Molecular cloning and sequencing of a pectinesterase gene from *Pseudomonas solanacearum*

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Two pectinesterase-positive *Escherichia coli* clones, differing in expression levels, were isolated from a genomic library of *Pseudomonas solanacearum*. Both clones contained a common DNA fragment which included the pectinesterase-encoding region. The different expression levels found with the two clones could be ascribed to different positioning of the pectinesterase gene with respect to a vector promoter. Restriction analysis, subcloning, and further exonuclease deletion mapping revealed that the genetic information for pectinesterase was located within a 1.3 kb fragment. A protein of 41 to 42 kDa was expressed from this fragment. Nucleotide sequence analysis of the respective region disclosed an open reading frame of 1188 bp. The deduced polypeptide had a calculated molecular mass of 41 004 Da, which is consistent with the determined size of the pectinesterase protein. The predicted amino acid sequence showed significant homology to pectinesterases from *Erwinia chrysanthemi* and tomato. In cultures of *E. coli* clones up to 30% of total pectinesterase activity was transported into the medium. However, no significant pectinesterase activity could be detected in the periplasm.

Introduction

Pectic enzymes are of great importance as pathogenicity factors in a broad range of plant diseases caused by micro-organisms (Basham & Bateman, 1975; Stephens & Wood, 1975; Collmer, 1986). Two main types of pectic enzymes can be distinguished: pectinesterases (PEs), which remove methoxyl residues from pectin, and a variety of depolymerizing enzymes, which cleave the galacturonic acid backbone either by β -elimination or by hydrolysis. Pectic depolymerases and PEs, in combination with cellulases and proteases, attack the middle lamellae and primary cell walls of plants and may lead to maceration and cell death.

Apart from their role in pathogenesis, pectic enzymes are also of considerable commercial value in the food industry. Crude enzyme preparations, exhibiting different pectinolytic, cellulolytic and proteolytic activities, are used, for example, to clarify fruit juices and to increase the yield of juice and solids from plant material.

In special cases the use of individual enzymes instead of crude extracts might have some advantages. PE free from depolymerases is of specific interest for preparing low-methoxyl pectin gels (Calesnick *et al.*, 1950) and for the clarification of cider (Rombouts & Pilnik, 1986). The cloning of various pectate lyase and polygalacturonase encoding genes from *Erwinia* spp. (Keen *et al.*, 1984; Reverchon *et al.*, 1985; Kotoujansky *et al.*, 1985; Zink & Chatterjee, 1985), *Yersinia pseudotuberculosis* (Manulis *et al.*, 1988) and *Pseudomonas solanacearum* AW (Schell *et al.*, 1988) has been described. Plastow (1988) was the first to report the cloning and characterization of a PE gene from *Erwinia chrysanthemi* B374. Ray *et al.* (1988) described the identification and sequencing of a cDNA clone from tomato encoding PE.

The occurrence of various types of highly active pectic enzymes has been described for *P. solanacearum* (Lange & Knösel, 1970; Hildebrand, 1971; Ofuya, 1984), which is one of the most important plant pathogens (Buddenhagen & Kelman, 1964). Since the objective of our work was the cloning and characterization of genes encoding pectinases of commercial importance, this organism was chosen as a potent source. Here we describe the isolation, subcloning and sequencing of a gene (*pme*) coding for a PE (EC 3.1.1.11) from the phytopathogenic strain *P. solanacearum* DSM 50905. We also present data on secretion of the PE protein from *Escherichia coli* clones.

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Abbreviations: PE, pectinesterase; *pme*, gene encoding PE; FID, flame ionization detector; CAT, chloramphenicol acetyltransferase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M37772.

Methods

Strains, plasmids and phages. *Pseudomonas solanacearum* was obtained from the Deutsche Sammlung für Mikroorganismen (DSM 50905). *E. coli* HB101 (ATCC 33694) and *E. coli* C600 (ATCC 23724) were used as hosts for recombinant plasmids. *E. coli* K38 (HfrC) was used for *in vivo* analysis of plasmid-encoded proteins. The plasmids pBR322 (Bolivar *et al.*, 1977), pBR325 (Bolivar, 1978) and pUC18 (Yanisch-Perron *et al.*, 1985) were used as vectors for the construction of the gene library and for subcloning experiments. Plasmids pT7-5, pT7-6 and pGP1-2 (Tabor & Richardson, 1985) were used for the analysis of proteins encoded by cloned DNA fragments. M13mp18 and M13mp19 (Norrand *et al.*, 1983) were used to generate single-stranded templates for sequencing reactions.

Cultivation conditions. *P. solanacearum* and *E. coli* cells were usually grown in LB medium (Maniatis *et al.*, 1982) at 30 °C and 37 °C respectively. For preparation of cell lysates, M9 mineral salts medium (Miller, 1972), supplemented with the appropriate amino acids (20 mg l⁻¹) and thiamin (2 mg l⁻¹), and containing glycerol (5 g l⁻¹) as the sole carbon source, was used. For the selection of plasmids, ampicillin (100 mg l⁻¹), tetracycline (10 mg l⁻¹) and kanamycin (40 mg l⁻¹) were added as required.

DNA isolation. High-molecular-mass chromosomal DNA of *P. solanacearum* was isolated by the method of Saito & Miura (1963). Large-scale preparations of plasmid DNA were done by the alkaline lysis method, with further purification by caesium chloride/ethidium bromide gradient centrifugation (Maniatis *et al.*, 1982). The method of Birnboim & Doly (1979) was used for small-scale extractions and rapid analysis of plasmid DNA.

Construction of a gene library of *P. solanacearum*. Chromosomal DNA was partially digested with *Sau*3AI and fractionated by agarose gel electrophoresis. Fragments 5–15 kb in size were recovered by electroelution (Maniatis *et al.*, 1982) and purified by DEAE-cellulose chromatography using Whatman DE52. The purified fragments were ligated into *Bam*HI-cleaved, dephosphorylated pBR322. The ligation mixture was used to transform *E. coli* HB101. Recombinant clones containing inserts were detected by their Ap^R/Tc^S phenotypes.

General recombinant DNA techniques. Restriction endonucleases and DNA-modifying enzymes were used under the conditions recommended by their suppliers. All cloning procedures as well as BAL31 digestions and modifications of DNA fragments were performed according to standard methods as described by Maniatis *et al.* (1982). Unphosphorylated *Xho*I-linkers (8-mer, Pharmacia) were used to religate plasmids following BAL31 deletion as described by Seth (1984). Specific DNA fragments were recovered from agarose gels by electroelution as described by Maniatis *et al.* (1982) or by electrotransfer onto NA45-DEAE-membranes (Schleicher & Schuell). In the case of small DNA fragments the 'freeze-squeeze' method of Thuring *et al.* (1975) was used.

Screening. The screening for pectinolytic clones was performed by a method based upon the 'cup-plate' assay of Dingle *et al.* (1953). Recombinant *E. coli* clones were grown for 2–3 d on M9-glycerol agar plates supplemented with 5 g pectin l⁻¹ (Sigma P-9135). The plates were then flooded with a saturated solution of copper acetate. Clear zones ('haloes') on a turbid background appear around colonies expressing pectinolytic activities.

Preparation of cell extracts. *E. coli* cells were grown in M9-glycerol medium to the late exponential growth phase and harvested by centrifugation. The cells were suspended in fresh, ice-cold M9 medium and disrupted by ultrasonication (B. Braun Labsonic 2000, 100 W, 2 × 30 s). The cell lysates were centrifuged for 20 min at 30000 g to

remove cell debris. Extracts were stored at 4 °C. The protein content was determined by the Lowry method.

Preparation of cell fractions. *E. coli* clones were grown in M9-glycerol medium to the stationary phase, and the cells were harvested by centrifugation. After removal of the supernatant, portions of 1.0 g (wet weight) of the pelleted cells were resuspended in 80 ml 30 mM-Tris (pH 7.3)/20% (w/v) sucrose. EDTA was added to a final concentration of 10⁻³ M, and the suspension was incubated with vigorous shaking (rotary shaker, 180 r.p.m.) for 7 min at room temperature. After centrifugation the Tris/sucrose/EDTA supernatant (TSE) was collected. For preparing the fraction containing the periplasmic enzymes the 'osmotic shock' method of Heppel (1971) was followed. The cytoplasmic fraction was obtained by ultrasonic treatment of the remaining spheroplasts and removal of cell debris. Protein was determined according to Bradford (1976) using a commercial reagent kit (BioRad).

Detection of enzyme activities. PE activity was assayed by determination of liberated methanol by gas chromatography (GC). Cell lysates were diluted with 50 mM-Tris/HCl pH 7.0 as appropriate. For control experiments the lysates were replaced by M9 medium or a solution of a commercial PE (Sigma P-1889). The reaction assay was set up by mixing 900 µl of a pectin stock solution (10 g l⁻¹) and 900 µl of diluted cell lysates in sterile sealable glass vials (Wheaton). After accurate sealing, the vials were incubated at 30 °C. Samples of 0.8 µl were taken at appropriate time intervals and injected into the gas chromatograph. The following conditions were used for GC analysis: glass column (1.8 m × 2 mm) containing Poropak Q (80–100 mesh), column temperature 160 °C, injector temperature 170 °C, FID temperature 210 °C, carrier gas nitrogen, 30 ml min⁻¹. One unit of PE is defined as 1 µmol methanol liberated min⁻¹ at 30 °C.

Depolymerase activity was determined by HPLC analysis of reaction products using the following conditions: Aminex resin-based column (BioRad HPX-87H), 300 × 7.8 mm, column temperature 65 °C, flow rate 0.7 ml min⁻¹, mobile phase 5 mM-H₂SO₄. Reaction assays were prepared in standard reaction tubes using pectin and pectate (Sigma P-1879) as substrates. Equal volumes (500 µl) of a substrate stock solution (10 g l⁻¹) and cell-free extract were mixed in Eppendorf tubes and incubated at 30 °C for various times up to 8 h. Reactions were stopped by heating to 95 °C for 3 min. Solids were removed by centrifugation, and samples of the supernatants were injected into the HPLC. Two commercial pectic depolymerases (Sigma P-5146, P-3026) were used in control experiments.

Chloramphenicol acetyltransferase activity was measured according to Shaw (1975) by following the formation of 5-thio-2-nitrobenzoate photometrically at 412 nm.

Activities of β-lactamase were estimated using the chromophoric substrate nitrocefin and monitoring spectrophotometrically the increase of absorbance at 482 nm as described by O'Callaghan *et al.* (1972).

Analysis of encoded proteins. A T7 RNA polymerase/promoter system was used for specific labelling of plasmid-encoded proteins (Tabor & Richardson, 1985). DNA fragments were cloned into the vector plasmids pT7-5 and pT7-6 downstream of the bacteriophage T7 φ10 promoter. These two plasmids differ in the orientation of their multiple cloning sites, and DNA fragments can therefore be cloned and analysed in both orientations. The recombinant plasmids were used to transform *E. coli* K38 harbouring plasmid pGP1-2; this plasmid contains the gene for T7 RNA polymerase under the control of the λ P_L promoter, which is regulated by the thermosensitive repressor cI857. After a period of normal cell growth at 30 °C, the transcription of the T7 RNA polymerase gene was induced by a temperature shift to 42 °C. The host RNA polymerase was inhibited by addition of rifampicin, and translated polypeptides were labelled with [³⁵S]methionine. Sub-

sequently, cells were lysed, and synthesized polypeptides were analysed by SDS-PAGE (Laemmli, 1970) and fluorography.

DNA sequencing. Single-stranded DNA templates were obtained by cloning deletion fragments into M13 derivatives mp18 and mp19, and sequencing was performed according to the dideoxy chain-termination method (Sanger *et al.*, 1977) using [α - 35 S]dATP (400 Ci mmol $^{-1}$; 14.8 TBq mmol $^{-1}$) and T7 DNA polymerase (Sequenase, USB). To overcome secondary structures in G+C-rich stretches, 7-deaza-dGTP or dITP were used alternatively in the sequencing reactions.

Sequence data were handled and analysed using the 'Gene Master' (BioRad) or GCG (Genetics Computer Group, University of Wisconsin) computer program packages.

Construction of pGST1. The 2.0 kb *EcoRV* fragment of pSAP13 was ligated with *EcoRV*-cleaved, dephosphorylated pBR325. Hybrid plasmids which contained the *pme* gene fragment in the proper orientation (under the control of the vector P2 promoter) were selected and designated as pGST1.

Results and Discussion

Cloning of the *pme* gene

A genomic library of *P. solanacearum* was prepared in *E. coli* HB101 using pBR322 as vector plasmid. Seven thousand *E. coli* clones were individually tested for pectinolytic activities using the pectin agar plate assay. Two putative clones showing a significant difference in the size of the produced haloes were found. The hybrid plasmids contained in these clones were designated as pSAP1 (6.1 kb insert; larger halo) and pSAP2 (10.5 kb insert; smaller halo). By retransformation of *E. coli* C600 with isolated plasmid DNA it was verified that pectinolytic activity was mediated by the respective plasmids.

Cell extracts of *E. coli* C600 clones harbouring pSAP1, pSAP2 or pBR322 were prepared and assayed for PE and pectic depolymerase activities by GC and HPLC analysis. No significant depolymerase activity on pectin or pectate could be detected under the conditions used. In tests for PE activity, methanol was liberated from pectin in reaction mixtures containing cell lysates of pSAP1 and pSAP2 clones. Control experiments with lysates of *E. coli* C600(pBR322) showed detectable but very low levels of liberated methanol, and similar effects were seen when cell lysates were replaced with M9 medium. Since methyl ester groups are very sensitive to saponification by dilute alkali, even at low temperature (Albersheim *et al.*, 1960), this slight background of methanol could be due to such effects. In accordance with the observation that pSAP1 clones produced larger haloes on pectin agar plates, a much higher specific activity of PE (about 14-fold) was found in the lysates of pSAP1 clones compared to pSAP2 clones (Fig. 1).

Restriction enzyme analysis and Southern blot hybridization experiments using pSAP1 and pSAP2 DNA

Fig. 1. PE activity in cell extracts of different *E. coli* clones. Cell extracts were prepared, and PE activity was determined by GC as described in Methods. The increase of liberated methanol as a function of incubation time is shown for *E. coli* C600 containing pSAP1 (■), pSAP2 (▲) or (control) pBR322 (□). The data are based on the content of total protein in the lysates.

showed partial identity between these plasmids, suggesting that they contain overlapping fragments of *P. solanacearum* DNA. The orientation of this common fragment is different with respect to the pBR322 vector part. A deletion derivative of pSAP12 (see Fig. 2) lacking the P2 promoter of the pBR322 vector part (pSAP121) exhibited a decrease in PE activity to the level of pSAP2 clones (data not shown). This indicates that the high level of expression of PE in clones containing pSAP1, pSAP11, pSAP12 and pSAP13 (Fig. 2) could be mediated by transcription of the *pme* gene from the P2 promoter of pBR322, whereas in pSAP2 and in pSAP121 a *P. solanacearum* promoter which can be recognized by *E. coli* RNA polymerase seems to be active. These data also provided evidence for the direction of transcription of the *pme* gene as indicated in Fig. 2.

A 1.34 kb fragment contains the entire information for PE

Based upon detailed restriction analysis, various subfragments of pSAP1 were cloned into pBR322 or pUC18. The clones obtained were investigated for their ability to express PE activity. The smallest fragment still conferring the PE phenotype upon *E. coli* clones was identified in pSAP13 (Fig. 2). pSAP13 was constructed by introducing a 2.0 kb *ClaI* fragment of pSAP1, containing the 0.3 kb *ClaI*–*Bam*HI fragment of pBR322 and the 1.7 kb *Sau*3AI–*ClaI* fragment of *P. solanacearum* DNA, into the *ClaI* site of pBR322. With respect to the pBR322 vector part, the orientation of the cloned fragment in pSAP13 is analogous to pSAP1.

Fig. 2. Restriction map of the *P. solanacearum* DNA fragment in pSAP1 and locations of cloned subfragments. The plasmids pSAP11, pSAP12 and pSAP15 resulted from deletion of certain fragments of pSAP1 and subsequent religation. pSAP13, pSAP14, pSAP16, pSAP17 and pSAP18 were constructed by subcloning restriction fragments into the vector plasmids pUC18 or pBR322. A detailed restriction map of the 1.7 kb fragment of pSAP13 and positions of relevant exonuclease BAL31 deletions are shown in the lower part of the figure. The strategy for deletions at the 5' end (pSAP1303–1307) was as follows. pSAP13 DNA was linearized with *EcoRI*. After incubation with BAL31 the DNA was digested with *HindIII*. Appropriate fragments were ligated into *SmaI/HindIII*-cut pUC18. For 3' deletions (pSAP1201–1202), pSAP13 was partially digested with *ClaI*. Following incubation with BAL31, the resulting fragments were religated by attachment of a *Xho*-linker. All recombinants were used to transform *E. coli* HB101 and assayed on pectin agar plates. The ability to express a functional PE is shown in the right-hand column. (**E. coli* clones harbouring pSAP1305 or pSAP1306 exhibited less PE activity on pectin agar plates than did other PE-positive clones. For details see text.) The region encoding the PE is represented by an open bar. Direction of transcription of the *pme* gene is indicated by an arrow. Restriction sites are indicated for: A, *ApaI*; Av, *AvaI*; B, *BamHI*; C, *ClaI*; K, *KpnI*; N, *NcoI*; P, *PstI*; Sc, *SacII*; S, *SalI*; Sa, *SmaI*; Sp, *SphI*. Sizes are given in kb.

Exonuclease BAL31 deletions were carried out from both ends of the 1.7 kb fragment. A set of clones selected after restriction analysis was assayed for PE activity. The results presented in Fig. 2 show that the removal of up to 0.26 kb at the 5' terminus (pSAP1303–1304) and of approximately 0.1 kb at the 3' end (pSAP1201) did not influence the expression of PE activity. Cells harbouring pSAP1305 or pSAP1306, carrying 5' deletions of about 0.33 kb and 0.34 kb respectively, exhibited a markedly lower PE activity on pectin agar plates. The further deletion of a small part at the 5' end (pSAP1307) or at the 3' terminus (pSAP1202) resulted in a complete loss of PE activity.

From these data it was concluded that the entire *pme* gene is located within a 1.34 kb fragment as indicated in Fig. 2. Furthermore, it is probable that the decreased PE activity in clones containing pSAP1305 or pSAP1306 is due to an effect on the promoter region.

Identification of the PE polypeptide

Proteins encoded by the 1.7 kb fragment of pSAP13 were analysed by expression studies using the T7 RNA polymerase/promoter system. The *pme* region was

prepared as a 1.9 kb fragment using the *EcoRV* and *HindIII* vector sites of pSAP13 and ligated into the *SmaI/HindIII*-digested plasmids pT7-5 and pT7-6 as shown in Fig. 3(a). The resulting hybrids were used to transform *E. coli* K38 harbouring pGPI-2 and the plasmid-encoded proteins were labelled as described in Methods. After fluorography of the SDS-polyacrylamide gel a single protein band could be detected with pSAP132 but not with pSAP133 (Fig. 3b). This protein, which we conclude to represent PE, has a molecular mass of about 42 kDa and was only expressed when the PE-encoding region was transcribed in the direction expected for transcription from the natural promoter. A plasmid-specific 41 kDa protein was also detected by SDS-PAGE analysis of total proteins present in cell lysates and culture supernatants of *E. coli* clones containing pSAP1 (results not shown).

Nucleotide sequence of the *pme* gene

The nucleotide sequence of the 1698 bp *Sau3AI*–*ClaI* fragment present in pSAP13 is given in Fig. 4. An open reading frame extending from the methionine codon (ATG) at position 287 to the termination codon at

Fig. 3. Detection of polypeptides encoded by the *P. solanacearum* DNA insert of pSAP13. (a) Hybrid plasmids constructed for expression mediated by T7 RNA polymerase. The PE-encoding region of pSAP13 was prepared as an *EcoRV*–*HindIII* fragment and ligated into *SmaI*/*HindIII*-cut pT7-5 and pT7-6 and is represented by the double-lined segment; the direction of transcription of the *pme* gene is indicated by the small arrow. The bacteriophage T7 ϕ 10 promoter and the gene coding for β -lactamase (*bla*) are also indicated. (b) Analysis of encoded proteins by SDS-PAGE and fluorography. Lane 1, pT7-5. Lane 2, pSAP133. Lane 3, pSAP132. The positions of unlabelled molecular mass marker proteins are shown on the left. From top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase. The arrow indicates the 42 kDa band in lane 3 which is absent if the direction of transcription is reversed (lane 2).

position 1475 was found which could encode a protein of 396 amino acids and a calculated molecular mass of 41004 Da. This fits well to the size of the identified polypeptide (41–42 kDa). A putative Shine–Dalgarno sequence (S/D; indicated in Fig. 4) can be found appropriately positioned 5' to the presumed translational start codon. The additional ATG found in the same reading frame at position 434 is not preceded by a reliable S/D sequence.

The G + C content of the *pme* gene was 65 mol% and corresponds to the overall G + C content of 66–67 mol% specified for the *P. solanacearum* chromosome (Holloway & Morgan, 1986). As with other *Pseudomonas* genes (Hadero & Crawford, 1986), the high G + C content could be attributed to a bias towards G and C nucleotides in the third position (88.2%). Furthermore, the restricted codon usage (46 of 61 codons are utilized; data not shown) resembles that of highly expressed genes in *E. coli*.

Computer analysis of the 5' non-coding region disclosed several putative promoters homologous to *E. coli* σ^{70} consensus sequences as indicated in Fig. 4. Analogous promoter structures have also been described for *Pseudomonas* spp. (Deretic *et al.*, 1989). The sequence

from bases 82 to 110, for instance, shows 67% homology with the –35 and –10 sequences of the σ^{70} consensus promoter. The spacing between the –35 and –10 regions is 16 bases, which is typical for strong *E. coli* promoters (Hawley & McClure, 1983). In addition, as described by Drew *et al.* (1985) for Gram-negative bacteria, an A + T-rich region was observed around this putative *pme* promoter. The A + T content of the promoter-flanking region from bases 80 to 160 is 54 mol%, whereas the overall A + T content for the *pme* gene is 35 mol%.

Downstream of the translational stop of *pme*, two inverted repeats which may form stem-loop structures (1500–1540, 1566–1588) can be seen. However, these repeats are not followed by T residues, and it is not clear whether these structures could represent transcriptional terminators.

Homologies between PEs

The primary structures of the PEs from *Erwinia chrysanthemi* (Plastow, 1988) and tomato (*Lycopersicon esculentum*; Ray *et al.*, 1988) have been reported. Both proteins are similar to the *P. solanacearum* enzyme with respect to their size, 366 and 390 amino acids, and their calculated subunit molecular mass, 39318 and 42506 Da, respectively.

By carrying out individual alignments of the deduced amino acid sequences of these proteins, the percentage of overall identity was calculated according to the algorithm of Needleman & Wunsch (1970). The homology between the two bacterial PEs was determined to be about 30%. Surprisingly, the homology between the bacterial enzymes and the tomato PE was in the same range. The highest homology score was found between the *E. chrysanthemi* PE and the tomato PE, about 32% identity, which corresponds to data published recently by Hinton *et al.* (1990). Good homologies were also identified between the deduced amino acid sequences of a tomato polygalacturonase and two bacterial polygalacturonases, the *pglA* gene product of *P. solanacearum* AW (Huang & Schell, 1990), and the *peh* gene product of *Erwinia carotovora* (Hinton *et al.*, 1990).

A three-way comparison between the amino acid sequences of all three PE polypeptides is shown in Fig. 5. Short stretches of highly conserved amino acids occur at several positions. Since some of these regions are well conserved in the prokaryotic and the eukaryotic enzymes, an implication of essential structures (enzymically active sites) of the PE molecules may be considered. However, biochemical indications about functional domains in PEs have not been experimentally determined. In Fig. 6 the relative spacings of the well-

Sau3AI

GATCGGC ACG ATG GAA AAA CTG ACG CCC GCT TGC CCG GAC ATC GGC CAT CCG GCT TGT AGA
 CCG AAG ACC ACC CGC ATA TAT TGC CGA AGC GCT TTT GAA CGA TTA TCA AAG TTG AAT CGG
 CAA TAA TCG TGC GCG CAA TCC GGT CCA AAT GAA CGC GAT CCT TAT AAT GGC GCT CCC GGC
 CAA TGT CCG CGC AGC GCC GCC CAA TTC CAT CAG TTG CAT ATT CCT CTA AAA AAC AAA ACC
 GCC TGA CAT OGG ATC AGG CCT GCC TTT GAC AGG AAA AAT CGA CAC Met Gln Ser Thr Thr
 CTG TAT TTG AAG ACC GCC GCG TTC CTG GGA GGG TGC TCC CTG TTT GCC GCC Thr ACA ACC
 Ala Ala Thr Ser Thr Ala Thr Arg Pro Gln Leu Ser Asn Ala Asp Ala Arg Ala Tyr Thr
 GCC GCG ACC AGC ACC GCC ACA CCG CCG CAA CTC AGC AAC GCC GAC GCA CGG GCC TAT ACC
 Ile Ala Ser Tyr Met Ala Ser Phe Gly Thr Ile Gly Ser Leu Thr Thr Asp Asn Trp Asp
 ATC GCC AGC TAC ATG GCG AGC TTC GGA ACG ATC GGC TCG CTG ACC ACC GAC AAC TGG GAT
 Pro Thr Gly Gly Val Gly Ala Val Ser Gly Phe Arg Ala Asn Tyr Ala Val Ala Trp Asp
 CCC ACC GGC GGC GTC GCG GCG GTC AGC GGC TTC CGG GCC AAC TAT GCG GTC GCC GCG GAC
 Gly Ser Ala Gln Tyr Lys Thr Val Gln Ala Ala Ile Asp Ala Ala Val Ala Asp Gly Gly
 GGC TCG GCG CAG TAC AAG ACG GTG CAG GCC GCG ATC GAC GCG GCC GTC GCC GAC GGC GGC
 Val Ala Arg Lys Tyr Ile Ser Val Lys Ala Gly Thr Tyr Asn Glu Leu Val Cys Val Pro
 GTG GCG CGC AAG TAC ATC AGC GTG AAG GCC GGC ACC TAC AAC GAA CTG GTC TGC GTG GTG CCC
 Glu Ser Ala Pro Pro Ile Thr Leu Tyr Ser Leu Asp Ala Asn Ala Asn Asn Thr Val Ile
 GAG TCG GCG CCC CCC ATC ACG CTG TAT AGC CTC GAT GCC AAC GCC AAC AAC ACG GTG ATC
 Val Tyr Asn Asn Ala Asn Pro Thr Pro Ala Ser Gly Ala Lys Thr Asn Pro Cys Met Gly
 GTC TAC AAC AAC GCC AAC CCG ACG CCC GCT TCA GGC GCA AAG ACG ACC CCC TGC ATG GGC
 Thr Ser Ser Asn Ala Thr Val Gly Thr Val Arg Ser Ala Thr Ala Met Val Arg Ala Ser
 ACC AGC AGC AAC GCG ACG GTC GGC ACG GTG CGC AGC GCC ACC GCC ATG GTG CGG GCA TCG
 Asn Phe Asn Ala Arg Asn Leu Thr Phe Lys Asn Ser Tyr Val Glu Gly Thr Phe Ala Asp
 AAC TTC AAC GCC CGC AAC CTG ACG TTC AAG AAC AGC TAC GTG GAA GGC ACC TTC GCC GAC
 Asn Asn Gln Ser Ala Val Ala Leu Ala Val Arg Gly Asp Lys Ala Ile Leu Glu Asn Val
 AAC AAC CAG TCC GCG GTG GCG CTT GCC GTG CGC GGC GAC AAG GCG ATC CTC GAA AAC GTC
 Ser Val Ile Gly Asn Gln Asp Thr Leu Tyr Leu Gly Ala Thr Asn Asn Thr Met Val Ile
 TCG GTC ATC GGC AAC CAG GAC ACG CTG TAT CTC GGC ACG AAC AAC ACC ATG GTG ATC
 Arg Ala Tyr Phe Lys Asn Ser Phe Ile Gln Gly Asp Thr Asp Phe Ile Phe Gly Ala Gly
 CGC GCC TAC TTC AAG AAC AGC TTC ATC CAG GGG GAT ACG GAC TTC ATC TTC GGT GCC GGT
 Thr Ala Val Phe His Gly Cys Thr Ile Gln Tyr Thr Ala Ala Arg Leu Gly Ala Arg Ala
 ACC GCG GTC TTC CAC GGC TGC ACG ATC CAG TAC ACA GCC GCG CGC CTC GGC GCG CGC GCC
 Thr Ser Tyr Val Phe Ala Pro Ser Thr Ala Pro Asp Asn Pro His Gly Phe Leu Ala Ile
 ACC AGC TAC GTC TTC GCA CCG AGC ACG GCG AAC GCT TCG AAC AAC AGC ACG CAT GGC TTC CTC GCC ATC
 Asn Ser Thr Phe Asn Ala Thr Gly Asn Ala Ser Asn Asn Ser Thr His Leu Gly Arg Ala
 AAC AGC ACG TTC AAC GCG ACC GGA AAC GCT TCG AAC AAC AGC ACG CAT CTG GGC CGC GCG
 Trp Asp Gln Gly Val Ser Gly Thr Ser Ala Tyr Ile Asn Gly Ser Ser Pro Asn Gly Gln
 TGG GAT CAG GGC GTG AGC GGC ACG TCG GCC TAT ATC AAC GGC AGT TCG CCC AAC GGC CAG
 Val Val Ile Arg Asp Ser Ser Leu Gly Ala His Ile Arg Leu Ala Asp Pro Trp Gly Pro
 GTG GTG ATC CGC GAT TCG TCG CTC GGC GCG CAT ATC CGG CTG GCC GAT CCG TGG GGC CCC
 Ser Thr Ala Gly Arg Pro Tyr Cys Ser Ser Lys Cys Ala Tyr Ser Ala Asn Arg Phe Phe
 TCG ACG GCG GGC GCG CCG TAT TGT TCG TCC AAG TGT GCG TAT TCG GCC AAT CCG TTC TTT
 Glu Tyr Asn Asn Thr Gly Ala Gly Ser Gly Asn *** GTG AGG TTG CCG TAG CCA TCG CCG
 GAG TAC AAC AAC ACG GGC GCG GGC AGC GGC AAC TGA GTG AGG TTG CCG TAG CCA TCG CCG
 GGA TGG GCC GCC CCT TGC AGG GGC CAT CGG CCC ATC CTG CCC GAT GAT CCG GCC GGC GGC
 TCA AGC GCG CTT TCT GGT GAA GCG CGC GGA ACG CTC CCC CGA AGG CTG GCT CAT CGT CTC
 CTG ATT CAT CCG GTC GAG CAC ACG CTT GGC CCG GTC CGT GCA ATC TTC GCC TTC CGC CTT
 GGT CGC AGC TCA TCG AT
 1698

EcPE	MLKT ..ISGTL... ALS.LIIAAS V.....
PsPE	MQSTTLYLKT AFLGGCSLF AATALAATST ATRPQLSNAD ARAYTIASYM ASFGTIGSLT TDNWD.....
LePE	MIN GTNLDELISR AKVALAMLAS VTTPN..... ..DEVLR PGLGKMPSWV S.SRDRKLME
<div> <div>region I</div> <div>100</div> </div>	
EcPE	...HQAQAAT TYNNAVSKSS SDGKTFKTTA DAIASAPA.G S.TPFVILIK NGVYNERLTI T....RNNLL
PsPE	.PTGGVGAVS GFRANYAVAA DGSAQYKTQV AAIDAADVG GVARKYISVK AGTYNELVCV PESAPPITLY
LePE	SSGKDIGA.. ..NAVVAKDQ TG..KYRTLA EAVAAAP.DK SKTRYVIYVK RGTYKENVEV ..SSRKMNL
<div> <div>200</div> </div>	
EcPE	.LKGESRNGA VIAAAT...A AGTLKSDGSK WGTA.GSSTI T.....I SAKDFSAQSL TIRNDFDFPA
PsPE	SLDANA.NNT VIVYNNANPT PASGAKTNPC M.GTSSNATV GTVRSATAMV RASNFNARNL TFKNSYVEGT
LePE	II.GDGMAT IIT.GSLNVV DGS..... ..TTFHSATL AAV..... .GKGFILQDI CION....TA
<div> <div>region II</div> <div>region III</div> </div>	
EcPE	NQAKSDSDSS KIKDTQAVAL YVTKSGDRAY FKDVSLVGQY DTLVYSGG... ..RSFFSD CRISGTVDFI
PsPE	FADNNQS... ..AVAL AV..RGDKAI LENVSVIGNQ DTLYLGTNN TMVIRAYFKN SFIQGDITDFI
LePE	GPAK..... ..HQAVAL RV..GADKSV INRCRIDAYQ DTLYHSQ... ..RQFYQS SYVTGTIDFI
<div> <div>region IV</div> <div>300</div> </div>	
EcPE	FGDGTALFNN C..DL.VSRY RADVKSGNVS GYLTPS.TN INQKYGLVIT NSRVIRESDS VP.AKSYG..
PsPE	FGAGTAVFHG CTIQYTAARL GAR.ATSYV. ...FAPS.TA PDNPHGFLAI NSTF....NA TGNASNNSTH
LePE	FGNAAVVFQK C..QL.VARK PGKYQQNMV. ...TAQGRID PNQATGTSIQ FCDIIASPD LKPVWKEPTY
<div> <div>region V</div> <div>400</div> </div>	
EcPE	LGRPWHPTTT FSDGRYADPN AIGQTVFLNT SMDNHI...Y GWDKMSG... ..KDKNGN TIWFPEDSR
PsPE	LGRAWDQGV SGT SAYINGSS PNGQVVRDS SLGAHRLAD PWGPSTAGRP YCSSKCAISA NRFF.....
LePE	LGRPWK... ..YSRTVMES SLGG...LID PSG..... WAEW...HGD ...FALKTLY
<div> <div>region VI</div> </div>	
EcPE	FFEYKSYGAG AAVSK..... DRRQLTD.AQ AAETQSKVL GD....WTPT LP.....
PsPE	..EYNNIGAG SGN.....
LePE	YGEFMNNGPG AGTSKRVKWP GYHVITPAE AMSFTVAKLI QGGSWLRSTD VAYVDGLYDY SDIKLLFVYV
EcPE	494
PsPE	
LePE	TRHL

Fig. 5. Comparison of the deduced amino acid sequences of the PEs of *P. solanacearum* (PsPE), *E. chrysanthemi* (EcPE; Plastow, 1988) and *L. esculentum* (LePE; Ray *et al.*, 1988). Identical residues are indicated by asterisks. Regions of high homology are boxed and numbered (I to VI).

Fig. 4. Nucleotide sequence of the 1.7 kb *Sau3AI*-*ClaI* *P. solanacearum* DNA fragment containing the *pme* coding region. Nucleotides are numbered starting from the *Sau3AI* site at the top. A Shine-Dalgarno (S/D) box and regions showing homology to the -35 and -10 consensus of *E. coli* σ^{70} promoters are indicated. The stop codon is marked by three asterisks. Palindromic sequences in the 3' non-coding region are indicated by arrows. The deduced amino acid sequence is shown along with selected endonuclease restriction sites.

Fig. 6. Relative positions of the highly homologous regions derived from a three-way comparison of the amino acid sequences shown in Fig. 5. Sizes and relative positions of regions I to VI and the amino- and carboxy-termini of each PE enzyme are indicated.

Table 1. Relative distribution of enzyme activities in subcellular fractions of *E. coli* cells

Stationary *E. coli* cultures were fractionated and assayed for PE, β -lactamase and CAT activity as described in Methods. Enzyme activities are expressed as a percentage of the total activity of the culture. Sup., supernatant; TSE, Tris/sucrose/EDTA fraction; Per., periplasmic fraction; Cyt., cytoplasmic fraction; ND, not detectable.

	PE				β -Lactamase				CAT			
	Sup.	TSE	Per.	Cyt.	Sup.	TSE	Per.	Cyt.	Sup.	TSE	Per.	Cyt.
<i>E. coli</i> C600(pGST1)	17.6	1.0	ND	81.4	35.9	2.9	54.3	6.9	0.2	1.5	0.2	98.1
<i>E. coli</i> C600(pSAP1)	30.1	1.2	ND	68.7	35.3	4.2	56.9	3.6	—	—	—	—
<i>E. coli</i> C600(pBR325)	—	—	—	—	28.9	1.3	57.1	12.7	0.1	0.3	0.1	99.5

conserved regions are compared. It is apparent that the relative positions of all conserved regions are very similar with the bacterial PE enzymes but less stringent with the tomato enzyme. Nevertheless, the spacings of the internal regions from III to IV (21–24 amino acids), and of the carboxy-terminal regions from V to VI (19–23 amino acids) respectively, seem to be well conserved in all the proteins.

Localization of PE activity in *E. coli* clones

The ability of *E. coli* clones to degrade pectin on pectin agar plates indicated that PE is present extracellularly. To distinguish between partial leakage, cell lysis and active secretion, we performed a more extensive analysis of the compartmentalization of PE in *E. coli*. For this purpose pGST1 was constructed. This plasmid contains the *pme* gene as well as the genes for β -lactamase and chloramphenicol acetyltransferase (CAT) as periplasmic and intracellular marker enzymes. *E. coli* clones harbouring pGST1, pSAP1 and pBR325 were grown in glycerol medium to the stationary phase ($OD_{550} = 2.0$) and fractionated as described in Methods. All fractions were assayed for PE activity and for activities of the marker

enzymes β -lactamase (periplasmic) and CAT (cytoplasmic). The results are summarized in Table 1. Almost all of the CAT activity remained in the cytoplasm, indicating that cell lysis does not occur. However, the detection of a significant amount of β -lactamase activity in the supernatants of all *E. coli* clones investigated provides evidence for (PE-independent) periplasmic leakage. PE activity was mainly found in the cytoplasm and in the supernatant, whereas no PE activity could be detected in the periplasm.

Periplasmic leakage has often been described for extracellular enzymes, e.g. *Erwinia* pectinases, which have been cloned and expressed in *Escherichia coli*. Due to correct recognition and processing of a functional signal peptide by the *E. coli* secretion system the majority of the enzyme accumulates in the periplasmic space and is further released, together with periplasmic marker enzymes, into the medium (Keen *et al.*, 1984; Collmer *et al.*, 1985). SDS-PAGE of cell lysates and culture supernatants of PE-positive *E. coli* clones and experiments using the T7 *in vivo*-labelling system provided no evidence that processing of the PE protein occurs in *E. coli* cells. In accordance with these observations the first few residues of the predicted amino acid sequence do not

comply with the 'minimal requirements' for signal peptides postulated by Von Heijne (1985).

Considering our results, the appearance of larger amounts of PE activity in culture supernatants could not be attributed to simple outer-membrane leakage. The lack of PE activity in the periplasmic fraction might indicate a different mechanism for transporting PE out of the cell. As the secretion of the PE protein would be a desired option for its biotechnological production, further investigations on overproduction and secretion of the PE are in progress.

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