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Characterization of a Novel Pectate Lyase from *Erwinia carotovora* subsp. *carotovora*

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The pectate lyase (Pel, EC 4.2.2.2) isoenzyme profile of *Erwinia carotovora* subsp. *carotovora* was characterized by isoelectric focusing, and the corresponding genes coding for four different exported Pels were cloned. The nucleotide sequence of the *pelB* gene encoding one of these isoenzymes was determined and was shown to contain 1,040-bp open reading frame coding for a 37,482-Da protein with a putative cleavable amino terminal signal peptide. Overexpression and selective labeling experiments with the *pelB* clone demonstrated the synthesis of a 35-kDa polypeptide, which is in accordance with the deduced size of the processed PelB. The predicted amino acid sequence of PelB was very similar to that of Pel-3 of another *E. c.* subsp. *carotovora* strain 71, but showed no similarity to other previously characterized pectinolytic enzymes. The *pelB* gene is located next to the previously characterized *pehA* gene encoding an endopolygalacturonase. The two genes are divergently transcribed from a common control region and are subject to similar global regulation by the central virulence regulator *expI*. Inactivation of *pelB* did not appear to reduce the virulence of the mutant strain, suggesting that *pelB* does not have a major role in pathogenicity. Unlike other Pels, PelB required partially methyl esterified pectin as substrate suggesting that PelB represents a novel isoform of pectate lyase.

Additional keywords: Erwinia autoinducer (EAI), global regulation.

Soft-rotting *Erwinia* species are enterobacteria capable of infecting many different plant species, including several economically important crops. These bacteria produce a variety of extracellular enzymes that are able to degrade plant cell wall polymers and thereby macerate the parenchymatous tissues of the host plant (Pérombelon and Kelman 1980). The production and secretion of particularly pectinolytic enzymes appears to be vital for the virulence of both *E. carotovora* and *E. chrysanthemi*. This is demonstrated by isolation of avirulent mutants that are pleiotropically affected in either extracellular enzyme production (*exp*, *aep*, and *rex* mutants) (Pirhonen et al. 1991, 1993; Murata et al. 1991; and Jones et al. 1993, respectively) or secretion of the enzymes to the external milieu (*out* mutants) (Andro et al. 1984; Thurn and Chatterjee 1985; Ji et al. 1987; Pirhonen et al. 1991). The *expI* and *rex* mutants have been shown to lack a freely diffusible signal molecule, the *Erwinia* autoinducer (EAI), which is normally required for coordinate activation of extracellular enzyme encoding genes and hence virulence (Pirhonen et al. 1993; Jones et al. 1993).

Pectinolytic enzymes seem to constitute the most important group of extracellular enzymes produced by soft-rotting *Erwinia* species. These bacteria produce both hydrolytic depolymerizing pectinolytic enzymes, which cleave the α-1,4-glycosidic bond in the substrate by hydrolysis (exo- and endo-polygalacturonases) and lyases which cleave by the β-elimination (pectate and pectin lyases) (Collmer and Keen 1986). The different *Erwinia* species appear to harbor a limited number of hydrolases, e.g., *E. carotovora* characteristically produces one major endopolygalacturonase, while *E. chrysanthemi* seems to produce either a single exopolygalacturonase or no hydrolytic enzymes at all (Collmer and Keen 1986). In contrast, both *E. carotovora* and *E. chrysanthemi* produce several different isoforms of pectate lyases, which seem to be encoded by independently expressed genes (Collmer et al. 1985; Collmer and Keen 1986; Willis et al. 1987; Hinton et al. 1989). The presence of these different Pel isoforms has been suggested to provide the bacteria the ability to infect many different plant species and tissues (Beaulieu et al. 1993). Accordingly, the Pels have been proposed to be essential for pathogenicity of *E. chrysanthemi* (Collmer and Keen 1986). The role of particular Pels in the bacterial virulence has been studied by creating directed mutations in the individual *pel* genes and assessing the virulence of these mutants. Inactivation of single (Boccara et al. 1988) or multiple *pel* genes (Ried and Collmer 1988; Beaulieu et al. 1993; Kelemu and Collmer 1993) leads to reduced virulence. However, even inactivation of all the known *pel* genes of *E. chrysanthemi* did not totally abolish the virulence and recent work by Kelemu and Collmer (1993) suggests the presence of additional Pel isoforms.

Many of the *pel* genes have been cloned and characterized (Keen and Tamaki 1986; Lei et al. 1987; Tamaki et al. 1988;

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Trollinger et al. 1989; Hinton et al. 1989; Yoshida et al. 1991; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992; Favey et al. 1992). Hinton et al. (1989) have described four Pel isoenzymes from *E. carotovora* subsp. *carotovora* SCRI193 (*E. c.* subsp. *carotovora*) strain. Two of these isoenzymes were shown to be extracellular while two appeared to be periplasmic. No periplasmic Pels have been described from *E. chrysanthemi*. Hinton et al. (1989) grouped all published Pels into three distinct families based on their sequence similarity (PLbc, PLade, and periplasmic PLs). The members of each family show extensive amino acid identity (70 to 90%), while only limited identity is exhibited between the families, with the periplasmic PL family being the most distinct. However, even the periplasmic enzymes still share significant similarity with other Pels. Yoder et al. (1993a) recently determined the three-dimensional structure of *E. chrysanthemi* PelC belonging to PLbc family and found a novel motif of parallel β-strands stabilized by amino acid side chain stacks. A similar structure was found in *E. chrysanthemi* PelE, that belongs to different Pel family (Yoder et al. 1993b). Yoder et al. (1993a) also suggested a location for a putative Ca^{2+} binding site in the active site of the enzyme, in accordance with the absolute requirement of Ca^{2+} ions for *Erwinia* Pel activity (Zink and Chatterjee 1985; Willis et al. 1987).

We have characterized the Pel profile from *E. c.* subsp. *carotovora* strain SCC3193 by isoelectric focusing (IEF) and found four extracellular Pel isoenzymes. We describe the molecular cloning and the nucleotide sequence of *pelB* gene. The *pelB* gene was found to be closely linked but divergently transcribed in relation to an endopolygalacturonase gene (*pehA*) (Saarilahti et al. 1990). We show that *pelB* appears to encode a new type of pectate lyase that is highly similar to the Pel-3 pectate lyase recently characterized from a related *E. c.* subsp. *carotovora* strain 71 (Liu et al. 1994) but has no significant similarity with any other Pel proteins. In contrast to other Pels, PelB appears to preferentially utilize partially methoxylated pectin as substrate.

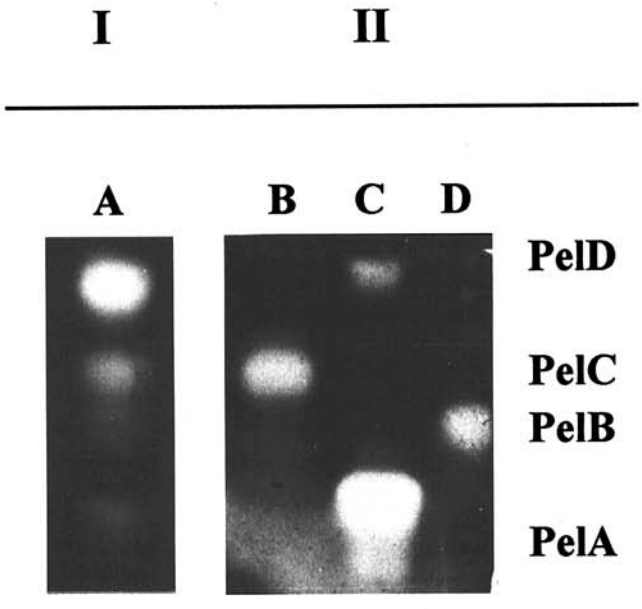

Fig. 1. The pectate lyase profile of *Erwinia carotovora* subsp. *carotovora* wild-type strain (SCC3193) and the activities coded by individual clones carried by *E. coli*. The proteins were separated by isoelectric focusing (IEF) and the localization of different Pel isoenzymes was visualized by activity stain overlay. The samples were: **A,** 20 µl of the culture filtrate prepared from overnight grown SCC3193 and the periplasmic proteins isolated from *E. coli* carrying **B,** pHSK17; **C,** pHSK20; and **D,** pMUT4:4 encoding PelC, Pels A and D, and PelB isoenzymes, respectively.

RESULTS

Characterization of the Pel isoenzyme profile by isoelectric focusing (IEF) and cloning of the Pel encoding genes.

The wild-type *E. c.* subsp. *carotovora* strain SCC3193 (Pirhonen et al. 1988) was analyzed for the production of different Pel isoenzymes. The culture supernatant was shown to contain four bands in IEF corresponding to basic Pel isoenzymes, PelD, PelC, PelB, and PelA (Fig. 1). Of these PelD and PelC appeared to be the most dominant and PelB a minor isoform as detected by IEF from culture supernatants of in vitro grown cells. We did not detect any neutral or acidic Pel isoforms from this strain. The genes corresponding to these different Pel isoenzymes were cloned from a plasmid library by screening for the enzyme activity on indicator plates. Characterization of the gene products by IEF showed that two of the Pel encoding genes (*pelA* and *pelD*) were carried by the same clone (pHSK20), while the gene corresponding to the PelC isoenzyme was carried on a separate clone (pHSK17) (Fig. 1). The PelB isoenzyme encoding gene was carried by a clone (pMUT4:0) together with *pehA* gene (Figs. 1 and 3A). The genomic fragment harboring *pelB* and *pehA* genes was subcloned as two separate plasmids pMUT4:4 (*pelB*) and pMUT4:3 (*pehA*). The activity of the PelB isoenzyme was visible only when 30% methoxylated pectin (Fig. 1) instead of PGA was used as the substrate in the agarose overlay (data not shown), suggesting that PelB represents a new type of Pel and prompted us to characterize PelB.

Characterization of the *pelB* gene product.

The *pelB* gene carried by a pMUT4:4 deletion derivative pRK8-2 was used to identify the *pelB* gene product using the

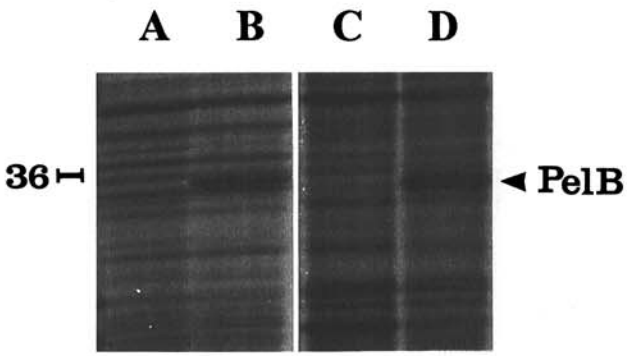

Fig. 2. Overexpression and selective labeling of *pelB* gene product by phage T7-expression system in *E. coli*. The proteins were separated by SDS-polyacrylamide (12%) gel and visualized by Coomassie blue staining (lanes A and B) or by fluorography (lanes C and D). The samples were: **A,** 20 µl of sonicates from DH5α/pBluescript II; **B,** DH5α /pRK8-2 and 20 µl of total proteins labeled with [^{35}S]-methionine, **C,** C600/pRK8-2 uninduced cells; and **D,** C600/pRK8-2 induced cells. The molecular weight marker (in kDa) is shown on the left.

phage T7 expression system (Tabor 1990). The total proteins from *Escherichia coli* DH5α carrying pRK8-2 plasmid or the control plasmid pBluescript II were characterized by SDS-PAGE (Fig. 2). Visualization of the separated proteins both by Coomassie blue staining of the gel and by selective labeling of pRK8-2 encoded proteins by [^{35}S] methionine followed by fluorography of the gels demonstrated the presence of 35-kDa protein (Fig. 2). The lack of this protein in similarly treated control samples from pBluescript II carrying cells or noninduced labeled cells carrying pRK8-2 suggested that the 35-kDa polypeptide is indeed the *pelB* gene product.

Determination of the *pelB* nucleotide sequence.

To characterize the *pelB* clone in more detail, we determined the nucleotide sequence of the 2.3-kb genomic fragment from pRK8-2 harboring the *pelB* gene (Figs. 3A and B). The sequence of both strands was determined by the Sanger dideoxy-chain termination method (Sanger et al. 1977). This fragment was shown to contain one 1,040-bp long open reading frame (ORF) between bases 277 and 1317 with a putative ribosome binding site (AGGA) located 11 bases upstream of the translation initiation codon at position 266. The translation stop codon (TAA) at position 1318 was followed by a putative transcription termination loop centered between positions 1383 and 1384. The loop was followed by some T residues suggesting a Rho-independent type of transcription termination (Fig. 3B). Translation of the *pelB* ORF results in a 347-aa polypeptide with a calculated molecular weight of 37,482. The hydropathy index of the amino terminal end of PelB was computed with the SOAP program (PC/GENE, IntelliGenetics, Mountain View, California). The N-terminal 21 aa had the characteristics of a potential prokaryotic signal peptide with one positive charge at position –19 (K) in the N-region, seven hydrophobic residues, although several times interrupted (Y-L-T-P-I-F-L-C-T-A-A-F-S-F), in the central H-region and two polar residues in the C-region ending with a characteristic consensus cleavage site (A-Q-A) (von Heijne 1985; 1987) (Fig. 3B). The predicted molecular weight of the processed PelB protein is 35,051, which is in good agreement with the observed size of the PelB polypeptide (Fig. 2).

***pelB* is transcribed divergently from the endopolygalacturonase gene (*pehA*).**

As discussed above the *pelB* gene was contained within the same genomic fragment in pMUT4:0 as the endopolygalacturonase encoding gene *pehA* described by Saarilahti et al. (1990) (Fig. 3A). Characterization of the nucleotide sequence of the region between *pelB* and *pehA* genes demonstrated that these two genes are divergently transcribed (Figs. 3A and 4). The transcriptional start sites for both genes were determined by primer extension analysis using RNA isolated from SCC3193 cells grown in L-medium (Fig. 4). The extension reaction was terminated sharply for *pelB* between G and C residues at 75 and 76 bp upstream from the PelB translation initiation codon (Fig. 4). No other significant start sites were detected. Analysis of the nucleotide sequence upstream from the detected transcription start site for *pelB* revealed the presence of a putative sigma 70 type promoter sequence with a reasonable –10 box (TATATA) and a poor –35 box (CCCTGAAT) positioned at –14 and –46, respectively (Fig. 4). For *pehA* the termination of the extension reaction was between C and G residues at 190 and 191 bp upstream from the translation initiation codon (Fig. 4). Few additional shorter extension products were also detected indicating instability of *pehA* specific mRNA, but the band described above was repeatedly the strongest (data not shown). When the sequence upstream from the putative transcription start site for *pehA* was studied, typical sequences for sigma 70 type promoter with a –10 region (TAATTT) and a –35 region (TGTTGATGT) at –13 and –42, respectively, were found (Fig. 4).

The *pelB* and *pehA* genes exhibit both coordinate global and differential gene specific regulation.

We have previously demonstrated that the expression of several extracellular enzyme encoding genes including *celVI* coding for a major cellulase, *pelC* coding for a pectate lyase

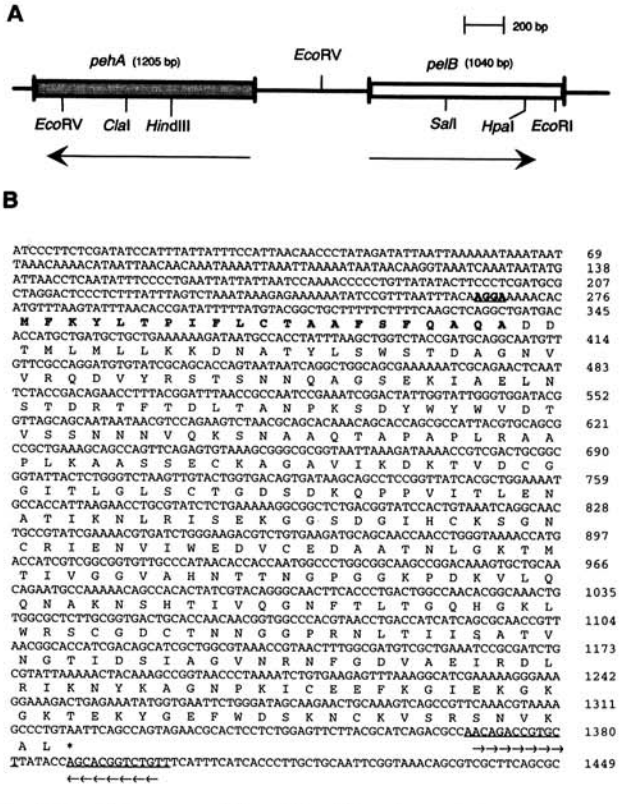

Fig. 3. Restriction map of the *pehA-pelB* region and the nucleotide and deduced amino acid sequence of *pelB*. **A,** *pehA* and *pelB* open reading frames have been marked by differentially shaded boxes and the sizes of the ORFs are indicated in parenthesis. The arrows indicate the direction of transcription. The nucleotide sequence of the whole genomic fragment shown has been determined. The sequence of *pehA* has been determined by Saarilahti et al. (1990). The restriction enzyme recognition and cleavage sites that were used in subcloning of pRK8 have been marked. **B,** The determined nucleotide sequence of *pelB* gene with numbers on the right referring to the nucleotide sequence. The putative ribosome binding site is marked with bold underlined letters and the putative transcription termination loop is underlined and marked with arrows. The deduced amino acid sequence is shown under the nucleotide sequence. The translation termination codon is indicated by an asterisk. The putative NH_2-terminal signal peptide is shown in bold. The nucleotide sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Database under the accession number X79232.

and *pehA* is under global control by *expI* (Pirhonen et al. 1993). To determine whether the expression of *pelB* was also subject to this global control, we characterized *pelB* expression in the avirulent *expI* mutant (SCC3065) (Fig. 5A). The results show that the *pelB* gene is not expressed in the *expI* mutant, demonstrating similar global control of *pelB* expression as previously described for *pehA, pelC,* and *celV1* (Pirhonen et al. 1993). However, the expression of *pelB* and *pehA* appears to be also independently regulated: Comparison of the expression of these genes in wild-type (SCC3193), *pelB* mutant (SCC5001), and *pehA* mutant (SCC4188) showed that inactivation of one of the genes did not markedly affect the expression of the other (Fig. 5B). Saarilahti et al. (1992) have previously demonstrated that *pehA* gene expression requires a functional positive regulator, PehR. Characterization of *pelB* expression, in the *pehR* mutant (SCC1935) showed that *pelB* expression was not reduced (data not shown). These data indicate that the two genes are also differentially regulated.

Function of PelB.

Several of the pectate lyases have been shown to have a role in virulence of *Erwinia* (Boccara *et al.* 1988; Ried and Collmer 1988; Beaulieu et al. 1993). Therefore, we wanted to determine the contribution of PelB for the virulence of SCC3193. To this aim we constructed a strain (SCC5001) in which the coding region of the *pelB* gene was interrupted by an interposon carrying the Cm resistance marker (Fig. 6A). To verify that *pelB* was interrupted by the interposon we characterized the organization of the *pelB* gene both in the SCC5001 mutant and in the wild-type strain SCC3193 by Southern hybridization (Fig. 6B). The results of the analysis confirmed that the *pelB* was indeed inactivated. The virulence of this marker exchange mutant (SCC5001) was compared to that of the wild-type strain (SCC3193) using several different test systems. We assessed the virulence of these strains on axenic tobacco seedlings grown with or without 10 mM $CaCl_2$, on stems of greenhouse grown potatoes and on potato tuber slices. We could not detect differences in any of the virulence tests (data not shown), demonstrating that under these test conditions the virulence of the PelB$^-$ mutant SCC5001 was similar to that of the wild-type strain. This suggests that the PelB protein does not play a major role in the virulence of *E. c.* subsp. *carotovora.*

Previously characterized Pels have been grouped into three distinct families on basis of their sequence similarities (Hinton et al. 1989). To determine whether PelB was related to these pectate lyase families or other pectinolytic enzymes, the predicted protein sequence of PelB was aligned with all other known pectinolytic enzyme sequences (PALIGN or CLUSTAL, PC/GENE, IntelliGenetics, Inc., Mountain View, California). We did not find any significant similarity between PelB and the previously described Pel families, suggesting that PelB might represent a novel type of pectate lyase. Recently, Liu et al. (1994) described a novel Pel from another strain of *E. c.* subsp. *carotovora.* Alignement of PelB with this new Pel, demonstrated that the two polypeptides were very similar. Comparison of the predicted amino acid sequences of PelB and Pel-3 revealed 93% aa identity between these two polypeptides. This similarity was not restricted to

Fig. 4. The nucleotide sequence of the *pelB* and *pehA* control region with the transcriptional start sites. The transcription start sites and the direction of transcription have been marked by arrows. The putative –10, –35 regions, and the putative ribosome binding sites have been underlined. The translation initiation codons and the direction of translation for both polypeptides are indicated by arrows. The star indicates the first nucleotide of the sequence presented in Figure 3B.

certain domains but was found throughout the entire length of the polypeptides. The lack of similarity of PelB and Pel-3 with previously characterized Pels or other pectinolytic enzymes clearly indicate that PelB and Pel-3 represent the members of a new Pel family.

To get more insight into the funtion of PelB, we characterized the enzymatic properties and substrate specificity of this enzyme (Fig. 7A and B). We had originally observed that the PelB isoenzyme was detectable in IEF when 30% methoxylated pectin but not when PGA was used as the substrate in agarose overlays. Using this pectin as substrate we characterized the pH optimum for a PelB crude enzyme preparation. PelB was not active at acidic or neutral pH. Detectable PelB activity was observed at pH 8.5 and maximal activity was reached at pH 9.5 (data not shown). This optimal pH was used in the following experiments. All *Erwinia* pectate lyases are reported to require Ca^{2+} ions for their activity (Zink and Chatterjee 1985; Willis et al. 1987). We characterized the optimal Ca^{2+} concentration for PelB by assaying the activity using 30% methoxylated pectin as substrate (Fig. 7A). In this analysis we used PelC which is more closely related to the PLade and PLcd families (Hinton et al. 1989) as a reference.

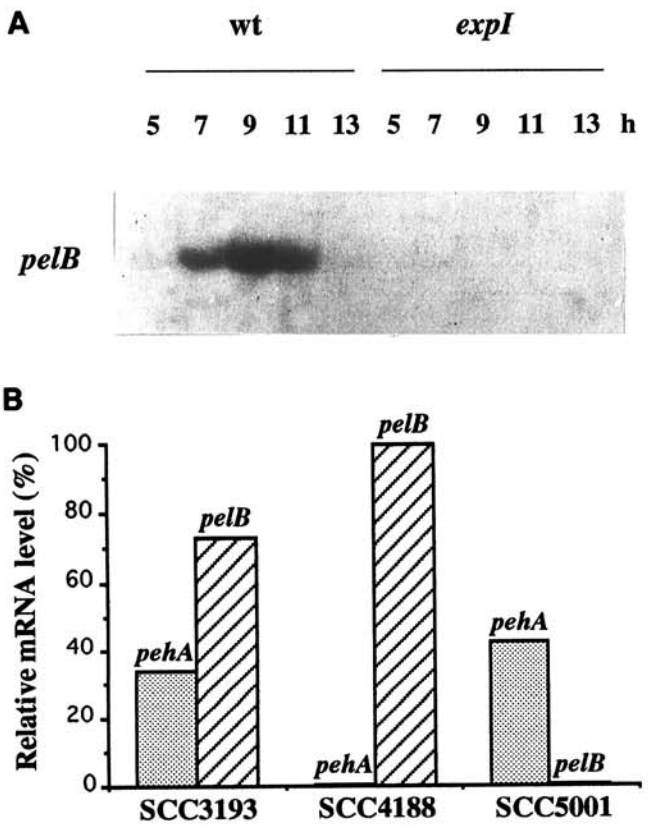

Fig. 5. Northern analysis of the *pelB* expression in *expI* mutant (SCC3065) and *pelB* and *pehA* expression in wild-type (SCC3193), *pehA* mutant (SCC4188), and *pelB* mutant (SCC5001). **A,** The accumulation of mRNA was followed during the growth and the samples were collected at indicated time points. The mRNA hybridizing to *pelB* gene is shown. The growth of the cells was as described by Pirhonen et al. (1993). **B,** Total RNA isolated at 11 h of growth after dilution was separated on agarose gel and blotted on nylon membrane. The filter was probed with a specific *SalI-Hin*dIII fragment hybridizing to both *pelB* and *pehA*. The relative amounts of RNA isolated from different backgrounds are shown as differentially shaded bars.

Detectable PelB activity was first observed at 1 mM Ca^{2+} concentration and the maximal activity was observed at 5 mM Ca^{2+} (Fig. 7A). Higher Ca^{2+} concentrations could not be tested as the substrate became insoluble. Some PelC activity was detectable in the absence of added Ca^{2+}, but it was clearly stimulated by Ca^{2+} addition reaching the maximal activity at 5 mM Ca^{2+} concentration (Fig. 7A). The results demonstrate that the requirement for Ca^{2+} ions was more strict for PelB than for PelC.

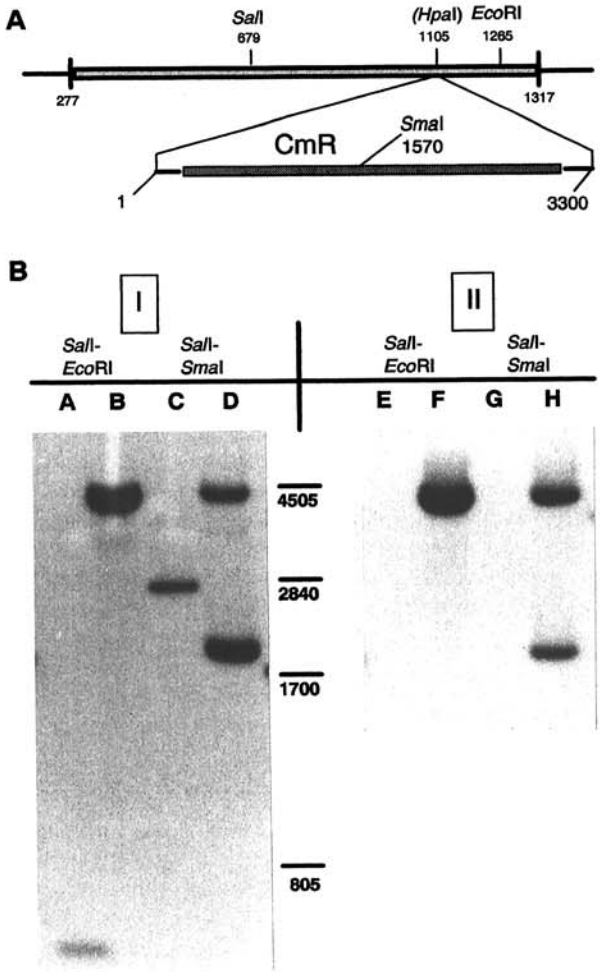

Fig. 6. The localization of the Cm resistance marker gene in *pelB* mutant (SCC5001) and the verification of the *pelB* organization in wild-type (SCC3193) and *pelB* mutant (SCC5001) by Southern analysis. **A,** The *pelB* ORF is shown with relevant restriction sites, *Hpa*I site that is inactivated in SCC5001 is in parenthesis. The numbering of the *pelB* gene is as in Figure 3B. The fragment carrying the Cm^R gene used in creating the SCC5001 marker exchange mutant with relevant *Sma*I site is shown. The region corresponding to the Cm^R gene is shown as a shaded box. **B,** Panel I shows the hybridization patterns of *SalI-Eco*RI digested SCC3193 (lane A), SCC5001 (lane B), and *SalI-Sma*I digested SCC3193 (lane C), SCC5001 (lane D) chromosomal DNA, probed with gene-specific 586 bp *SalI-Eco*RI *pelB* fragment. A single fragment of 586 and 2,600 bp from SCC3193 digests (lanes A and C) and a single 3,886-bp *SalI-Eco*RI fragment (lane B) which is cut in two 1,996 and 3,900 bp fragments in *SalI-Sma*I digest (lane D) of SCC5001 chromosomal DNA are recognized with the *pelB* probe. Panel II shows the hybridization patterns of similar digests, probed with Cm^R gene specific fragment. Lanes E and G show that the Cm^R gene does not hybridize to SCC3193 chromosomal DNA and lanes F and H show that bands identical with lanes B and D are recognized. The molecular size markers in bp are shown between panels I and II.

To characterize the apparent requirement for methoxylated substrate and to test whether the degree of methoxylation was crucial for PelB, we analyzed PelB activity by using pectin with increasing methoxylation degree as substrate (Fig. 7B). The PelB activity was evaluated on PGA as well as on 30, 68, and 93% methyl esterified pectin. PelB was not active on PGA but it was clearly active on all methoxylated substrates, with the maximal PelB activity exhibited on 68% methoxylated pectin (Fig. 7B). This is in clear contrast to PelC, which was most active on PGA and showed reduced activity with methoxylated substrates (Fig. 7B). We also compared PelB activity on 68% methoxylated pectin with activity on 95% methoxylated Link pectin (McMillan et al. 1993) by using the kinetic Pel assay described by Tsuyumu and Chatterjee (1984). The result showed that PelB could even use the 95% methoxylated Link pectin as substrate, although it was clearly more active on 68% methoxylated pectin (data not shown). Thus both types of enzyme assays show that PelB can cleave pectin, but the activity is reduced when a substrate with higher methylation degree is used. Taken together our data demonstrate that PelB is a novel type of pectate lyase, preferring partly methyl esterified pectin as substrate.

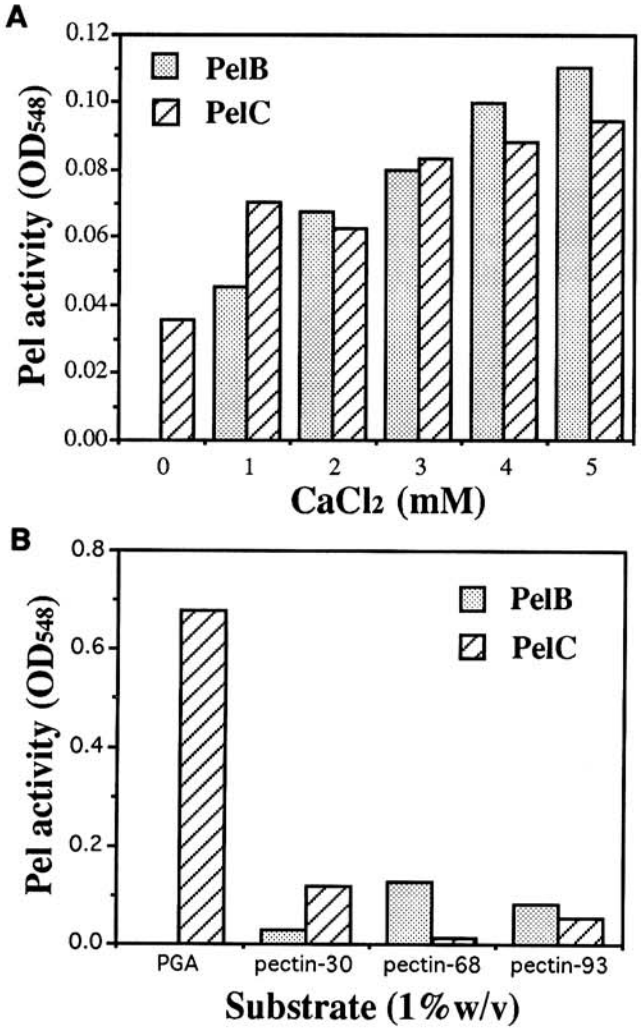

Fig 7. Requirement for Ca^{2+} and substrate specificity of PelB. The enzymatic activity of PelB was characterized as described in detail in materials and methods with PelC as a reference. Equal amounts of the crude enzyme preparations (see Materials and Methods) were added to the assay mixtures. **A,** PelB and PelC activities were assayed at indicated $CaCl^2$ concentrations using pectin-30 as substrate. **B,** PelB and PelC activities were characterized on different substrates at pH 9.5 and 5 mM $CaCl_2$: Polygalacturonic acid (PGA), 30% methyl esterified pectin (pectin-30), 68% methyl esterified pectin (pectin-68), and 93% methyl esterified pectin (pectin-93). The approximate methyl esterification degrees of respective substrates are given by Sigma Chemical Co. PelB and PelC activities were expressed as OD_{548} and indicated as differentially shaded bars.

DISCUSSION

The plant pathogenic enterobacterium *E. c.* subsp. *carotovora* strain SCC3193 was found to produce four extracellular basic pectate lyases (Fig. 1). One of the corresponding genes, *pelB* was found to be carried on the same chromosomal fragment as the previously characterized *pehA* gene coding for an endopolygalacturonase (Saarilahti et al. 1990) (Fig. 3A). These two genes were shown to be closely linked and divergently transcribed from different strands of a common control region. We localized the transcriptional initiation sites for both of these genes (Fig. 4). The primer extension reactions were terminated between G and C residues at 75 and 76 bp (*pelB*) and between C and G residues at 190 and 191 bp (*pehA*) upstream of the translation start codons locating the transcriptional start sites of *pelB* and *pehA* within 302 bp from each other. Both of the transcription start sites were preceded by sequences resembling typical *E. coli* sigma 70 promoters. Expression of both of the *pelB* and *pehA* genes were shown to require a functional *expI* gene (Fig. 5A and Pirhonen et al. 1993). The *expI* gene codes for an autoinducer synthase needed for the production of a small diffusible signal molecule (*Erwinia* autoinducer, EAI) similar to *Vibrio fischeri* autoinducer [*N*-(3-oxohexanoyl) homoserine lactone] (*Vibrio* autoinducer, VAI) (Pirhonen et al. 1993). Accumulation of this autoinducer in cultures of *E. c.* subsp. *carotovora* is responsible for the growth phase-dependent activation of the expression of extracellular enzyme encoding genes. Based on the detected analogy with *V. fischeri lux*-regulation (Pirhonen et al. 1993; Jones et al. 1993), the EAI is suggested to form a complex with a DNA binding protein, which would mediate the effect of the autoinducer by activating the transcription of the target genes. Devine et al. (1989) and Shadel and Baldwin (1992) have analyzed the binding of *V. fischeri* LuxR protein to the regulatory regions of the genes that are controlled by VAI-LuxR complex and have identified a sequence forming the binding site. Fuqua et al. (1994) and Fuqua and Winans (1994) have extended this analysis by aligning several other promoter regions of similarly regulated genes and found a putative consensus sequence forming a loop structure. Since both *pelB* and *pehA* genes are under global *expI* control resembling LuxR-LuxI type of regulation, we analyzed the region between these two genes for secondary structures resembling *lux* boxes. Several loop structures could be found, but none of the sequences matched the consensus sequence. Thus, the binding site for the putative global regulator remains to be demonstrated. In addition to the common *expI*-mediated global control, *pelB* and *pehA* genes seem to be also subject to differential gene-specific regulation. Saarilahti et al. (1992) have characterized a *pehA* specific positive regulator called *pehR*. We studied if the expression of *pelB* was affected in *pehR* mutant (SCC1935) and

found that in this mutant *pelB* expression was not reduced (data not shown). Furthermore, there appears to be a difference in the temporal pattern of expression of *pelB* and *pehA* genes as well as in their responsiveness to external stimuli. We have recently shown (Flego et al., submitted) that in wild type the *pehA* gene is turned on first and continues to be expressed until early stationary phase, while *pelB* is turned on later and reaches the maximum level at early stationary phase when the *pehA* specific mRNA production is already ceasing (c.f., Fig. 5A). Recent work by Flego et al. (in preparation) suggest a differential response of *pelB* and *pehA* expression to Ca^{2+}: Inhibitory for *pehA* and stimulatory for *pelB*. Taken together the expression data for the *pelB* and *pehA* genes demonstrate both global and gene-specific regulation most likely mediated by sequences in the region between these two genes. Some of these sequences might overlap and thereby serve the regulation of both of the genes.

The Pels have been suggested to have a major role in the virulence of the soft-rotting *Erwinia* species. However, as reported by Saarilahti et al. (1992) and Boccara and Chatain (1989) other pectinolytic enzymes such as endopolygalacturonase (PehA) and pectin methylesterase (Pem), respectively, also contribute the virulence of this plant pathogen. Even the involvement of cellulase in virulence of *E. c.* subsp. *carotovora* was recently demonstrated by Walker et al. (1994) and Mäe et al. (submitted). The role of individual Pels in virulence has been characterized by creating targeted mutations into *pel* genes (Boccara et al. 1988; Ried and Collmer 1988; Beaulieu et al. 1993; Kelemu and Collmer 1993). As shown by Boccara et al. (1988) certain isoenzymes are more crucial for the disease development (PLa and PLd), but the inactivation of one or even several *pel* genes still causes at most only a reduced virulent phenotype. Therefore, it was not unexpected that inactivation of *pelB* gene in the marker exchange mutant did not appear to reduce the ability to cause disease on the plants tested. It is possible that the PelB isoenzyme is crucial in conditions when the bacteria are infecting plants that have an increased degree of methyl esterification in the cell wall pectin as PelB appears to prefer this kind of substrate (see below). We used tobacco (*Nicotiana tabacum* 'Samsun') leaves and potato (*Solanum tuberosum* 'Bintje') stems and tubers in our virulence tests. The methylation degree in these tissues is possibly too low to show the difference in maceration capacity between mutant and wild type. Indeed, McMillan et al. (1993) have shown recently that somatic potato hybrids (*Solanum brevidens* × *S. tuberosum* subsp. *tuberosum*) with higher degree of methylation in their cell walls are more resistant towards *E. chrysanthemi*, *E. c.* subsp. *atroseptica,* and *E. c.* subsp. *carotovora* infections. This resistance was clearly shown to correlate with the degree of methyl esterification of pectin in infected tissues. It would be interesting to compare the virulence of the wild type and *pelB* mutant strains on such somatic potato hybrids.

The basic PelB isoenzyme described in this report is clearly a lyase capable of degrading pectin (Figs. 1 and 7B). However, it does not show similarity to the previously described pectinolytic enzymes families including three distinct Pel families characterized by Hinton et al. (1989) but shows extensive similarity to the Pel-3 pectate lyase from a new Pel family (Liu et al. 1994). The extensive similarity of PelB and Pel-3 (93% identical amino acids) indicates a common origin for these two proteins. Interestingly, Kelemu and Collmer (1993) have recently characterized yet another set of in planta induced independently regulated Pels from *Erwinia chrysanthemi* EC16. Beaulieu et al. (1993) have also reported the presence of another set of Pels in *E. chrysanthemi* 3937 strain. Unfortunately, no sequence data allowing comparisons between SCC3193 PelB and in planta induced Pels are yet available. However, since SCC3193 PelB is not particularly induced by celery extract (data not shown) and is produced when the cells are grown in L medium without induction it is unlikely that SCC3193 PelB would belong to this new group of in planta induced Pels.

The PelB isozyme appears unique in its substrate specificity: It has a preference to partially methyl esterified pectin. PelB activity analyzed by using pectin with increasing methoxylation degree as substrate showed that PelB was distinct from another Pel (PelC) of *E. c.* subsp. *carotovora* more related to the PLade and PLcd families. PelB was most active on partially methoxylated (68%) substrate and showed no activity on PGA while PelC was most active on PGA exhibiting lower activity on methoxylated substrates. However, PelB is not a pectin lyase which characteristically favor highly methoxylated pectin as substrate and do not require Ca^{2+} ions for activity (Itoh et al. 1982; Tsuyumu and Chatterjee 1984). From the data presented in this report we conclude that we have characterized the member of a new Pel family. The pectinolytic enzyme arsenal of the soft-rotting *Erwinia* species thus appears even more complex, comprising many different classes of enzymes with related activity. This astonishing complexity could reflect both the powerful macerating capacity and the wide host range of these plant pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The *Escherichia coli* host strains used for plasmid maintenance and DNA preparation were DH5α (Hanahan 1983) and XL1-blue. The latter was purchased from Stratagene (La Jolla, California) together with a pBluescript II Exo III/Mung Bean DNA deletion kit. The *E. coli* strain C600 was used for overexpression of *pelB* and the selective labeling of the plasmid encoded proteins (Tabor 1990). The *E. c.* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen et al. 1988) and the *expI* mutant strain SCC3065 (Pirhonen et al. 1991) have been described. The vector used in the construction of pRK8 was the first version of pBluescript (I). The plasmids used are described in Table 1. Transfer of the plasmids was by standard transformation technique, by electroporation technique using a Biorad gene pulser (Bio-Rad Laboratories, Richmond, California) according to the manufacturer's instructions or by T4GT7 transduction as described previously (Pirhonen and Palva 1988; Pirhonen et al. 1991). The transducing bacteriophage T4GT7 has been described by Wilson et al. (1979).

Growth conditions, growth media, and chemicals.

E. coli was propagated in L medium (Miller 1972) at +37°C and *E. c.* subsp. *carotovora* in the same medium at +28°C. For the Northern (RNA) analysis (Fig. 5B) *E. c.* subsp. *carotovora* was grown in celery extract described by

Murata et al. (1991). The Pel indicator plates were as described by Pirhonen et al. (1991). Chloramphenicol (Cm) and kanamycin (Km) were added to media at 25 μg/ml and ampicillin (Ap) at 150 μg/ml. The IPTG (isopropyl-β-D-thiogalactoside) was from Kodak (Rochester, New York) and was added to 85 μg/ml. The restriction enzymes, T4 ligase, exonuclease III, the multiprime DNA labeling kit and the radiolabeled compounds were from Amersham International (UK). Mung bean nuclease was from Stratagene and the sequencing kit from United States Biochemicals Corp. (Cleveland, Ohio). The 30% esterified pectin (P-9135), 68% esterified pectin (P-9436), 93% esterified pectin (P-9561), polygalacturonic acid (PGA) (P-1879), ruthenium red (R-2751), and CAPS (C-2632) were from Sigma Chemical Co. (St. Louis, Missouri). The Link pectin (95% esterified) described by McMillan et al. (1993) was a generous gift from M. Pérombelon.

Genetic techniques.

The *pelB* marker exchange mutant was constructed as follows: First a *pelB* gene-specific 586-bp *Sal*I-*Eco*RI fragment was cloned into *Sal*I-*Eco*RI digested pBluescript II vector (the construct was named pRK9). Then the chloramphenicol resistance gene (Cm^R) was taken as *Hin*dIII-*Hin*dIII interposon specific fragment from pHP45Ω-Cm plasmid, isolated from agarose gel, blunted by Klenow reaction and ligated with *Hpa*I digested pRK9. A *Sal*I-*Pst*I fragment carrying *pelB* interrupted with Cm^R was further cloned into *Sal*I-*Pst*I digested pAM34 resulting in pRK13. For construction of the *pehA* marker exchange mutant the Cm^R marker was isolated as described for *pelB*, but it was ligated as a *Hin*dIII fragment into pHLU102 resulting in pDFL1. A 7-kb *Bam*HI-*Hin*dIII partially digested fragment from pDFL1 was isolated from an agarose gel, blunted, and cloned into a blunted *Bst*EII site in pHSK24, generating pDFL3. pRK13 and pDFL3 were each transduced into SCC3193 and Ap^R–Cm^R transductants were selected. The exchange recombinations with the chromosome was essentially done as described by Roeder and Collmer (1985) except that L medium was used instead of low phosphate medium. Isolation of marker exchange mutants was by screening for Cm^R-Ap^S colonies and the results were confirmed by Southern hybridization. The marker exchange mutants were named SCC5001 and SCC4188 for *pelB* and *pehA*, respectively.

DNA analysis, cloning techniques, deletion analysis, determination of the nucleotide sequence and primer extension analysis.

Unless otherwise stated, isolation of plasmid DNA or chromosomal DNA and gel analyses of the plasmid DNA was by established procedures as described in Sambrook et al. (1989). The genes coding for the different Pel activities were cloned by ligating partially *Sau*3A digested SCC3193 chromosomal DNA with either pUC18 (pHSK17 and pHSK20) or pMUT201 (pMUT4:0). The ligation mixtures were transformed to *E. coli* DH5α competent cells. The transformants were screened on Pel indicator plates and the potential positive clones were analyzed by isolating the periplasmic proteins (shockate) that were separated by IEF and stained for the activity. The 2,300-bp *pelB* insert was obtained as a *Xba*I-*Kpn*I fragment from pMUT4:4, blunted in a Klenow reaction and cloned into the *Sma*I site in pBluescript I polylinker. This new clone was called pRK8. To create unidirectional deletions extending into the *pelB* gene, the polylinker was double cleaved with appropriate restriction enzymes (*Sac*I-*Bam*HI and *Hin*dIII-*Kpn*I) on each side of the insert. The created 3′ or 5′ overhangs were digested with exonuclease III (Henikoff 1984), single-stranded DNA was removed with mung bean nuclease, the blunt ends were ligated and the plasmids were transformed into *E. coli* strain XL1-blue. Overlapping deletion plasmids that covered the whole insert were isolated and sequenced. The sequencing was performed on both strands by the Sanger dideoxy-chain termination method (Sanger et al. 1977) using the Sequenase DNA sequencing kit (United States Biochemicals). The sequencing of the region between *pelB* and *pehA* genes was partly performed by using the oligonucleotides 5′ AAATATCGGTGTTAAATACTTAAACATGTGT 3′ and 5′ TTTTATTATCCTGCTTATAGTTTCATT-

Table 1. Plasmids used and constracted in this study

Plasmid	Relevant characteristics	Reference or source
pUC18	Cloning vector (Ap^R)	Yanisch-Perron et al. 1985
pBluescript I	Cloning vector (Ap^R)	BRL
pBluescript II SK	Cloning vector (Ap^R)	BRL
pAM34	(Ap^R)	Gil and Bouche 1991
pMUT201	Cloning vector (Cm^R)	Karlsson et al. 1991
pHLU102	*lacZ* promoter probe vector	Lång and Palva 1993
pHP45Ω-Cm	(Cm^R)	Fellay et al. 1987
pHSK24	pUC18 carrying *pehA*	Saarilahti et al. 1990
pHSK17	pUC18 carrying *pelC* as a *Sau*3A fragment	Saarilahti et al., unpublished
pHSK20	pUC18 carrying *pelA/pelD* as a *Sau*3A fragment	Saarilahti et al., unpublished
pMUT4:0	pMUT201 carrying *pehA/pelB* as a *Sau*3A fragment	This work
pMUT4:3	pBluescript II carrying *pehA* from pMUT4:0	This work
pMUT4:4	pBluescript II carrying *pelB* from pMUT4:0	This work
pRK8	2.3 kb *Xba*I-*Kpn*I fragment carrying *pelB* from pMUT4:4 in pBluescript I	This work
pRK8-2	a deletion derivative of pRK8 with *pelB* under T7 promoter	This work
pRK9	586 bp *Sal*I-*Eco*RI fragment of *pel*B in pBluescript II	This work
pRK10	*Eco*RV-*Sal*I fragment of *pelB* in pBluescript II	This work
pRK11	*Eco*RV-*Dra*II fragment of *pelB* in pBluescript II	This work
pRK13	*Sal*I-*Eco*RI fragment from pRK9 carrying Ω-Cm in pAM34	This work
pDFL1	Ω-Cm cloned as a *Hin*dIII fragment from pHP45Ω-Cm into pHLU102	This work
pDLF2	7.0 kb *Bam*HI-*Hin*dIII fragment harboring the Ω-Cm from pDFL1 inserted into *Bst*EII site in *pehA* of pHSK24	This work

GTT 3′ designed for the primer extension analysis of *pelB* and *pehA*, respectively, as the sequencing primers and partly subclones carrying the two halves of the control region as *Eco*RV-*Sal*I and *Eco*RV-*Dra*II fragments in pBluescript II (clones pRK10 and pRK11, respectively) using standard pBluescript II primers. The primer extension analysis was as described by Nurk et al. (1993). RNA was isolated from SCC3193 cells grown in L medium to OD_{600} 0.848 and 1.86 for *pehA* and *pelB,* respectively. Sequencing reactions labeled with (α-^{32}P)-dATP and primed with the above described oligonucleotides were run in parallel with the primer extension products and used as size markers.

The isolation of chromosomal DNA, total RNA, and the Southern and Northern blot analysis.

Chromosomal DNA from SCC3193 and SCC5001 was isolated and 5 µg of the DNA was digested with appropriate restriction enzymes and run on 0.8% agarose gel, blotted on nylon membrane (Hybond-N, Amersham International, UK) essentially as described by Sambrook et al. (1989) and hybridized overnight at +65°C in 5× Denhardt's, 5× SSP, 0.2% SDS, 500 mg of denatured herring sperm DNA per milliliter. A 586-bp *Sal*I–*Eco*RI fragment of *pelB* was used as *pelB*-specific probe and the *Hin*dIII-*Hin*dIII interposon specific fragment was used as probe for Cm^R marker. The probe fragments were labelled with (α-^{32}P)-dATP by random priming. After hybridization, the membranes were washed in standard washes (6× SSC—0.5% SDS, 3× SSC—0.5% SDS, 1× SSC—0.5% SDS at +65°C for 15 min) and exposed to X-ray film. RNA was isolated as previously described for gram-negative bacteria (Gilman 1987). Ten micrograms of total RNA was denatured in formamide and formaldehyde, separated by formaldehyde gel electrophoresis. Blotting and hybridizations were performed as described for the Southern blot analysis. We used a 1,380-bp *Bam*HI–*Sal*I fragment of pRK8 as the *pelB-pehA* probe. The probe fragments were labeled with (α-^{32}P)-dATP by random priming. After hybridization, the membranes were washed and exposed as described for the Southern blot technique. The mRNA hybridizing to *pehA–pelB* probe was quantified by PhosphorImager and the data was processed with the Image Quant (Molecular Dynamics, Sunnyvale, California) program.

Isoelectric focusing (IEF).

Ultrathin IEF of Pel isoenzymes was done by using a LKB Multiphor II apparatus (Pharmacia, Uppsala, Sweden) and LKB Ampholines (pH range 9 to 11) (Pharmacia, Uppsala, Sweden) essentially as described in Willis et al. (1987) or in the manufacturer's instructions. Detection of the enzyme activity was by using an ultrathin overlay of 0.25% (w/v) 30% esterified pectin –1% (w/v) agarose which was stained with 0.05% (w/v) ruthenium red essentially as described by Bertheau et al. (1984) and Collmer et al. (1985).

Isolation of periplasmic proteins of *E. coli* (shockate), preparation of crude enzyme samples and Pel assay.

The proteins from *E. coli* periplasm were selectively isolated as described by Heppel (1971) and Palva (1978) and used for the IEF. The crude enzyme samples were cell lysates from C600/pRK8-2 and DH5α/pHSK17 cultures for PelB and PelC, respectively, obtained by disruption of cells by brief ultrasonic treatment. The samples were stored frozen at –20° C until assayed. The Pel assay was based on thiobarbituric assay described by Sherwood (1966). However, instead of 100 mM Tris-HCl pH 8.5, 200 mM CAPS, pH 9.5, was used as buffer and the samples were incubated 2 h at +37°C with the substrate. The Pel activity was expressed as OD_{548}. A crude extract prepared from induced C600/pBluescript II was used as a control and the measured background values were subtracted from the OD_{548} values.

Overproduction of PelB and detection of pRK8-2 encoded proteins.

The overexpression of *pelB* and the selective labeling of the PelB protein was done by phage T7 RNA polymerase/promoter system described by Tabor (1990). The construct used was one of the deletions made for DNA sequencing (pRK8-2) in which the *pelB* gene is located under the control of the T7 RNA polymerase promoter carried by pBluescript I. After growth and heat induction (+42°C 30 min) the cells were centrifuged and resuspended into Tris-HCl (pH 8.5—5 mM $CaCl_2$ buffer and broken by a brief ultrasonic treatment. The labeled cells were directly resuspended into 4× sample buffer (40%, w/v, glycerol-20%, w/v, β-mercaptoetanol—10%, w/v, SDS—0.02%, w/v, bromphenol blue in 0.25 M Tris-HCl, pH 6.8) and boiled. The proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 12% slab gel (Laemmli 1970) and were visualized by either Coomassie blue staining or fluorography of stained and dried gels.

The virulence assay.

The virulence of SCC3193 and SCC5001 was compared on axenic tobacco seedlings (*Nicotiana tabacum* 'Samsun') cultivated essentially as described by Pirhonen et al. (1991) either with or without 10 mM $CaCl_2$. The inoculation of overnight grown cells was as described by Pirhonen et al. (1993) and the symptom development was followed as by Pirhonen et al. (1991). The potato tubers (*Solanum tuberosum* 'Bintje') were commercial potatoes and the virulence was tested on 1-cm-thick surface sterilized tuber slices by inoculating cells grown overnight into pits bored into tuber medulla. The slices were incubated at +28°C at 100% humidity and the development of symptoms was evaluated after 24 h. The virulence on greenhouse-grown potato stems was essentially tested as described by McMillan et al. (1993) and symptom development was followed for 48 h.

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