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Characterization of *Erwinia chrysanthemi* PY35 *cel* and *pel* Gene Existing in Tandem and Rapid Identification of Their Gene Products

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Genomic DNA of the phytopathogenic *Erwinia chrysanthemi* PY35 was partially digested with *Sau*3AI, ligated into the *Bam*HI site of pBluescript II SK+, and introduced into *E. coli*. One clone that was able to hydrolyse carboxymethylcellulose and polygalacturonic acid was selected. A 2.9 kb fragment containing the *pel*L1 gene (pPY300) and *cel*5Z gene (pPY401) in tandem was subcloned and sequenced. The *pel*L1 and *cel*5Z genes had open reading frames of 1,278 bp and 1,281 bp encoding 425 and 426 amino acid residues with calculated molecular weights of 45,649 Da and 46,473 Da, respectively. *pel*L1 and *cel*5Z carried a typical prokaryotic signal peptide of 24 and 41 amino acid residues, respectively. The apparent molecular masses of the proteins when expressed in *E. coli* cells were approximately 43 kDa (PelL1) and 42 kDa (Cel5Z) as assessed by PGA–SDS–PAGE and CMC–SDS–PAGE.

The enterobacterium *Erwinia chrysanthemi* causes soft rot disease of various plants. Its pathogenicity is due to its ability to secret several extracellular enzymes that include pectate lyases, cellulases, and proteases. These extracellular enzymes attack cell walls and membranes of plants leading to plant tissue maceration (1). As many as eight *E. chrysanthemi pel* genes have been characterized, *pel*A, *pel*B, *pel*C, *pel*D, *pel*E, *pel*I, *pel*L, and *pel*Z. 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) (2–4). Two *E. chrysanthemi cel*Y gene and *cel*Z gene have been previously reported (5, 6). Thus, pectate lyases and cellulases in particular are major determinants of the pathogenicity of these bacteria. The digestion of cell wall components by cellulase and pectate lyase in co-operation may directly enhance the bacterial penetration of the plant tissue and, furthermore, facilitate the degradation of cell wall materials by other cell wall degrading enzymes and/or aid in the release of cell wall components that could be directly utilized to nourish the bacterium.

Collmer *et al.* (7) visualized bands of pectate lyase activity after PAGE in the presence of SDS by blotting the slab gels onto polygalacturonic acid agar. However, diffusion of renatured enzymes out of the polyacrylamide gel was inefficient and slow. Activity staining after PGA–SDS–PAGE appeared to be a better procedure for the characterization of the activities of these enzymes. This technique entails enzyme activity and high resolution of proteins based on their size in the presence of SDS. In the course of our work with pectate lyase of *Erwinia* sp. we modified Mckeon's *in situ* procedure (8) for the detection of pectate lyase.

E. chrysanthemi PY35, used in the present study, was originally isolated from Chinese cabbage tissue showing soft-rot symptoms. In order to understand the roles played by the pectic and cellulolytic isozymes of *E. chrysanthemi* PY35 in pathogenicity, it is necessary to study each one. In this report we describe the cloning and sequencing of the genes in tandem encoding beta-(1,4)-glucangluconohydrase (EC 3.2.1.4) and pectate lyase (EC 4.2.2.2) as well as the molecular weights of the recombinant proteins employing CMC–SDS–PAGE and PGA–SDS–PAGE zymograms. The enzymatic properties and the complete protein sequences of the pectate lyase and CMCase, referred to as *pel*L1 and *cel*5Z, are presented.

Abbreviations used: *Ech, Erwinia chrysanthemi;* CMC–SDS–PAGE, carboxymethylcellulose–sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PGA–SDS–PAGE, polygalacturonate–sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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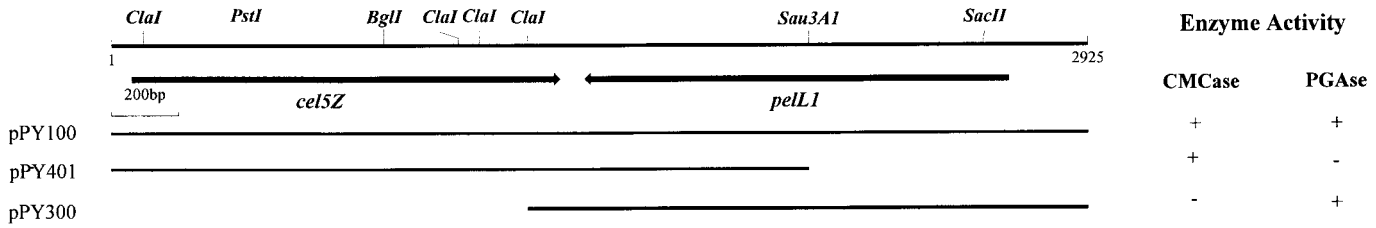

FIG. 1. Physical map of the *Erwinia chrysanthemi* PY35 *cel*5Z gene and *pel*L1 gene. The cleavage sites of the restriction enzymes *Sau*3AI, *Pst*I, *Sac*II, *Cla*I, and *Bgl*I are shown. pPY300 (*pel*L1) and pPY401(*cel*5Z) were constructed by subcloning a 2,9 kb fragment of pPY100 into pBluescript II SK+, respectively.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Culturing conditions and media used for *Ech* PY35 have been previously described (9). *E. coli* cells and recombinant *E. coli* cells were cultured in LB medium containing appropriate antibiotics; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml.

Extracellular enzyme assay. To detect cellulase and pectate lyase activity from *E. coli* XL1-Blue harboring the cloned *cel* and *pel* gene, bacterial colonies were grown on LB medium supplemented with 1% (w/v) CMC and 0.7% sodium polypectate, respectively. After incubation for 3 days, the plate for CMCase was stained with 0.1% (w/v) Congo Red solution for 30 min, rinsed with water, washed twice with 1 M NaCl, and then stained with 0.1 N HCl for the CMCase activity. Positive clones exhibiting extracellular cellulase activity were surrounded by a yellow halo against a red background (10). To visualize the halos formed due to pectate lyase activity, the plates were flooded with 10% of a saturated solution of copper acetate for 30 min. After excess stain was washed off, a halo against a blue background became visible (11).

Recombinant DNA techniques. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were used as described by Sambrook *et al.* (12). Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD) and Boehringer Mannheim (Indianapolis, IN). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

DNA sequencing. Nucleotide sequencing was done by the dideoxy-chain termination method using the PRISM Ready Reaction Dyedeoxy terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT). The samples were analyzed with an automated DNA sequencer (Model 310; Applied Biosystems. The DNA sequences reported here have been deposited in the GenBank database under Accession Number AF208495 (*cel*5Z) and AF171228 (*pel*L1).

Preparation of cell extract. Recombinant *E. coli* cells harboring the *cel*5Z gene and *pel*L1 gene were cultured at 37°C for 48 h in a 3 ml LB medium supplemented with 0.1% CMC and 0.1% polygalacturonic acid. Whole cell extracts from these cultures were prepared by sonication. For sonication, the cells were grown to logarithmic phase and concentrated 10-fold by resuspension in 100 mM Tris-HCl (pH 7). The cell extracts were mixed with sample buffer [62 mM Tris-HCl (pH 6.8), 10% glycerol, 0.025% bromophenol blue, 5% β-mercaptoethanol, and 2% SDS] at a ratio of 1:1 (v/v), heated at 95°C for 3 min for SDS–PAGE. Protein concentrations were determined by the method of Bradford (13).

Identification of the pelL1 and cel5Z products. For the identification of *pel*L1 gene product, the electrophoresis was performed in 0.75 mm gels in a vertical slab gel unit following a modified method of Mckeon (8). The separating gel contained 10% acrylamide, 0.33% bisacrylamide, and 0.1% polygalacturonic acid and 0.01% fibrinogen (PGA–SDS–PAGE). The protein sample (30 μg) was electrophoresed at 75 volts. Then the protein in SDS gel was renatured by incubation overnight in three periodic rinses of 250 ml of 10 mM Tris-HCl (pH 7.5) and 1% Triton-X 100. Finally the gel was incubated in 10 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ at 37°C overnight. Then the gel was immersed in 0.1% Toluidine Blue O in the same buffer for 5 min and destained for approximately 1 h with three changes of Tris-Cl buffer. Carboxymethylcellulose–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (CMC–SDS–PAGE) for the identification of *cel*5Z product was performed as described by Park *et al.* (14, 15).

RESULTS

Isolation and restriction map of cel5Z and the pelL1 gene. A shot-gun method was used to clone the CMCase and pectate lyase gene cluster. The genomic DNA of *E. chrysanthemi* PY35 was partially digested with *Sau*3AI. Three to five kb fragments were then ligated into the *Bam*HI site of pBluescript II SK+ and the construct introduced into the *E. coli* XL1-Blue host. The bacteria were then plated on CMCase and pectate lyase activity indicator medium. From among three thousand transformants, one of them contained a 2.9 kb fragment (pPY100) that has pectate lyase and CMCase activity. The size of the inserted DNA and the orientation of restriction cleavage sites were determined. The inserted DNA of pPY100 contains the restriction sites for *Sau*3AI, *Cla*I, *Sac*II, *Pst*I, and *Bgl*I. A 1.6 kb fragment (pPY300) and 2.0 kb fragment (pPY401) were defined and designated *pel*L1 and *cel*5Z, respectively (Fig. 1), since each fragment was sufficient for pectate lyase and CMCase activity, respectively as determined on polygalacturonic acid and CMC indicator medium. Many individual isozymes of pectate lyase and endoglucanase of Erwinias have been reported, but this is the report of a clone harboring a *cel* gene and *pel* gene in tandem. Other like pectate lyases (16), proteases (17), and Out (18) genes of *Erwinia* sp. have been described in tandem structure. The functional significance for this tandem arrangement of the *pel* and *cel* genes has remained elusive thus far.

Deduced amino acid sequence. The 1.6 kb inserted fragment in pPY300 (*pel*L1) was sequenced using the

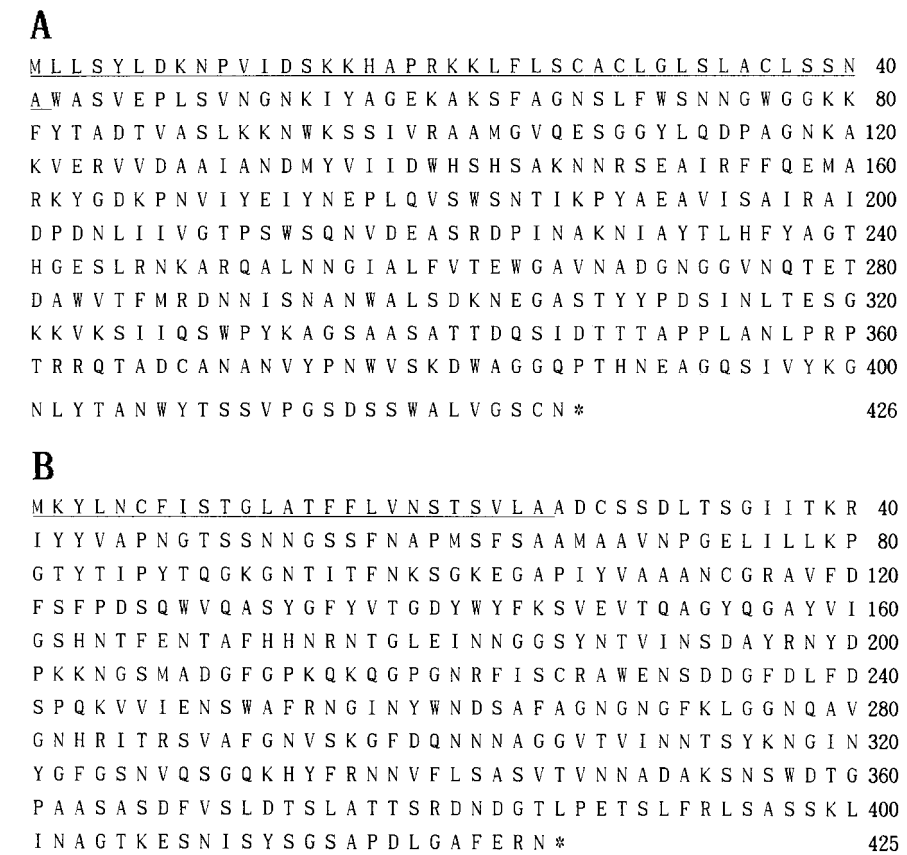

FIG. 2. Deduced amino acid sequences of the *cel*5Z (A) and *pel*L1 genes (B). Regions of the signal peptides are underlined. Amino acid sequence and hydrophobicity analyses were determined with the PC/GENE program.

dideoxy chain-termination method. The open reading frame contains 1,278 nucleotides and encodes a protein of 425 amino acid residues with a predicted molecular weight of 45,649 Da. The ATG initiation codon at nucleotide position 238 is preceded by a putative Shine-Dalgarno sequence, GAGG. The open reading frame ends with the ochre stop codon TAA at position 1,515 (Accession Number AF171228). The first 25 amino acids of PelL1 separated from the rest of the protein by a potential cleavage site in front of Ala_{26} have the typical features of a prokaryotic signal peptide. This sequence is likely to function in *E. coli* XL1-Blue in the export of PelL1 to the periplasm (Fig. 2B and Table 1).

The *cel*5Z gene of 2.0 kb DNA fragment (pPY401) has open reading frames of 1,281 bp encodes a protein of 425 amino acid residues. Thus the inferred protein has molecular masses of 46,473 Daltons. The ATG initiation codon at nucleotide position 60 is preceded by a putative Shine-Dalgarno sequence, GGAGA. The open reading frame ends with the ochre stop codon TAA at

TABLE 1

Molecular Weights of the Pre- and the Processed Forms of Cel5Z and PelLI

	Molecular weight of protein form (Da)/number of amino acids[a]			
	Precursor M.W.[b]	Signal peptide M.W.[c]	Calculated M.W.[d]	Apparent M.W.[e]
Cel5Z	46,473/426	4,450/41	42,041/385	42,000 ± 500/−
PelLI	45,649/425	2,741/24	42,925/421	43,000 ± 500/−

[a] Molecular weight and [c] hydrophobicity analysis of the signal peptide were calculated with the PC/GENE program.
[b] Protein of the primary gene product before signal modification.
[d] Protein after post-translational modification of the primary gene product.
[e] Actual proteins were electrophoresed by using CMC–SDS–PAGE and PGA–SDS–PAGE.

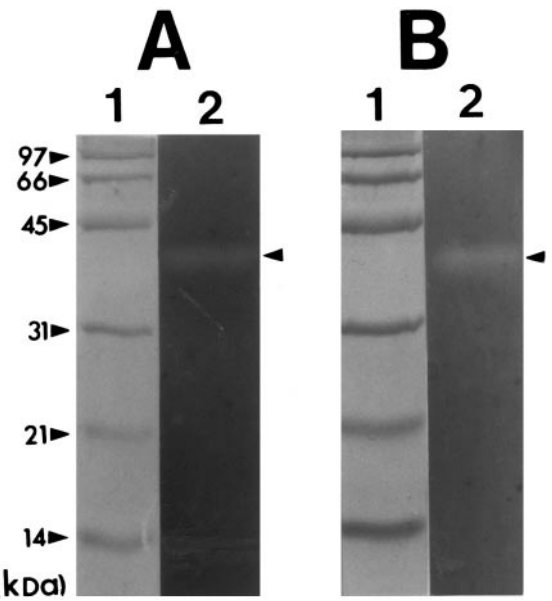

FIG. 3. Detection of pectate lyase and CMCase activity by PGA–SDS–PAGE and CMC–SDS–PAGE. (A) Lane 1 of the gel containing the molecular weight marker. Lane 2 contains the extract of transformed *E. coli* expressing PelL1; (B) lane 1 of the gel containing the molecular weight marker. Lane 2 contains the extract of transformed *E. coli* expressing Cel5Z; the gels containing the molecular weight standards were cut off after electrophoresis and stained with 0.025% Coomassie blue R-250. Molecular weight markers used were phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

position 1,338 (Accession Number AF208495). The amino acid composition analysis of the proteins suggests that they are highly hydrophilic. The most probable cleavage site for a putative signal peptide is between the amino acids at position 41 and 42 for Cel5Z (Fig. 2A and Table 1).

Identification of the pelL1 and cel5Z product. To facilitate the characterization of the pectate lyase, we developed a direct activity staining technique that allows rapid and specific detection of pectate lyase in polyacrylamide slab gel. The technique takes advantage of the ability of very small amount of pectate lyase to degrade polygalacturonic acid. After electrophoresis the proteins in the gel are allowed to renature, and the gel is then stained with 0.1% (w/v) Toluidine Blue O. This leaves a white halo around a band where polygalacturonic acid has been degraded. The pattern was reproducible. A protein band with the apparent molecular weight of about 43,000 Da, as determined in comparison to the mobilities of protein standards, produced a halo (Figs. 3A and 4). Since the predicted *pel*L1 gene product consists of 425 amino acids with an estimated molecular mass of 45,649 Da, the size of the PelL1 protein identified by the PGA–SDS–PAGE zymogram corresponds well with the predicted size; cleavage of the signal peptide would produce a protein with a molecular weight of about 43,000 Da. The difference in the molecular weight between the unprocessed and the processed forms of the pectate lyase is a reduction of about 2,700 Da in molecular weight, which agrees with the prediction based on the deduced amino acid sequence (Table 1). In order to determine whether the inclusion of polygalacturonic acid into the gel had any effect on the mobility of the enzymes, samples were also run in gels without the substrate and blotted onto agarose gel. No difference in the apparent molecular weights was observed (data not shown). It can further be seen that the cloned pectate lyase and molecular weight marker staining can be performed on the same gel, which facilitates the matching and identification of protein and active band.

For the identification of the *cel*5Z gene product, CMC–SDS–PAGE described by Park *et al.* (14) was performed. After electrophoresis the gel was renatured, stained with 0.1% Congo red, and then destained in 0.5% sodium chloride solution. This left a yellow halo zone where CMC was degraded. The band with apparent molecular weight of about 42,000 Da as determined from the mobilities of protein standards produced a strong halo zone A final treatment with 0.1 N HCl turned the background dark blue which facilitates photographic documentation (Figs. 3B and 4). Since the predicted *cel*5Z gene product consists of 426 amino acids with an estimated molecular mass of 46,473 Da, this corresponded well with the sizes of the Cel5Z identified by the CMC–SDS–PAGE zymograms. The cleavage of the leader peptide would produce pro-

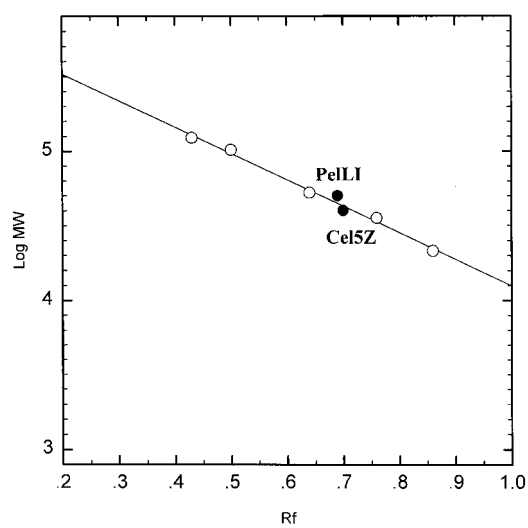

FIG. 4. Molecular weights of Cel5Z and PelL1. The molecular weights of Cel5Z and PelL1 were estimated by plotting the log of the molecular weights of standard markers, Cel5Z and PelL1 vs the relative mobility (Rf).

tein with a molecular weight about 42,000 Da, smaller than the calculated molecular weight of 46,473 Da for the unprocessed polypeptide. The difference in mobility between the unprocessed and the processed form of CMCase corresponds to a reduction in the molecular weight of about 4,400 Da which agrees well with the prediction based on the deduced amino acid sequence (Table 1).

DISCUSSION

Why has *Erwinia* acquired multiple genes encoding pectate lyases and CMCase? This poses an interesting question in particular since cell-wall component of some plant, which theoretically seems to be the natural substrate for pectate lyase and CMCase, is not chemically homogeneous (19).

To facilitate our genetic approach to the analysis of *Ech* pathogenicity, we needed a virulent, genetically amendable strain. *Ech* PY35 proved to be a convenient strain from a genetical, physiological, and pathological point of view (9). Previous data had shown that *Ech* produced eight pectate lyases (2–4) and two CMCases (5, 6); PelE and CelZ appeared to be the major CMCase and pectate lyase produced by *Ech*. We demonstrated that a clone that was able to hydrolyse carboxymethylcellulose and polygalacturonic acid contained the *cel*5Z and *pel*L1 genes in tandem. The *pel*L1 and *cel*5Z genes had open reading frames of 1,278 bp and 1,281 bp encoding 425 and 426 amino acid residues, respectively.

Pectate lyase and CMCase from *Ech* can be detected after SDS–PAGE *by in situ* activity staining. The results of the PGA–SDS–PAGE for the pectate lyase and the CMC–SDS–PAGE for the CMCase revealed a clear enzyme activity band, respectively (Fig. 3). The calculated molecular weights of the preform and the processed form of PeL1 and Cel5Z was 45,649 Da and 46,473 Da, and the apparent molecular weight of the processed form was estimated to be approximately 43 kDa and 42 kDa, respectively (Fig. 4). It is likely that *in situ* renaturation is only possible with monomeric enzyme composed of identical subunit. The method is not applicable to enzymes, whose activity requires participation of subunits with different molecular weights (20). As far as the pectate lyase and CMCase from *Erwinia* are concerned, these techniques do not appear to have serious limitations. Moreover, the sequences of the *pel* gene and *cel* gene revealed that they encode only a single polypeptide chain. The proteins of PelL1 and Cel5Z seem to lack any glycosylation. Langsford *et al.* (21) have demonstrated a role for glycosylation in *Cellulomonas fimi* endo-glucanases by showing that the glycan protects the extracellular enzyme from proteolysis.

The markedly higher sensitivity of the *in situ* technique as compared to the agar replica procedure reflects the peculiarity of enzyme renaturation in polyacrylamide gels. This method is by at least two orders of magnitude more sensitive than the agar replica technique (14, 20). The renatured enzymes may be physically anchored to the gel by the folding of the polypeptides around the gel matrix as the enzymes are renatured. This makes the diffusion of renatured enzymes out of the gel slow and inefficient as compared to native enzymes. Embedding the polymeric substrate into the separation gel circumvents this problem. Moreover, the presence of the substrate may help to refold the enzyme upon removal of the detergent (22). The *in situ* zymogen technique is convenient for preliminary characterization of recombinant clones carrying *Erwinia* genes involved in cellulose and polygalacturonic acid degradation. This *in situ* method has therefore many advantages. First, activity staining for pectate lyase and CMCase with standard protein staining can be performed in the same gel, respectively. Second, pectate lyase and CMCase activity can be measured and the protein bands with different molecular masses presumably arising from proteolytic processing can be readily confirmed. Third, cell extracts of samples of a culture can be directly analyzed. Finally, the separated proteins do not have to diffuse out of the gel.

The variety of cell-wall degrading enzymes may result from separate functional adaptations in the enzymatic activities of the bacterium to plant conditions that the bacterium may encounter during the first step of infection or that may develop during tissue maceration. The current concept is that pectic and cellulolytic enzymes released by phytopathogens are involved in the enzymatic hydrolysis of plant cell walls in cooperation. It is generally believed that these enzymes serve as cell wall-modifying enzymes, since their actions may also render other polysaccharide components in the cell walls more susceptible to hydrolysis. A study of the exact role of these enzymes is essential to the understanding of the mechanisms of host-parasite interaction in the disease cycle. We are now addressing the characterization of the *cel*5Z and *pel*L1 by introducing reporter transposon insertions into these two genes in order to explore their relative levels of expression and roles during pathogenesis.

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