

Extracellular Secretion of Pectate Lyase by the *Erwinia chrysanthemi* Out Pathway Is Dependent upon Sec-Mediated Export across the Inner Membrane

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The plant pathogenic enterobacterium *Erwinia chrysanthemi* EC16 secretes several extracellular, plant cell wall-degrading enzymes, including pectate lyase isozyme PeLE. Secretion kinetics of ³⁵S-labeled PeLE indicated that the precursor of PeLE was rapidly processed by the removal of the amino-terminal signal peptide and that the resulting mature PeLE remained cell bound for less than 60 s before being secreted to the bacterial medium. PeLE-PhoA (alkaline phosphatase) hybrid proteins generated in vivo by *TnphoA* insertions were mostly localized in the periplasm of *E. chrysanthemi*, and one hybrid protein was observed to be associated with the outer membrane of *E. chrysanthemi* in an *out* gene-dependent manner. A gene fusion resulting in the substitution of the β -lactamase signal peptide for the first six amino acids of the PeLE signal peptide did not prevent processing or secretion of PeLE in *E. chrysanthemi*. When *peLE* was overexpressed, mature PeLE protein accumulated in the periplasm rather than the cytoplasm in cells of *E. chrysanthemi* and *Escherichia coli* MC4100 (pCPP2006), which harbors a functional cluster of *E. chrysanthemi out* genes. Removal of the signal peptide from pre-PeLE was SecA dependent in *E. coli* MM52 even in the presence of the *out* gene cluster. These data indicate that the extracellular secretion of pectic enzymes by *E. chrysanthemi* is an extension of the Sec-dependent pathway for general export of proteins across the bacterial inner membrane.

Gram-negative bacteria secrete to their milieu various proteins which can play important roles in pathogenesis, degradation of extracellular polymers, and other interactions with the environment (5, 21). The molecular mechanisms by which gram-negative bacteria secrete proteins across the outer membrane are not well understood, but there appear to be two pathways, one Sec independent and the other Sec dependent. The first type is exemplified by the secretion of *Escherichia coli* hemolysin and *Erwinia chrysanthemi* protease (22, 51). The secreted proteins of this group lack amino-terminal signal peptides. Instead, targeting sequences in the carboxyl-terminal portion of the proteins are recognized by three secretion gene products (HylB, HlyD, and TolC) which are postulated to form a complex between the inner and outer membranes (22, 32). The second type is exemplified by the secretion of *Klebsiella pneumoniae* pullulanase and *E. chrysanthemi* pectic enzymes and cellulases. Secreted proteins of this group possess amino-terminal signal peptides which apparently function in *E. coli* in facilitating translocation of the proteins across the bacterial inner membrane (2, 7, 15, 27, 40, 41, 45). Extracellular secretion of these proteins, however, requires the function of accessory secretion genes, such as the *out* genes of *E. chrysanthemi* and *Erwinia carotovora* (1, 26, 37, 50), the *xcp* genes of *Pseudomonas aeruginosa* (14), and the *pul* genes of *K. pneumoniae* (12). Recent reports of homology between the *out*, *xcp*, and *pul* genes suggest that gram-negative bacteria use a conserved mechanism to secrete proteins by this pathway (14, 16).

The Sec-dependent extracellular secretion systems are more common in gram-negative bacteria, secrete more pro-

teins, and appear to be biochemically more diverse. For example, *K. pneumoniae* secretes a single lipoprotein, pullulanase, which is acylated after the removal of the signal peptide (41). Further secretion of pullulanase across the outer membrane requires secretion genes *pulC-O* and *pulS* (10, 11, 44, 46). Pullulanase, after crossing the outer membrane, anchors on the outer surface of the membrane before being slowly released to the medium (8). In contrast, the plant soft-rotting enterobacterium, *E. chrysanthemi*, secretes an array of plant cell wall-degrading enzymes, including exo-poly- α -D-galacturonosidase, pectin methylesterase, cellulase, and at least four isozymes of pectate lyase (5). Unlike pullulanase, these proteins are secreted without any apparent covalent modifications (26). Similar arsenals of plant cell wall-degrading enzymes are secreted by many other plant pathogenic bacteria, including *E. carotovora* (18, 19, 52), *Xanthomonas campestris* pv. *campestris* (13), and some plant pathogenic *Pseudomonas* spp. (33, 47).

In *E. chrysanthemi*, all pectic enzymes and cellulase are secreted through a common pathway encoded by the *out* genes. This is indicated by two observations: (i) single chromosomal mutations (*out* mutations) block extracellular secretion of all of these enzymes (1, 26, 50), and (ii) an *out* gene cluster isolated from *E. chrysanthemi* EC16 enables *E. coli* (which normally accumulates foreign extracellular proteins in the periplasm) to specifically secrete all *E. chrysanthemi* pectic enzymes (16).

The presence of signal peptides at the amino termini of the extracellular *E. chrysanthemi* proteins suggests that the first step in the secretion of these proteins is Sec mediated. This hypothesis is supported by two observations: (i) when genes encoding *E. chrysanthemi* pectic enzymes and cellulases are expressed in *E. coli*, the proteins are found in the periplasm with their signal peptides removed (2, 3, 7, 15, 27, 31, 40),

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and (ii) mutations in the *out* genes result in the accumulation of these extracellular proteins in the periplasm of *E. chrysanthemi*, again with their signal peptides removed (1, 26, 50). Since these observations are based on cells either lacking or defective in *out* genes, it is not certain whether operation of the Out pathway would provide an alternative route across the inner membrane for extracellular secretion of pectic enzymes and cellulases. In this study, we directly address the involvement of Sec-mediated translocation across the inner membrane in PelE secretion in both *E. chrysanthemi* and Out⁺ *E. coli*(pCPP2006). A recent report by Pugsley et al. (43) has provided similar evidence that extracellular secretion of the *Klebsiella* pullulanase by *E. coli* is Sec dependent: (i) *secA*, *secB*, *secD*, *secE*, *secF*, and *secY* were required for processing of the prepullulanase signal peptide, and (ii) drastic alteration of the signal peptide caused pullulanase to remain cytoplasmic.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in King's medium B (29) at 28 to 30°C. Appropriate antibiotics were added at concentrations of 20 µg/ml (nalidixic acid), 50 µg/ml (kanamycin and spectinomycin), and 100 µg/ml (ampicillin).

General DNA manipulations. DNA restrictions, ligations, and other modifications followed the basic protocols of Maniatis et al. (35) or the manufacturers' instructions. Enzymes and related chemicals were purchased mainly from Bethesda Research Laboratories, IBI, and Boehringer Mannheim. DNA sequencing was done with pBluescript SK(+) from Stratagene and the T7 Sequencing Kit from Pharmacia LKB. *E. chrysanthemi* was transformed by electroporation with the Gene Pulser (Bio-Rad), as previously described (15).

Labeling and immunoprecipitation. For pulse-chasing of PelE in *E. chrysanthemi*, AC4150 was first grown overnight in 5 ml of M9 medium supplemented with 0.2% polygalacturonate (Pfaltz & Bauer, Inc.) and 0.5% Casamino Acids and then was grown to an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.7 in M9 minimal medium (35) supplemented with 0.2% polygalacturonate and a mixture of various amino acids (except methionine) at concentrations of 50 µg/ml. Cells were pelleted and washed once in fresh M9 medium and resuspended in 5 ml of the same medium plus an amino acid mixture containing all the essential amino acids except methionine. The resuspended culture was shaken for an additional 30 min. A 0.5-ml aliquot of this culture was pulsed with [³⁵S]methionine (at 20 µCi/ml, specific activity of >800 Ci/mmol; Amersham) for 30 s or 2 min, and it was then chased with excess unlabeled methionine for various times. Pulsing or chasing was stopped by the addition of crushed ice (approximately 0.5 ml) to the labeling mixture. Cells were sedimented by centrifugation for 2 min in a microcentrifuge at 4°C and washed once in an ice-cold solution of 0.01 M Tris-HCl (pH 7.0). PelE antiserum, preabsorbed with an *E. coli* HB101 lysate, was used in the subsequent immunoprecipitation, which was done essentially as described by Ito et al. (25). Labeling of MC4100 (SecA⁺) and MM52 (SecA⁻) cells containing pPEL7421 and/or pCPP2006 and the subsequent immunoprecipitation were performed according to the procedure described by Oliver and Beckwith (38). All immunoprecipitates were separated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. The gels were dried and autoradiographed with X-ray film.

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics	Reference
<i>E. coli</i>		
CC118	<i>araD139</i> Δ(<i>ara leu</i>)7697 Δ <i>lacX74 phoA</i> Δ20 <i>galE</i> <i>galK thi rpsE rpoB</i> <i>argE</i> (Am) <i>recA1</i>	36
DH5α	F ⁻ <i>andA1 hsdR17</i> (r _K ⁻ m _K ⁻) <i>supE44 thi-1 recA1 gyrA96</i> <i>relA1</i> φ80d <i>lacZ</i> Δ <i>M15</i>	BRL ^a
MC4100	F ⁻ Δ(<i>lac</i>) <i>U169 araD136 relA</i> <i>rpsL thi</i>	38
MM52	MC4100 <i>ts51</i>	38
<i>E. chrysanthemi</i>		
AC4150	Nal ^r derivative of EC16	4
AC4200	<i>out</i> mutant of AC4150	50
CUCPB5006	Δ(<i>pelB pelC</i>)::28-bp Δ(<i>pelA</i> <i>pelE</i>) derivative of AC4150	15
Plasmids		
pBluescript SK	Amp ^r	Stratagene
pPEL748	pINIII A-2 carrying <i>pelE</i>	28
pPEL7421	pUC8 carrying <i>pelE</i>	28
pPEPA1-5	A series of plasmids with <i>TnphoA</i> inserted in the <i>pelE</i> gene in pPEL7421, generating hybrid proteins PelE-PhoA1-5	This work
pCPP19	Cosmid vector, Sp ^r /Sm ^r	2a
pCPP2006	pCPP19 carrying a functional cluster of <i>out</i> genes from <i>E.</i> <i>chrysanthemi</i> EC16	16
pSH7	pBR322 derivative, after elimination of the <i>EcoRI</i> site and introduction of a new <i>EcoRI</i> site at the former <i>ScaI</i> site	This work
pSH8	pSH7 carrying the 1.8-kb <i>EcoRI</i> fragment from pPEL7421, PelE ⁺	This work
pBLPL10	pSH8 carrying a Bla-PelE signal peptide fusion	This work

^a BRL, Bethesda Research Laboratories.

Gene fusions between *pelE* and *phoA*. Gene fusions between *pelE* and *phoA* (encoding *E. coli* alkaline phosphatase) were generated by *TnphoA* mutagenesis of a cloned *pelE* gene. Transfection of λ::TnphoA into *E. coli* CC118 containing pPEL7421 and selection for blue colonies on plates containing 300 µg of kanamycin per ml and 100 µg of 5-bromo-4-chloro-3-indolyl phosphate per ml essentially followed the procedure reported by Manoil and Beckwith (36). Blue colonies were transferred with toothpicks to pectate semisolid agar plates (48) to confirm the loss of the pitting phenotype caused by TnphoA insertions in the *pelE* gene. The sizes of the PelE-PhoA hybrid proteins produced by PelE⁻/PhoA⁺ colonies were determined with SDS-polyacrylamide gels immunoblotted with antisera raised against PhoA or PelE.

Construction of PelE proteins with a hybrid signal peptide. Hybrid proteins containing the β-lactamase signal peptide in place of amino-terminal portions of the PelE signal peptide were constructed by the following procedure. The *EcoRI* site in pBR322 was destroyed by digestion with *EcoRI*, treatment with mung bean nuclease, and ligation of the resultant blunt ends. The modified plasmid was digested with

ScaI, treated with calf intestine alkaline phosphatase, and then ligated with *EcoRI* linkers to create pSH7. This plasmid was digested with *EcoRI*, treated with calf intestine alkaline phosphatase, and ligated with the 1.8-kb *EcoRI* *pelE*⁺ fragment from pPEL7421 to produce pSH8. The *MluI* site located in the signal peptide-encoding region of *pelE* (28) in pSH8 was digested with *MluI* and then with BAL 31 for various lengths of time. The BAL 31-digested plasmids were then religated and transformed into *E. coli* DH5 α . The transformants were screened for the elimination of *HincII* and *DraI* sites located downstream of the β -lactamase signal peptide-encoding region by restriction mapping, for pectolytic activity on pectate semisolid agar plates, and finally for the size of encoded hybrid proteins by immunoblotted SDS gels.

Subcellular localization of PelE-PhoA hybrids. Bacteria carrying various pPEL7421 derivatives producing PelE-PhoA hybrids were grown to late logarithmic phase (OD₆₀₀ of 0.7 to 1.0) at 30°C in King's medium B or M9CA medium supplemented with 0.2% polygalacturonate and 1.0 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were separated from medium by centrifugation and washed once in an ice-cold solution of 0.01 M Tris-HCl (pH 7.0). The periplasmic fraction was isolated by the procedure of Witholt et al. (53). The resulting spheroplasts were lysed by repeated freeze-and-thaw cycles, and the unlysed cells were removed by low-speed centrifugation (5,000 \times *g* for 20 min). Membranes were pelleted by centrifugation at 27,000 \times *g* for 1 h, and inner and outer membranes were separated by sucrose gradient centrifugation (39). Membrane banding similar to that described by Osborn and Munson (39) was observed with *E. coli* CC118 and *E. chrysanthemi* AC4150, except that in AC4150 the M band was frequently split into two close bands. Proteins in each fraction were concentrated either by ultrafiltration in Centricon tubes or by precipitation with 5% trichloroacetic acid followed by washing in ice-cold 90% acetone. All protein samples were suspended in 0.01 M Tris-HCl (pH 7.0). The amount of the protein sample from different subcellular fractions to be loaded in each well of an SDS-10% polyacrylamide gel was adjusted to represent an equal volume of the initial culture. Immunoblotting was performed with GeneScreen Plus nylon membranes under conditions recommended by the manufacturer (NEN Research Products, DuPont). Rabbit antisera raised against PelE and PhoA and goat anti-rabbit antibody conjugated with alkaline phosphatase were used to visualize appropriate proteins in the blots.

RESULTS

Kinetics of PelE secretion in *E. chrysanthemi*. If PelE secretion is a two-step process involving sequentially the Sec and Out pathways, then pulse-labeled, mature PelE should be present briefly in a cell-bound fraction of wild-type *E. chrysanthemi* cells that are actively secreting PelE. Figure 1 shows the result of a pulse-chase experiment performed to monitor the process of PelE secretion in *E. chrysanthemi* AC4150. With a brief pulse of 30 s followed by immunoprecipitation, a 43-kDa protein, corresponding to mature PelE, appeared in the cell fraction. The mature PelE was detected in the medium only after a 2-min pulse. Almost all of the mature PelE in the cell was chased into the medium within 60 s. The pre-PelE was not detected under the conditions with which we performed the pulse-chase experiment. These data indicate that in the first step, pre-PelE was translocated across the inner membrane and then processed

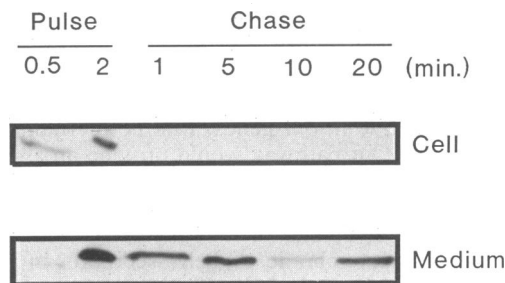


FIG. 1. Pulse-chasing of PelE during its extracellular secretion by logarithmically growing *E. chrysanthemi*. Labeling and immunoprecipitation steps are explained in the text.

to its mature form within 30 s. Subsequently, the mature PelE was translocated across the outer membrane in the second step, which took approximately another 60 s. Virtually all of the synthesized PelE was secreted in about 2 min by *E. chrysanthemi*.

Analysis of PelE-PhoA hybrid proteins in *E. coli* CC118, *E. chrysanthemi* AC4150, and Out⁻ *E. chrysanthemi* AC4200. PelE-PhoA hybrids were constructed by *TnphoA* mutagenesis to determine whether targeting sequences in PelE would enable PhoA to be secreted and to determine the subcellular location of hybrids whose movement through the Out pathway was blocked. As a periplasmic protein, PhoA would not be expected to interfere with the first step of PelE export and could possibly be secreted through the outer membrane of *E. chrysanthemi* if a targeting sequence from PelE were supplied. However, the data from an immunoblot of five hybrid proteins with molecular masses of 55, 66, 70, 73, and 76 kDa (Fig. 2) in *E. chrysanthemi* show that the amino terminus (including about 70% of the whole PelE polypeptide) was not

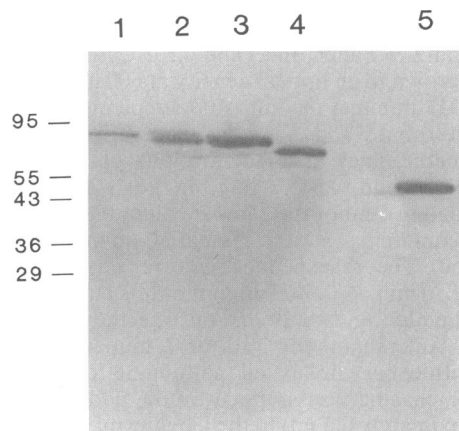


FIG. 2. Immunoblotted SDS-polyacrylamide gel of the PelE-PhoA hybrid proteins produced by *E. coli* CC118 cells harboring plasmids pPEPA1 through pPEPA5. The five plasmids carried in-frame fusions between *pelE* and *phoA* that had been generated by *TnphoA* insertions. Proteins in sonicated cell extracts were resolved by electrophoresis through an SDS-10% polyacrylamide gel and then transferred to a Gene Screen Plus nylon membrane. PelE-PhoA hybrid proteins were detected by using polyclonal rabbit antiserum to PhoA and goat anti-rabbit antibody conjugated with alkaline phosphatase. The lane numbers correspond to the number assigned to each plasmid in the series; e.g., lane 1 shows the hybrid protein produced by pPEPA1. The locations and molecular masses in kilodaltons of marker proteins are shown to the left of the gel.

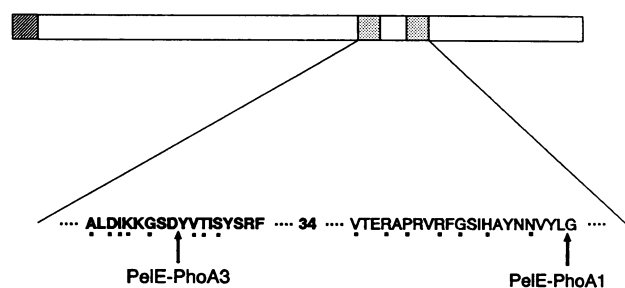


FIG. 3. Locations of the *TnphoA* insertions in the *pelE* gene which resulted in the production of the hybrid proteins PelE-PhoA1 and PelE-PhoA3. The two regions that are conserved among six extracellular pectate lyase isozymes of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* are shown with predicted amino acid sequences (49). The conserved amino acids are denoted with dots underneath them. Arrows indicate the positions of *TnphoA* insertions, which were determined by DNA sequencing.

sufficient for the secretion of the hybrids by *E. chrysanthemi*. All five hybrids were retained in the cell, mostly in the periplasm (data not shown).

Previous analysis of the nucleotide sequences of at least six *pel* genes revealed two highly conserved regions, each consisting of about 20 amino acid residues (Fig. 3) (49). To ascertain the presence of these sequences in the hybrids, the exact locations of the insertions in PelE-PhoA1 and PelE-PhoA3 were determined by DNA sequence analysis of the *pelE::TnphoA* junctions and are shown in Fig. 3. PelE-PhoA1 included these two conserved regions but was not secreted by *E. chrysanthemi*.

PelE-PhoA1 and PelE-PhoA2 were less stable in *E. chrysanthemi* than in *E. coli* CC118 because of apparent proteolytic degradation. Consequently, further analysis of hybrid proteins in *E. chrysanthemi* was done only with PelE-PhoA3, which was stable in both *E. chrysanthemi* and its *out* mutants. PelE-PhoA3 was localized exclusively in the periplasm of *E. coli* CC118 during logarithmic growth (Fig. 4). Neither the cytoplasmic fraction nor the membrane fraction accumulated this hybrid to a detectable level at that growth

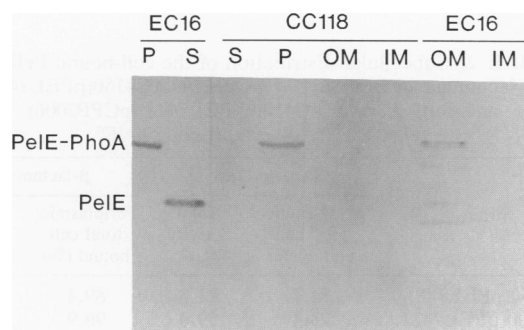


FIG. 4. Immunoblot demonstrating the subcellular localization of PelE-PhoA3 in *E. coli* CC118 and *E. chrysanthemi* AC4150 cells. Subcellular fractionation of cultures grown to late logarithmic phase was done as described in Materials and Methods. Proteins in each fraction were then resolved by electrophoresis through an SDS-10% polyacrylamide gel, transferred to a GeneScreen Plus nylon membrane, and detected by using polyclonal rabbit antiserum to PelE and goat anti-rabbit antibody conjugated with alkaline phosphatase. S, culture supernatant; P, periplasm; IM, inner membrane; OM, outer membrane.

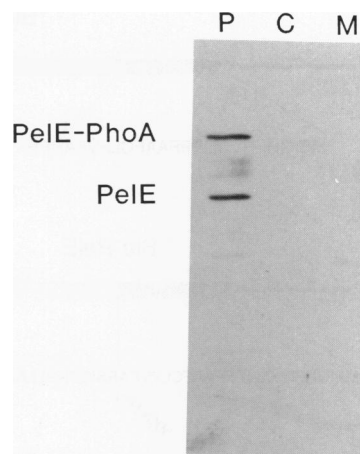


FIG. 5. Immunoblot demonstrating the subcellular localization of PelE-PhoA3 in the *Out⁻* *E. chrysanthemi* mutant AC4200. Samples were prepared and analyzed as described in the legend to Fig. 4. P, periplasm; C, cytoplasm; M, total membrane.

stage. In contrast, PelE-PhoA3 was detected in both periplasmic and membrane fractions in *E. chrysanthemi*. Interestingly, abortive secretion of the hybrid protein did not interfere with normal secretion of the native PelE. When the total membrane pellets of *E. coli* CC118 and *E. chrysanthemi* AC4150 were separated into inner and outer membrane fractions by sucrose gradient centrifugation, PelE-PhoA3 was detected mostly in the outer membrane fraction but not in the inner membrane fraction (Fig. 4). Some of the PelE-PhoA3 hybrid protein also associated with the M bands, which may have consisted of both the outer and the inner membranes (data not shown). The association of PelE-PhoA3 with the outer membrane of *E. chrysanthemi* AC4150, but not with that of *E. coli* CC118, suggested that *out* gene products in AC4150 might mediate this association. To test this possibility, pPEPA3, which directs the synthesis of PelE-PhoA3, was transformed into AC4200, an *out* mutant of *E. chrysanthemi* (50). The transformant was then grown in M9CA medium to stationary phase. The cells were washed and fractionated into membrane, periplasmic, and cytoplasmic samples. Figure 5 shows that PelE-PhoA3 in this *out* mutant was no longer associated with the membrane but, rather, accumulated in the periplasm with PelE (along with a few other proteins that weakly cross-react with anti-PelE antibody).

Secretion of a PelE protein synthesized with a hybrid β -lactamase-PelE signal peptide. Four of the first 12 amino acids in the PelE signal peptide are positively charged. To determine whether this or other features of the PelE signal peptide function in sorting PelE to the *Out* pathway, we sought to study the secretion behavior of PelE hybrids in which the β -lactamase signal peptide was substituted for at least a portion of the PelE signal peptide. Our approach was to use BAL 31 digestions initiated at a unique *Mlu*I site in *pelE* (Fig. 6) to generate a collection of deletion derivatives in which the β -lactamase signal peptide was fused with a truncated PelE signal peptide. The deletion products were first screened for loss of *Dra*I and *Hinc*II sites in the sequences encoding the mature β -lactamase. The pectolytic phenotypes of *E. coli* DH5 α cells producing these fusion products were then determined on pectate semisolid agar plates. The ability of such colonies to sink into the medium

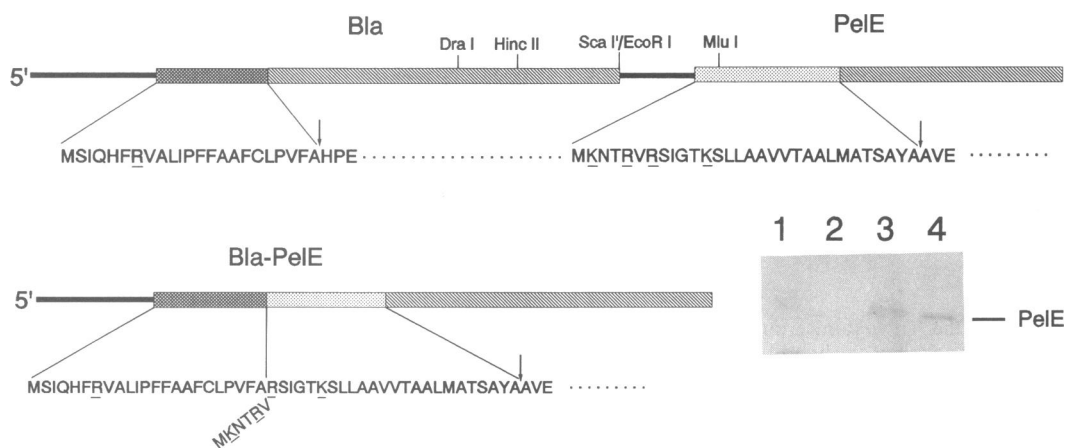


FIG. 6. Signal peptide sequences of Bla, PelE, and Bla-PelE fusion. Arrows indicate the processing sites. *Dra*I and *Hinc*II are in the amino terminus-encoding region of the β -lactamase mature protein; *Mlu*I is in the PelE signal peptide-encoding region. Heavily shaded and stippled boxes are the signal peptides of Bla and PelE, respectively; hatched boxes are the amino terminal regions of the mature Bla and PelE. The positively charged amino acid residues in the signal peptides are underlined. The insert shows an immunoblot of an SDS-10% polyacrylamide gel that was immunostained with anti-PelE antibody as described in the legend to Fig. 4. Preparation of fractions is described in the text. Lanes: 1, CUCPB5006 cell-bound fraction; 2, CUCPB5006 culture supernatant; 3, CUCPB5006(pBLPL10) cell-bound fraction; 4, CUCPB5006(pBLPL10) culture supernatant.

requires that the altered PelE retain enzymatic activity and would be enhanced by the export of the protein to the *E. coli* periplasm. Surprisingly, all of the hybrid proteins with pectate lyase activity in *E. coli* retained most of the PelE signal peptide. DNA sequence analysis of one of these, pBLPL10, revealed that the β -lactamase signal peptide had replaced the first six amino acids (including two of the four positively charged residues) of the PelE signal peptide (Fig. 6). Immunoblotted SDS gel analysis of the periplasmic fractions of *E. coli* DH5 α (pBLPL10) and *E. coli* DH5 α (pSH8) indicated that the level of Bla-PelE hybrid protein production by pBLPL10 was approximately 15% of the level of PelE production by pSH8 and that the two proteins had the same mobility, which indicates that the Bla-PelE hybrid was properly processed to a mature protein in *E. coli* (data not shown). Plasmid pBLPL10 was transferred to *E. chrysanthemi* CUCPB5006 (Δ pelE) by electroporation. An immunoblotted SDS gel of cell-bound and culture supernatant fractions of CUCPB5006 and CUCPB5006(pBLPL10) revealed that mature PelE was secreted to the medium of the cells producing the hybrid (Fig. 6, lane 4), but because there were cross-reacting proteins in the cell-bound fraction of CUCPB5006, we cannot eliminate the possibility that some of the PelE was retained.

Presence of overproduced PelE in the periplasm of AC4150 and Out⁺ *E. coli*. In order to determine in which subcellular compartment unsecreted pectate lyase would be localized when the Out machinery is saturated, we overexpressed the *pelE* gene in both *E. chrysanthemi* AC4150 and Out⁺ *E. coli*(pCPP2006), which contains a functional cluster of *out* genes from *E. chrysanthemi* EC16 (16). If PelE is secreted in one step through junctions between the inner and outer membranes, the unsecreted pectate lyase should accumulate mostly in the cytoplasm. If, on the other hand, PelE crosses the inner and outer membranes in two discrete steps, the unsecreted PelE should accumulate mostly in the periplasm. pPEL748 (28), which carries the *pelE* gene under the control of the triple *lac* UV5 promoters of pINIII, was introduced into *E. chrysanthemi* AC4150, and pPEL7421, which carries *pelE* under the control of the *lac* promoter in pUC18 (28),

was introduced into *E. coli* MC4100(pCPP2006). These two *pelE*⁺ plasmids were chosen because they supported high levels of PelE production in both bacteria. pPEL748 produces substantially more PelE than pPEL7421 in *E. coli* (28) and in *E. chrysanthemi* (data not shown). The transformants were grown to an OD₆₀₀ of 1.1 to 1.5 in King's medium B broth supplemented with 0.1% polygalacturonate and 1 mM IPTG. Under this condition, AC4150(pPEL748) retained 15 to 20% of the pectate lyase activity in the cell. *E. coli* MC4100(pPEL7421, pCPP2006) retained 40 to 50% of the pectate lyase activity in the cell (Table 2). Most of the cell-bound pectate lyase was found to cofractionate with the osmotic shock fluid, indicating that the cell-bound pectate lyase was mainly in the periplasm of both AC4150 and the Out⁺ *E. coli* (Table 2).

SecA dependence of PelE secretion in Out⁺ *E. coli*. A critical test of the involvement of the Sec machinery in the

TABLE 2. Subcellular distribution of the cell-bound PelE and β -lactamase in Out⁺ *E. chrysanthemi* AC4150(pPEL748) and Out⁺ *E. coli* MC4100(pPEL7421, pCPP2006) cells that are overexpressing *pelE*^a

Strain	Pectate lyase		β -lactamase	
	Periplasmic/ total cell bound (%)	Total activity (U) ^b	Periplasmic/ total cell bound (%)	Total activity (U)
AC4150(pPEL748)	71.7	11.3	89.4	14.3
MC4100(pPEL7421, pCPP2006)	73.4	59.0	96.9	292.5

^a Bacteria were grown at 30°C to late logarithmic phase (OD₆₀₀ of 1.0 to 1.2) in King's medium B broth supplemented with appropriate antibiotics. Cells were collected after centrifugation and washed twice in an ice-cold solution of 10 mM Tris-HCl (pH 7.5) and 30 mM NaCl. The periplasmic fraction was prepared from washed cells by the method of Heppel (17), except that lysozyme (final concentration of 60 μ g/ml) was added at the plasmolysis step. Enzymatic activities of PelE and β -lactamase were assayed spectrophotometrically at 235 and 240 nm, respectively (6, 7).

^b Total enzyme activity units are expressed as micromoles of product generated per minute per milligram of bacterial protein.

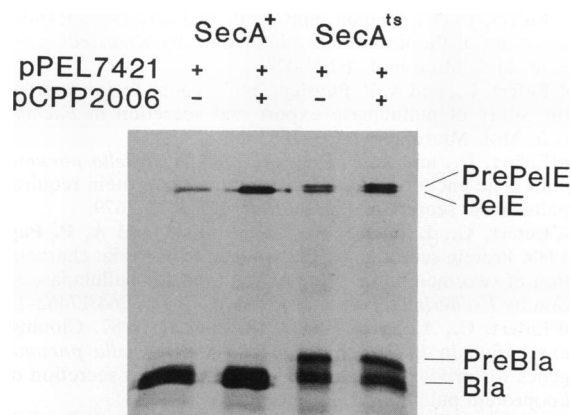


FIG. 7. SDS-polyacrylamide gel of [³⁵S]methionine-labeled MC4100 (SecA⁺) and MM52 (SecA⁻) harboring pPEL7421 (*pelE* and *bla* genes) and pCPP2006 (*E. chrysanthemi* *out* genes). Bacteria were grown at 30°C to an OD₆₀₀ of 0.2 to 0.4 and then at 37°C (nonpermissive temperature for MM52) for 3.5 h. A 0.5-ml portion of each culture was labeled with [³⁵S]methionine. Labeling and immunoprecipitation procedures are explained in the text. Symbols: PrePeIE, the precursor of PeIE; PreBla and Bla, the precursor and mature β-lactamase, respectively.

extracellular protein secretion is to use conditional mutations [*secA*(Ts)-like] that would block protein export across the inner membrane under nonpermissive conditions to determine whether the secretion of extracellular proteins is also affected. Although such mutants are available only in *E. coli*, the Out⁺ phenotype of *E. coli* strains carrying pCPP2006 makes such a test possible. At the permissive temperature (30°C), only mature PeIE and β-lactamase were present in both SecA⁺ and SecA⁻ strains (data not shown). After a temperature shift to 37°C for 3.5 h (nonpermissive for MM52), the SecA⁺ *E. coli* strain (MC4100) contained only the mature PeIE and β-lactamase, indicating that both pre-PeIE and pre-Bla were processed in this strain the same as at the permissive temperature (Fig. 7, lanes 1 and 2). In contrast, the precursors of PeIE and Bla accumulated in the *secA*(Ts) *E. coli* strain (MM52) under the nonpermissive temperature (Fig. 7, lanes 3 and 4). The accumulation of the PeIE precursor was independent of the presence of the *out* genes (carried on pCPP2006). The fact that the presence of the *E. chrysanthemi* *out* genes did not affect the SecA dependence of *pelE* processing in *E. coli* strongly supports the hypothesis that the secretion of PeIE across the inner membrane is via the Sec machinery.

DISCUSSION

We have made five observations which support the hypothesis that Out-mediated secretion of pectate lyase isozyme PeIE to the milieu of *E. chrysanthemi* is preceded by translocation across the inner membrane by the Sec pathway for general protein export. (i) Mature PeIE was observed in a cell-bound fraction during active PeIE secretion. (ii) Abortive secretion of a PeIE-PhoA hybrid resulted in the accumulation of hybrid protein in the periplasm and outer membrane but not in the cytoplasm and inner membrane. (iii) A PeIE protein synthesized with a hybrid β-lactamase-PeIE signal peptide was still secreted to the medium. (iv) When the Out pathway was saturated by overexpression of the *pelE* gene in *E. chrysanthemi* or Out⁺ *E. coli*(pCPP2006)

cells, mature PeIE accumulated in the periplasm. (v) The processing of the PeIE precursor was SecA dependent even in an Out⁺ cell. Our data argue against a one-step secretion pathway involving either putative membrane adhesion zones, as has been suggested for the secretion of exotoxin A by *P. aeruginosa* (34), or the Sec-independent secretion pathway used, for example, by hemolysin (22). Most importantly, the processing of pre-PeIE was SecA dependent, not Out dependent, and mature PeIE or PeIE-PhoA hybrids accumulated in the periplasm rather than the cytoplasm of cells overexpressing *pelE* or containing *pelE::phoA* fusions.

It is important to note that we cannot eliminate the possibility that the effect of SecA deficiency on pre-PeIE processing was the indirect result of the loss of the Out apparatus from the bacterial envelope. However, three observations argue against this. (i) The processing of PeIE (and presumably the export and processing of Out proteins in the envelope) was only partially blocked (Fig. 7). (ii) No quantitative difference in pre-PeIE processing was observed between Out⁺ and Out⁻ SecA⁻ cells. (iii) The SecA deficiency had a similar effect on the processing of pre-Bla. Also, it seems unlikely that PeIE is sorted into the Out pathway until it has completed Sec-mediated translocation across the inner membrane, since the Sec pathway continues to function in the general export of proteins like β-lactamase to the bacterial envelope at the same time that extracellular proteins are being secreted to the medium. Thus, PeIE secretion is apparently a two-step process, and entry into the Out pathway must occur after passage through the Sec pathway, although not necessarily after release to the periplasm.

The targeting signals for the secretion of extracellular proteins via the Sec-dependent pathway have yet to be identified for any gram-negative bacterium. Our approach involving gene fusions between PeIE and PhoA failed to identify any targeting sequences for Out-mediated secretion in *E. chrysanthemi*. Previous sequence comparisons among at least six *pel* genes encoding extracellular pectate lyase isozymes of *E. chrysanthemi* and *E. carotovora* revealed two highly conserved regions, each consisting of about 20 amino acid residues (Fig. 3) (49). The PeIE-PhoA1 hybrid protein, although including both of these regions and 70% of the PeIE amino terminus, was not secreted by *E. chrysanthemi*. This suggests that either this portion of the PeIE polypeptide was insufficient for PeIE export or PhoA has a structure (probably the dimer conformation) that is incompatible with translocation across the outer membrane. Similar results were observed for Pga-PhoA hybrid proteins in *Pseudomonas solanacearum* (24). In *K. pneumoniae*, some PulA-PhoA hybrids are at least partially translocated across the outer membrane but are not released to the medium, whereas a PulA-Bla hybrid was efficiently secreted (9, 30). However, one must also face the possibility that there is no simple targeting sequence for this class of extracellular proteins but, rather, that the overall structure of the secreted protein is important. This possibility is supported by the observation that no consensus sequences have been identified among pectate lyases, exo-poly-α-D-galacturonidase, cellulases, and pectin methylesterase, which are all secreted by *E. chrysanthemi* via the Out pathway (3, 15, 19, 40, 49). Extracellular secretion of the *E. chrysanthemi* pectic enzymes appears to be representative of the secretion of a large family of extracellular proteins that possess amino terminal signal peptides, including the polygalacturonase of *P. solanacearum* (24), pullulanase of *K. pneumoniae* (42), aerolysin of *Aeromonas hydrophila* (23), and enterotoxin of *Vibrio*

cholerae (20). It is also apparent that different extracellular proteins in this class may exhibit different secretion kinetics and/or covalent modifications. For example, aerolysin and cholera toxin accumulate in the periplasm before being released to the medium, whereas PeE is rapidly released to the medium following processing (20, 54).

The observation of related secretion processes among different gram-negative bacteria is consistent with the recent finding of similarity among the secretory genes of *E. chrysanthemi* (16), *K. pneumoniae* (42), *P. aeruginosa* (14), and *E. carotovora* subsp. *carotovora* (46a). Despite an underlying similarity, the secretion systems of these bacteria appear to be highly specific for the proteins they secrete (16). For example, the *Erwinia* Out pathway distinguishes not only between envelope proteins and extracellular proteins but also between the extracellular proteins of related bacteria (16). Thus, the secretion of extracellular proteins that possess amino-terminal signal peptides by gram-negative bacteria appears to involve two conserved pathways and a factor that confers specificity in the recognition of extracellular proteins.

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ADDENDUM IN PROOF

A paper just published (A. P. Pugsley, I. Poquet, and M. G. Kornacker, *Mol. Microbiol.* 5:865-873, 1991) reports that the two steps in the secretion of pullulanase from *E. coli* can be uncoupled by inducing expression of the cloned *pulC-O* operon after a pool of cell-bound pullulanase has been formed. The secretion of pullulanase under this condition provides further evidence that there are two distinct steps in the secretion of proteins from gram-negative bacteria by the Sec-dependent pathway.

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