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Clp and RpfF Up-regulate Transcription of *pelA1* Gene Encoding the Major Pectate Lyase in *Xanthomonas campestris* pv. *campestris*

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Exopolysaccharide and several extracellular enzymes of *Xanthomonas campestris* pv. *campestris* (Xcc), the causative agent of black rot in crucifers, are virulence determinants. In this study, two Xcc annotated extracellular pectate lyase genes, *pelA1* and *pelA2*, belonging to family 1 of the polysaccharide lyase, were characterized. Sequence and mutational analyses have demonstrated that *pelA1* encodes the major pectate lyase, whereas *pelA2* is not transcribed. Using the 5′ RACE method, the *pelA1* transcription initiation site was mapped at nucleotide G, 103 nt upstream of the *pelA1* start codon. Promoter analysis demonstrated that polygalacturonic acid and $CaCl_2$ induce the expression of *pelA1*. Transcriptional fusion assays also indicated that Clp (cAMP receptor protein-like protein) and RpfF (an enoyl-CoA hydratase homologue that is required for the synthesis of *cis*-11-methyl-2-dodecenoic acid, a low molecular weight diffusible signal factor, DSF) positively regulate *pelA1* transcription. Gel retardation assays showed that Clp exerts a positive control over expression of *pelA1* by direct binding to the upstream Clp-binding site. In conclusion, the present research demonstrated that *pelA1* codes for the major pectate lyase in Xcc strain Xc17 and that its expression is up-regulated by Clp and RpfF. This is the first study to characterize pectate lyase gene expression in Xcc.

KEYWORDS: Cyclic AMP receptor protein-like protein; promoter-*lacZ* reporter assay; pectate lyase; transcription; *Xanthomonas campestris*

INTRODUCTION

The Gram-negative plant pathogenic *Xanthomonas campestris* pv. *campestris* (Xcc) is the causative agent of black rot in crucifers, a disease that causes tremendous agricultural losses (*1*). It is capable of producing large amounts of an exopolysaccharide and secreting several extracellular enzymes (such as protease, endoglucanase, pectate lyase, and polygalacturonase), which have been considered to be important virulence determinants (*2*, *3*).

The production of these extracellular products is coordinated by a cell−cell communication mechanism through diffusible signal factor (DSF), which is dependent on RpfF, a putative enoyl-CoA hydratase, for synthesis and the RpfC/RpfG two-component system for perception and signal transduction (*4*, *5*). A recent study also indicated that global transcription factor cAMP receptor protein-like protein (Clp) is essential for DSF regulation of virulence factor production (*6*). Gel retardation and transcriptional fusion assays have shown that Clp exerts a positive control over expressions of *engA* (encoding the major endoglucanase) and *pehA* (encoding the major polygalacturonase) by direct binding to the upstream Clp-binding sites, whereas *prt1* (encoding the major protease) without a Clp-binding site is

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Table 1. PCR Primers Used in This Study

primer	sequence [a]	position [b]	direction and use [c]
	pelA1 Gene Amplification		
−211*Xho*I	5′-CTCGAGCATGCGCGATCACACATCCT-3′	−211	F, promoter analysis
−148*Pst*I	5′-CTGCAGATTGCAGTGTCCTCGCTGCT-3′	−148	F, complementation
−146*Pst*I	5′-AACTGCAGTGTCCTCGCGATTGTCTCCACG-3′	−146	F, promoter analysis and gel retardation
−98*Pst*I	5′-CTGCAGTCTTCTCTTGAATGTCCCGC-3′	−98	F, promoter analysis and gel retardation
−70*Pst*I	5′-AACTGCAGCGTGACTGCGCGTGCACATCGA-3′	−70	F, promoter analysis and gel retardation
−27*Pst*I	5′-AACTGCAGATTGCGATGCTCCTAGTATC-3′	−27	F, promoter analysis and gel retardation
+42*Xba*I	5′-GCTCTAGAGCGCGGAACAGACTTTTCAG-3′	+42	R, promoter analysis
+134*Xba*I	5′-GCTCTAGACGGCGGCCGCCGTTGAAAATTT-3′	+134	R, promoter analysis
+104*Nde*I	5′-CATATGAAGCCGAAATTCTCAACGGC-3′	+104	F, mutant construction and confirmation
+182*Eco*RV	5′-GATATCGATCCTGCACTGGAAGTTGC-3′	+182	F, mutant confirmation
+1234*Xho*I	5′-CTCGAGCAGCTTTCCGGCACCTG-3′	+1234	R, mutant construction and confirmation
+1237*Xba*I	5′-TCTAGATTACAGCTTTCCGGCACCTG-3′	+1237	R, complementation
+134B	5′-CGGCGGCCGCCGTTGAGAAT-3′ (5′ biotin labeled)	+134	R, gel retardation
290R	5′-CGGCAGCGTTCTTGACCGTG-3′	+290	R, TIS determination and mutant confirmation
373R	5′-GCCTTCGCTGACGTCGATCA-3′	+373	R, TIS determination
559R	5′-ATCCCAGACCGGCTGCGGAT-3′	+559	R, TIS determination
	pelA2 Gene Amplification		
−274*Xho*I	5′-CTCGAGCTGACGTCAAGCAAAAGCA-3′	−274	F, promoter analysis
+29*Xba*I	5′-TCTAGACGACCCACAACTGTCGATGC-3′	+29	R, promoter analysis

[a] Added restriction enzyme sites are underlined. [b] Position of the 5′-end of the oligonucleotides relative to the TIS of *pelA1* or the translation start site of *pelA2* gene. [c] F, forward direction; R, reverse direction.

controlled in an indirect manner (*7*−*9*). The flagellin gene, *fliC*, the heat shock *groESL* genes, the secondary polygalacturonase gene *pglA*, and the type II secretion structural gene *xpsE* are also controlled by Clp (*10*−*13*). Transcriptional fusion assays have shown that RpfF positively regulates *pehA* transcription (*7*).

Five pectate lyase genes (*pelA1*, *pelA2*, *pelB*, *pelE*, and *hrpW*) have been annotated in the fully sequenced genomes of Xcc strains ATCC33913, 8004, and B100 (*14*−*16*) as well as in strain Xc17, in which the genome sequence is almost complete (http://xcc.life.nthu.edu.tw). None of these genes have been studied in detail, although three forms of pectate lyases in Xcc strain 8004 have been reported (*17*, *18*). It was found that a *pelB* mutant derived from Xc17 showed no change in pectate lyase activity on plate assays (*19*), indicating that PelB is not the major pectate lyase in Xcc. More recently, three pectate lyase encoding sequences (*pelA1*, *pelA2*, and *pelB*) in Xcc ATCC33913 were cloned and expressed in *Escherichia coli*, and the recombinant PelB was purified and crystallized (*20*, *21*). There has been limited information concerning pectolytic enzymes in other xanthomonads. Only a single isoform of pectate lyase with a p*I* of 8.8 in *X. campestris* pv. *vescatoria* (Xcv) strain XV56 has been reported (*22*). The causal agent of cotton blight, *X. campestris* pv. *malvacearum* (Xcm) strain B414, also contains one isoform of pectate lyase with an estimated molecular mass of 41 kDa and a p*I* of 9.7 (*23*). The aim of the present study is to evaluate the transcriptional regulation of *pelA1* and *pelA2* in Xcc strain Xc17. This is the first study to characterize the pectate lyase gene expression in Xcc. Knowledge obtained from this study is expected to facilitate further investigations on the expression and function of pectate lyase in Xcc, as well as the understanding of transcriptional regulation mechanisms of this phytopathogen.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions. *E. coli* DH5α (*24*) was the host for DNA cloning. Xc17 was a virulent wild-type strain isolated in Taiwan (*25*). Strains derived from Xc17 by marker exchange have included PLA (*pelA1* mutant), PLAH (*pelA1* and *pehA* double mutant) isolated in this study, *clp* mutant AU56E (*26*), *pehA* mutant MH172, and *rpfF* mutant RM17F (*7*). Luria−Bertani (LB) broth and L agar (*27*) were the general-purpose media for cultivating *E. coli* and Xcc at 37 and 28 °C, respectively. XOLN was a basal salt medium containing 0.0625% tryptone and 0.0625% yeast extract (*28*). Xylose (2%), polygalacturonic acid (PGA, 0.5%, grade II, Sigma Chemical Co.), and pectin with a 30, 60, or 90% degree of esterification (0.5%, Sigma Chemical Co.) were supplemented as required. The following antibiotics were added when necessary: ampicillin (50 μg/mL), kanamycin (50 μg/mL), gentamycin (15 μg/mL), and tetracycline (15 μg/mL).

DNA Techniques. Enzymes were purchased from Promega (Madison, WI) and Roche Diagnostics GmbH (Mannheim, Germany). Standard protocols have been described elsewhere (*29*). PCR was carried out as previously described (*8*) using primers listed in **Table 1**. After PCR amplification, the amplicons were separately ligated into the PCR cloning vector yT&A (Yeastern Biotech Co., Ltd., Taipei, Taiwan), which had the M13 forward and reverse primers for sequence verification to ensure that no mutations resulted during PCR. DNA sequences on both strands were determined by the dideoxy chain termination method (*30*). After DNA sequence confirmation, the fragments were each excised from the respective plasmids and cloned into appropriate sites of the various vectors for specific purposes. Transformation of *E. coli* was performed according to the standard method (*29*) and that of Xcc by electroporation (*31*).

Mutant Construction. Procedures for construction of PLA (*pelA1* mutant) were as follows. The 1059 bp *Nde*I−*Xho*I fragment containing the Xc17 *pelA1* gene was cloned in the *E. coli* vector pOK12 (*32*), which contains P15A *ori* and cannot be maintained in Xcc, giving pOKpelA. A Gm^r cartridge from pUCGM (*33*) was inserted into the unique *Hin*cII site within the pOKpelA insert, giving rise to pOKpelAG. The resultant plasmid, pOKpelAG, was electroporated into Xc17, allowing for double crossover via homologous recombination through the identical regions in the chromosome and the plasmid to replace the wild-type sequence. For the PLAH (*pelA1* and *pehA* double mutant) construction, the pOKpelNH356, containing the 356 bp *Not*I−*Hin*cII fragment internal to the Xc17 *pelA1* gene cloned into pOK12, was electroporated into *pehA* mutant MH172 following a single crossover. Insertion of the Gm^r cartridge or pOKpelNH356 into *pelA1* was confirmed by PCR. Briefly, genomic DNA isolated from PLA or PLAH was used as template, and the Gm^r cartridge or pOKpelNH356 inserted *pelA1* region was amplified by primer pairs +104*Nde*I/+1234*Xho*I or +182*Eco*RV/290R shown in **Table 1**.

Construction of PelA1-Expression Plasmid pRKpelA. The PelA1-expression plasmid, pRKpelA, was generated by cloning the 1385 bp DNA fragment encompassing the upstream 251 bp fragment plus the

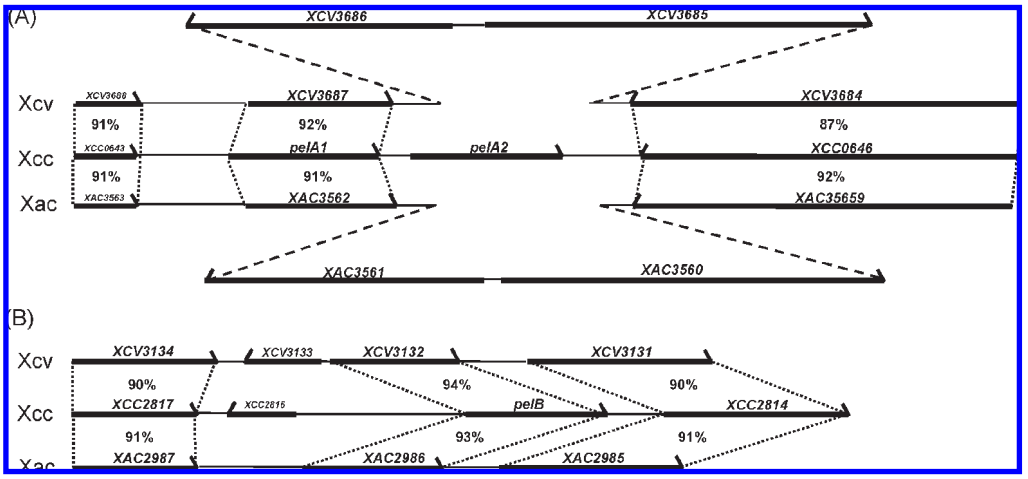

Figure 1. Comparative analysis of the organization of pectate lyase genes of the polysaccharide lyase family 1 in *Xanthomonas*. The *pelA1* (**A**), *pelA2* (**A**), and *pelB* (**B**) in Xcc were compared to their homologues from *X. campestris* pv. *vesicatoria* (Xcv) and *X. axonopodis* pv. *citri* (Xac). The percentage values refer to the amino acid identity of each homologue using Xcc gene product for comparison.

entire coding region of the Xc17 *pelA1* into the *Pst*I−*Xba*I sites of the broad-host-range vector pRK415 (*34*). The *pelA1* gene was oriented in the same direction as the *lac* promoter in pRK415.

Plate Assay for Pectate Lyase Activity. Pectate lyase activity assay was performed on agar plates containing PGA as substrate as previously described (*35*). Strains to be assayed were grown overnight in LB medium, washed once, and diluted with sterile distilled water to OD_{550} = 1; 3 μL was deposited onto the surface of the XOLN plates containing xylose (2%) and PGA (0.5%). After 2 days of incubation, the plates were stained with 0.2% ruthenium red for 1 h and destained with distilled water for 10 min. PGA is a polymer of D-galacturonate residues with α-1,4 glycosidic linkages. The free acid groups of the polymer bind to ruthenium red to form a red complex, but monomers and oligomers do not. Polymer degradation by pectate lyase depletes the plate of stain-binding material so that clearing indicates enzyme activity.

Pathogenicity Test. To test for pathogenicity, cells from an overnight culture were diluted with sterile distilled water to OD_{550} of 1.0, and 3 μL was used as the inoculum for the pathogenicity tests on 2-week-old potted cabbage seedlings according to previously described procedures (*25*). The testing was carried out in three independent experiments with six replicates.

Mapping the 5′ End of *pelA1* mRNA. The 5′ rapid amplification of cDNA ends (RACE) system (*36*) was used to determine the transcription initiation site (TIS) using Invitrogen version 2.0 kit (Invitrogen Corp., Carlsbad, CA). Total RNA was isolated from Xc17 (midexponential phase) by the Qiagen RNA extraction system (Qiagen Inc., Valencia, CA). The Abridged Anchor Primer (AAP) and Abridged Universal Amplification Primer (AUAP) were used in combination with the gene-specific primers. The gene-specific primers for RT-PCR, nested PCR1, and nested PCR2 were 559R, 373R, and 290R, respectively. The sequences and locations of these primers are shown in **Table 1**. The PCR products were ligated into the yT&A vector for sequence verification.

Transcriptional Fusion Assay. Six P*pelA1-lacZ* transcriptional fusion constructs were generated by cloning PCR fragments into the broad-host-range-promoter-probing vector pFY13−9, which used *lacZ* as the reporter (*37*). Constructs pFY−211+134, pFY−146+134, pFY−98+134, pFY−70+134, pFY−70+42 and pFY−27+134 carried nt −211/+134, −146/+134, −98/+134, −70/+134, −70/+42, and −27/+134 regions relative to the *pelA1* TIS, respectively. The *pelA2* upstream region was also cloned using the same strategy to give pFYpelA2, which contained −274 to +29 (303 bp) relative to the *pelA2* putative translational start site. These constructs were separately introduced into different Xcc strains by electroporation. 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 regular intervals, and the β-galactosidase activity was assayed as previously described (*27*), with the enzyme activity expressed in Miller units (*27*).

Gel Retardation Assay. The DNA probes used for gel retardation were prepared by PCR amplification of the desired Xc17 *pelA1* upstream regions, using 5′-end biotinylated synthetic oligonucleotides as the primers (**Table 1**). The amplicons were purified from agarose gels. The recombinant Clp protein, the binding conditions, and detection procedures were as previously described (*8*).

RESULTS

Xcc Has Three Pectate Lyase Genes with Products Belonging to Polysaccharide Lyase Family 1. The open reading frame numbers of the five annotated pectate lyase genes in Xcc strains ATCC33913, 8004, and B100 are (i) XCC0644, XC_3591, and XCCB100_3711 for *pelA1*; (ii) XCC0645, XC_3590, and XCCB100_3710 for *pelA2*; (iii) XCC2815, XC_1298, and XCCB100_1344 for *pelB*; (iv) XCC1219, XC_3023, and XCCB100_3086 for *hrpW*; and (v) XCC0122, XC_0126, and XCCB100_0131 for *pelE*, respectively (*14*−*16*). There are also such homologues, XC672, XC673, XC988, XC5582, and XC2923, in the genome sequence of strain Xc17 (http://xcc.life.nthu.edu.tw). These gene products belong to three families of the polysaccharide lysase based on sequence similarity (http://afmb.cnrs-mrs.fr/CAZY/index.html): PelA1, PelA2, and PelB belong to family 1; HrpW belongs to family 3; and PelE belongs to family 10.

Genome analysis of the genes belonging to polysaccharide lyase family 1 (*pelA1*, *pelA2*, and *pelB*) indicated that there is similar organization in these Xcc strains (*14*−*16*). In Xcc strain ATCC33913, *pelA1* and *pelA2* are linked together with 237 bp intergenic space (**Figure 1A**). The genes encompassing them are *XCC0643* (474 bp, encoding ribosomal-protein-alanine acetyltransferase) and *XCC0646* (2835 bp, encoding valyl-rRNA synthetase). The former is located upstream of *pelA1* in the same direction with 701 bp intergenic space, and the latter is downstream of *pelA2* in the opposite orientation and separated by 576 bp. As shown in **Figure 1A**, there are such compatible homologues with 87−92% shared amino acid sequence identities in both *X. campestris* pv. *vesicatoria* (Xcv) strain 85-10 and *X. axonopodis* pv. *citri* (Xac) strain 306 (*15*, *38*). The *pelA2* homologue is missing in Xcv and Xac. In addition, genes *XCV3686* and *XAC3561*, coding for soluble lytic murein transglycosylase, and *XCV3685* and *XAC3560*, coding for TonB-dependent receptor, are located downstream of the *pelA1* homologue. These two genes appear elsewhere in the chromosome in Xcc.

The organization of *pelB* and its flanking genes is also similar in these xanthomonads. The genes encompassing the *pelB* in Xcc

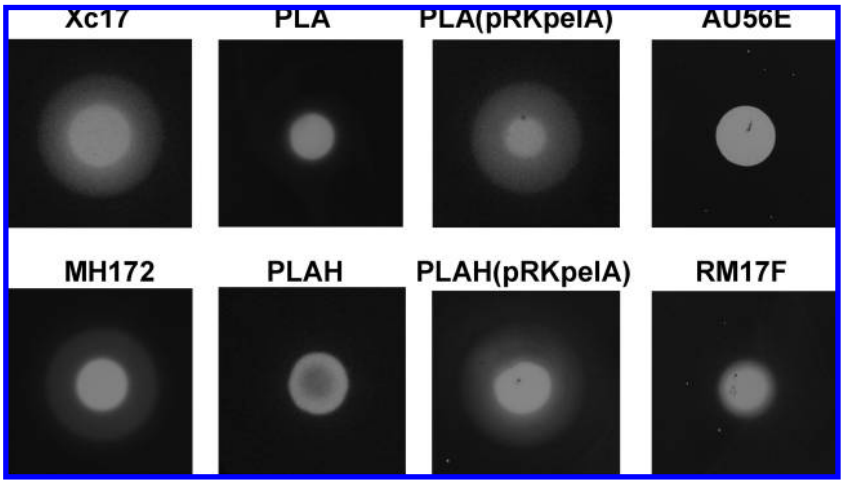

Figure 2. Plate assay for pectate lyase in different Xcc strains. The pectolytic activity was evaluated using XOLN basal medium supplemented with 2% xylose and 0.5% PGA. Clearing zones were visualized after 0.2% ruthenium red staining. Consistent results were obtained from three experiments with triplicate samples. Strains shown here are Xc17 (wild-type), PLA (*pelA1* mutant), PLA(pRKpelA) (PLA complemented with *pelA1*), AU56E (*clp* mutant), MH172 (*pehA* mutant), PLAH (*pelA1* and *pehA* double mutant), PLAH(pRKpelA) (PLAH complemented with *pelA1*), and RM17F (*rpfF* mutant).

Table 2. Bacteria Growth Rates and Symptom Development of Different Xcc Strains

			growth rate[a]				
strain	mutation	medium[b]	6 h	24 h	30 h	48 h	appearance of symptom[c]
Xc17	none	LB	1.97	7.10	7.06	6.70	7−8
		XOLN + glycerol	0.65	1.14	1.31	1.47	
		XOLN + xylose	1.13	2.27	2.53	2.49	
		XOLN + glucose	1.55	2.77	3.22	3.17	
		XOLN + PGA	0.98	1.26	1.22	1.20	
PLA	*pelA1*	LB	1.90	6.72	7.10	6.22	8−9
		XOLN + glycerol	0.63	1.11	1.34	1.20	
		XOLN + xylose	1.04	2.08	2.31	2.57	
		XOLN + glucose	1.39	2.82	2.79	2.84	
		XOLN + PGA	0.90	1.19	1.21	1.12	
PLAH	*pelA1* and *pehA*	LB	1.63	6.20	6.33	6.46	8−9
		XOLN + glycerol	0.69	1.06	1.19	1.46	
		XOLN + xylose	1.02	2.08	2.27	2.36	
		XOLN + glucose	1.24	3.03	3.06	3.13	
		XOLN + PGA	0.78	0.87	0.90	0.93	

[a] Cells were grown in different media, and OD_{550} measurements were carried out at 6, 24, 30, and 48 h, respectively. [b] The carbon sources added were 2% for glycerol, xylose, and glucose and 0.5% for PGA. [c] Symptom appearances were observed on different days after inoculation.

strain ATCC33913 are *XCC2816* (519 bp, encoding *IS*1480 transposase) and *XCC2814* (1386 bp, encoding amino acid transporter) (**Figure 1B**). *XCC2816* is located upstream of *pelB* in the opposite direction with a 1302 bp intergenic space, whereas *XCC2814* is located downstream of *pelB* in the same orientation and separated by 430 bp. As shown in **Figure 1B**, there are *pelB* and *XCC2814* homologues in Xcv and Xac, with 90−94% shared amino acid identities with *pelB* and *XCC2814*. In Xcv there is a gene (576 bp, encoding a hypothetical protein) located upstream of the *Xcc pelB* homologue that shares no sequence similarity with *XCC2816*. There are *Xcc2817* (a 942 bp proline imino-peptidase gene, upstream of *pelB*) homologues in both Xcv and Xac, which share about 90% amino acid identity with *Xcc2817*, and all of them have the same direction relative to *pelB* and its homologues in these three *Xanthomonas*.

PelA1 Is the Major Pectate Lyase in Xcc. PLA (*pelA1* mutant) and PLAH (*pelA1* and *pehA* double mutant) constructed by insertional mutation exhibited significantly reduced pectate lyase activity compared with Xc17 (wild-type) or MH172 (*pehA* mutant) on plate assay using PGA as substrate (**Figure 2**). In PLA or PLAH with cloned *pelA1*, PLA(pRKpelA), or PLAH(pRKpelA), wild-type-level pectate lyase activity was restored (**Figure 2**). This implies that PelA1 is the major pectate lyase of Xcc.

The two mutant strains (PLA and PLAH) and Xc17 grew at comparable rates and reached similar final OD_{550} values in LB (6.20−7.10) and XOLN (1.06−1.47 with glycerol, 2.08−2.57 with xylose, 2.77−3.22 with glucose) after entering the stationary phase. The growth rate of *pelA1* and *pehA* double-mutant PLAH was slightly reduced (OD_{550} = 0.93) compared with *pelA1* mutant PLA (OD_{550} = 1.12) as well as wild-type strain Xc17 (OD_{550} = 1.20) at 48 h when PGA was used as the sole carbon source (**Table 2**).

To test whether PelA1 is required for pathogenicity in Xcc, *pelA1* mutant PLA was used to infect cabbage with wild-type virulent strain Xc17 used for comparison. The rate of development of symptoms around the cuttings of the leaf edges inoculated with PLA was slightly slower than that of those inoculated with Xc17. Moreover, we evaluated the virulence of PLAH, a double mutant that impaired both major PGA-degrading

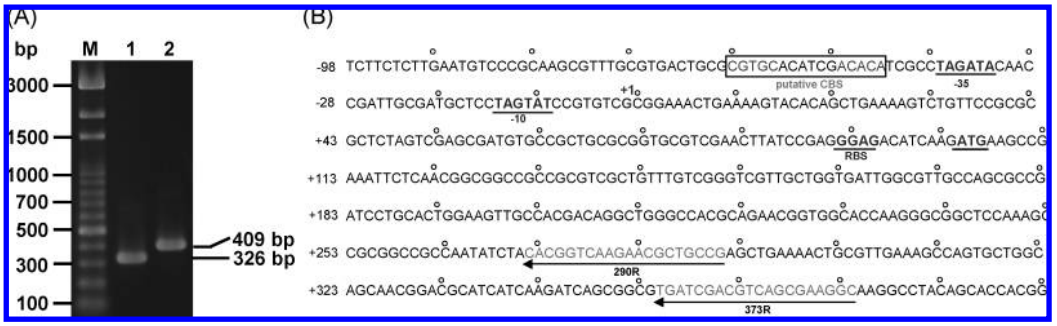

Figure 3. (**A**) Mapping of the 5′ end of Xcc *pelA1* transcript by 5′ RACE method. Fragments were amplified by PCR using the Abridged Universal Amplification Primer (Life Technologies, Inc.) in combination with gene specific primer 290R (lane 1) or 373R (lane 2). Lane M, size markers. (**B**) Sequence of the *pelA1* upstream region. Shown are putative Clp-binding site (CBS), +1 (the determined transcription initiation site, TIS), predicted −10/−35 sequences, putative ribosomal binding site (RBS), start codon (ATG), and positions of 290R and 373R.

Table 3. Promoter Activities Expressed from Different Transcriptional Fusion Constructs in Xc17

	pelA1 Xc17(pFY−211+134)		*pelA2* Xc17(pFYpelA2)	
inducer[a]	OD_{550}	β-galactosidase[b] (Miller units)	OD_{550}	β-galactosidase[b] (Miller units)
none	1.95	77.05 ± 3.67	2.18	7.53 ± 0.49
PGA	2.35	67.26 ± 7.06	2.12	5.77 ± 0.65
$CaCl_2$	2.20	72.50 ± 3.09	2.41	6.64 ± 2.64
PGA + $CaCl_2$	2.20	314.15 ± 18.73	2.15	7.49 ± 0.62
pectin (30%)	2.14	88.82 ± 6.72	2.00	11.94 ± 0.68
pectin (60%)	2.15	78.82 ± 0.74	2.14	7.71 ± 0.55
pectin (90%)	2.01	76.67 ± 6.20	2.04	5.45 ± 0.14
pectin (30%) + $CaCl_2$	2.13	108.67 ± 1.24	2.17	6.93 ± 3.23
pectin (60%) + $CaCl_2$	1.98	95.88 ± 5.00	2.28	10.49 ± 2.43
pectin (90%) + $CaCl_2$	2.10	83.45 ± 8.11	2.15	3.82 ± 0.79

[a] Cells were grown in XOLN supplemented with xylose (2%) with PGA or pectin and in the presence or absence of $CaCl_2$. The concentrations of PGA or pectin with 30, 60, or 90% degree of esterification were 0.5%, and the $CaCl_2$ added here was 1 mM. [b] Measurements were carried out at 24 h. The results are presented as the mean ± standard deviation (n = 3).

enzymes, and the results showed only minor differences between the wild type and the mutant. As shown in **Table 2**, the leaf symptoms caused by PLA and PLAH strains appeared 1−2 days later than those caused by the wild-type strain Xc17. The lesion lengths caused by these different strains were about 2 cm at 14 days after inoculation.

Xcc *pelA1* encoded a protein of 377 aa (including signal peptide) with a calculated molecular mass of 40193 Da and a p*I* of 8.76. As a secreted protein, it had an N-terminal signal sequence of 26 aa with a possible cleavage site, ASA^{26}−$D^{27}P$, as predicted by Signal P software (*39*). Sequence analysis revealed that PelA1 has highest shared identity (93%) with Xcm strain B-414 pectate lyase (*23*). Multiple sequence alignment indicated that there are 27 invariant amino acids, of which 7 are Gs and 14 have chemical properties compatible with catalytic function in extracellular pectate lyases (*40*, *41*). They are well conserved in PelA1 and are situated at T44, T112, D166, D179, H180, D209, T218, S220, K229, T250, R262, R267, H272, and K376, in which K229, R262, and R267 are conserved catalytic residues and D166 and D209 are conserved Ca^{2+}-binding residues. When compared with strain ATCC33913, the Xc17 PelA1 possesses three amino acid substitutions: A66T, V72A, and A263V, in strains ATCC33913 and Xc17.

Mapping the 5′ End of the *pelA1* Transcript. To map the TIS of the Xcc *pelA1*, the 5′ RACE method was employed. Sequencing of the two fragments generated by nested PCR, 326 and 409 bp (**Figure 3A**), showed that transcription of *pelA1* was initiated at nucleotide G, 103 nt upstream from the translation start codon (**Figure 3B**). A putative ribosome-binding site (GGAG) was present 9 nt upstream of the translation start codon (**Figure 3B**). A possible sigma 70 promoter with a −10 box (TAGTAT) and a −35 box (TAGATA) was located at −8 and −33 (with a spacer of 19 nt) relative to the TIS, respectively. A predicted Clp-binding site CGTGCN_6ACACA, with 7/10 matching (underlined bases) the consensus sequence (*8*), was located at −59/−44 relative to the *pelA1* TIS (**Figure 3B**).

Expression of *pelA1* Is Induced by PGA and $CaCl_2$ and Requires Clp and RpfF. In preliminary experiments, there was no detectable β-galactosidase activity in Xc17(pFY−211+134) when XOLN plus xylose was used (**Table 3**). When XOLN containing xylose, or supplementary PGA or $CaCl_2$ only, was used as culture medium, there was also no detectable enzyme activity in Xc17 carrying this construct (**Table 3**). When both PGA and $CaCl_2$ were present in XLON−xylose medium, significant incremental expression was found. This indicated that both PGA and $CaCl_2$ are essential for *pelA1* expression. Owing to similar levels of activity for different added calcium concentrations ranging from 0.1 to 1.0 mM, 0.5 mM $CaCl_2$ was selected for assay. In addition, the levels were found to increase following cell growth until about 24−48 h (data not shown); therefore, the β-galactosidase levels were measured at 24 h throughout this study.

The *pelA1* promoter activity in Xc17(pFY−211+134) was also determined when cells were grown in XOLN containing xylose and pectin (0.5%) with different degrees of esterification and with or without $CaCl_2$. No significant differences in growth rates or final yields were observed between the strains when the cells were grown under the same conditions (**Table 3**). As shown in **Table 3**, when $CaCl_2$ was omitted, similar levels of activity, between 77 and 89 U, were obtained whether esterified pectin was at the 30, 60, or

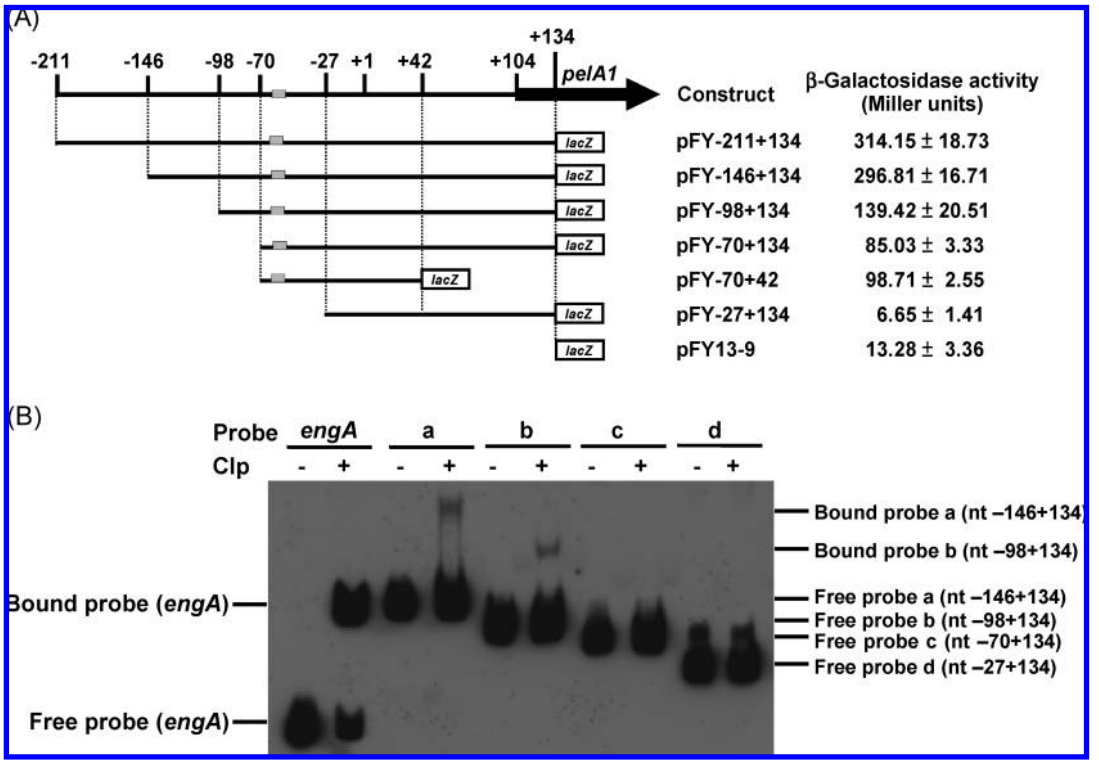

Figure 4. (**A**) Transcriptional fusion constructs used for deletion mapping of the Xcc *pelA1* promoter region. Horizontal lines represent regions cloned to form the constructs. The region containing the putative CBS is thickened. Numbers with pFY prefix are nucleotide positions relative to the *pelA1* TIS. (**B**) Gel retardation assay of Xcc *pelA1*. Primers used for PCR amplification are listed in **Table 1**. The fragments were used in EMSA with (+) or without (−) Clp protein (0.5 μg). The *engA* promoter region, −81 to +22 relative to TIS, was used as the positive control. Three independent experiments were performed, and similar results were obtained.

90% level, similar to the results for PGA only (67 U). The levels of β-galactosidase activity were 109, 96, and 83 U in medium containing both $CaCl_2$ and esterified pectin at the 30, 60, and 90% levels, respectively.

To locate the promoter region of *pelA1*, another five P*pelA1*-*lacZ* transcriptional fusion constructs together with pFY−211+134 (**Figure 4A**) were used to assay promoter activity in the wild-type Xc17 in XOLN−xylose medium in the presence of PGA and $CaCl_2$. As shown in **Figure 4A**, the highest levels of enzyme, 314 and 297 U, were detected in Xc17(pFY−211+134) and Xc17(pFY−146+134). The levels of β-galactosidase in Xc17 (pFY−98+134), Xc17(pFY−70+134), and Xc17(pFY−70+42) were lower (139, 85, and 99 U, respectively) than that expressed by Xc17(pFY−146+134) (297 U) (**Figure 4A**). The remaining transformant, Xc17(pFY−27+134), displayed the same levels of enzyme as Xc17(pFY13−9) without the *pelA1* promoter (13 U).

The *clp* mutant AU56E, as well as *rpfF* mutant RM17F, gave a nearly invisible clearing zone on the plate assay, as shown in **Figure 2**. To test for the involvement of Clp and RpfF in the transcription of *pelA1*, the transcriptional fusion construct pFY−146+134 was introduced into AU56E and RM17F, and the resultant strains were subjected to β-galactosidase assays in XOLN medium plus xylose, PGA, and $CaCl_2$. The β-galactosidase levels were 20 and 34 U for AU56E(pFY−146+134) and RM17F(pFY−146+134), respectively, indicating that both Clp and RpfF are required for transcription of the *pelA1* gene.

Gel Retardation Assay Shows That Clp Binds to the *pelA1* Promoter Directly. The findings that *pelA1* transcription is reduced in the *clp* mutant and that there is a predicted Clp-binding site upstream of *pelA1* suggest that Clp binds directly to the Clp-binding site to activate *pelA1* transcription. To demonstrate this binding, a gel retardation assay was performed using biotinylated probes, regions −146/+134 (probe a), −98/+134 (probe b), −70/+134 (probe c), and −27/+134 (probe d) relative to *pelA1* TIS. As a positive control, assay using the *engA* promoter, which is directly regulated by Clp (*8*), was performed in parallel. As shown in **Figure 4B**, Clp binds to probe b, in addition to probe a, but not to probe c or d, indicating that region −98/+134 possesses the complete sequence for Clp to bind directly and is where the predicted Clp-binding site (−59/−44) is located.

The Upstream Region of *pelA2* Has No Promoter Activity. To characterize the expression of *pelA2*, the upstream region of *pelA2* was also PCR-amplified and cloned into the promoter-probing vector pFY13−9 with *lacZ* as the reporter, giving rise to transcriptional fusion pFYpelA2 as described under Materials and Methods. There was no detectable promoter activity in Xc17 (pFYpelA2) containing *pelA2* upstream region from −274 to +29 bp relative to the predicted translation start codon under our assay conditions (**Table 3**). Using RT-PCR, no *pelA2* transcript was detected (data not shown).

DISCUSSION

Until now, it was only known that the extracellular proteins from the culture supernatant of Xcc strain 8004 separated by fast protein liquid chromatography have three peaks with pectate lyase activity (I, II, and III), with form III predominant (*17*). After subjection to SDS-PAGE analysis, the estimated molecular masses of each isozyme are 33 kDa for I, 36.5 kDa for II, and 41 kDa for III (*17*). Together with the previous analysis and plate assay (**Figure 2**), as well as calculated molecular mass, it was suggested that *pelA1* encodes the major pectate lyase in Xcc, which may be isozyme III (*17*) in this bacterium. The residual pectate lyase activity retained by PLA (*pelA1* mutant) was

presumably due to secondary PelB or other unidentified pectate lyase(s). There is no available evidence to demonstrate which gene might code for the 33 kDa isozyme I, the minor pectate lyase in Xcc strain 8004 (*17*).

Mutation of the major pectate lyase gene *pelA1* has no effect on growth rate even when PGA is used as the sole carbon source, indicating that it is not the sole gene for metabolizing PGA in Xcc. A similar result was found for the major polygalacturonase gene *pehA* mutant (*7*). The ability of *pelA1* and *pehA* double mutant to use PGA was slightly impaired. These results suggest that the two major PGA-degrading enzymes, coded by *pelA1* and *pehA*, contribute more to the growth of bacteria in the minimal medium using PGA as the sole carbon source than do the individual mutants.

The ability to degrade pectin may facilitate pathogen invasion into the cells of host plants and is useful for pathogens in terms of virulence. To date, it is only known that the mutant strain lacking pectate lyase isozyme I retains the same pathogenicity as the wild type in inoculated seedlings, but the nucleotide sequences of isozyme I remain unclear (*18*). There is no evidence to demonstrate whether the three isoforms of pectate lyase exert synergistic effects either in pathogenesis or in bacterial nutrition. The pathogenicity results in this study showed that *pelA1* plays a minor role in the disease. This is similar to the observation for *pehA* mutant, in which symptoms appear 7−8 days postinfection, which is about 1 day more than for Xc17 (*7*). Three pectate lyase isozymes have been reported; inactivation of only one pectate lyase gene cannot determine conclusively the function of pectolysis in pathogenicity. However, a similar situation has been found in the *pelA1* and *pehA* double mutant in which both major PGA-degrading enzymes have been knocked out. It was thought that Xcc genome annotation reveals different kinds of extracellular enzymes as well as numerous virulence determinants that are associated with bacterial pathogenesis. Therefore, the result that losing one or two PGA-degrading enzymes does not cause a distinctive difference in Xcc virulence was expected. Nevertheless, a possible role for these genes in some accessory mechanism of pathogenicity cannot be excluded. The occurrence of multiple isoforms probably has physiological significance and may permit pathogenesis in a variety of different conditions and hosts.

Usually, pectate lyase genes are either constitutively expressed (*42*) or inducible by pectic compounds (*43*, *44*). The effects of media on the level of pectate lyase activity in Xcc have previously been evaluated, and pectate lyase has been induced within 2 h of addition of PGA to 24 h cultures of wild-type strain 8004 (*17*). In this study, a reporter assay indicated that neither PGA nor pectin alone is enough for *pelA1* expression; and in the presence of $CaCl_2$, PGA is a better inducer than pectin of *pelA1* expression. It was also suggested that the −146/+134 region contains the complete *pelA1* promoter sequence and is capable of its maximal-level expression, whereas the −27/+134 region excluding the −35 box is not sufficient for expressing significant levels of *pelA1* promoter activity. In addition, expression from constructs pFY−98+134, pFY−70+134, and pFY−70+42, the insert of which contained the complete −10 and −35 regions and a putative Clp-binding site, gave lower activity (29−47% of pFY−146+134), suggesting that region −145/−71 can somehow enhance the promoter activity. A similar situation was seen in Xcc *engA* in which construct containing −81/+22 gave only 70% of the activity at maximal-level expression (*8*). The transcriptional fusion analyses in *clp* mutant AU56E(pFY−146+134) and *rpfF* mutant RM17F(pFY−146+134) were also consistent with the plate assay showing nearly no pectate lyase activities in the *clp* and *rpfF* mutants.

Gel retardation indicated that the predicted Clp-binding site upstream of *pelA1* is functional. Because region −70/+134 (probe c) also contains the Clp-binding site, but no detectable DNA−protein complex, only the −70/+134 region in *pelA1* upstream was not sufficient for Clp binding in our in vitro assay. This result was consistent with the transcriptional fusion assay in which the in vivo promoter activity of Xc17(pFY−70+134) was only 29% of that of Xc17(pFY−146+134). The region −146/−70 may play an enhancing role in binding of Clp to Clp-binding site to form a stable Clp-DNA complex in vivo to reach maximal level expression of this gene.

Gene organization analysis showed no *pelA2* homologue in other *Xanthomonas* in comparable chromosome location (**Figure 1A**). In addition, neither *pelA2* promoter activity nor its transcript was detected via reporter assay or RT-PCR in Xcc strain Xc17 in the present study. Together with the comparative analysis of the organization of pectate lyase genes in *Xanthomonas*, it was suggested that *pelA2* may be a pseudogene which came about due to gene duplication in Xcc during evolution. It is possible that *pelA2* is a nonfunctional pectate lyase gene in Xcc. The loss of this function would have no crucial effect because the pectate lyase gene plays no determinant role in pathogenicity. Another possibility is that the homologous DNA includes a functional pectate lyase gene, but the prerequisites for its expression were not fulfilled under the testing conditions. A recent microarray analysis showed that 94 of the 165 RpfF/DSF-regulated genes including *pelA1* and *pelA2* subjected to Clp regulation had similar expression patterns in Xcc strain XC1 (*6*, *45*). A more recent study indicated that heterologous expression of the *pelA2* sequence as active pectate lyase required its subcloning in *E. coli* Rosetta cells (*21*). One possibility for these apparent differences is the genetic variations between strains, similar to the previously observed discrepancy in cell aggregation; a mutation in the *rpfF* gene causes the formation of prominent aggregates in Xcc strain 8004 but not in XC1 (*45*). The precise role of this gene may need further study.

ABBREVIATIONS USED

AAP, Abridged Anchor Primer; AUAP, Abridged Universal Amplification Primer; CBS, Clp-binding site; Clp, cAMP receptor protein-like protein; DSF, diffusible signal factor; PGA, polygalacturonic acid; 5′ RACE, rapid amplification of cDNA ends; TIS, transcription initiation site; Xac, *Xanthomonas axonopodis* pv. *citri*; Xcc, *Xanthomonas campestris* pv. *campestris*; Xcm, *Xanthomonas campestris* pv. *malvacearum*; Xcv, *Xanthomonas campestris* pv. *vesicatoria*.

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