, 1997). The expression of *ahpC* is inducible by H_2O_2, organic hydroperoxides and superoxide generators (menadione and paraquat) in an OxyR dependent manner. In an attempt to evaluate the use of mini-Tn7 vector in gene expression analysis in Xcc, *ahpC* promoter was transcriptionally fused to *lacZ* in pUC18-mini-Tn7T-Gm-lacZ as described in the Materials and Methods. The recombinant plasmid pTn7T-P_{ahpC}-lacZ was introduced into Xcc wild-type and the transformants were selected for Gm^r phenotype. Insertion of Tn7 elements containing *ahpC* promoter-*lacZ* fusion and curing of pBBR1-FLP2 from the transformants giving Xcc::mini-Tn7T-P_{ahpC}-lacZ, was verified as described in an earlier section. The *ahpC* promoter activity was monitored by measuring β-galactosidase activity. Xcc::mini-Tn7T-P_{ahpC}-lacZ was cultivated in SB medium until the cells reached exponential phase of growth before bacterial cultures were being challenged 100 μM menadione (MD), 100 μM H_2O_2, 100 μM cumene hydroperoxide (CHP) 100 μM *tert*-butyl hydroperoxide (BHP). As illustrated in Fig. 5, treatment with MD, H_2O_2, CHP and BHP increased β-galactosidase activity by 3.0, 2.4, 4.5 and 3.3 folds, respectively, relative to wild-type level. The expression pattern of *ahpC* in Xcc was similar to previously characterized and closely related *ahpC* from *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1997). Our data presented the usefulness of mini-Tn7 vectors as tools to assess gene expression profile in plant pathogenic *Xanthomonas*.

Application of the mini-Tn7 vectors in other *Xanthomonas*

We further tested the functionality of the mini-Tn*7* system in other *Xanthomonas* spp., specifically *Xanthomonas oryzae* pv. *orzyae* (Xoo). The pUC18-mini-Tn*7*T-Gm delivery plasmid and pTNS2 helper plasmid were introduced into Xoo by electroporation using previously described conditions (Mongkolsuk *et al.*, 1996). The mini-Tn*7* insertion sites were determined in 35 transformants by PCR using genomic DNA templates isolated and two pairs of primers (*glmS1*XoUp, 5'-CGACCGCCTGCTGGAAAA3', and Tn*7*R or *glmS1*Xo Down, 5'-ATCTTCGACGCTCAACAG3', and Tn*7*L). Amplification of a 280 bp fragment demonstrated that insertion of the mini-Tn*7*T-Gm element in all transformants occurred downstream of *glmS1* (*xoo0746*). Furthermore, sequence analysis of the PCR products indicated that the mini-Tn*7* insertion site (*att*Tn*7*) was 25 bp downstream of *glmS1*. No insertions were observed at *glmS2* (*xoo3917*)(data not shown). We also analyzed the published genomic sequences of all *Xanthomonas* spp. and determined that *X. campestris* pv. *vesicatoria* (Thieme *et al.*, 2005), *X. oryzae* pv. *oryzae* (Xoo) (Lee *et al.*, 2005), and *X. axonopodis* pv. *citri* (da Silva *et al.*, 2002) all contain two putative *glmS* genes. However, upon searching for putative *att*Tn*7* sites surrounding the two *glmS* genes, *att*Tn*7* sites could

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only be identified downstream of the respective *glmS1* genes (Fig. 1c). Based on the results obtained with the mini-Tn*7* system in Xcc and Xoo, it is likely that the mini-Tn*7* system will work in other *Xanthomonas* spp. and that the mini-Tn*7* will probably insert downstream of *glmS1*. Additionally, the excision of *aac*C1 from Xoo::mini-Tn*7*T-Gm was tested by transforming the strain with pBBR1-FLP2. The results from the PCR analysis using *glmS1*XoUp and *glmS1*XoDown primers and DNA from Xoo::mini-Tn*7*T/pBBR1-FLP2 demonstrated the excision of the Gm^r marker through FLP-FRT recombination (data not shown). As described in the earlier section 3.2, pBBR1-FLP2 could be cured from the resulting strain with sucrose counter-selection. Taken together, our data suggest the possible applications of mini-Tn*7* system and a new vector for FLP-FRT system for making unmarked mutation in a variety species of xanthomonads. This will be useful for genetic analysis of industrially important and phytopathogenic bacteria, *Xanthomonas* spp.

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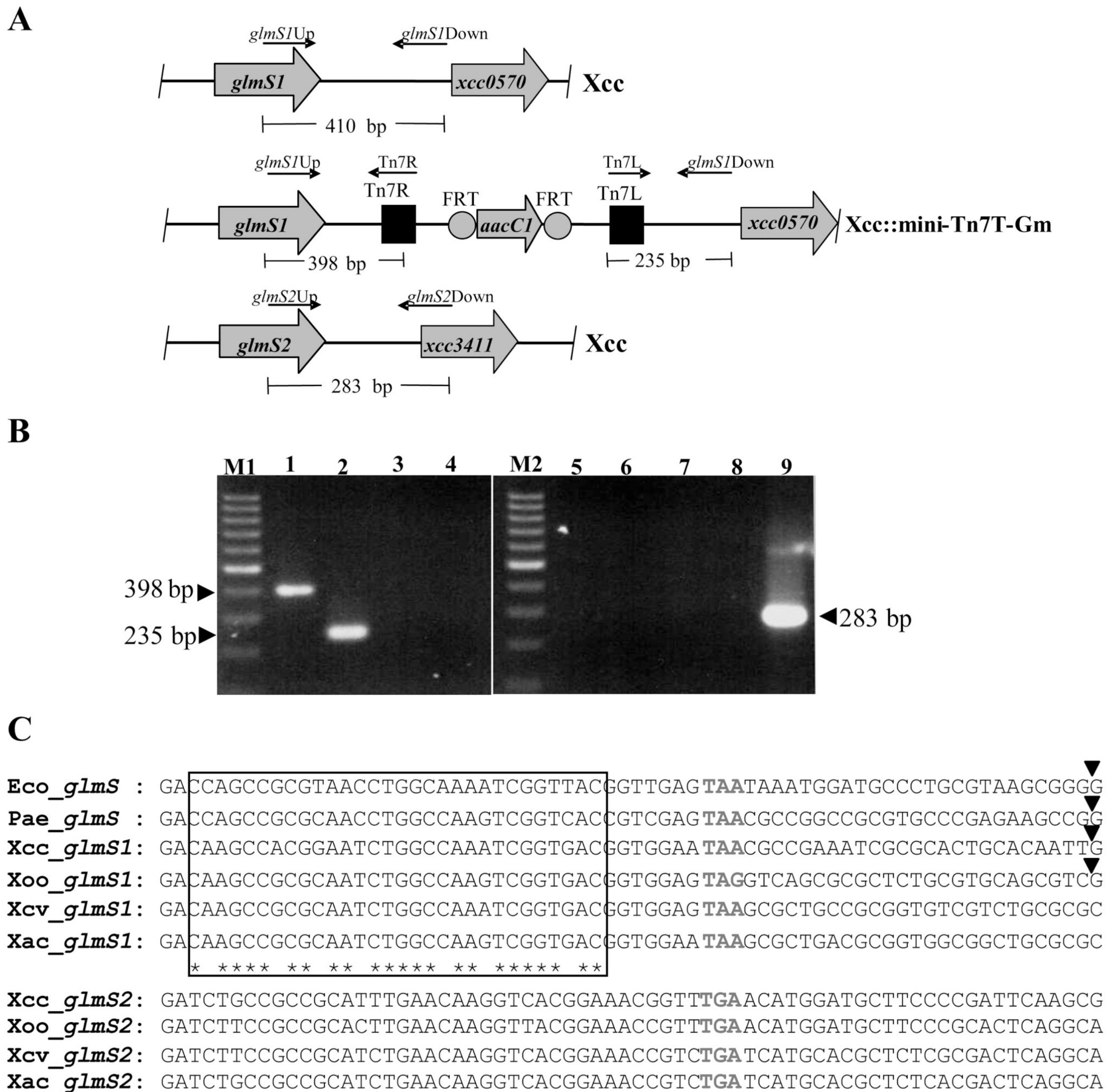

Fig. 1. Transposition of mini-Tn*7* into the *X. campestris* pv. *campestris* (Xcc) chromosome

(A) Physical maps of Xcc *glmS1* and *glmS2* loci (top and bottom) and an integration event at glmS1 (Xcc::mini-Tn*7*T-Gm; middle). Arrows denote the positions and orientations of the indicated oligonucleotide primers. Tn*7*R and Tn*7*L, right and left end of Tn*7*, respectively; *FRT*, FLP recombinase target; *aacC*1, Gm acetyltransferase-encoding gene.

(B) PCR amplification to confirm the transposition of the mini-Tn7T-Gm. Genomic DNA of a representative Gm^r transformant was used as the template in PCR reactions with primer pairs specific for *glmS1* and *glmS2* as follows: lane 1, *glmS1*Up and Tn*7*R; lane 2, *glmS1*Down and Tn*7*L; lane 3, *glmS1*Up and Tn*7*L; lane 4, *glmS1*Down and Tn*7*R; lane 5, *glmS2*Up and Tn*7*R; lane 6, *glmS2*Down and Tn*7*L; lane 7, *glmS2*Up and Tn*7*L; lane 8,

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*glmS2*Down and Tn*7*R; and lane 9, *glmS2*Up and *glmS2*Down. M1 and M2 are 100-bp molecular weight ladder (Fermentas, Canada) for lanes 1 to 4, and lanes 5 to 9, respectively. (C) Alignment of the nucleotide sequences of the *glmS* 3' and downstream sequences. The sequences analyzed were the *glmS* regions of *E. coli* (Waddell & Craig, 1989) (Eco) and *P. aeruginosa* (Choi & Schweizer, 2006) (Pae), and the putative *glmS1* and *glmS2* regions of Xcc [*xcc0569* and *xcc3411* (da Silva *et al.*, 2002)], Xoo [*xoo0746* and *xoo3917* (Lee *et al.*, 2005)], *X. campestris* pv. *vesicatoria* [*xcv3754* and *xcv0770* (Thieme *et al.*, 2005)] and *X. axonopodis* pv. *citri* [*xac3637* and *xac0714* (da Silva *et al.*, 2002)]. *att*Tn7 associated sequences (boxed) were identified based on a previous study with *E. coli* (Waddell & Craig, 1989) and asterisks represent sequence identity in all species. Arrowheads indicate mini-Tn*7* insertion sites. Bold and gray letters denote the stop codons of the respective *glmS* genes.

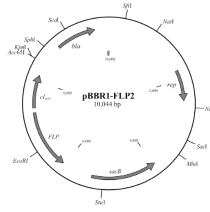

Fig. 2. Map of FLP recombinase expressing plasmid

The physical map of pBBR1-FLP2 (GenBank accession number FJ797950) shows the locations and orientations of the genetic determinants involved in replication (*rep*), mobilization (*mob*), ampicillin/ carbenicillin resistance (*bla*, β-lactamase-coding gene), FLP-*FRT* recombination (*FLP*, Flippase-coding gene) and the counter-selectable marker (*sacB, Bacillus subtilis* levansucrase-encoding gene). Some unique restriction enzyme sites are shown. cI_{857}, gene encoding a temperature sensitive λ repressor.

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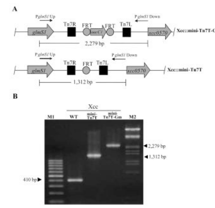

Fig. 3. FLP-mediated excision of antibiotic resistance gene

(A) Physical maps of Xcc::mini-Tn*7*T-Gm and Xcc::mini-Tn*7*T showing the insertion at the *glmS1* locus. Arrows represent the positions and orientations of oligonucleotide primers used in PCR analysis of excision events. Tn*7*R and Tn*7*L, right and left end of Tn7, respectively; *FRT*, FLP recombinase target; *aacC*1, Gm acetyltransferase-encoding gene. (B) PCR amplification to confirm the excision of the Gm^r marker. Genomic DNAs of Xcc::mini-Tn*7*T-Gm and Xcc::mini-Tn7T were amplified with *glmS1*Up and *glmS1*Down primers. Arrowheads show the migration of expected DNA fragments whose sizes are indicated in kilobases. M1 and M2 are a 100 bp DNA ladder and the λDNA/*Eco*RI+*Hin*dIII markers (Fermentas, Canada), respectively. WT, Xcc wild-type.

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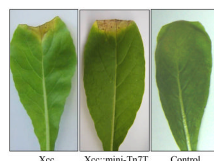

Fig. 4. Virulence testing in *X. campestris* pv. *campestris* strains

The virulence of Xcc and Xcc::mini-Tn*7*T strains was tested on a susceptible host, Chinese radish (*Raphanus sativus*) (Dow *et al.*, 2003). The infection of the tested plant was performed using the leaf clipping method (Dow *et al.*, 2003). Lesion lengths were measured 14 days post-inoculation. Control is the leaf without bacterial inoculation.

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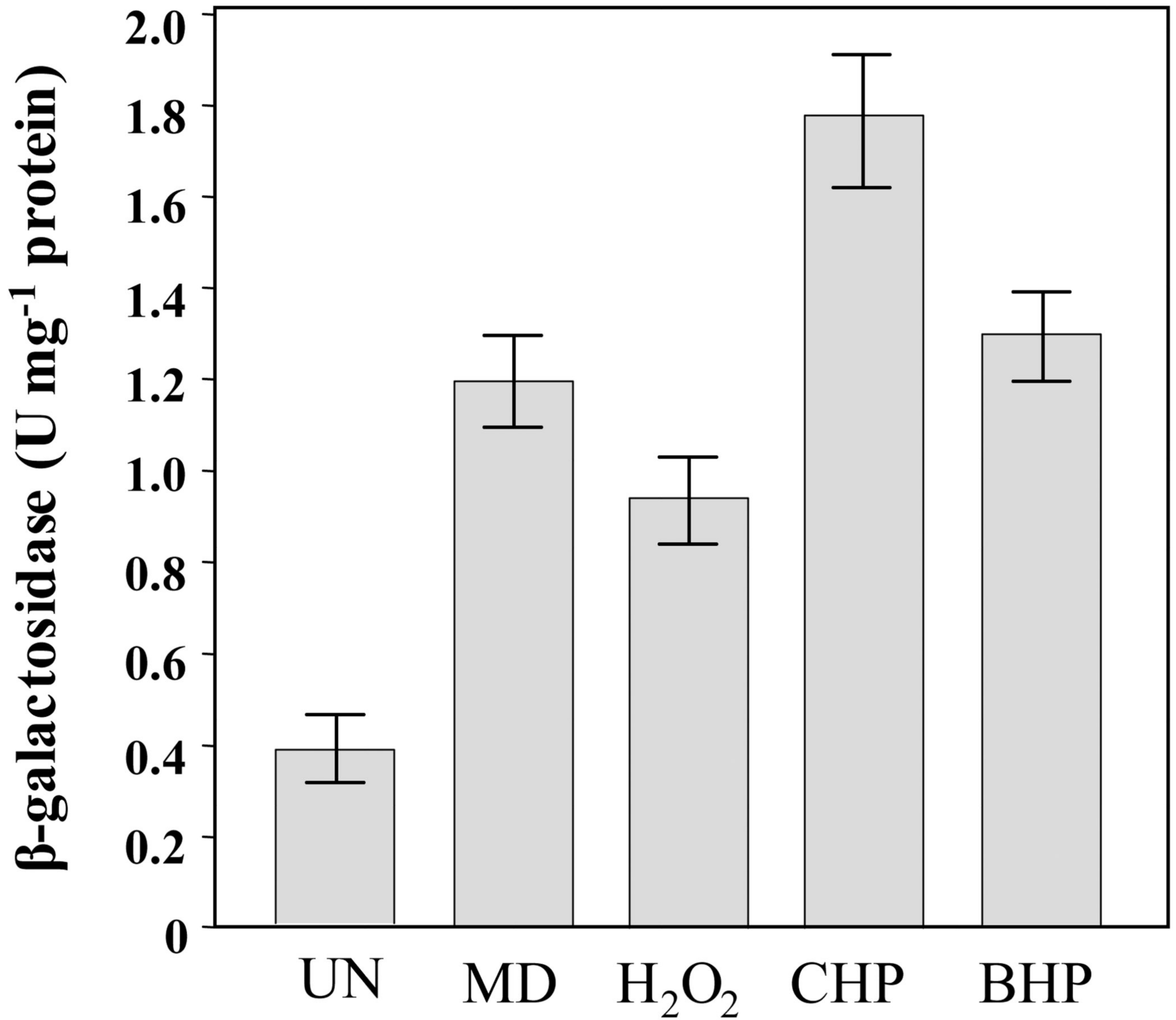

Fig. 5. Expression analysis of *ahpC* in *X. campestris* pv. *campestris*

Xcc::mini-Tn7T-P_{ahpC}-lacZ (the *ahpC* promoter transcriptionally fused to *lacZ* reporter inserted into chromosome on a mini-Tn7 vector) cultures were grown either uninduced or induced with 100 µM of menadione (MD), H_2O_2, cumene hydroperoxide (CHP) or *tert*-butyl hydroperoxide (BHP) for 30 min. The β-galactosidase activity in clear lysates was determined and expressed as international units per mg protein. UN, un-induced.