J. Microbiol. Biotechnol. (2004), 14(5), 1047–1051

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Analysis of *cel* and *pel* Genes from *Pectobacterium chrysanthemi* PY35 for Relatedness to Pathogenicity

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Received: September 9, 2003
Accepted: December 19, 2003

Abstract The phytopathogenic bacterium *Pectobacterium chrysanthemi* secretes multiple isozymes of plant cell wall disrupting enzyme such as pectate lyase and cellulase. The *cel* gene, existing in tandem with the *pel* gene, was isolated previously [10]. The role of Cel5Z and PelL1 in *P. chrysanthemi* PY35 pathogenicity on potato tissues was assessed by mutagenizing cloned *cel* gene and *pel* gene in tandem and recombining them with the chromosomal alleles. Strains with the Km cassette interposon in *pel*L1 or a double mutant showed a delay in the appearance of symptoms, suggesting that *P. chrysanthemi* PY35 pectate lyase PelL1 may play a minor role in soft-rot pathogenesis.

Key words: *Pectobacterium chrysanthemi*, *cel* gene, *pel* gene, double mutant, pathogenicity

Pectobacterium chrysanthemi, previously classified as *Erwinia chrysanthemi*, is one of the pathogenic enterobacteria causing soft-rot disease in plants. This bacterium is capable of degrading the macromolecules that compose the structure of plant cell wall and middle lamellae, resulting in the maceration of the plant tissue. This macerating capacity of the organism comes from the secretion of a set of extracellular enzymes that include both cellulolytic enzymes and pectinolytic enzymes. These enzymes occur in multiple enzymatic forms. The bacterial synthesis of isozymes may ensure a more efficient degradation of polysaccharides present in the plant cell wall or provide a more advantageous regulatory strategy to the bacteria [2].

Some *E. chrysanthemi* enzymes such as the pectinases, proteases, and cellulases exist in multiple enzymatic forms [7]. As many as eight of *E. chrysanthemi* 3937 endo-Pels have been characterized (PelA, PelB, PelC, PelD, PelE, PelI, PelL, and PelZ) [13]. The corresponding genes are organized in four clusters on the bacterial chromosome (*pel*A-*pel*E-*pel*D, *pel*B-*pel*C-*pel*Z, *pel*I, and *pel*L). A mutant of *E. chrysanthemi* 3937 with five mutated pectate lyases (pectate lyase ABCDE) was noninvasive but still able to macerate the inoculated leaves of *Saintpaulia* plants and to produce limited maceration activities on potato tubers, suggesting other depolymerizing enzyme activities produced by *E. chrysanthemi* [1].

The occurrence of multiple isozymes has complicated the biochemical and genetic analyses of the roles of the enzymes. The role of the tandem structure of the *cel* gene and *pel* gene of *P. chrysanthemi* has not been reported, so far. The *cel*5Z gene and *pel*L1 gene (2.9 kb), existing in tandem from *P. chrysanthemi* PY35, were cloned and characterized in our laboratory [9–11]. In this paper, the role of the *cel*5Z gene and *pel*L1 gene of *P. chrysanthemi* PY35 in pathogenicity toward potato tissues was assessed by mutagenizing cloned *cel*5Z gene, *pel*L1 gene and recombining them with the chromosomal alleles.

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were cultured in LB or in M63 minimal medium supplemented with the required metabolites [14]. The enzyme activities for extracellular cellulase and pectate lyase were detected using the agar diffusion method [4, 5, 10]. Carboxymethylcellulose (CMC)-SDS-PAGE and polygalacturonate (PGA)-SDS-PAGE were performed as described previously [10, 12]. The *cel*5Z gene (accession number AF208495) and *pel*L1 gene (accession number AF171228) encoding the endo-1,4-β-D-glucanase and pectate lyase from *P. chrysanthemi* PY35, respectively, were used for the experiment of marker-exchange. Primer design for site-directed mutagenesis was performed as described in Reference [8]. Bacterial cells for the pathogenicity tests were prepared as previously described [14].

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Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	References/ sources
Bacterial strains		
E. coli DH5α	*polA*12, *lacZ*53, *rpsL*151, *thyA*36, *rha*-5,*deoC*2, λ-, IN(*rrn*D-*rrn*E)	MBI
P. chrysanthemi PY35	Wild-type	[10]
PY35 *cel*5Z::Ω	*P. chrysanthemi* PY35, *cel*5Z::Ω	This study
PY35 *pel*L1::Km	*P. chrysanthemi* PY35, *pel*L1::Km	This study
PY35 *cel*5Z::Ω-*pel*L1::Km	*P. chrysanthemi* PY35, *cel*5Z::Ω, *pel*L1::Km	This study
Plasmids		
pBluescript II SK+	Cloning vector, Amp^r, T3 primer, M13-20 primer	Stratagene
pBR322	Cloning vector, Amp^r, Tet^r	MBI
pHP45Ω	Donor of the Ω fragment, Amp^r, Spc^r, Sm^r	[6]
pUC4K	Donor of the kanamycin fragment, Amp^r, Km^r	Amersham
pPY100	2.9 kb partial *Sau*3AI fragment containing *cel*5Z and *pel*L1 from *P. chrysanthemi* PY35	[10]
pPY200	2.6 kb partial *Sac*II self-ligation fragment of pPY100 in pBluescript II SK+	This study
pPY300	1.7 kb *Cla*I self-ligation fragment of pPY100 in pBluescript II SK+	This study
pPY400	2.5 kb *Pst*I-*Sau*3AI ligation fragment of pPY100 in pBluescript II SK+	This study
pPY500	1.5 kb partial *Sac*II self-ligation fragment of pPY100 in pBluescript II SK+	This study
pPY600	2.2 kb *Pst*I-*Sac*II ligation fragment of pPY100 in pBluescript II SK+	This study
pCEL5Z::Ω	PPY500, *cel*5Z::Ω in pBluescript II SK+	This study
pPELL1::Km	PPY300, *pel*L1::Km in pBluescript II SK+	This study
pBR-CEL5Z::Ω	*Eco*RI fragment containing *cel*5Z::Ω from pCEL5Z::Ω in pBR322	This study
pBR-PELL1::Km	*Eco*RI fragment containing *pel*L1::Km from pPELL1::Km in pBR322	This study

Isolation of *cel*5Z::Ω Mutant and *pel*L1::Km Mutant

Site-directed *in vitro* mutation of *cel*5Z (pPY500) to generate the *Bam*HI site was performed using 26-mer synthetic oligonucleotide primers; 5'-GAAAGAGTGGTGGATCCCG-CAATCGC-3' (forward) and 5'-GCGATTGCGGGATCCAC-CACTCTTTC-3' (reverse) according to the manufacturer's specifications of site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Also, the *Bam*HI site of *pel*L1 (pPY300) was generated using 25-mer synthetic oligonucleotide primers; 5'-CGGCTTCCGGATCCGATTTCGTCAG-3' (forward) and 5'-CTGACGAAATCGGATCCGGAAGCCG-3' (reverse). The 2 kb *Bam*HI fragment of pHP45Ω was ligated into the *Bam*HI site of the site-mutagenized pPY500 and the construct was introduced into the *E. coli* DH5α host (Fig. 1). The bacteria were then plated on CMCase activity indicator medium containing ampicillin (50 μg/ml), streptomycin (25 μg/ml), and spectinomycin (50 μg/ml). None of the 150 isolated antibiotic resistant transformants showed CMCase activity. The inserted 2 kb pHP45Ω fragment into the site-mutagenized pPY500 was confirmed with restriction enzyme mapping and sequencing, which was defined and designated as *cel*5Z::Ω.

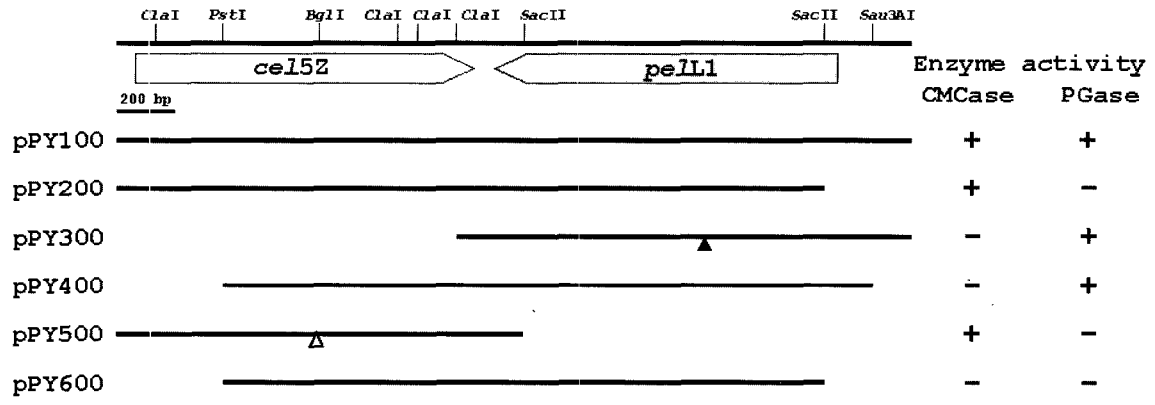

Fig. 1. Restriction map of the *Pectobacterium chrysanthemi* PY35 *cel*5Z gene (accession number AF208495) and *pel*L1 gene (accession number AF171228) in tandem.

The cleavage sites of the restriction enzymes *Bgl*I, *Cla*I, *Pst*I, *Sac*II, and *Sau*3AI are shown. Sites for the site-directed mutagenesis and insertion of omega (△) and Km (▲) cassettes are indicated.

Also, the 1.6 kb *Bam*HI fragment of pUC4K was ligated into the *Bam*HI site of the site-mutagenized pPY300 and transformed. The bacteria were then plated on pectate lyase activity indicator medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml). None of the 170 isolated antibiotic resistant transformants showed pectate lyase activity. The inserted fragment was confirmed, and the size of the inserted DNA and the orientation of restriction cleavage sites were also determined. The inserted 1.6 kb of pUC4K fragment into the site-mutagenized pPY300 was defined and designated as *pel*L1::Km.

Construction of the *cel*5Z::Ω Mutant, *pel*L1::Km Mutant, and *cel*5Z::Ω-*pel*L1::Km Mutant in *P. chrysanthemi*

Roeder and Collmer [14] suggested that effective marker-exchange mutagenesis necessitated delivery of the mutated gene on an unstable replicon. Although pBR322 is maintained stably in *P. chrysanthemi* in media containing sufficient phosphate, the plasmid is lost when cultures are grown in phosphate-limited media. So, the fragment of *cel*5Z::Ω in pBluscriptII SK+ was cut with *Eco*RI and then ligated into the *Eco*RI site of the pBR322. This plasmid (pBR-CEL5Z::Ω) was introduced into the wild-type *P. chrysanthemi* PY35 by electroporation and integrated into the chromosome by marker-exchange recombination. Streptomycin/spectinomycin-resistant colonies were selected from transformants after successive culture in M63 medium containing low phosphate. In order to inactivate the *pel*L1 gene, the fragment of *pel*L1::Km in pBluscriptII SK+ was cut with *Eco*RI and then ligated into the *Eco*RI site of the pBR322. The resulting plasmid (pBR-PELL1::Km) was introduced into the wild-type *P. chrysanthemi* PY35 and the *cel*5Z mutant *P. chrysanthemi* PY35 cells by electroporation. Kanamycin-resistant colonies were selected from transformants after successive culture in M63 medium containing low phosphate. Then, the inserted DNA was integrated into the chromosome of the *cel*5Z mutant *P. chrysanthemi* PY35 by marker-exchange recombination after successive culture in M63 medium containing low phosphate [14]. Transformants carrying the integrated plasmid were selected by determining their resistance to kanamycin for the *pel*L1::Km mutant, and their resistance to kanamycin, streptomycin, and spectinomycin for the *cel*5Z::Ω and *pel*L1::Km double mutant, because kanamycin-, spectinomycin-, and streptomycin-resistance can only be obtained by integration of the plasmid into the host chromosome in M63 medium containing low phosphate (Fig. 2). Integration was confirmed by characterization of the chromosomal DNA by PCR and DNA sequencing [6].

Identification of *cel*5Z::Ω Gene and *pel*L1::Km Gene Products

Previously, a direct activity staining technique was developed for rapid and specific detection of pectate lyase and endoglucanase in polyacrylamide slab gel [10]. After electrophoresis, the proteins in the gel are allowed to renature, and the gel is then stained with 0.1% (w/v)

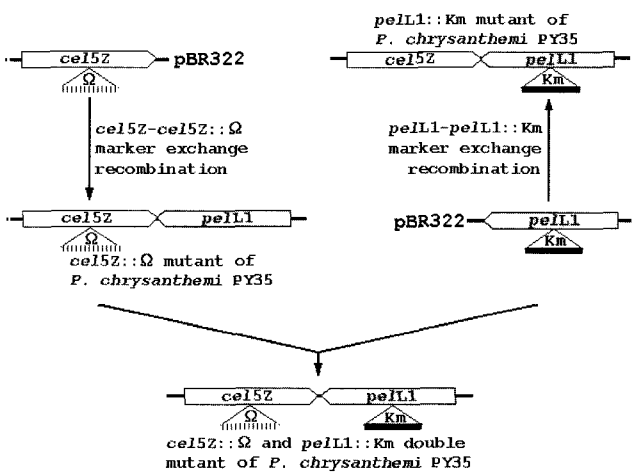

Fig. 2. Marker-exchange mutagenesis of the *cel*5Z gene and *pel*L1 gene mutant of *P. chrysanthemi* PY35.

The *cel*5Z gene and the *pel*L1 gene in pBR322 were inactivated by insertion of the *Bam*HI fragment of the pHP45Ω into the *Bam*HI site of the *cel*5Z and by insertion of the *Bam*HI fragment of the pUC4K into the same site of the *pel*L1 gene, respectively. The *cel*5Z::Ω mutant and the *pel*L1::Km mutant in *E. coli* were introduced into *P. chrysanthemi* PY35 genome by exchange recombination. The *pel*L1::Km mutant was introduced into the *cel*5Z::Ω mutant *P. chrysanthemi* PY35 for the double mutant by a second exchange recombination.

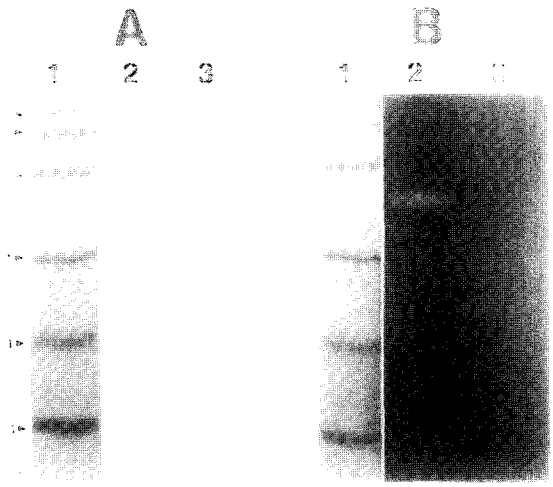

Fig. 3. Detection of CMCase and pectate lyase activity by CMC-SDS-PAGE and PGA-SDS-PAGE.

A, Lane 1 (also B) containing molecular weight standards: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400); Lanes 2 and 3 containing PelL1 and PelL1::Km of *P. chrysanthemi* PY35 expressed in *E. coli* DH5α, respectively. B, Lanes 2 and 3 containing the Cel5Z and the Cel5Z::Ω of *P. chrysanthemi* PY35 expressed in *E. coli* DH5α, respectively.

Toluidine blue O for pectate lyase and 0.5% (w/v) Congo red for endoglucanase. The result of CMC-SDS-PAGE and PGA-SDS-PAGE for the detection of the *cel*5Z::Ω and the *pel*L1::Km gene products confirmed that the *cel*5Z gene and *pel*L1 gene were inactivated in *E. coli* (Fig. 3).

Pathogenicity of *cel*::Ω and *pel*::Km Mutants of *P. chrysanthemi* PY35

Pathogenicity tests were performed for the *cel*5Z::Ω mutant, the *pel*L1::Km mutant, and the *cel*5Z::Ω-*pel*L1::Km double mutant of *P. chrysanthemi* PY35, which are the marker-exchange products. The symptoms were distinct 3 days after inoculation. The physical appearance of the soft-rot tissue caused by the *cel*5Z::Ω mutant of *P. chrysanthemi* PY35 was similar to that caused by the wild-type (data not shown). But the *pel*L1::Km mutant and the *cel*5Z::Ω-*pel*L1::Km mutant of *P. chrysanthemi* PY35 showed a delay in the appearance of symptoms. This fact suggests that *P. chrysanthemi* endoglucanase (Cel5Z) was not essential for the pathogenicity, and pectate lyase (PelL1) may play a minor role in soft-rot disease development (Fig. 4).

The reason why *Pectobacterium* acquired multiple genes encoding 1,4-β-D-endoglucanases and pectate lyases poses an interesting question. Multiple isozymes may allow a more complex regulatory strategy for degradation of plant cell wall in diverse environments, or simply provide higher levels of CMCase and pectate lyase activities through an increased gene dosage. The exact roles of these enzymes should be investigated to understand the mechanisms of the plant-pathogen interaction in the disease cycle. Further study is anticipated to find the reason for multiple cell wall degrading enzymes. The full characterization of the *cel* and *pel* genes of *P. chrysanthemi* PY35 should bring us closer to finding the answer.

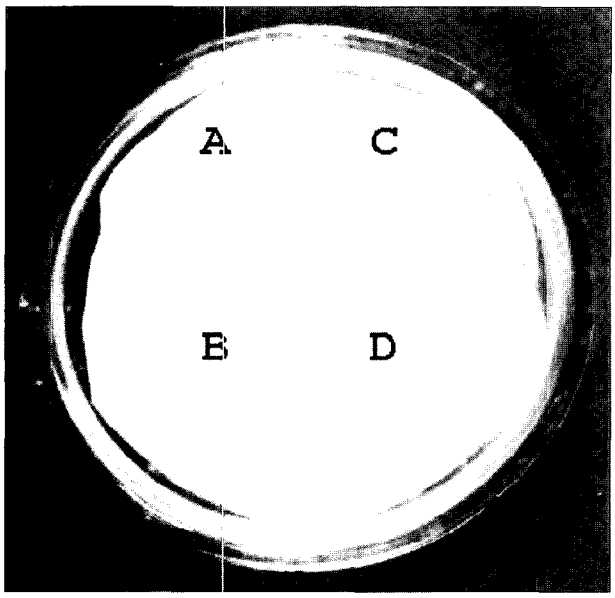

Fig. 4. Pathogenicity of the *cel*5Z and *pel*L1 mutant strains and the wild-type *P. chrysanthemi* PY35 on potato tubers.
(A) *E. coli* for the negative control, (B) bacteria (10^5) of the wild-type *P. chrysanthemi* PY35, (C) the *pel*L1::Km mutant of *P. chrysanthemi* PY35, and (D) the *cel*5Z::Ω and *pel*L1::Km mutant of *P. chrysanthemi* PY35 were inoculated on potato tissues. Disease symptoms were observed after 3 days.

Acknowledgments

This work was supported by KRF Grant (2000-042-G00006), BioGreen 21 Program, ARPC, and partially by a grant from 21C Frontier MGACP, MST (Grant MG02-0101-003-1-0-1), Republic of Korea. W.J.L. is the recipient of a BK21 fellowship from the Ministry of Education (2004).

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