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Cloning and Sequencing of the *pel*CI Gene Encoding Pectate Lyase of *Erwinia carotovora* subsp. *carotovora* LY34

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Abstract : Phytopathogenic *Erwinia carotovora* subsp. *carotovora (Ecc)* LY34 causes plant tissue maceration by secretion of pectinolytic enzymes such as pectate lyase (PL) existed as multiple isoenzyme form. Genomic DNA from *Ecc* LY34 was digested with *Sau*3AI and ligated into the *Bam*HI site of pBluescript II SK$^+$. Among them, a clone hydrolyzing polypectate was selected and its DNA was digested with *Bam*HI. Through the subsequent subcloning the resulting 3.1 kb fragment, corresponding to a *pel*CI, was subcloned into pLYPA 100. The structural organization of a *pel*CI gene encoding a 374 amino acid residues consists of an open reading frame (ORF) of 1,122 bp commencing with a ATG start codon and followed by a TAA stop codon. PelCI contained a typical prokaryotic signal peptide of 22-amino acid. Since the deduced amino acid sequences of PelCI protein was very similar to those of PelIII of *Erwinia carotovora* subsp. *carotovora*, and to those of Pel3 of *Erwinia carotovora* subsp. *atroseptica*, and to those of PelC of *Erwinia carotovora* subsp. *carotovora*, it belong to the same family PLbc group. The 374-amino acid PelCI had a calculated Mr of 40,507 and pI of 7.60.
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Introduction

Erwinias are phytopathogenic enterobacteria which cause soft-rot on a variety of important crops.[1] Their pathogenicity is due to their ability to produce several extracellular enzymes including pectinases, cellulases and proteases.[2] These extracellular enzymes attack plant cell walls and membrane components and lead to plant tissue maceration. Among pectinases, pectate lyase (PL) seems to be one of the most important determinants of the pathogenicity. The enzymes secreted by *Ecc* include cellulase, protease, pectate lyase, and polygalacturonase.[3,4]

Some enzymes from *Ecc* such as pectinases, proteases, and cellulase are multiple enzymatic forms.[5] *Ecc* produces two to four isoenzymes of PL,[6] while *E. chrysanthemi* produces at least five PLs.[7~10] Bacterial syntheses of isoenzymes may help additionally to ensure the degradation of the complex polysaccharides present in the plant cell wall or provide a more advantageous regulatory strategy to the bacteria. On the other hand, the occurrence of multiple isoenzymes has complicated the biochemical and enzymatic analyses of each of them since the very similar molecular weights of isoenzymes caused serious problems in purifying them to homogeneity.

Mechanism of bacterial phytopathogenicity has been greatly facilitated by use of modern genetic techniques. These techniques have allowed the role of suspected factors to be rigorously tested and have also led to the cloning of a number of pathogenicity genes whose precise functions remain to be determined. The role of specific PL isoenzymes from *Erwinia chrysanthemi* in causing plant tissue maceration has been studied.[11~13] The EMBL Data Library included more than 50 entries for sequences encoding PLs. Some them are plant genes for enzymes which have been hypothesized to participate in pollen tube growth in tomato[14] and tobacco.[15]

Strain LY34, used in the present study was originally isolated from chinese cabbage tissue containing soft-rot symptoms; it caused soft rot of potato tubers and chinese cabbages. Previous work in our laboratory suggested that *Ecc* LY34 secreted five cellulase isoenzymes and several pectate lyase isoenzymes.[3] To study the role of the pectic and the cellulolytic isoenzymes of *Ecc* LY34 to pathogenicity, it is necessary to study each one of these genes. In this report we describe the cloning of

Key words : *Erwinia carotovora* subsp. *carotovora (Ecc)*, pectate lyase (PL), *pel*CL gene
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a pectate lyase (EC 4.2.2.2) from *Ecc* strain LY34. The properties of the pectate lyase, here referred to as Pel-CI, and the complete nucleotide sequence of the corresponding structural gene are presented. The deduced primary structure of the protein has been compared with the sequences of other pectate lyases.

Materials and methods

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1. The plasmid pLYPA100 (Fig. 1) carries the segment of *pel*CI gene from *Erwinia carotovora* subsp. *carotovora* LY34.

Media and growth conditions

The cultural conditions and the media used for *Ecc* LY34 have been previously described.[3] The *E. coli* strains were cultured in a LB medium containing the appropriate antibiotics; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml.

Enzymes and chemicals

Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, Md.) and Boehringer Mannheim (Indianapolis, Ind.). Components of the media were purchased from Difco (Detroit, Mich.). Other chemicals were purchased from Sigma (St. Louis, Mo.).

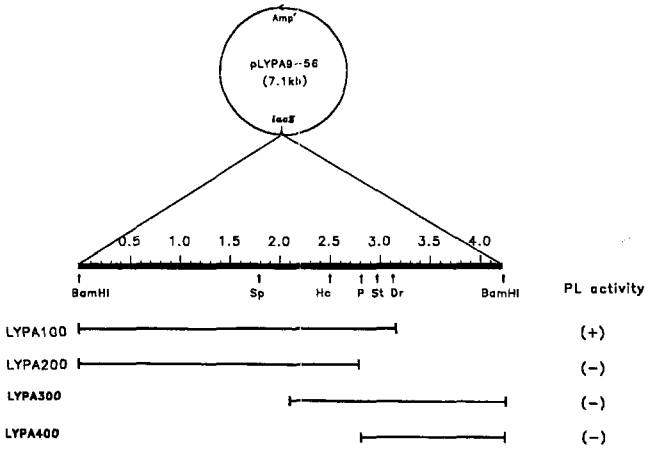

Fig. 1. Physical map of the *Erwinia carotovora* subsp. *carotovora* LY34 pectate lyase (PL) gene. The cleavage sites of restriction enzymes *Dra*I, *Pst*I, and *Hin*cII are shown. pLYPA9-56 was constructed by cloning a 4.2kb *Sau*3AI fragment of *Ecc* LY34 into the *Bam*HI site of pBluescriptII SK$^+$ vector. pLYCA100 (*pel*CI) was derived by cloning the 3.1 kb *Bam*HI-*Sma*I fragment of pLYPA9-56 into the corresponding sites of pBluescript II SK$^+$ vector. *Dra*I site is the amino-terminal end. Abbreviations for restriction enzymes and ORF sites are as follows. Hc, *Hin*cII; P, *Pst*I; Dr, *Dra*I; St, start codon; Sp, stop codon.

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Sources
Erwinia carotovora subsp. *carotovora* LY34	Wild type	This work
E. coli HB101 DH5α	ϕ80d *lacZ*ΔM15:*end*A1, *rec*A1; *hsd* R17(r-k,m+k), *sup*E44, *thi*1, λ *gyr*A, *rel* A1, F', Δ(*lac*ZYA-*arg*F)U169	Bethesda Research Laboratories, Inc. (Gaithersburg, Md)
XL1-Blue	*rec*A-, *rec*A1, *lac*Z-, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, F' *pro*AB, *lac*Iq *lac*ZΔM15, Tn10	Stratagene
Plasmid pBluescript II KS$^+$ & pBluescript II SK$^+$	Cloning vector, Amp^r, T3 primer, M13-20 primer	Stratagene
pLYPA9-56 (*pel*CI)	4.2kb partial *Sau*3AI fragment of *pel* gene from *Erwinia. carotovora.* subsp. *carotovora*	This work
pLYPA100	3.1kb *Bam*HI-*Dra*I fragment of pLYPA9-56 carrying the *pel* gene in pBluescript II SK$^+$	This work
pLYPA200	2.8kb *Bam*HI-*Pst*I fragment of pLYPA9-56 in pBluescript II SK$^+$	This work
pLYPA300	1.7kb *Hin*cII-*Bam*HI fragment of pLYPA9-56 in pBluescript II SK$^+$	This work
pLYPA400	1.4kb *Bam*HI-*Pst*I fragment of pLYPA9-56 in pBluescript II SK$^+$	This work

Pectate lyase enzyme assay

For routine measurements of pectate lyase activity of *Ecc* LY34 or *E. coli* containing pectate lyase DNA clones, a plate assay was employed with sodium polypectate as a substrate in the medium. For *E. coli* containing the pectate lyase clones, 0.7% sodium polypectate was used previously described by Lim[16] and for *Erwinia*, 0.25% sodium polypectate was used in TY medium.[16] To visualize the halos formed due to pectate lyase activity, the plates were flooded with 10% saturated solution of copper acetate for 30 min. The excess stain was removed and the halo was visualized against a blue background. The observations were recorded within 30 min.

Recombinant DNA techniques

Standard procedures for agarose gel electrophoresis, purification of DNA from agarose gels, restriction endonuclease digestions, DNA ligation, and other cloning-related techniques were used as described by Sambrook *et al.*[17]

Southern analysis

Genomic DNA isolated from a *Ecc* LY34 was digested with *Bam*HI, *Eco*RI, *Pst*I, and *Hind*III fractionated on a 0.5% agarose gel, and transferred to nylon membrane. Non-radiolabeled probes were prepared from *Hinc*II-*Dra*I digested 0.6 kb of LYPA100. The probes were labeled by the random octamer primer technique using biotin-14-dCTP according to the manufacturer's specifications (Tropix, Massachusettes, Inc). Homologous hybridization was carried out at 65℃ for 14 hrs.

DNA sequencing

The 3.1 kb *Bam*HI-*Dra*I fragment from pLYPA100 was cloned into the pBluescript II SK+ vector. A series of deletion of pLYPA100 was performed by using exonuclease III (Promega). Nucleotide sequences were determined for both strands by dideoxy-chain termination method using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) with one of M13, T3, T7 primer or synthetic oligonucleotide primers. Samples were analyzed by an automated DNA sequencer (model 373A; Applied Biosystems, Inc.). DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information with the BLAST network service and subjected to multiple sequence alignment by the CLUSTAL method using the PC/GENE program (IntelliGenetics, Inc.).

DNA sequence accession number.

The DNA sequence reported here have been deposited in the GenBank database under accession number AF026033.

Results

Isolation and restriction map of *pel*CI gene

Cloning of a pectate lyase gene was done by short gun method. The total DNA of *Ecc* LY34 was digested partially by *Sau*3AI for ten minutes. Partially digested 3 to 5 kb fragments of total DNA were ligated to the *Bam*HI site of pBluescriptII SK^+ vector and transformed to *E. coli* host XL1-Blue on indicator media for pectate lyase activity. pLYPA9-56 containing 4.2 kb insert DNA showed white activity ring on polypectate media (Fig. 2). The plasmid DNA pLYCA7-39 was digested with several restriction enzymes. The size of inserted DNA and each orientation of restriction cleavage sites were determined (Fig. 3). The insert DNA in pLYPA9-56 plasmid contained a single restriction site for each of *Pst*I and *Hinc*II. Subsequent subcloning of the 4.2 kb insert in pLYPA9-56 showed that the 3.1 kb *Bam*HI-*Dra*I fragment in the plasmid pLYPA100, named *pel*CI gene was sufficient for pectate lyase activity as determined on polypectate indicator media. It is seemed that pectate lyase protein is secreted in *E. coli* in a fashion similar to that of *Ecc.* (Fig. 2). The pectate lyase activity produced by this fragment was independent of the orientation of the insert in the vector, strongly suggesting that the expression of this pectate lyase gene was due to its own promotor.

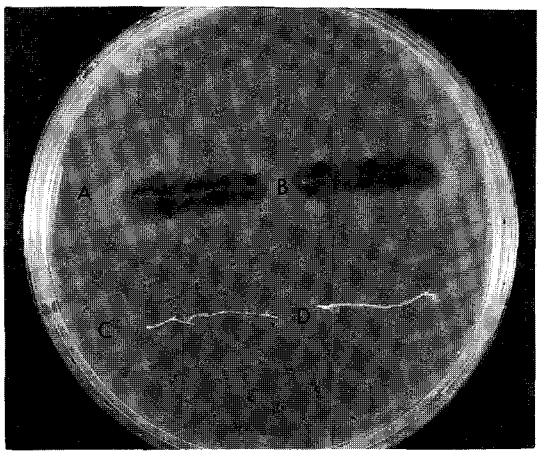

Fig. 2. Detection of pectate lyase-positive clone by agar diffusion method. The cells were incubated for 4 days at 37℃. Lane A, a positive control, *Erwinia carotovora* subsp. *carotovora* LY34; lane B, pLYPA100; laneC, a negative control, *E. coli*; lane D, a negative control, SK(+).

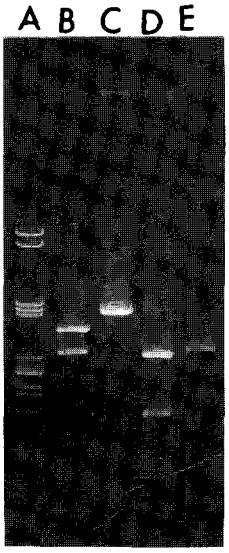

Fig. 3. Restriction endonuclease digestion pattern of 4.2 kb insert from pLYPA9-56. Lane A, λ/*Pst*I; lane B, pLYPA9-56/*Eco*RI+*Xba*I; lane C, pLYPA300/*Hinc*II; lane D, pLYPA300/*Hinc*II+*Xba*I; lane E, SK+/*Bam*HI.

Hybridization experiment

Chromosomal DNA was isolated from *Erwinia carotovora* subsp. *carotovora* LY34, digested completely

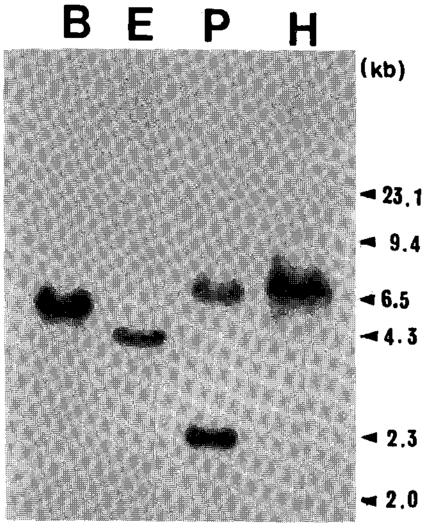

Fig. 4. Southern analysis of the *pel*CI gene from *Erwinia carotovora* subsp. *carotovora* LY34. The genomic DNA from *Erwinia carotovora* subsp. *carotovora* LY34 was digested with *Bam*HI (lane B), *Eco*RI (lane E), *Pst*I (lane P), *Hind*III (lane H) separated on 0.5% agarose gel, transferred onto a nylon membrane, and hybridized with *Hinc*II-*Dra*I digested 0.6 kb fragment of LYPA100 as a probe DNA. The probes were labeled by the random octamer primer technique using biotin-14-dCTP according to the manufacturer's specifications (Tropix, Massachusettes, Inc). Homologous hybridization was carried out at 65°C for 14 hrs.

with *Bam*HI, *Eco*RI, *Pst*I, and *Hind*III and probed with *Hinc*II-*Dra*I, 0.6 kb fragment of pLYPA100 digested. A single band was observed. It is possible that a single copy of this sequence might exist in the *Ecc* LY34. The probe hybridized to both a 6.6-kb *Pst*I-*Pst*I fragment and a 2.2-kb *Pst*I-*Pst*I fragment since this region was part of the *pel*CI gene due to *Pst*I cutting (Fig. 4).

Nucleotide sequence of *pel*CI

The 3.1 kb *Bam*HI-*Dra*I fragment from pLYPA100 was sequenced. It contained one complete ORF. Fig. 5 shows the *pel*CI structural gene along with its flanking regions. There is an open reading frame composed of 1, 122 nucleotides encoding a protein of 374 amino acids with a predicted molecular weight of 40,507. The ATG initiation codon at nucleotide position 218 is preceeded by a putative Shine-Dalgarno (SD) sequence, AGGAGA. The open reading frame is ended by the ochre stop codon TAA at position 2,341. The first 22 amino acids of PelCI with a predicted cleavage site in front of Asp_{23} showed typical features of a prokaryotic signal peptide both in size and distribution of hydrophobic and hy-

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   1 TTTAAAATAGATACTTATTAAGCATTGATGTTTCCATCATTATTCATCCGATAGATTTAATACCTAAATATTGAATTATTTTATTTTTATGATAAGACTTAATCGGGTTTTCAATAAACC

 121 TTGATTTCATTTTTATTGAAACAACACAATAATATAATCTGGGTTATGTGGATCATTTATACCCAAACAAAAATTCAATTCCAAGGAGAGTACCTTAATGAAATACCTACTGCCTTCTGC
                                                                                               SD          M  K  Y  L  L  P  S  A
                                                  ↓
 241 AGCCGCTGGGCTGTTATTGCTTGCGGCCCAACCAACGATGGCGGCAAATACGGGGGGTTATGCCACCACTGACGGTGGTGACGTTGCCGGTGCCGTGAAAAAAACAGCGCGCTCCATGCA
       A  A  G  L  L  L  L  A  A  Q  P  T  M Asp A  N  T  G  G  Y  A  T  T  D  G  G  D  V  A  G  A  V  K  K  T  A  R  S  M' Q

 361 AGATATTATTGATATCATCGAAGCCGCGAAGCTGGATTCCAATGGCAAGAAAGTGAAAGGTGGCGCTTACCCGCTTGTCATCACCTATAACGGTAATGAAGACGCGCTGATCAAAGCCGC
       D  I  I  D  I  I  E  A  A  K  L  D  S  N  G  K  K  V  K  G  G  A  Y  P  L  V  I  T  Y  N  G  N  E  D  A  L  I  K  A  A

 481 TGAGGCCAACATCTGCGGCCAGTGGAGTAAGGATGCCCGAGGTGTAGAAATCAAAGAATTCACCAAAGGGATTACCATCATCGGAACCAATGGATCGTCCGCCAACTTCGGGATCTGGCT
       E  A  N  I  C  G  Q  W  S  K  D  A  R  G  V  E  I  K  E  F  T  K  G  I  T  I  I  G  T  N  G  S  S  A  N  F  G  I  W  L

 601 GGTTAAATCACCCGATATCGTTATCCGCAATATGCGTTTCGGCTACTATCCTGGCGGCGCGCAGGACGGCGATGCGATTCGTATCGATAACACGCCGAACGTCTGGATTGACCACAACGA
       V  K  S  P  D  I  V  I  R  N  M  R  F  G  Y  Y  P  G  G  A  Q  D  G  D  A  I  R  I  D  N  T  P  N  V  W  I  D  H  N  E

 721 GATCTTCGCGAAAAACTTTGAATGCGCAGGCACAAAAGACGGTGACACCACATTCGAATCCGCGATTGATATCAAGAAAGCCTCAACCAACGTGACGGTGTCGTACAACTACATTCACGG
       I  F  A  K  N  F  E  C  A  G  T  K  D  G  D  T  T  F  E  S  A  I  D  I  K  K  A  S  T  N  V  T  V  S  Y  N  Y  I  H  G

 841 CATCAAAAAAGTCGGTCTGAGCGGCTTCAGCAGCAGCGATACGGGCCGTGACCTGACTTACCATCACAATATTTACGATGACGTTGACGCTCGCCTCCCGCTGCAACGTGGCGGCCAGGT
       I  K  K  V  G  L  S  G  F  S  S  S  D  T  G  R  D  L  T  Y  H  H  N  I  Y  D  D  V  D  A  R  L  P  L  Q  R  G  G  Q  V

 961 TCACGCGTACAACAACCTGTATACTGGCATCACCAGTTCTGGCCTGAACGTGCGTCAGAAAGGGATTGCGCTGATCGAACGTAACTGGTTCGAGAATGCGAAAAACCCGGTGACCTCACG
       H  A  Y  N  N  L  Y  T  G  I  T  S  S  G  L  N  V  R  Q  K  G  I  A  L  I  E  R  N  W  F  E  N  A  K  N  P  V  T  S  R

1081 TTACGACGGTTCCAACTTCGGTACCTGGGAGCTGCGTAATAACAACGTCATGAGCCCGGCTGATTTCGCTAAATACAACATCACCTGGGATAAAGATTCCAAGCCCTACGTGAATGCAGA
       Y  D  G  S  N  F  G  T  W  E  L  R  N  N  N  V  M  S  P  A  D  F  A  K  Y  N  I  T  W  D  K  D  S  K  P  Y  V  N  A  E

1201 AGACTGGAAAAGCACTGGCACATTCGCTTCTGTTCCTTACAGCTACTCTCCGGTTTCGGCACAGTGCGTGAAGGACAAACTGGCGAACTATGCTGGCGTGAACAAAAATCTTGCGGTGCT
       D  W  K  S  T  G  T  F  A  S 'V  P  Y  S  Y  S  P  V  S  A  Q  C  V  K  D  K  L  A  N  Y  A  G  V  N  K  N  L  A  V  L

1321 GACGGCAGCAAATTGCAACTAATTGCGATGTGTAAAGCATGAAAGGTAAATAAGCGGACTTCAGGCTCCGACCCTCATTGTCCTGTGCTAACCGG
       T  A  A  N  C  N  -
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Fig. 5. Nucleotide and deduced amino acid sequence of the *pel*CI gene of *Erwinia carotovora* subsp. *carotovora* LY34 with its flanking regions is shown. The putative Shine-Dalgarno (SD) region is indicated. Initiation and termination codons are shaded. The cleavage site of signal sequence is shown by an arrow.

drophilic amino acid residues.

Amino acid sequence homology between *pelCI* and other *pel* gene

An amino acid sequence comparison using SwissProt files revealed that PelCI shares significant sequence similarity with previously reported *Erwinia carotovora* strain ER PelIII,[18] and *Erwinia carotovora* subsp. *atroseptica* C18 Pel3,[19] and *Erwinia carotovora* subsp. *carotovora* strain SCR193 PelC.[20] The deduced 374-amino acid sequences of *pelCI* gene are very similar to those of PelIII (374 amino acids) of *Erwinia carotovora* Er strain with 98.7% identity and 99% similarity, to those of PelC (374 amino acids) of *Erwinia carotovora* subsp. *carotovora* strain SCR193 with 97.9% identity and 98.2% similarity, and to those of Pel3 (374 amino acids) of *Erwinia carotovora* subsp. *atroseptica* C18 with 85.3% identity and 90.9% similarity respectively (Fig. 6).

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PelCI     MKYLLPSAAAGLLLLAAQPTMAANTGGYATTDGGDVAGAVKKTARSMQDI   50
PelIII    MKYLLPSAAAGLLLLAAQPTMAANTGGYATTDGGDVAGAVKKTARSMQDI   50
PelC      MKYLLPSAAAGLLLLAAQPTMAANTGGYATTDGGDVAGAVKKTARSMQDI   50
Pel3      MKYLLPSAAAGLVLLAAQPTMAANTGGYATTDGGDVSGAVKKTARSLQEI   50
PelX      MKYLLPSAAAGLLLLAAQPTMAANTGGYATTDGGDVSGAVKKTARSLQEI   50
          ************.**********************.*********.*.*

PelCI     IDIIEAAKLDSNGKKVKGGAYPLVITYNGNEDALIKAAEANICGQWSKDA  100
PelIII    IDIIEAAKLDSNGKKVKGGAYPLVITYNGNEDALIKAAEANICGQWSKDA  100
PelC      IDIIEAAKLDSNGKKVKGGAYPLVITYNGNEDALIKAAENDICGQWKKDA  100
Pel3      VDIIEAAKKDSSGKAVKGGAYPLVITYNGNEDALIKAAEANICGQWSKDP  100
PelX      VDIIEAAKKDSSGKAVKGGPYPLVITYNGNEDALIKAAEANICGQWSKDP  100
          ******* **.** ****.*******************..*****.**.

PelCI     RGVEIKEFTKGITIIGTNGSSANFGIWLVKSPDIVIRNMRFGYYPGGAQD  150
PelIII    RGVEIKEFTKGITIIGTNGSSANFGIWLTKSSDIVIRNMRFGYMPGGAQD  150
PelC      RGVEIKEFTKGITIIGTNGSSANFGIWLTKSSDIVIRNMRFGYMPGGAQD  150
Pel3      RGVEIKEFTKGITILGTNGSSANFGIWMVNSSNVIVRNMRFGYMPGGAKD  150
PelX      RGVEIKEFTKGITILGTNGSSANFGIWMVNSSNVIVRNMRFGYMPGGAKD  150
          **************.************...*.....******* ****.*

PelCI     GDAIRIDNTPNVWIDHNEIFAKNFECAGTKDGDTTFESAIDIKKASTNVT  200
PelIII    GDAIRIDNTPNVWIDHNEIFAKNFECAGTKDGDTTFESAIDIKKASTNVT  200
PelC      GDAIRIDNTPNVWIDHNEIFAKNFECAGTKDGDTTFESAIDIKKASTNVT  200
Pel3      GDAIRIDNSPNVWIDHNEIFAKNFECAGTPDNDTTFESAVDIKKASTNVT  200
PelX      GDAIRIDNSPNVWIDHNEIFAKNFECAGTPDNDTTFESAVDIKKASTNVT  200
          ********.******************** *.*******.**********

PelCI     VSYNYIHGIKKVGLSGFSSSDTGRDLTYHHNIYDDVDARLPLQRGGQVHA  250
PelIII    ISYNYIHGIKKVGLSGFSSSDTGRDLTYHHNIYDDVNARLPLQRGGQVHA  250
PelC      VSYNYIHGIKKVGLSGFSSSDTGRDLTYHHNIYDDVNARLPLQRGGQVHA  250
Pel3      VSYNYIHGVKKVGLSGSSNTDTGRNLTYHHNIYSDVNSRLPLQRGGQVHA  250
PelX      VSYNYIHGVKKVGLSGSSNTDTGRNLTYHHNIYSDVNSRLPLQRGGQVHA  250
          .*******.******* *..****.********.**..************

PelCI     YNNLYTGITSSGLNVRQKGIALIERNWFENAKNPVTSRYDGSNFGTWELR  300
PelIII    YNNLYTGITSSGLNVRQKGIALIERNWFENAKNPVTSRYDGSNFGTWELR  300
PelC      YNNLYTGITSSGLNVRQKGIALIERNWFENAKNPVTSRYDGSNFGTWELR  300
Pel3      YNNLYGGIKSSGFNVRQKGIALIESNWFENALNPVTARNDDSNFGTWELR  300
PelX      YNNLYDGINSSGFNVRQKGIALIIRN-MHNALNPVATRDGDANFGTWDLR  299
          *****.**.***.********** .* ..** ***..* ...*****.**

PelCI     NNNVMSPADFAKYNITWDKDSKPYVNAEDWKSTGTFASVPYSYSPVSAQC  350
PelIII    NNNVMSPADFAKYNITWDKDSKPYVNAEDWKSTGTFASVPYSYSPVSAQC  350
PelC      NNNVMSPADFAKYNITWDKDTKPYVNAEDWKSTGTFASVPYSYSPVSAQC  350
Pel3      NNNITSPSDFAKYNITWGKPSTPHINADDWKSTGKFPAVPYSYSPVSAQC  350
PelX      NNNITSPADFAKYNITWDKATTPYINAEDWKSTGTFSAVPSSYSPFSAQC  349
          ***. **.**********.* ..*..**.******.*..** **** ****

PelCI     VKDKLANYAGVNKNLAVLTAANCN  374
PelIII    VKDKLANYAGVNKNLAVLTAANCN  374 (98.7%, 99% homology)
PelC      VKDKLANYAGVNKNLAVLTAANCN  374 (97.9%, 98.2% homology)
Pel3      VKDKLASYAGVGKNQAVLTAANCK  374 (85.3%, 90.9% homology)
PelX      -KDKW------------------  353 (84.1%, 90.6% homology)
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Fig. 6. Alignment of the predicted amino acid sequences of PelCI with those of corresponding proteins of other organisms. Alignment was derived by the CRUSTAL program. Identical and conserved amino acids in equivalent positions are indicated by asterisks and dots, respectively. The pectate lyase PelCI are compared to those from PelIII of *Erwinia carotovora* subsp. *carotovora* strain ER,[18] and to Pel3 of *Erwinia carotovora* subsp. atroseptica C18,[19] and to PelC of *Erwinia carotovora* subsp. *carotovora* strain SCR193,[20] and PelX of *Erwinia carotovora* subsp. *carotovora* (Unpublished).

Phylogenetic analysis of PelCI protein

The known 15 different gene sequences in pectate lyase group were extracted from the GenBank and EMBL database. The deduced amino acid sequences were then aligned with each other to maximize the sequence similarity by using the PC/GENE protein alignment program. We have constructed phylogenetic trees of pectate lyase proteins by the PC/GENE CLUSTAL method using public databases, as shown in Fig. 7. The phylogenetic tree showed that the PelCI protein appeared genetically similar to PelIII of *Erwinia carotovora*

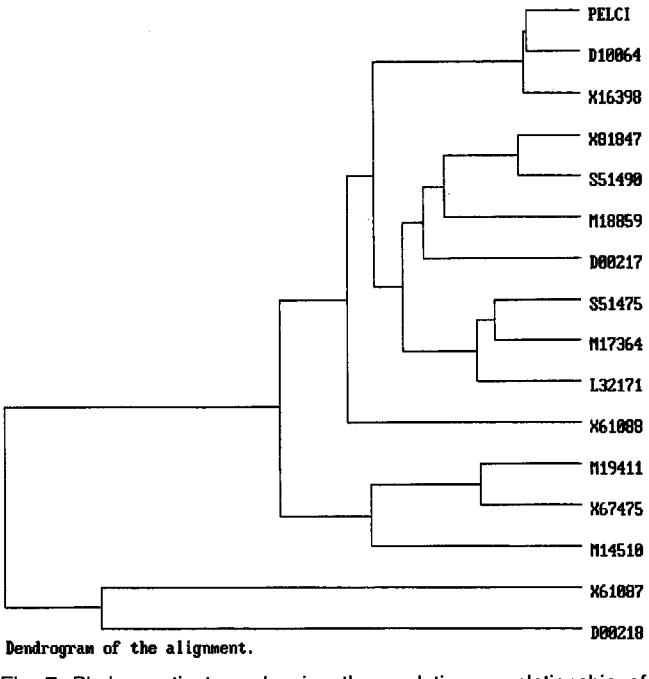

Fig. 7. Phylogenetic tree showing the evolutionary relationship of the pectate lyase amino acid sequences. The estimated genetic distance between sequences is proportional to the length of horizontal lines connecting one sequence to another. GenBank accession numbers for these sequences are as follows. D10064, *Erwinia carotovora* subsp. *carotovora* PelIII; X16398, *Erwinia carotovora* subsp. *carotovora* PelC; X81847, *Erwinia carotovora* subsp. *atrosetica* Pel3; S51490, *Erwinia carotovora* subsp. *carotovora* PelI; M18859, *Erwinia carotovora* subsp. *carotovora* PelA; D00217, *Erwinia carotovora* subsp. *carotovora* PALI; S51475, *Erwinia carotovora* subsp. *carotovora* PelII; M17364, *Erwinia carotovora* subsp. *carotovora* PelB; L32171. *Erwinia carotovora* subsp. *carotovora* Pel-1; X61088, *Erwinia carotovora* subsp. *carotovora* PelX-like; M19411, *Erwinia chrysanthemi* PAL; X67475, *Erwinia chrysanthemi* PelBC; M14510, *Erwinia chrysanthemi* PAI; X61087, *Erwinia carotovora* subsp. *carotovora* PelX; D00218, *Erwinia chrysanthemi* Pal.

strain ER,[18] and to Pel3 of *Erwinia carotovora* subsp. *atroseptica* C18,[19] and to PelC of *Erwinia carotovora* subsp. *carotovora* strain SCR193.[20]

Discussion

The obstacles to genetic analysis of structural genes in the case of the various phenotypes conferred by multiple isoenzymes can be readily overcome by manipulating the genes after molecular cloning. We have initiated this approach to the genetic pectate lyase isoenzyme in *Erwinia carotovora* subsp. *carotovora*. The unusual multiple enzymes suggest that pectate lyase is important in life cycle of *Erwinia carotovora* subsp. *carotovora*. Multiple enzymes may allow for a more complex regulatory strategy, allow degradation of pectate lyase in diverse environments, or simply provide higher levels of pectate lyase production through increased gene dosage. The reason why erwinias have acquired multiple genes encoding PLs is an intriguing question. It is possible that pectate, which has been supposed to be the natural substrate for PL, is chemically homogeneous. Multiple genes encoding PL could, however, result in elevated synthesis of the required enzymes for efficient depolymerization of pectate. Alternatively, it seems likely that the various PL isoenzymes hydrolyse α-(1→4) bonds situated in different physical environments within the heterogeneous cell wall pectin.[15]

To facilitate our genetic approach to the analysis of *Ecc* pathogenicity determinants we had to select a virulent, genetically amendable strain. *Ecc* LY34 strain proved to be the convenient strain from genetical, physiological and pathological points of view.[3] We located on a more detailed restriction map the gene for the isoenzyme of pectate lyase produced by *Ecc* LY34. The restriction sites in multiple cloning sites of pBluescript, the vector in which the pelCI gene was cloned, allowed us to excise a fragment for use as a DNA sequencing (Fig. 5) and a hybridization probe. The hybridization result suggested that only a single copy of *pel*CI sequence could be found in the *Ecc* LY34 (Fig. 4). Previous work has shown that *Ecc* produces at least three PL isoenzymes. Lie *et al.*[21,22] cloned three *pel*s which are tandem cluster in the same transcriptional direction in *Ecc* EC strain, and sequenced the nucleotides of *pel*A, *pel*B and *pel*C. The comparison of nucleotide sequences of *pel*CI with those of *pel*A or *pel*B or *pel*C suggested that *pel*CI might correspond to pelC. Hinton *et al*[20] also cloned and sequenced *pel*C of *Ecc* SCRI193 and there was 99.0% homology between PelCI and PelIII in the amino acid sequence. Moreover, the homology extended not only to structural genes but also to 5'- and 3'-noncoding regions. Yoshida *et al.*[18]analyzed that the *pel*III of *Ecc* Er strain was about 5.6 kb down stream of *pel*I, *pel*X and both *pel*I and *pel*III had individual transcription units in the same direction. Also Bartling *et al.*[15] have cloned 7.5 kb region of *E. c. atroseptica* which had three *pel* gene (*pel*1, *pel*2. *pel*3) in tandem. Therefore, it is possible that another *pel* gene of LY34 exists in tandem to the upstream of *pel*CI. However, the structural organization of the corresponding genes was not cloned. The tandem organization has also been described for *Erwinia* genes encoding proteases[23] and Out proteins.[24] The functional significance for this tandem arrangement remains elusive thus far.

The sequencing of several *pel* genes has allowed the identification of three distinct gene families. The PLbc family comprises genes which encode extracellular PLs produced by *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi*. The PLade family is made up of genes which encode extracellular PLs from *E. chrysanthemi* alone.[20] The third family only includes two intracellular PLs, from *Yersinia pseudotuberculosis* ICPB3821 (PLY) and *Ecc* EC153 (PL153).[25,26]E The *pel*CI gene from *Ecc* LY34 belongs to the PLbc family of extracellular PLs. PelCI shares 84.1-98.7% overall amino acid identity with other members of family (Fig. 6). There is a greater homology between PelCI of LY34 and Pel3 of *Erwinia carotovora* subsp. *atroseptica* C18 than has been observed between any *pel* genes from the same or different *Erwinia* strains, implying that the strains *Ecc* LY34 and *Erwinia carotovora* subsp. *atroseptica* C18 are very closely related. (Fig. 7). It is interesting to speculate on the role that the PLs of the periplasmic family have in the virulence of *Ecc*. It has been reported that members of this family are not required for efficient tissue maceration : the PL153 gene, and its analogue from the *Ecc* strain DB71, have been inactivated without a significant effect upon maceration ability.[26] In contrast, similar experiments with extracellular isoenzyme PLe of *Echr* demonstrated a crucial role in tissue maceration.[12,27]

The current concept is that pectic and cellulolytic enzymes released by phytopathogens are the important factors involved in enzymatic hydrolysis of plant cell walls. It is believed that these enzymes serve as wall-modifying enzymes, since their action renders other polysaccharide components in cell walls more susceptible to hydrolysis. A study of the exact role of

these enzymes is essential to an understanding of the mechanisms of host-parasite interaction in the disease cycle. Further work is currently under way in our laboratory to determine the role of multiple cell-wall degrading enzymes, by cloning more *pel* and *cel* genes of *Ecc* and characterizing them, and the construction of genetically modified strains by marker exchange.

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Erwinia carotovora subsp. *carotovora* LY34에서 *pel*CI 유전자 클로닝

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초 록 : Pectate lyase isoenzymes을 분비하는 *Erwinia carotovora* subsp. *carotovora* LY34는 식물조직을 연화시키는 연부균이다. 이 균주로부터 게놈 DNA를 분리하여 *Sau*3AI 제한효소로 부분 절단한 다음 pBluescript SK$^+$ 벡터에 클로닝하여 pectate lyase를 분비하는 클론을 분리하였다. 분리 결과 4.2 kb 크기의 DNA 단편을 가지고 있었으며 이를 다시 재클로닝하여 3.1 kb 크기의 *pel*CI 유전자를 함유하는 pLYPA100을 구하였다. 이 유전자의 DNA 염기서열을 분석한 결과 374 개의 아미노산을 구성하는 1,122 bp의 ORF를 확인하였다. 시작코돈과 종결코돈은 ATG와 TAA였으며 초기 서열 22개의 아미노산으로 구성된 전형적인 원핵세포의 signal peptide가 존재하였다. PelCI의 단백질 염기서열을 다른 단백질과 유사성을 분석한 결과 *Erwinia carotovora* subsp. *carotovora* Er 균주의 PelIII, *Erwinia carotovora* subsp. *carotovora* SCR193 균주의 PelC 및 *Erwinia carotovora* subsp. *atroseptica* C18 균주의 Pel3과 유사하였으며 PLbc family에 속하였다. PelCI의 분자량은 40,507, pI는 7.60으로 계산되었다.

찾는 말 : *Erwinia carotovora* subsp. *carotovora*, pectate lyase, *pel*CI 유전자
*연락저자