Two *Xanthomonas* Extracellular Polygalacturonases, PghAxc and PghBxc, Are Regulated by Type III Secretion Regulators HrpX and HrpG and Are Required for Virulence

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Submitted 13 September 2007. Accepted 11 January 2008.

Xanthomonas campestris pv. *campestris*, the causal agent of black rot disease, produces a suite of extracellular cell-wall degrading enzymes (CWDE) that are involved in bacterial virulence. Polygalacturonase (PG) is an important CWDE and functions to degrade the pectic layers of plant cell walls. Although previous studies have documented the virulence functions of PG in *Erwinia* and *Ralstonia* species, the regulation of PG genes still needs to be elucidated. In this study, we identified two novel PG genes (*pghAxc* and *pghBxc*) encoding functional PG from *X. campestris* pv. *campestris* 8004. The expressions of these two PG genes are regulated by the type III secretion regulators HrpX and HrpG and the global regulator Clp. These PG genes could be efficiently induced in planta and were required for the full virulence of *X. campestris* pv. *campestris* to *Arabidopsis*. In addition, these PG were confirmed to be secreted via the type II secretion system in an Xps-dependent manner.

Additional keywords: hydrolysis, pectin, pathogenesis.

The type II secretion system (T2SS) allows most gram-negative bacteria to deliver extracellular hydrolytic enzymes and toxins to their surroundings and hosts, many of which are responsible for pathogenesis in plants and animals (Sandkvist 2001a). Among the virulence factors secreted by the T2SS of phytopathogens, cell-wall degrading enzymes (CWDE), such as polygalacturonases (PG), cellulases, xylanases, and proteases, have been studied due to their clear or potential functions in bacterial pathogenesis, and comprehensive studies on the structures and functions of CWDE have been undertaken. CWDE function to break down the components of host cell walls and may play a crucial role in virulence and bacterial nutrition (Dow et al. 1990, 1998; Gonzalez and Allen 2003; Huang and Allen 1997; Kamoun and Kado 1990; Liu et al. 2005; Ray et al. 2000).

PG are hydrolytic enzymes of CWDE that selectively degrade the pectic polymers, the major components of higher-plant middle lamellae and primary cell walls. PG are classified by activity as endo-PG (EC 3. 2. 1. 15) and exo-PG (EC

3. 2. 1. 67). Endo-PG randomly hydrolyze polygalacturonic acid, releasing oligosaccharidic chains of variable length, whereas exo-PG release either galacturonate monomer, in the case of exoPG (EC 3.2.1.67), or dimer (digalacturonate), in the case of exo-poly-a-D-galacturonosidase (EC 3.2.1.82), starting from the nonreducing end (Huang and Allen 1997). PG are produced by a wide range of fungal and bacterial phytopathogens to overcome the physical barriers composed of pectins and to provide abundant carbon resources inside the plant host. Their activities cause symptoms including leaf spot, soft rot, and wilt (Collmer and Keen 1986; Huang and Allen 1997). Although some aspects of expression regulation and the roles of pectolysis of PG have been studied in phytopathogens such as *Erwinia* spp. and *Ralstonia solanacearum* (Allen et al. 1997; Gonzalez and Allen 2003; Huang and Allen 1997; Hugouvieux-Cotte-Pattat et al. 1996, 2002; Nasser et al. 1999; Valls et al. 2006), the regulation and function of PG in *Xanthomonas* spp. have not been elucidated. Additionally, according to our alignment results, PG of *Xanthomonas campestris* pv. *campestris* appeared to differ from those reported, based on their nucleic-acid and amino-acid alignments. These differences may provide clues as to some new aspects of expression and function.

In many *Xanthomonas* species, HrpG and HrpX are two upstream regulators of *hrp* genes in the type III secretion system (T3SS). HrpG regulates the expression of HrpX, an AraC-like transcription activator that controls a genome-wide regulon including *hrp* genes and some effector genes (Buttner and Bonas 2002; Wengelnik et al. 1999). The HrpX-regulated promoters often carry a conserved motif termed the “plant-inducible promoter (PIP) box” and a -10 box (consensus, TTCGB-N₁₅-TTCGB-N₃₀₋₃₂-YANNNT) (Buttner and Bonas 2002; Furutani et al. 2006; Koebnik et al. 2006; Noel et al. 2003). The PIP box has been used as an effective marker to identify genes belonging to the HrpX regulon from the genome database. Recently, the specific binding of HrpX to corresponding PIP boxes has been reported (Koebnik et al. 2006).

Clp is a global regulator in *X. campestris* pv. *campestris* that encodes a homolog of the cAMP receptor protein (Crp) in *Escherichia coli* and functions as a regulator in exopolysaccharide and extracellular enzyme production (de Crecy-Lagard et al. 1990; Hsiao et al. 2005; Hsiao and Tseng 2002). Recent studies showed that Clp was involved in the transcriptional expression of about 299 genes, including a few that encode transcription factors (He et al. 2007).

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X. campestris pv. *campestris*, the causal agent of black rot disease, infects large numbers of crucifer plants (Williams 1980) and has been used as a model pathogen on studies of bacterial-plant interactions. *X. campestris* pv. *campestris* produces a range of extracellular enzymes that are collectively essential for pathogenesis (Dow and Daniels 1994). We have sequenced the whole genome sequence of *X. campestris* pv. *campestris* 8004, and analysis of the HrpX regulon showed that two genes of PG (*pghAxc* and *pghBxc*) contained a perfect PIP box, implying they might be regulated by HrpX. In this study, we demonstrated that PghAxc and PghBxc are functional PG and were required for the full virulence of *X. campestris* pv. *campestris* in *Arabidopsis*. *pghAxc* and *pghBxc* were independently regulated by the T3SS regulator HrpX/HrpG and the global regulator Clp.

RESULTS

Characterization of two putative PG genes, *pghAxc* and *pghBxc*.

Genome analysis of the genes belonging to the HrpX regulon in *X. campestris* pv. *campestris* 8004 revealed two putative

PG genes, *pghAxc* (Xc0705) and *pghBxc* (Xc1849), encoding the pectin-degrading enzymes, which have been assigned to the secreted extracellular CWDE of the T2SS (da Silva et al. 2002). However, both genes contained a perfect PIP box and a -10 box in their promoter regions (TTCGtttcacggcagccgTT CGC-N₃₀-TAGAGT in *pghAxc* and TTCGgcttgccggtgccgacTT CGC-N₃₁-GATGGT in *pghBxc*), suggesting they might be HrpX-regulated (Furutani et al. 2006; Koebnik et al. 2006). Pfam analysis showed that PghAxc and PghBxc contained the glyco_hydro_28 (PF00295) domains, which belong to glycosyl hydrolase family 28, which is associated with cell-wall degradation. Alignments of the PghAxc and PghBxc of *X. campestris* pv. *campestris* with the PG formerly studied from *Erwinia* spp. and *R. solanacearum* showed divergences but with some conserved catalytic domains; PghAxc is homologous with PehB (exo-PG, *R. solanacearum*) and PghBxc is homologous with PehA/pglA (endo-PG, *R. solanacearum*). However, PG in *Xanthomonas* species share nearly 80% similarity, suggesting that they may evolve conservatively for host-plant cell-wall degradation.

To characterize enzymatic function of endo- and exo-PG, viscometric assays and reducing sugar ends assays were performed. Measurement of viscosity demonstrated that the *pghAxc* mutant decreased the polygalacturonate substrate viscosity, comparing the wild-type strain and the *pghBxc* mutant (Table 1), suggesting that the PghBxc is an endo-PG. The reducing sugar ends assay showed that the enzyme activity of *pghAxc* mutant decreased to nearly 45% of the wild type, suggesting the PghAxc is an exo-PG (Table 1).

Expression of *pghAxc* and *pghBxc* are regulated by HrpX and Clp.

The perfect PIP boxes suggest HrpX-dependent expression. Previous studies have shown that Clp, a global transcription activator, regulates the transcriptions of many extracellular enzymes of *X. campestris* pv. *campestris* (de Crecy-Lagard et al. 1990; Hsiao and Tseng 2002; Hsiao et al. 2005). To test this

Table 1. Polygalacturonase (PG) activities of *Xanthomonas campestris* pv. *campestris* 8004 strains

Strains	PG activities	
	Viscosity ^a	Reducing sugar ends generated ^b
8004 (wild type)	100	150 ± 8.6
XCM31 (<i>pghA</i> mutant)	85 ± 4.1	76 ± 5.2
XCM32(<i>pghB</i> mutant)	14 ± 5.5	110 ± 6.4

^a Change of the substrate viscosity as a percentage of wild-type level, indicating the primarily endo-PG activity as measured by change in viscosity of a polygalacturonic acid substrate solution.

^b Indicating the exo-PG activity by detecting the reducing sugar ends generated. Measured as units per 10⁹ CFU.

Fig. 1. Transcriptional expression of *pghAxc* and *pghBxc* are regulated by HrpX and Clp. **A**, Promoter-β-glucuronidase (GUS) activity assays of *pghAxc* and *pghBxc* in strains *Xanthomonas campestris* pv. *campestris* 8004, XCM13 (the *hrpX* mutant), and XCM21 (the *clp* mutant) in minimal medium MMXC (Wang et al. 2007). pL6GUS was introduced into *X. campestris* pv. *campestris* 8004 and was used as a negative control. Error bars represent standard deviations. 1 unit = 1 nmol 4-methyl-umbelliferone produced per minute per bacterium. **B**, Reverse transcription-polymerase chain reaction analysis of *pghAxc* and *pghBxc* expression in wild type and the *hrpX* and *clp* mutants. Equal amounts of total RNA pretreated with DNase I (RNase free) were used for first-strand cDNA synthesis. 16s RNA was used as control.

transcriptional dependency, we cloned the promoter regions of *pghAxc* and *pghBxc* and fused them with β -glucuronidase (*GUS*) genes. *GUS* activities were assayed in wild type (*X. campestris* pv. *campestris* 8004), the *hrpX* mutant (XCM13), and the *clp* mutant (XCM21) strains in minimal medium (Wang et al. 2007). The results showed that the transcription activities of both *pghAxc* and *pghBxc* were impaired in the mutants of *hrpX* and *clp*. In particular, the activities in the *hrpX* mutant were decreased to nearly 10% that of the wild-type *X. campestris* pv. *campestris* 8004 (Fig. 1A). Furthermore, we also found that *pghAxc* possesses higher transcriptional activity than *pghBxc* in *X. campestris* pv. *campestris*. These results were further confirmed by reverse transcription-polymerase chain reaction (RT-PCR), which was performed using cDNA synthesized from the total RNA from *X. campestris* pv. *campestris* 8004 and the mutants of *hrpX* and *clp* cultured in minimal medium as templates (Fig. 1B).

HrpG is involved in the regulation of *pghAxc* expression and is dependent on HrpX.

In *Xanthomonas* spp., it has been known that HrpG and HrpX are two linked upstream regulators of the T2SS, in which HrpG directly regulated the expression of HrpX (Buttner and Bonas 2002; Wengelnik et al. 1999). To investigate the regulation of HrpG on the two PG, we performed the promoter-*GUS* activity test in the wild-type strain, XCM11 (the *hrpG* mutant), XccOHRpG (*hrpG* overexpressed in wild type), and XCM13OHRpG (the *hrpX* mutant strain with *hrpG* overexpressed). The results showed that mutation in the *hrpG* gene decreased the transcriptional activities of *pghAxc*. Overexpression of *hrpG* was able to enhance the transcriptional activities of *pghAxc*, while with the mutated *hrpX*, the transcriptional activities of *pghAxc* decreased to a very low level (Fig. 2). The changes of the *pghBxc* transcript levels in these strains were not significant (data not shown). This result may be due to the weak expression of *pghBxc*. Cross-testing between HrpG/HrpX and Clp showed no significant relationship between them (data not shown), suggesting that regulation of PG by HrpG/HrpX and Clp might be independent in *X. campestris* pv. *campestris*.

Fig. 2. HrpG regulates the transcriptional expression of *pghAxc* with the dependency on HrpX. Promoter- β -glucuronidase (*GUS*) activity assays of *pghAxc* in *Xanthomonas campestris* pv. *campestris* 8004, XCM11 (the *hrpG* mutant), XccOHRpG (*hrpG* overexpressed in wild-type *X. campestris* pv. *campestris* 8004), and the *hrpX*-mutated strain with *hrpG* overexpressed in minimal medium MMXC (Wang et al. 2007). pL6GUS was used as a negative control. Error bars represent standard deviations. 1 unit = 1 nmol 4-methyl-umbelliferone produced per minute per bacterium.

pghAxc and *pghBxc* can be efficiently induced in planta.

It has been reported that bacterial genes with a PIP box were mostly able to be induced in planta (Buttner and Bonas 2002). RT-PCR was performed to monitor the expression of *pghAxc* and *pghBxc* genes in planta using total RNA isolated from infected *Arabidopsis* (Col-0) leaves from 0 to 60 h after inoculation. The PCR products of *pghAxc* (639 bp) were clearly amplified from the RNA samples collected at 36, 48, and 60 h after inoculation compared with faint products at 12 and 24 h. However, faint products of *pghBxc* (960 bp) were only amplified from the *Arabidopsis* leaves 24 h after inoculation (Fig. 3A). The PCR products of *pghAxc* amplified at 0 h, and products of *pghBxc* at 0 and 12 h were barely detectable (Fig. 3A). Tests have also been done using the *hrpX* and *clp* mutant strains (XCM13 and XCM21) to inoculate *Arabidopsis*. However, we found that expressions of PG genes in planta could barely be detected with the mutated *hrpX* or *clp* (Fig. 3B). These results further confirmed that the transcription of PG genes was dependent on the regulation of HrpX and Clp. Taken together, these results suggest that both PG genes can be efficiently induced in planta and are dependent on HrpX and Clp.

PghAxc and PghBxc are secreted via the T2SS in an Xps-dependent manner.

Previous studies show the PG of phytopathogens belong to the CWDE, which are secreted to the milieu via the T2SS (Liu et al. 2005; Sandkvist 2001b). The *pghAxc* gene encodes an exo-PG of 47 kDa, and *pghBxc* encodes an endo-PG of 60 kDa. Analysis showed both of the two PG have N-terminal signal peptides (Center for Biological Sequence Analysis SignalP server). We computationally defined the signal peptide of PghAxc to the first 22 amino acids and PghBxc to 28 amino acids, which suggested the T2SS secretion of those two PG.

Fig. 3. Expression patterns of *pghAxc* and *pghBxc* in planta using **A**, *Xanthomonas campestris* pv. *campestris* 8004 and **B**, strains XCM13 and XCM21 as the inoculants. Total RNA of uninoculated *Arabidopsis* leaves (mock-inoculated) and leaves 0, 12, 24, 36, 48, and 60 h after inoculation were used for first-strand synthesis in reverse transcription-polymerase chain reaction (RT-PCR). Amplification products of RT-PCR using *pghAxc*- and *pghBxc*-specific primers and 16s RNA-specific primers were visualized on an agarose gel with ethidium bromide. The products of 16s RNA were used as a control.

Immunoblots were performed to test whether or not PghAxc and PghBxc are secreted via the T2SS. The *pghAxc* and *pghBxc* were fused by Myc tag at the C-terminus and were analyzed by the anti-Myc antibody. The constructs for providing the PG-Myc (pPghAMyc and pPghBMyC) were transformed into the strains of wild type (8004), the *xpsE* mutant (XCM25), and the *xpsD* (XCM26) mutant. XpsE and XpsD are two key structural proteins of the *X. campestris* pv. *campestris* T2SS that structure the channel of the T2SS and determine secretion of the extracellular enzymes (Chen et al. 1996; Ray et al. 2000). Immunoblots of the proteins secreted by these strains in

the minimal medium showed that in the cell-free supernatant of *X. campestris* pv. *campestris* 8004, both PghAxc and PghBxc could generate remarkable signals of detection (Fig. 4A). As opposed to the relatively substantive secretion of these two PG by the wild type, the secretion of these two PG by the two mutants (*xpsE* and *xpsD*) was barely detectable in the culture, which suggests that PghAxc and PghBxc belong to the T2SS and secrete in an Xps-dependent manner (Fig. 4A). Furthermore, the PG activity assays using cell-free supernatant of the *xpsE* and *xpsD* mutants showed very low hydrolytic activities as compared with the wild-type strain *X. campestris* pv.

Fig. 4. PghAxc and PghBxc are secreted via the type II secretion system in an Xps-dependent manner. **A**, Immunoblot of secreted polygalacturonase (PG)-Myc proteins in *Xanthomonas campestris* pv. *campestris* 8004, XCM25 (the *xpsE* mutant), and XCM26 (the *xpsD* mutant). Strains were cultured in minimal medium and the secreted proteins in culture fluids were collected. Equal amounts of concentrated secreted proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gels and Myc antibodies were used for detection. **B**, Activity assays of the secreted PG in the culture fluids of wild-type and the *xpsE* and *xpsD* mutants. Error bars represent standard deviations. A unit liberates 1 μ mol galacturonic acid from polygalacturonic acid per minute.

Fig. 5. **A**, Activity assays of the secreted polygalacturonases (PG) in the culture fluids of *Xanthomonas campestris* pv. *campestris* 8004, XCM13 (the *hrpX* mutant), XCM21 (the *clp* mutant), XCM31 (the *pghAxc* mutant), XCM32 (the *pghBxc* mutant), and XCM321 (the *pghAxc* and *pghBxc* double mutant). Error bars represent standard deviations. A unit liberates 1 μ mol galacturonic acid per minute. **B** and **C**, Bacterial growth curves of strains used in PG activity assays. Strains were cultured in minimal medium using polygalacturonic acid as the sole carbon source, and samples were taken every 12 h. This experiment was repeated five times, and error bars represent standard deviations.

campestris 8004, which further confirms the secretion dependency of PghAxc and PghBxc on the Xps proteins of the T2SS (Fig. 4B).

The hydrolytic activities of PghAxc and PghBxc.

To test the activities and virulence of these two PG (PghAxc and PghBxc), we generated the mutants *pghAxc* and *pghBxc* as well as the double mutant. Activity assays were performed in *X. campestris* pv. *campestris* 8004, XCM31 (the *pghAxc* mutant), XCM32 (the *pghBxc* mutant), and XCM321 (the double mutant). All the mutants showed lower hydrolytic activities compared to the wild type, especially the *pghAxc* mutant and the double mutant (Fig. 5A). The results of these activity assays suggested that PghAxc contributed more to the full *X. campestris* pv. *campestris* PG activities than did PghBxc. Furthermore, we also recorded the dynamic population growth of these strains in minimal medium using the polygalacturonic acids as the sole carbon source. The results showed that the

wild type always had a growth advantage compared with the mutants, which is consistent with the activity assays. In addition, dynamic growth of XCM13 (the *hrpX* mutant), XCM21 (the *clp* mutant), and XCM25 (the *xpsE* mutant) showed that, as opposed to the increased growth of the wild-type *X. campestris* pv. *campestris* 8004, the growth of the three mutants trended downwards after being cultured for 36 h in minimal medium (Fig. 5B). This result suggested that mutation of these genes would disadvantage the growth of bacteria in the minimal medium using polygalacturonic acids as the sole carbon source.

pghAxc and *pghBxc* are required for full virulence of *X. campestris* pv. *campestris* in *Arabidopsis*.

Dip-, spray-, and infiltration-inoculation methods were used in pathogenicity tests. We have tested several ecotypes of *Arabidopsis* and have found that the ecotype *Kendle* is most suitable for the assay. The inoculants for the pathogenicity

Fig. 6. A, Typical symptoms of disease elicited by *Xanthomonas campestris* pv. *campestris* strains using dip- and **B**, spray-inoculation methods in *Arabidopsis*. The inoculants were *X. campestris* pv. *campestris* 8004 (1), the *pghAxc* mutant (5), and complemented strain (2), the *pghBxc* mutant (6) and complemented strain (3), the *pghAxc* and *pghBxc* double mutant (4), and mock-inoculated (10 mM MgCl_2) (7). Bacterial density was 3×10^8 CFU/ml for dip and spray inoculation.

tests were *X. campestris* pv. *campestris* 8004, XCM31 (the *pghAxc* mutant), XCM32 (the *pghBxc* mutant), and XCM321 (the *pghAxc* and *pghBxc* double mutant). The results showed that the wild type caused distinctive necrosis symptoms compared with the mutants and the double mutant, all of which elicited weak symptoms with a minor wilt in both dip- (Fig. 6A) and spray-inoculation assays (Fig. 6B). The mutant-complemented strains of both *pghAxc* and *pghBxc* were able to elicit the same distinctive necrosis symptoms as the wild type (Fig. 6). However, no differences could be found between the wild type and the mutants using the infiltration method (data not shown). These results suggest that *pghAxc* and *pghBxc* are required for full virulence of the bacteria in *Arabidopsis*. Moreover, we also assayed the bacterial growth in *Arabidopsis* by

infiltration inoculation, and the results showed only minor differences between the wild type and the mutants (data not shown).

DISCUSSION

Earlier studies of PG in *Erwinia* spp. and *R. solanacearum* mainly focused on the aspects of the enzyme activity and virulence (Gonzalez and Allen 2003; Huang and Allen 1997; Lei et al. 1985; Liu et al. 2005). In this study, two novel PG genes from *X. campestris* pv. *campestris* have been characterized. Their hydrolytic enzyme activity and virulence functions have been more comprehensively assayed and the regulation of their expression has been defined, which provides evidence of some linkages between the T2SS and the T3SS. Furthermore, we showed that the expressions of these two PG in planta are strongly inducible.

Alignments of the amino-acid sequences of all the PG from the three phytopathogens showed a low identification of PghAxc (about 30%) and PghBxc (about 17%) to the homologs from *Erwinia* spp. and *R. solanacearum*, suggesting that these proteins have a degree of novelty. However, within the *Xanthomonas* species, those two PG are relatively conserved (over 70% sequence identity). These facts suggested that PG in different pathogenic species were divergent and may evolve differently according to host preference and environmental surroundings. In addition, the computational comparison among all the published sequences of PG also revealed numbers of conserved regions, suggesting they may be required for the common functions of substrate binding and hydrolysis of PG.

Although some genes not belonging to the T3SS are thought to belong to the HrpX regulon (da Silva et al. 2002), only one gene encoding the extracellular (CysP2) T2SS enzyme has so far been demonstrated to be regulated by HrpX (Furutani et al. 2004). CysP2 is an extracellular protease that contains an imperfect PIP box in its promoter region. Using GUS assays, RT-PCR, and silver-stain approaches, the authors demonstrated the HrpX-dependency of *cysP2*'s expression (Furutani et al. 2004). In our study, we found two novel PG from *X. campestris* pv. *campestris* with HrpX-dependent expression. HrpX is the key regulator in the T3SS that controls the expression of

Table 2. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference or source
<i>Xanthomonas campestris</i> pv. <i>campestris</i> strains		
8004	Wild-type; Rif ^r	Turner et al. 1984
XCM13	<i>hrpX</i> pK18 knockout mutant; Rif ^r , Km ^r	Wang et al. 2007
XCM21	<i>clp</i> pK18 knockout mutant; Rif ^r , Km ^r	This study
XCM31	<i>pghAxc</i> pK18 knockout mutant; Rif ^r , Km ^r	This study
XCM31C	<i>pghAxc</i> mutant containing pHMpghAxc	This study
XCM32	<i>pghBxc</i> pK18 knockout mutant; Rif ^r , Km ^r	This study
XCM32C	<i>pghBxc</i> mutant containing pHMpghBxc	This study
XCM321	<i>pghAxc</i> and <i>pghBxc</i> double mutant; Km ^r and Sp ^r	This study
XCM25	<i>xpsE</i> <i>Tn5</i> -insertion mutant; Rif ^r , Km ^r	Sun et al 2003
XCM26	<i>xpsD</i> <i>Tn5</i> -insertion mutant; Rif ^r , Km ^r	Sun et al 2003
Plasmids		
pGEM-T easy	PCR cloning vector, Amp ^r	Promega
pHM1	Broad-host range <i>cos</i> <i>parA</i> IncW derivative of pRI40 Spr, Strr	Innes et al. 1988
pHMpghAxc	pHM1 containing full length of <i>pghAxc</i> gene	This study
pHMpghBxc	pHM1 containing full length of <i>pghBxc</i> gene	This study
pHMpghAxcMyc	pHM1 containing Myc-tagged <i>pghAxc</i>	This study
pHMpghBxcMyc	pHM1 containing Myc-tagged <i>pghBxc</i>	This study
pK18mob	Suicide plasmid in <i>X. campestris</i> pv. <i>campestris</i> ; Km ^r	Schafer et al. 1994
pKpA	<i>pghAxc</i> knockout construct	This study
pKpB	<i>pghBxc</i> knockout construct	This study
pK18hrpX	<i>hrpX</i> knockout construct	This study
pKnockout-Ω	Suicide plasmid in <i>X. campestris</i> pv. <i>campestris</i> ; Sp ^r	Lab collection
pKopB	<i>pghBxc</i> knockout construct derived from pKnockout-Ω; Sp ^r	This study
pL6GUS	pLAFR6 contains promoterless <i>uidA</i> gene in multiple cloning sites, promoter-probe vector	Lab collection
pPEGGUS	pLAFR6 carrying <i>pghAxc</i> promoter fused with <i>uidA</i>	This study
pPPGGUS	pLAFR6 carrying <i>pghBxc</i> promoter fused with <i>uidA</i>	This study
pRK2013	ColE1 replicon TraRK ⁺ Mob ⁺ ; Km ^r	Lab collection

^a Rif = rifampicin, Km = kanamycin, Amp = ampicillin, Sp = spectinomycin, Str = streptomycin, Gm = gentamycin, Tc = tetracycline; ^r = resistant.

Table 3. Primers used in this study^a

Gene target	Primer sequence (5' → 3')	Restriction site added
<i>pghAxc</i>		
Axck-F	CAGCGCATTTACGCTCTAC	...
Axck-R	CAACATTGGCAAGCACCAGG	...
Axcp-F	CCCAAGCTTGGAGATGGTGCAGTTCCAG	HindIII
Axcp-R	CGGGATCCACGTAGTCACCCGAAGAAG	BamHI
Axcm-F	CAAGCTTCTTCGGGTGACTACGTCATG	HindIII
Axcm-R	GGTACCTTACAGATCTTCTTCAGAAATA AGTTTTTGTTcCAACGCAGGAAAGTGC GGTG	KpnI
<i>pghBxc</i>		
Bxck-F	ATCCCACCCAATCCAAACGC	...
Bxck-R	GAAGCATCGGACTTGATACGC	...
Bxcp-F	CCCAAGCTTGTACGGCTTGTTCCTTA G	HindIII
Bxcp-R	CGGGATCCAGTGGGAGAGAGGTGAC	BamHI
Bxcm-F	CAAGCTTCACTGGAGTCAAACGGAATG	HindIII
Bxcm-R	GAATTCTTACAGATCTTCTTCAGAAATA AGTTTTTGTTTCGACGGCTGCGGCGCCG TCGG	EcoRI

^a Primers were designed according to the *Xanthomonas campestris* pv. *campestris* 8004 genomic DNA sequence. a. Suffixes F and R represent forward and reverse primers, respectively. ... = ??; k = knock-out primer. p = β-glucuronidase activity primer. m = Myc primer.

hrp and some effector genes. The fact that HrpX is also involved in the regulation of some extracellular enzymes of the T2SS suggests that HrpX controls the expression of the majority of genes containing a PIP box in *Xanthomonas* spp. and, therefore, may be involved in regulation of many *Xanthomonas* genes. This regulation of HrpX suggested that the signal transduction networks of pathogens are cross-linked and that the T3SS and T2SS may cooperate via various regulators to promote the virulence of the pathogen in the host. However, the PG activity assay showed that, in XCM13 (the *hrpX* mutant) and XCM321 (the *pghAxc* and *pghBxc* double mutant), PG activity (up to approximately 30% of the wild-type strain) could be detected, suggesting that additional functional PG exists in *Xanthomonas* spp. HrpG, a two-component response regulator, is the upstream regulatory factor of HrpX and controls the regulatory cascade of the T3SS in *Xanthomonas* spp. The results showed that the regulatory activity of HrpG is weaker than that of HrpX. Moreover, with the *hrpX* gene mutated, HrpG would lose most of its regulatory controls on the PG even if *hrpG* is overexpressed. These results suggested that HrpG was indirectly involved in PG regulation but the regulation was HrpX-dependent. Therefore, we speculated that the regulation HrpG on the *pghAxc* may be achieved via HrpX and that the PG belonged to the HrpG/HrpX regulon.

Clp of *X. campestris* pv. *campestris* is a member of the conserved global-regulator family. The most representative protein of this family is Crp of *E. coli*. This regulator has been extensively studied and has been shown to directly regulate about 192 genes (He et al. 2007; Martinez-Antonio and Collado-Vides 2003; Zheng et al. 2004). Similar to Crp, Clp in *X. campestris* pv. *campestris* also influences the expression of a number of genes, especially the genes in the T2SS (He et al. 2007). A recent study showed direct binding of Clp to the promoter region of *engXCA*, which is a cellulase secreted via the T2SS (Hsiao et al. 2005). Another example of a Clp-regulated extracellular enzyme is PRT1, which is a protease secreted via the T2SS that is responsible for *X. campestris* pv. *campestris* virulence (Dow et al. 1990; Hsiao and Tseng 2002). Our data showed that *pghAxc* and *pghBxc* are regulated by Clp, which agreed with previous studies showing that Clp is involved in the expression of extracellular enzymes of T2SS (He et al. 2007; Hsiao et al. 2005; Martinez-Antonio and Collado-Vides 2003; Zheng et al. 2004). However, analysis of the promoter regions of PG genes only identified the conserved sequences of Clp binding sites 'TGTGA-N6-TCACA' (Dong and Ebright 1992) in the promoter of *pghBxc*. Therefore, it is reasonable to predict that *pghBxc* may be regulated by Clp directly by binding to the box and that *pghAxc* may affect regulation in another indirect way. A similar situation has also been found in Clp-regulated PRT1 (Dow et al. 1990; Hsiao and Tseng 2002). Some regulatory proteins may directly regulate the expression of *pghAxc* and, in turn, those regulators may require the function of Clp. That is to say, Clp regulation of PG genes may be achieved in a cascade manner. From the reports of He and associates (2007), some genes belonging to the T3SS are regulated by Clp, which is also evidence that the T2SS and T3SS are cross-linked.

The PG of phytopathogens have been classified to the CWDE secreted via the T2SS. This is mainly based on the N-terminal signal peptide of PG and the severe effects on extracellular enzyme activity by deficiencies of the T2SS. *Xanthomonas* protein secretion (*xps*) genes encode structural proteins to form the secretion channel of the T2SS, which is essential for the secretion of the T2SS extracellular enzymes (Hu et al. 1998; Ray et al. 2000). We provided direct evidence of T2SS-dependent secretion of PG using immunoblot detection in the culture fluids of wild-type and *xps* mutants.

The ability to degrade pectin is useful for pathogens in virulence. During pathogenesis, plant cell walls act as the first barrier of defense against bacterial invasion and multiplication. The enzymatic activities of pectin degradation may facilitate pathogen invasion into the cells of its host plants by digesting the pectin layers of plant cells. Our data showed that the virulence of *pghAxc* and *pghBxc* mutants was attenuated in *Arabidopsis* using dip- and spray-inoculation methods. Dip and spray inoculation showed the distinctive differences in virulence between the wild type and mutants, while the infiltration method did not. This fact suggested that the virulence function of PghAxc and PghBxc may mainly execute in the early stage of bacterial pathogenesis, which is the process for pathogens to overcome the pectin barriers of plant cells. Furthermore, the expression of those two PG could be more efficiently induced in planta 36 h postinoculation, which also suggested that the PG were required for the bacterial virulence in the early stage of pathogenesis in planta. Additionally, in the infiltration-inoculation method, the bacteria are syringed directly into the intercellular spaces of plant cells, which artificially overcomes the epidermis of the leaves. Alternatively, the barely detectable differences in disease symptoms using infiltration inoculation may be explained by functional redundancy. The *X. campestris* pv. *campestris* genome annotation has revealed different isoforms of CWDE as well as numerous virulence factors that are associated with bacterial pathogenesis. Therefore, the result that losing one or two CWDE did not cause a distinctive difference on the *X. campestris* pv. *campestris* virulence could be expected.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 2. *X. campestris* pv. *campestris* 8004 was grown in NYGB medium (Tsuchiya et al. 1982) or in a plant-mimicking minimal medium, MMXC (MMX [Daniels et al. 1984], plus 10 mM sucrose, 10 mM fructose, 4 mM sodium glutamate, and 0.03% casamino acids) at 28°C. *E. coli* strains were grown in Luria-Bertani medium at 37°C. Antibiotics were used at the following concentrations: ampicillin at 100 µg/ml, kanamycin at 50 µg/ml, rifampicin at 50 µg/ml, tetracycline at 15 µg/ml for *X. campestris* pv. *campestris* 8004 and 50 µg/ml for *E. coli*, and spectinomycin at 100 µg/ml for *X. campestris* pv. *campestris* 8004 and 50 µg/ml for *E. coli*.

Mutant generation.

To obtain knock-out mutants, the truncated fragments of *pghAxc* and *pghBxc* were amplified with primers Axck and Bxck and were cloned into pGEM-T Easy vectors (Promega, Madison, WI, U.S.A.). After digestion by *EcoRI*, the fragment was cloned into the knock-out vector pK18mob (Schafer et al. 1994), giving pKpA and pKpB. These two knock-out constructs were introduced into *X. campestris* pv. *campestris* 8004. By homologous recombination, the corresponding genes in the *X. campestris* pv. *campestris* 8004 genome were inserted using the pK18mob vector and separated into two functionless truncated fragments. For the double mutant, the truncated fragment of *pghBxc* was cloned into another knock-out vector pKnock-out-Ω with spectinomycin resistance (Windgassen et al. 2000), giving pKopB. Then the pKopB construct was introduced to the *pghAxc* knock-out mutant and was selected by adding both kanamycin and spectinomycin into the agar medium. Southern blot and PCR were used to confirm all the mutant candidates. The knock-out mutant of *hrpX* (XCM13) was obtained similarly (Wang et al. 2007).

DNA manipulation and plasmid construction.

DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, subcloning, electroporation, PCR, Southern, and Western blot analyses were performed according to standard procedures (Sambrook et al. 1989). The PCR primers were listed in Table 3. All the PCR products used in cloning were first cloned into the pGEM-T Easy vector (Promega) and were verified by sequencing.

To construct *pgh* promoter-GUS fusion constructs, the whole promoter region of *pgh* was amplified with primers Axc_p and Bxc_p. The fragments were cloned into the promoter probe plasmid pL6GUS with *Hind*III and *Bam*HI, giving pPEGGUS and pPPGGUS. To express the Myc-tagged PG protein, the whole *pgh* gene was Myc-tagged at the 3'-terminus by PCR, using primers Axc_m and Bxc_m, and then, was cloned into the vector pHM1, giving pPghAMyc and pPghBMyC. The two constructs were introduced into wild type, giving the *xpsE* (XCM25) and *xpsD* (XCM26) mutants for secretion detection.

Protein preparations and enzyme activity assays.

Extracellular proteins were prepared collecting the supernatants of *X. campestris* pv. *campestris* cultures grown in MMXC medium for 72 to 96 h. For viscosity assays, the supernatants were concentrated approximately 30-fold by freeze drying and were dialyzed at 4°C overnight against 0.5 mM acetate buffer, pH 5.0. Endo-PG activity was measured as rate of viscosity change in a substrate solution containing 1% polygalacturonic acid in 50 mM sodium acetate, pH 5.0. Concentrated culture supernatant (1 ml) was added to 14 ml of substrate solution in an Ubbelohde Viscometer Capillary at 28°C.

Detection of secretory PG proteins of *X. campestris* pv. *campestris* in MMXC.

For the secretion assay, strains of *X. campestris* pv. *campestris* 8004 and *xpsE* (XCM25), and *xpsD* (XCM26) mutants carrying pPghAMyc or pPghBMyC, respectively, were cultured in NYGB medium for 30 h at 28°C. The procedures for protein preparation and immunoblot were performed as before (Wang et al. 2007).

Promoter activity assay and RT-PCR analysis.

For GUS activity assays, *X. campestris* pv. *campestris* 8004 and mutant strains were cultured in MMXC to an optical density at 600 nm (OD_{600}) = 0.5. Bacteria cells were diluted and disrupted in sonic buffer (20 mM Tris HCl, pH 7.0, 10 mM 2-mercaptoethanol, 5 mM EDTA, and 1% Triton X-100). GUS activity was assayed as described previously (Jefferson et al. 1987). One unit was defined as 1 nmol of 4-methyl-umbelliferone produced per minute per bacterium. For RT-PCR analysis in vivo, the bacteria were cultured exactly as for the GUS activity assay (OD_{600} = 0.5) and were infiltrated into leaves of *Arabidopsis*. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA were quantified by measuring the OD_{260} and were qualified by gel electrophoresis. Before synthesis of the first strands, total RNA was digested with RNase-free DNase I (TaKaRa, Dalian, China) to remove potential traces of genomic DNA. cDNA synthesis and PCR were conducted with a Superscript one-step RT-PCR kit (Invitrogen) and ex-*Taq* DNA polymerases (TaKaRa) with the primers Axck and Bxck.

RT-PCR assay.

Arabidopsis leaf samples were harvested from uninoculated plants and inoculated plants at 0, 12, 24, 36, 48, and 60 h after inoculation. Total RNA from inoculated *Arabidopsis* leaf tissues

was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript first-strand synthesis system (Invitrogen) after digestion with RNase-free DNase I (TaKaRa). PCR reactions were carried out using the synthesized cDNA products as templates with primers Axck and Bxck specific for *pghAxc* and *pghBxc*, respectively. The products of RT-PCR were detected by electrophoresis on a 1.0% agarose gel and were stained with ethidium bromide.

Pathogenicity assay.

Arabidopsis grown in a greenhouse for over 1 month were used for pathogenicity tests. After washing twice with 10 mM MgCl₂, bacteria at the correct optical densities were inoculated into the leaves by dip and spray inoculation at 3×10^8 CFU per milliliter and infiltration at 1×10^6 CFU per milliliter.

PG activity (reducing sugar ends assays) and dynamic population-growth assays.

To test the PG activity and dynamic population growth, we used MMXG medium (K₂HPO₄, 10.5 g/liter; KH₂PO₄, 4.5 g/liter; (NH₄)₂SO₄, 1.0 g/liter; MgSO₄, 1 mM; polygalacturonic acid, 0.5% (wt/vol), pH 6.0) modified from MMX with polygalacturonic acid as the sole carbon source. Extracellular protein preparations were made by collecting the supernatants from 72- to 96-h *X. campestris* pv. *campestris* cultures grown in MMXG minimal medium. For PG assays, these supernatants were concentrated and measured quantitatively as reducing sugar ends released, using the method of Huang and Allen (1997). One unit of PG activity was defined as the amount of enzyme that releases 1 mmol of oligogalacturonate from PGA per minute. For dynamic population-growth assays, the *X. campestris* pv. *campestris* strains were cultured first in NYG for 30 h, and then, were washed with 10 mM MgCl₂. Strains were diluted to OD_{600} = 0.1 and were cultured in MMXG minimal medium. Every 12 h, 500- μ l samples of each strain were taken for OD_{600} measurements.

ACKNOWLEDGMENTS

This work was funded by the Knowledge Innovation Program of the Chinese Academy of Sciences (grant KSCX2-YW-N-005) and the National Natural Science Foundation of China (grant 30228002).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Center for Biological Sequence Analysis SignalP server:
www.cbs.dtu.dk/services/SignalP
 Pfam website: www.sanger.ac.uk