

Erwinia carotovora subsp. carotovora EC1株の複数のポリ ガラクトナーゼを生産するDNA領域の核酸塩基配列の決 定

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Determination of Nucleotide Sequence of a Region Producing Multiple Polygalacturonases from *Erwinia carotovora* subsp. *carotovora* EC1

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Abstract

DNA region consists of 1804 bases (b) was shown to produce four polygalacturonases (Peh) in *Erwinia carotovora* subsp. *carotovora* (E.c.c.) EC1. The sequence of this region was determined in both directions. In this DNA fragment, there was one open reading frame (ORF1) that encodes for a protein of 402 amino acids with a calculated molecular weight of 42,646 which is in the range of previously reported molecular weight of E.c.c. Peh. The sequence of the amino acids deduced from ORF1 showed 90% homology with that of reported E.c.c. Peh. A possible signal peptide of 26 amino acids was found. Several shorter ORF were also found from both strands. Possible role of remaining 595 b for the production of four Peh was discussed.

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Key words : multiple polygalacturonases, *Erwinia carotovora* subsp. *carotovora*, different pI, DNA sequence.

INTRODUCTION

Erwinia carotovora subsp. *carotovora* and *E. chrysanthemi* are the main causal agents of soft-rot of a variety of cultivated plants. In the host-parasite interaction, these *Erwinia* species produce a larger amount of extracellular enzymes, including pectic enzymes, cellulases, proteases, hemicellulases and phospholipases^{3,11)}. Among them, production of pectic enzymes has been considered as the main factor that causes plant tissue maceration^{1,5,12,23)}. Though pectate lyase (Pel) has been considered as the main factor for the development of disease symptom in soft-rot *Erwinia* species, the role of polygalacturonase (Peh) has been unclear. *Endo*-Peh, which cleaves α -1,4-glycosidic linkages of pectate to yield oligomers of varying sizes, is found in *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* but not in *E. chrysanthemi*¹¹⁾.

It has been reported that most strains of *E. carotovora* subsp. *carotovora* (E.c.c.) studied to date produce one to three *endo*-Pel isozymes^{5,26,28)} and single *endo*-Peh^{4,14,19)}. Inactivation of the *peh* gene of E.c.c. Ecc71 by marker exchange mutagenesis²⁶⁾ suggests that this strain produces only single *endo*-Peh and at least one *exo*-Peh. The *endo-pel* and *endo-peh* genes have been sequenced, and their deduced amino acid sequences have been reported^{4,7,13-15,19)}.

Previously, we have reported that a clone, pEC301, of E.c.c. EC1 produces four Peh with pI 9.5, 6.0, 5.6 and 5.0⁹⁾. Here we report the determination of nucleotide sequence of this clone and deduced amino acid sequence from a possible ORF. From the analysis of the deletions and subclones, the minimum region required to produce four Peh was found out in 1.8 kb *Ksp*632I fragment. The possible mechanisms were discussed to explain how such a short DNA fragment can produce four forms of Peh.

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MATERIALS AND METHODS

Bacterial strains, phage and plasmids, media and growth conditions. Descriptions of bacterial strains, phage and plasmids used in this study are given in Table 1. Cells were grown in LB medium or 2×YT medium²⁰. When required, the LB medium was solidified with agar (15 g/liter). *Escherichia coli* cells were incubated at 37°C. When required, kanamycin (Km) and ampicillin (Ap) was added at the final concentration of 75 µg/ml and 25 µg/ml, respectively. Polygalacturonate-yeast extract agar (PYA, pH 6.8) supplemented with 1 mM EDTA was used for the detection of Peh activity.

Table 1. Bacterial strains, phage and plasmids

Strain, phage or plasmid	Genotype or description	Source
<i>Escherichia coli</i>		
MV1184	<i>araΔ (lac-proAB)rpsLthi(φ80lacZΔM15)Δ(srl-recA)306::Tn10(tet^r)</i> F' [<i>traD36proAB⁺lacI^qlacZΔM15</i>]	14
Phage Lambda M13KO7		18
Plasmids		
pUC118	Plasmid for production of single stranded DNA.	16
pEC301	Clone containing a 5.8 kb <i>EcoRI</i> DNA fragment from E.c.c. EC1 in pUC118.	This study
pEC401	5.8 kb <i>EcoRI</i> DNA fragment from Ecc EC1 but in opposite orientation respect to the m.c.s. of pUC118.	This study
pEC405	1.5 kb <i>Ksp632I</i> - <i>ApaLI</i> fragment from pEC301 in pUC118.	This study
pEC404	3.4 kb <i>SalI</i> fragment from pEC301 in pUC118.	This study
pEC302	1.8 kb <i>Ksp632I</i> DNA fragment from pEC301 in pUC118	This study

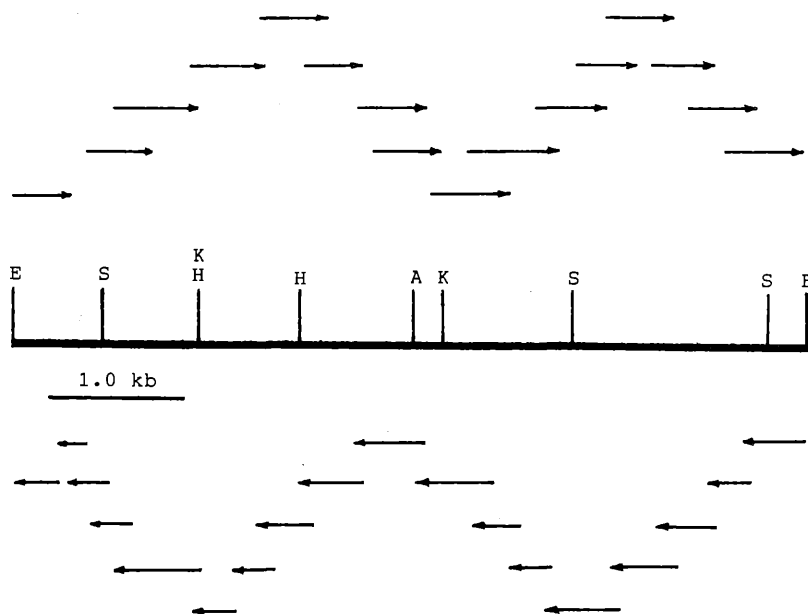


Fig. 1. Sequencing strategy for *peh* genes. The 5.8 kb *EcoRI* fragment of pEC301 is represented by a solid black line. The arrows represent determined sequence. Upper arrows correspond to the overlapped deletions from the left, and lower arrows correspond to the overlapped deletions from the right. Abbreviations: E, *EcoRI*; S, *SalI*; H, *HindIII*; K, *Ksp632I*; A, *ApaLI*.

1 CTG TGA AGA GAG GAA
 16 AAG CTT ACC ACC CGC TGG CTT CTG CGC ATT GAC GGG ATT CGT TTT
 |
 HindIII
 61 GTT TCC ACG TTT CGT TGA TAC TTC ATT TAA TTT TTT GGT GAT ATT
 106 TTA ACC ATG CGT AAA GTT GAA TGC TTT CCG AAT AAG CAT TGG AAT
 151 AAT TTT ATG TAA ATG GAC TTT TCA TTA ATC TAC ATA GGC ATG TGT
 196 GAC AAT TTT TAG TTG CTA TAA AGG CGT TTT ATT TTT ATT ATT AAG
 -35 -10
 241 CAA AAT GAA AGC GTA ATG CCT TTT GGG GAT GGA AAA GTT GTG GAT
 Kdgr
 286 GCT TAT AAG GTA AGG TTT GAC ATG GAA TAT CAA TCA GGC AAG CGA
 S.D. Met Glu Tyr Gln Ser Gly Lys Arg 8
 331 GTT TTA TCA TTA TCA CTG GGG CTT ATC GGT TTG TTT AGT GCA TCG
 Val Leu Ser Leu Ser Leu Gly Leu Ile Gly Leu Phe Ser Ala Ser 23
 376 GCA TTT GCT TCT GAT TCC CGA ACG GTG AGT GAA CCG AAA GCA CCG
 Ala Phe Ala Ser Asp Ser Arg Thr Val Ser Glu Pro Lys Ala Pro 38
 421 TCT TCC TGT ACG GTG CTT AAA GCG GAC AGT AGT ACG GCC ACG AGT
 Ser Ser Cys Thr Val Leu Lys Ala Asp Ser Ser Thr Ala Thr Ser 53
 466 ACA ATT CAA AAA GCG CTG AAT AAT TGC GGG CAA GGA AAA GCG GTA
 Thr Ile Gln Lys Ala Leu Asn Asn Cys Gly Gln Gly Lys Ala Val 68
 511 AAG CTG AGC GCA GGG AGT TCA TCC GTT TTT CTG AGC GGT CCG CTT
 Lys Leu Ser Ala Gly Ser Ser Ser Val Phe Leu Ser Gly Pro Leu 83
 556 TCT CTA CCT TCT GGC GTG AGC TTA TTA ATC GAC AAA GGG GTA ACC
 Ser Leu Pro Ser Gly Val Ser Leu Leu Ile Asp Lys Gly Val Thr 98
 601 CTA CGT GCT GTG AAT AAT GCC AAG TCT TTT GAA AAT GCG CCC TCA
 Leu Arg Ala Val Asn Asn Ala Lys Ser Phe Glu Asn Ala Pro Ser 113
 EcoT22I
 646 TCC TGT GGC GTG GTG GAT ACA AAC GGT AAA GGC TGC GAT GCA TTT
 Ser Cys Gly Val Val Asp Thr Asn Gly Lys Gly Cys Asp Ala Phe 128
 691 ATT ACC GCC ACA AGC ACG ACG AAT AGC GGA ATC TAT GGG CCG GGC
 Ile Thr Ala Thr Ser Thr Thr Asn Ser Gly Ile Tyr Gly Pro Gly 143
 HindIII
 736 ACC ATT GAT GGA CAG GGG GCC GTG AAG CTT CAG GAT AAA AAG GTG
 Thr Ile Asp Gly Gln Gly Ala Val Lys Leu Gln Asp Lys Lys Val 158
 781 AGC TGG TGG GAT CTG GCT GCC GAT GCC AAA GTG AAA AAG TTA AAA
 Ser Trp Trp Asp Leu Ala Ala Asp Ala Lys Val Lys Lys Leu Lys 173
 826 CAG AAT ACC CCC CGG CTG ATT CAG ATT AAT AAG AGC AAG AAT TTC
 Gln Asn Thr Pro Arg Leu Ile Gln Ile Asn Lys Ser Lys Asn Phe 188
 871 ACG CTG TAT AAC GTC TCT CTC ATT AAT TCT CCG AAC TTC CAT GTG
 Thr Leu Tyr Asn Val Ser Leu Ile Asn Ser Pro Asn Phe His Val 203

(Continued on next page)

Fig. 2. Nucleotide sequence of 1.8 kb *Ksp6321* fragment and deduced amino acid sequence of ORF1. The putative Shine-Dalgarno sequence (S.D.), -35 and -10 regions of promoter and KdgR box are underlined. The potential inverted repeat sequence following the termination codon is indicated by facing arrows.

Plate assay of polygalacturonate. Subclones producing Peh were detected on PYA in the presence of 1 mM EDTA as described by Saarilahti *et al.*¹⁹⁾

General DNA manipulations. Plasmid DNA was isolated and manipulated by using standard techniques²⁰⁾. Restriction enzymes, T4 DNA ligase and related reagents were purchased from Nippon Gene, New England Biolab and Boehringer Mannheim.

Sequence of the *peh* genes. Plasmids pEC301 and pEC401 contains the 5.8 kb *Eco*RI DNA fragment inserted in different orientations on pUC118. Series of deletions of the insert in pEC301 and in pEC401 were constructed by digestion with Exonuclease III using a deletion kit (Takara Shuzo). The nucleotide sequence of these deletions was determined by automatic sequencer (Applied Biosystems Model 370A). Ambiguous portions were rechecked manually by dideoxy chain termination method^{20,25,27)} using [α -³²P] dCTP. Edition of DNA sequence and of deduced protein sequence was performed by GENETYX (SDC). Homology search was done by GENETYX-CD (SDC).

RESULTS

*Nucleotide sequence of the *peh* gene*

The entire nucleotide sequence of the 5.8 kb *Eco*RI DNA fragment of pEC301 which produces four polygalacturonases was determined from both directions. The strategy of the sequencing is shown in Fig. 1. From the search of open reading frames, several candidates were found. Among them, only one open reading frame (ORF1) encodes for a protein of 402 amino acids with a calculated molecular weight of 42,646 which is in the range of previously reported molecular weight of E.c.c. Peh (Fig. 2). The translation start with ATG is at position 307 and is preceded by a putative ribosome binding site AAGG. A possible KdGR box¹⁶⁾ was found from 241 to 264 b upstream region from the translational start point. A possible sigma-70 type promoter was founded at position 208 (TTGCTA, -35 region) and position 231 (TATTAT, -10 region) with 17-base spacing. A termination codon TAA was found at position 1513, followed by one inverted repeated sequence with a loop size of 8 nucleotides and a stem size of 9 nucleotides. This palindromic structure without poly-T tail may be involved in ρ -factor dependent termination of the transcription. From the comparison with deduced amino acid sequences of two other *endo*-Peh of E.c.c.^{14,19)}, Peh1 showed 90% overall homology in amino acid sequence with two reported *endo*-Peh from the other strains of *E. carotovora* (Fig. 3). Presence of a signal peptide containing 26 amino acids at N-terminal was also suggested. Thus, the mature protein is released from Peh1 as a protein of 376 amino acid with a calculated molecular weight of 39,918. However, no other ORF, which encodes for protein of molecular weight in the range of reported Peh, was found.

Comparison between Peh1 and other pectinases

The deduced amino acid sequence of the ORF1 was compared with those of bacterial, fungal and plant pectinases for overall homology. It was confirmed that Peh1 has higher homology with bacterial Peh than with pectate lyase (Pel)^{5,7,10,13,15,18,23,24)}, pectin lyase (Pn1)^{2,17)} and pectin methylesterase (Pme)²²⁾. When a multiple alignment was done considering the gaps among amino acid sequences of Peh from *Lycopersicon esculentum* (tomato)²¹⁾, *Pseudomonas solanacearum*⁶⁾, the other strains of E.c.c.^{14,19)} and Peh1, high homologous region among all bacterial Peh including Peh1 was found (Fig. 3). But, identity of Peh from tomato did not show significant homology with other Peh from bacteria even in this region. When the conservative changes of amino acids are considered, all these Peh showed considerable similarity.

DISCUSSION

A subclone pEC302 containing only 1.8 kb *Ksp*632I DNA fragment produced four Peh. If the sizes of reported *peh* genes are applicable for these four Peh, it is impossible to locate four complete genes on 1.8 kb *Ksp*632I fragment. One possibility to explain how the 1.8 kb fragment produces four different Peh may be that ORF1 (1209b) produces the protein (Peh1) and remaining 595b produces three shorter polypeptides possibly as the modifiers for Peh1. In fact, several shorter ORF were found in the remaining

LEPG	234	V A S N L M I N A S A K S P N T D G V H V S N T Q
PSPG	301	K V T P A T C F T P E T V K N T D G F D P G Q S T
CPGA	247	- - - - - P S T A R N T D G I D P M S S K
CPGB	247	- - - - - P S T A R N T D G I D P M S S K
PEH1	247	- - - - - P S T A R N T D G I D P M S S K
LEPG	259	Y I Q I S D T I I G T G D D C I S I V S G S Q N V
PSPG	326	N V V L A Y S Y I N T G D D H V A V K A - - S S G
CPGA	272	N I T I A Y S N I A T G D D N V A I K A Y K G R A
CPGB	272	N I T I A Y S N I A T G D D N V A I K A Y K G R A
PEH1	272	N I T I A H S N I S T G D D N V A I K A Y K G R S
LEPG	284	Q A T N I - - - T C G P G H G I S I G S L G S
PSPG	351	P T R N L L F A H N H F Y Y G H G L S I G S E T N
CPGA	297	E T R N I S I L H N D F G T G H G M S I G S E T -
CPGB	297	E T R N I S I L H N D F G T G H G M S I G S E T -
PEH1	297	E T R N I S I L H N E F G T G H G M S I G S E T -
LEPG	309	G N S E A Y V S N V T V N E A K I I G A E N G V R
PSPG	376	T G V S N M L V T D L T M D G N D S S A G N G L R
CPGA	322	M G V Y N V T V D D L K M N G T T - - - N G L R
CPGB	322	M G V Y N V T V D D L K M N G T T - - - N G L R
PEH1	322	M G V Y N V T V D D L V M T G T T - - - N G L R
LEPG	334	I K T W Q G G S G Q A S N I K F L N V E M Q D V K
PSPG	401	I K S D A S R G G K V T N I V Y D G I C M R N V K
CPGA	347	I K S D K S A A G V V N G V R Y S N V M K N V A
CPGB	347	I K S D K S A A G V V N G V R Y S N V M K N V A
PEH1	347	I K S D K S A A G V V N G V R Y S N V M K N V A
LEPG	359	Y P I I I D Q N Y C D R V E P C I Q Q F S A V Q V
PSPG	426	E P L V F D P F Y S S V K G S L Y P N F T N I V
CPGA	372	K P I V I D T V Y E K K E G S N V P D W S D I T F
CPGB	372	K P I V I D T V Y E K K E G S N V P D W S D I T F
PEH1	372	K P I V I D T V Y E K K E G S N V P D W S D I T F

Fig. 3. Alignment of the repeated sequences between deduced amino acid sequences of Peh from tomato, *Pseudomonas solanacearum*, and E.c.c. Identical amino acids are closed in boxes and conservative changes are indicated by a period. Abbreviations: LEPG, Peh from *Lycopersicon esculentum*²¹; PSPG, Peh from *Pseudomonas solanacearum*⁶; CPGA, Peh from E.c.c. strain SCC3193¹⁹; CPGB, Peh from E.c.c. strain EC¹⁰; PEH1, Peh from E.c.c. strain EC1.

595b of the 1.8 kb *Ksp632I* fragment. These polypeptides may interact with the Peh from the ORF1 to modify the *pI*. Another possibility is that four genes overlap each other. However, no such ORF which encodes the proteins of molecular weights in the range of reported one, was found.

When the *phoA* gene was inserted in the putative promoter region for short ORF located upstream of ORF1 using *TnphoA*⁸⁾, all four Peh activity was completely knocked out (data not shown). From this result, it was suggested that the transcription of this short ORF is required to produce the remaining three Peh.

Though the deduced amino acid sequence of Peh1 showed 90% of overall homology with that of Peh from *E. carotovora* subsp. *carotovora* SCC3193¹⁹⁾, *pI* of Peh1 was slightly lower than *pI* 10.19 for Peh1. More interestingly, they claim that the clone of *pehA* showed single Peh, while our pEC302 produced multiple forms of Peh. The region other than ORF1 on pEC302 may be involved for the formation of multiple forms. The comparison to overall homology between Peh1 and Peh from another strain (EC) of *E. carotovora* subsp. *carotovora*¹⁴⁾ produced 90% of homology too, but no data of IEF was shown. Thus, we do not know if that clone produces multiple Peh.

When an alignment between amino acid sequences from eukaryotic and prokaryotic Peh was done, considering the gaps and the conservative changes, all five Peh compared showed considerable similarity. This result indicates that prokaryotic and eukaryotic Peh may have a common origin, in spite of the long evolutionary distance.

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和 文 摘 要

Jorge Jhoncon・酒井富久美・露無慎二： *Erwinia carotovora* subsp. *carotovora* EC1 株の複数のポリガラクトナーゼを生産する DNA 領域の塩基配列の決定

Erwinia carotovora subsp. *carotovora* (E.c.c.) EC1 株では、1804 塩基からなる DNA 断片が等電点を異にする 4 種のポリガラクトナーゼ (Peh) を生産した。この領域の塩基配列を両方向から決定したところ、他の E.c.c. 菌株で報告されている Peh と同程度の分子量 42,646 となる 402 個のアミノ酸をコードする翻訳領域 (ORF1) が存在した。ORF1 から読まれるアミノ酸の配列は既報の E.c.c. の Peh と約 90% の相同性を示した。また、26 個のアミノ酸からなるシグナルペプチドと考えられる領域も見いだされた。この他、短い ORF も存在していた。4 種の Peh を生産するための ORF1 を除いた 595 塩基の役割について論じた。