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External Loops at the C Terminus of *Erwinia chrysanthemi* Pectate Lyase C Are Required for Species-Specific Secretion through the Out Type II Pathway

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The type II secretion system (main terminal branch of the general secretion pathway) is used by diverse gram-negative bacteria to secrete extracellular proteins. Proteins secreted by this pathway are synthesized with an N-terminal signal peptide which is removed upon translocation across the inner membrane, but the signals which target the mature proteins for secretion across the outer membrane are unknown. The plant pathogens *Erwinia chrysanthemi* and *Erwinia carotovora* secrete several isozymes of pectate lyase (Pel) by the *out*-encoded type II pathway. However, these two bacteria cannot secrete Pels encoded by heterologously expressed *pel* genes from the other species, suggesting the existence of species-specific secretion signals within these proteins. The functional cluster of *E. chrysanthemi out* genes carried on cosmid pCPP2006 enables *Escherichia coli* to secrete *E. chrysanthemi*, but not *E. carotovora*, Pels. We exploited the high sequence similarity between *E. chrysanthemi* PelC and *E. carotovora* Pel1 to construct 15 hybrid proteins in which different regions of PelC were replaced with homologous sequences from Pel1. The differential secretion of these hybrid proteins by *E. coli* (pCPP2006) revealed M118 to D175 and V215 to C329 as regions required for species-specific secretion of PelC. We propose that the primary targeting signal is contained within the external loops formed by G274 to C329 but is dependent on residues in M118 to D170 and V215 to G274 for proper positioning.

Secretion of extracellular proteins is essential for virulence in many bacterial pathogens. Of the three major secretion pathways present in gram-negative bacteria, the type II pathway, or main terminal branch of the general secretion pathway, is used for the largest and most diverse group of proteins (41). The first type II pathway genes to be identified were those in the *pul* cluster, required for secretion of the starch-degrading enzyme pullulanase by *Klebsiella oxytoca*. Pugsley and coworkers demonstrated that *pulS* and a 13-gene operon, consisting of *pulC* to *pulO*, are necessary and sufficient for secretion of pullulanase across the outer membrane (40). Subsequent research has demonstrated that components of this secretion pathway are conserved among diverse gram-negative bacteria (39), including many pathogens of plants and animals.

Erwinia chrysanthemi and *Erwinia carotovora* are agents causing soft rot diseases in a variety of plant hosts. Characteristic symptoms of tissue maceration and cell death are caused by extracellular pectate lyase (Pel) and polygalacturonase, which are secreted along with other cell wall-degrading enzymes, including cellulases and pectin methyl esterase (4). Secretion of these enzymes by the type II pathway is essential for virulence, as *out* mutants, which are blocked in the pathway, do not elicit symptoms on host plants (26). A cosmid clone, pCPP2006, which complemented *E. chrysanthemi out* mutants was identified from a library of strain EC16 DNA, partially sequenced, and found to contain 12 genes, *outC* to *outM* and *outO*, highly similar to the *pul* cluster (14, 28). *out* genes have also been cloned from *E. carotovora*, but only the cosmid from *E. chrysanthemi* enables *Escherichia coli* to secrete cloned *Erwinia* enzymes (33, 45). Genes homologous to the *pul* and *out* clusters have been cloned from other pathogens, including *xps* of *Xanthomonas campestris* (10, 18) and *eep* of *Pseudomonas solanacearum* (21), involved in secretion of plant cell wall-degrading enzymes, *xcp* of *Pseudomonas aeruginosa* for secretion of elastase, exotoxin A, lipase, and alkaline phosphatase (2, 3, 11), *tcp* of *Vibrio cholerae* for secretion of cholera toxin (34), and *exe* in *Aeromonas hydrophila* for secretion of aerolysin (17, 20).

Exoproteins employing the type II pathway appear to cross the bacterial envelope in two stages. Synthesized with an N-terminal signal peptide, they are directed first to the Sec machinery for export across the cytoplasmic membrane (15, 42). Translocation of the mature protein across the outer membrane follows rapidly, but the mechanism of transport and the specific targeting features which distinguish exoproteins from those proteins remaining in the periplasm are poorly understood. Comparisons of the sequences of the diverse proteins which use the type II pathway have revealed no obvious regions of similarity (41). Deletion analyses, linker insertions, point mutations, and construction of hybrids with β-lactamase or alkaline phosphatase have all been used in the search for discrete targeting regions in the primary sequence (5, 9, 12, 15, 19, 24, 25, 35, 53). Studies on *K. oxytoca* pullulanase and *P. aeruginosa* exotoxin A have led to identification of relatively limited domains sufficient for targeting β-lactamase fusion proteins across the outer membrane (30, 31, 48). However, the observations that (i) two noncontiguous regions are required for targeting of pullulanase (48) and (ii) additional regions of exotoxin A either enhance or independently promote secretion (31) support the hypothesis that targeting features may be dependent on higher-order structure for their formation and/or proper positioning. For other exoproteins studied, including two from *E. chrysanthemi*, the vast majority of deletions and fusion constructs resulted in loss of all secretion

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capability, possibly because the structural context of the targeting signal had been disrupted (15, 43).

The *Erwinia out*-encoded type II pathway provides an attractive system for identification of targeting signals on exoproteins. Multiple enzymes employ the Out type II pathway, providing the opportunity for development of a general targeting model using proteins with various structures and sequences. Furthermore, three-dimensional structures have been determined for two *E. chrysanthemi* Pels, enabling structural analyses of any targeting regions that are identified (27, 54). Finally, species-specific secretion of *E. chrysanthemi* and *E. carotovora* Pels provides a novel avenue for identification of targeting regions in these proteins. *E. chrysanthemi* and *E. carotovora* secrete similar arsenals of plant cell wall-degrading enzymes, but the exoproteins of one species cannot be secreted by the other (14, 44). Furthermore, the cluster of *E. chrysanthemi out* genes carried on cosmid pCPP2006 retains this specificity for *E. chrysanthemi* Pels when functioning in *E. coli* (14).

Here, we have constructed hybrid proteins in which one region of a secreted Pel is substituted with the same region from a nonsecreted homolog, to systematically test each region for involvement in species-specific targeting while minimizing changes to their overall structural context. By mapping the regions required for species-specific secretion on the known structure of PelC, the locations of these regions within the protein structure were determined. Using this approach, we have demonstrated that elements within the regions V215 to C329 near the C terminus and M118 to D175 in the central part of the PelC protein are required for targeting PelC to the Out machinery. It is proposed that the primary signal determining species-specific secretion is contained within external loops near the C terminus whereas the required regions in the β-helix core are necessary for their proper positioning.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5α (13) was used as the standard strain for propagating recombinant plasmids and assaying for protein secretion. DH5α was grown in Terrific broth (47) at 37°C for isolation of plasmids and in King's B medium (22) at 30°C for protein secretion assays. The following concentrations of antibiotics were used where appropriate: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; and kanamycin, 50 μg/ml.

Recombinant DNA techniques. General procedures for isolation, analysis, and manipulation of DNA fragments were as described by Sambrook et al. (47). Subcloning was routinely performed by digesting vector and insert DNA with appropriate restriction enzymes, separating fragments by electrophoresis through 0.7% agarose gels, purifying the DNA with the Prep-a-Gene kit (Bio-Rad Laboratories, Richmond, Calif.), and ligating the vector and insert according to standard procedures. Before ligation, incompatible restriction sites were cloned by blunting the incompatible ends with T4 DNA polymerase (New England Biolabs, Beverly, Mass.) as described in reference 1.

Plasmids and subclones used in construction of PelC-Pel1 chimeras. Plasmids carrying *E. carotovora pel1* and *E. chrysanthemi pelC*, used for construction of the PelC-Pel1 chimeras, were made as follows. The *Nsp*I site in pBluescript II SK(−) and pBluescript KS(−) (Stratagene, La Jolla, Calif.) was deleted by digesting each with *Nsp*I, removing the 3′ overhangs with T4 DNA polymerase, and religating. The *Kpn*I site was subsequently deleted from each plasmid by the same procedure. The *Eco*RV site was deleted from pBluescript KS(−) *Nsp*I$^-$ *Kpn*I$^-$ by digesting with *Eco*RV and *Hin*dIII, filling in the *Hin*dIII 5′ overhang, and religating. The *Eco*RI fragment containing the *pel1* open reading frame from pAKC617 (7) was cloned into the *Eco*RI site of both pBluescript SK *Nsp*I$^-$ *Kpn*I$^-$ and pBluescript KS *Nsp*I$^-$ *Kpn*I$^-$ *Eco*RV$^-$ to create pAKC692 and pAKC693, respectively. For both pAKC692 and pAKC693, *pel1* is transcribed in the orientation opposite the *lac* promoter. pCPP2192, used for construction of pCPP2180, pCPP2183, pCPP2185, and pCPP2186, was made by cloning the *Xba*I-*Sph*I fragment from pPEL405 (52) into the *Xba*I-*Pst*I site of pBluescript SK. pCPP2193 was created by cloning the *Nci*I-*Xho*I fragment from pPEL403 (52) into the *Sty*I-*Xho*I site of pAKC617.

Site-directed mutagenesis of residues in the C-terminal branch. In preparation for site-directed mutagenesis, a 1.4-kb *Xba*I-*Hin*dIII fragment was cloned into the same sites in pRSET5A (49). Mutagenesis of divergent residues in the *pelC* region encoding the C-terminal branch to the corresponding sequences in *pel1* was performed by PCR using the overlapping extension method (32), with modifications described by Kita et al. (23). Following selection of desired *pelC* mutants, the genes were sequenced in entirety to confirm fidelity, using a Sequenase version 2.0 kit from United States Biochemical.

Assays for Pel activity and secretion. Pel secretion was assayed by fractionating 1 ml of culture at late logarithmic phase into cell and supernatant fractions by centrifugation. The cell pellets were washed once in cold fresh medium and sonicated in 1 ml of cold medium for 4 min with a model W-225R sonicator (Heat Systems Ultrasonics Inc., Plainview, N.Y.) at a duty cycle of 40% and an output of 4. Culture supernatants and sonicated cell pellets were assayed by an A_{235} assay and expressed in micromoles of unsaturated product liberated per minute per milligram of protein (8). Protein concentrations were determined by the Bradford assay (6). To account for nonspecific leakage, β-lactamase activity was determined for both cell and supernatant fractions, using the chromogenic cephalosporin compound nitrocefin (Glaxo, Greenford, Middlesex, England) and monitored at 540 nm. The percentage of Pel activity specifically secreted was determined by subtracting the percentage of total β-lactamase activity in the supernatant from the percentage of total Pel activity in the supernatant. In control experiments, with cells containing *pel* genes but no *out* genes, the percentage of total β-lactamase in the supernatant was 10 to 20% higher than the total percentage of Pel activity in the supernatant, indicating that use of β-lactamase as a periplasmic marker gives a conservative estimate of secretion efficiency.

Isoelectric focusing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and activity staining of the PelC-Pel1 chimeric proteins. Three-milliliter cultures of *E. coli* DH5α cells carrying pCPP2193, pAKC617, pCPP2194, pCPP2195, and each of the 14 chimeric constructs were centrifuged, washed with 5 ml of H_2O, centrifuged again, and sonicated for 5 min to break open the cells. Cell debris was removed by centrifugation, samples were concentrated as needed in Centricon microconcentrators (Amicon, Beverly, Mass.) and then washed with two starting volumes of H_2O to remove salts. Isoelectric focusing was performed on a PhastSystem (Pharmacia, Uppsala, Sweden), using gels rehydrated with 60% 3-10 and 40% 9-11 ampholytes (Sigma). pCPP2194 and pCPP2195 were run on premade sodium dodecyl sulfate-polyacrylamide gels, using the PhastSystem. Activity staining was performed as previously described (46), with the addition of 1% Triton X-100 to the wash buffer.

Computer analyses of protein sequences and structures. The PelC and Pel1 sequences were compared by using the FASTA program (36). The PelC structure was analyzed by using RasMol v2.5 (Roger Sayle, Biomolecular Structures Group, Glaxo Research & Development). Atomic coordinates for PelC and PelE were kindly provided by Frances Jurnak. Molecular modeling of Pel1 using the Pel1 sequence and PelC structure predicts a very high degree of similarity in overall folding (20a).

RESULTS

***E. carotovora* Pel1 is highly similar to *E. chrysanthemi* PelC but is not secreted by the *E. chrysanthemi* type II pathway.** The amino acid sequences of the *E. chrysanthemi* PelC and *E. carotovora* Pel1 mature proteins are 71% identical (Fig. 1). The sequence lengths differ by 1 residue, with mature PelC being 353 and Pel1 352 residues in length. When PelC is expressed in Out$^+$ *E. coli* DH5α(pCPP2006), 40 to 60% of the protein is specifically secreted to the supernatant, with efficiency increasing with reduced levels of expression. When Pel1 is expressed in DH5α(pCPP2006), no activity is observed in the supernatant.

Fourteen chimeric proteins constructed from *E. chrysanthemi* PelC and *E. carotovora* Pel1 retain Pel activity and have intermediate isoelectric points. Alignment of the coding regions for PelC and Pel1 revealed four conserved restriction sites in the two sequences. Their locations relative to the PelC sequence are as follows: *Nsp*I centered over the codon for M118, *Eco*RV centered over D170, *Hpa*I centered over V215, and *Kpn*I centered over G274 (Fig. 1). Using these sites, the sequences have been divided into five regions, designated A, B, C, D, and E (Fig. 1 and 2). Construction of hybrid genes from *pelC* and *pel1* required a set of parent clones for which the four conserved restriction sites were absent from the vector, the inserts were flanked by other restriction sites useful for subcloning, and the genes were expressed at appropriate levels. Four such clones were made as follows. (i) The *Nsp*I and *Kpn*I sites were deleted from pBluescript SK, and the *Nsp*I, *Kpn*I, and *Eco*RV sites were deleted from pBluescript KS. (ii) The *Eco*RI fragment from pAKC617 carrying the *pel1* gene driven by the *tet* promoter from pBR322 was cloned into the *Eco*RI

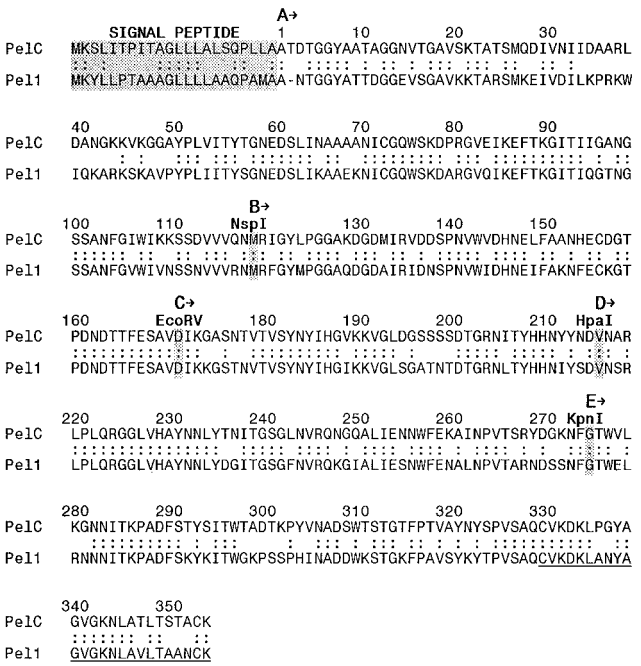

FIG. 1. Alignment of amino acid sequences for *E. chrysanthemi* PelC and *E. carotovora* Pel1. The N-terminal signal peptide is shaded, and the residues composing the C-terminal branch are underlined. Locations of four conserved restriction sites relative to the amino acid sequences are shaded and labeled. These sites have been used to divide the two sequences into five separate regions, labeled A, B, C, D, and E above the restriction sites.

site of pBluescript SK *Nsp*I$^-$ *Kpn*I$^-$ and pBluescript KS *Nsp*I$^-$ *Kpn*I$^-$ *Eco*RV$^-$ in orientations opposite the *lac* promoter to create pAKC692 and pAKC693, respectively. (iii) The *Nci*I-*Xho*I fragment carrying *pelC* from pPEL403 was cloned into the *Sty*I-*Xho*I sites of pAKC693 to create pCPP2193 such that *pelC* is driven by the *tet* promoter in pBluescript KS *Nsp*I$^-$ *Kpn*I$^-$ *Eco*RV$^-$. (iv) pCPP2192 was created by cloning the *Xba*I-*Sph*I fragment from pPEL405 into the *Xba*I-*Pst*I sites of pBluescript SK *Nsp*I$^-$ *Kpn*I$^-$ *Eco*RV$^-$. Using the four conserved sites described above and other sites flanking the coding regions, we constructed 14 hybrid genes in which different parts of *pelC* were replaced with homologous sequences from *pel1* (Fig. 2). For example, pCPP2178, in which regions B to E of *pelC* are replaced with *pel1* sequences, was constructed by cloning the *Nsp*I-*Xho*I fragment from pAKC693 into the *Nsp*I-*Xho*I sites of pCPP2193. All exchanges were verified by using diagnostic restriction digests.

E. coli DH5α cells carrying each of the 14 hybrids were grown to stationary phase and lysed, and total protein was concentrated 2- to 10-fold. Samples were run on an isoelectric focusing gel and activity stained. As seen in Fig. 3, all of the chimeric proteins from Fig. 2 retained Pel activity. The band for pCPP2185, although not as clear as the others, can be distinguished at the top of the gel, in close agreement with its predicted isoelectric point of 9.98. Hybrid proteins showed isoelectric points differing from PelC and Pel1, further confirming their status as novel proteins.

Three of the 14 PelC-Pel1 chimeric proteins retain secretion ability. Each of the 14 chimeric proteins made from PelC and Pel1 was expressed in *E. coli* DH5α carrying pCPP2006 and tested for its ability to be secreted. As shown in Fig. 2, proteins encoded by pCPP2183, where region A has been replaced, and pCPP2190, where region C has been replaced, were secreted at only slightly lower levels than wild-type PelC from pCPP2193. pCPP2197, in which both regions A and C of PelC are replaced with Pel1 sequences, was secreted at approximately one-quarter of wild-type levels. These results suggest that residues 1 to 118 at the N terminus of PelC and 170 to 215 in the central core of the protein are not absolutely required for species-specific targeting to the *E. chrysanthemi* Out system. Substitution of region B with the homologous sequences from Pel1 resulted in slightly higher levels of secretion than for wild-type Pel1, but when this region was replaced together with region C or both regions A and C, secretion ability was completely lost, indicating that it is in some way required for species-specific targeting. Replacement of PelC regions D and E, alone or in combination, resulted in complete loss of secretion.

Species-specific targeting of PelC is controlled by external loops at the C terminus as well as several noncontiguous turns of the β-helix core. As shown in Fig. 4, the PelC protein is composed of a parallel β-helix core covered at the N terminus by a short α-helix and at the C terminus by three loops (54). The results presented in Fig. 2 indicate that all or part of the three-loop cap at the C terminus (region E) is required for species-specific targeting as are elements within the 2.5 turns at the C-terminal end of the β-helix (region D) and 1.5 turns (region B) in the central part of the helix. The three-loop cap contained in region E is stabilized primarily by region D, although the third loop, or C-terminal branch (16), defined by C329 to C352, extends down the external face of the helix, passing over regions C and B in the β-helix core (Fig. 4). Structural analysis of PelC suggests that region B may influence the conformation of the three-loop cap by virtue of its direct interaction with the C-terminal branch.

The C-terminal branch defined by C329 to C352 is not a primary determinant of species-specific targeting. To better define the parts of the C-terminal region required for species-specific targeting, the third loop (C terminal branch) was chosen as a target of mutagenesis. Divergent residues 335 and 336, 346, and 349 to 351 in PelC were replaced with the corresponding sequences from Pel1 by site-directed mutagenesis. The resulting construct, PelC/1-335-351, was efficiently secreted by *E. coli*(pCPP2006), suggesting that the C-terminal branch does not play a direct role in species-specific targeting (Fig. 2).

DISCUSSION

The type II secretion system is present in diverse gram-negative bacteria and is required for secretion of virulence proteins by many pathogens. *E. chrysanthemi* and *E. carotovora* secrete similar Pels, using type II secretion pathways whose individual components share a high degree of sequence conservation; however, the two species cannot reciprocally secrete their Pel proteins (14). This observation reveals that species-specific secretion signals are embedded within otherwise similar proteins. Thus, the *E. chrysanthemi* Out system fails to secrete Pel1 from *E. carotovora* EC71 even though Pel1 is 71% identical to *E. chrysanthemi* PelC. By constructing 15 hybrid proteins in which different regions of PelC were substituted with homologous sequences from Pel1, the C-terminal cap, excluding the C-terminal branch, and several turns in the β-helix core were identified as regions required for species-specific secretion of PelC.

Most attempts to define targeting signals through the use of deletions or gene fusions with alkaline phosphatase or β-lactamase have failed to define discrete regions required for targeting (9, 19, 24, 25). For example, the two functional domains from *E. chrysanthemi* endoglucanase Z were stable when expressed independently, but neither retained secretion ability

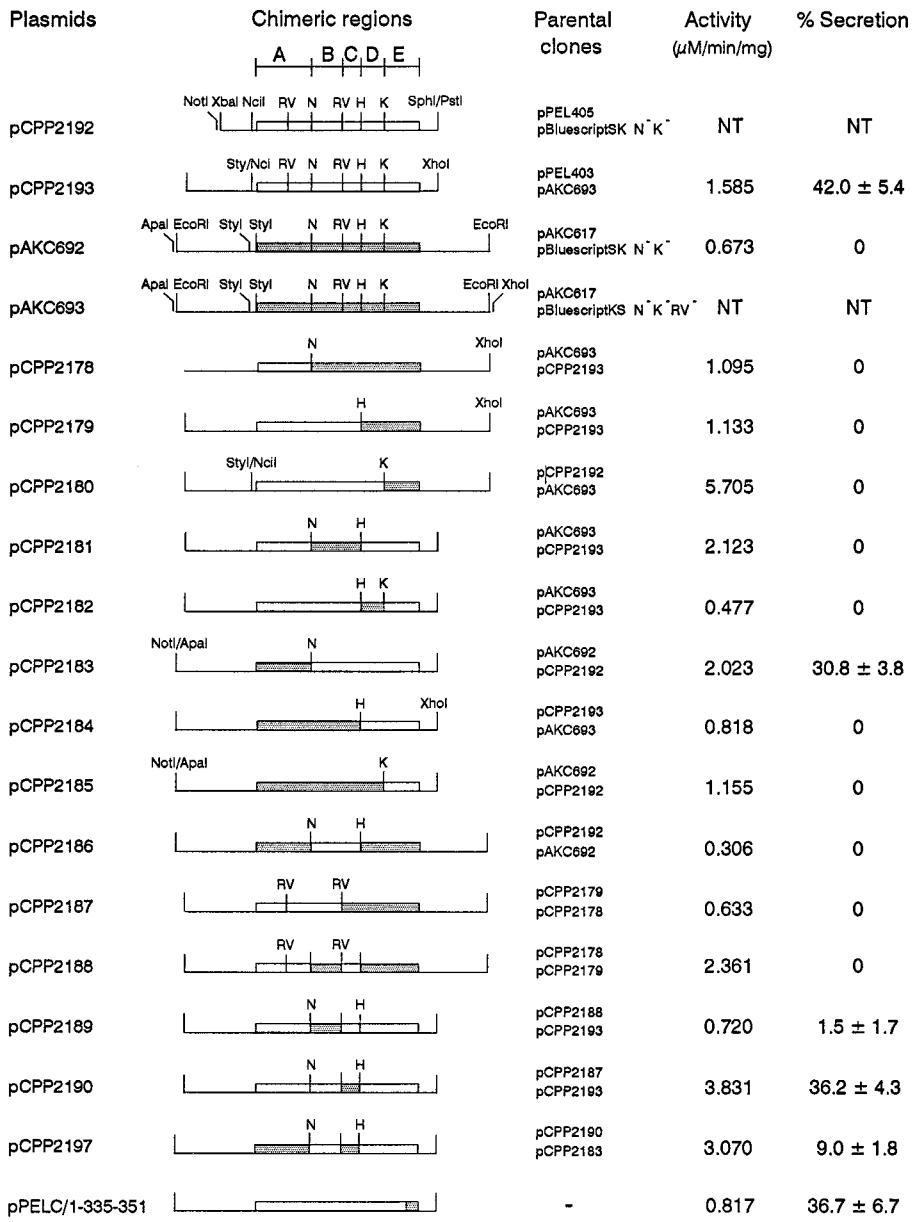

FIG. 2. Diagram of 2 *pelC* clones, 2 *pel1* clones, and 15 *pel1-pelC* hybrid constructs with parental clones, with the activity and percent secretion by Out$^+$ *E. coli* shown for each. Regions derived from *pelC* are indicated as open bars, and regions derived from *pel1* are shaded. Regions are designated A, B, C, D, and E according to the key at the top. Abbreviations for the four conserved sites used in construction of each hybrid: RV, *Eco*RV; N, *Nsp*I; H, *Hpa*I; K, *Kpn*I (all other sites are indicated by full names). Parental clones for each construct are shown with the source of the insert listed first and the vector-containing part of the construct listed below. For example, the hybrid clone pCPP2178 was constructed by inserting the *Nsp*I-*Xho*I fragment from pAKC693 into the *Nsp*I-*Xho*I sites of pCPP2193. For pPELC/1-335-351, the region corresponding to the *pel1* sequence was introduced by site-directed mutagenesis. Activity is shown as micromoles of unsaturated product liberated per minute per milligram of protein. Percent total extracellular Pel activity was obtained by determining the percentage of total Pel activity in the supernatant minus the percentage of total β-lactamase activity in the supernatant. Values indicated represent the averages of four separate trials for each clone. NT, not tested.

(43). Although specific regions have been implicated in the secretion of *P. aeruginosa* exotoxin A and *K. oxytoca* pullulanase, their actual role has been difficult to establish. In *P. aeruginosa* exotoxin A, residues 60 to 120 were found to be sufficient for targeting a β-lactamase fusion protein across the outer membrane. However, a deletion construct of exotoxin A containing the N-terminal 30 amino acids attached to the C-terminal 370 residues is also secreted (31), suggesting that targeting of the native protein may involve more than a single region within the primary sequence. Studies with *K. oxytoca* pullulanase–β-lactamase fusions revealed that two noncontiguous regions composed of residues 1 to 78 and 735 to 814 are sufficient and jointly necessary to promote secretion of β-lactamase across the outer membrane (48). However, deletion of either of these regions from native pullulanase only partially reduces secretion.

The elusiveness of a discrete element in the primary sequence involved in targeting has led to the proposal that the secretion signal could be dependent on higher-order structure for its formation. For example, targeting signals could be de-

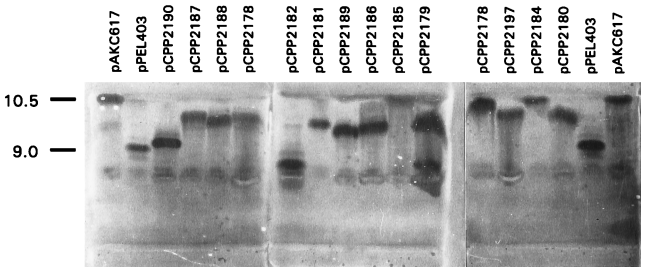

FIG. 3. Activity-stained isoelectric focusing profiles for PelC, Pel1, and 14 PelC-Pel1 hybrids. Names of each are indicated at the top, and the isoelectric points for PelC (pPEL403) and Pel1 (pAKC617) are indicated on the left. Faint artifactual bands are visible in each lane at the site of sample application (below 9.0), with intensities proportional to the amount of protein loaded.

termined by a patch signal located in one region of the final structure but composed of amino acids from diverse parts of the linear sequence. Alternatively, the signal could be defined by a region of primary sequence but highly dependent on the overall structure of the protein for proper presentation to the secretion machinery (39, 48). Disruption of overall structure, the likely reason that other attempts to identify targeting regions have been unsuccessful, is minimized by the PelC-Pel1 hybrid approach described here.

Using the PelC-Pel1 hybrid approach, elements within the following three regions of PelC were identified as being required for species-specific targeting: (i) region E, composed of the loops at the C-terminal end of the β-helical core of the protein; (ii) region D, containing the four helical turns partially covered by the C-terminal loops; and (iii), region B, containing 1.5 helical turns in the central portion of the β-helical core. Further mutagenesis of region E revealed that the C-terminal branch is not directly involved in species-specific targeting. The PelC-Pel1 hybrids could not be used to reciprocally identify regions in Pel1 required for species-specific targeting because the *E. carotovora out* gene cluster is not functional in *E. coli* (29) and an appropriate Pel-deficient *E. carotovora* strain is not available.

Several lines of evidence indicate that proteins secreted by the type II pathway are secreted in a largely folded conformation. It has been shown that disulfide bonds are made prior to secretion, and cholera toxin, a multimeric protein secreted by the *V. cholerae* type II pathway, is assembled in the periplasm prior to secretion (5, 37, 38, 50, 55). Assuming that the Pel proteins are folded prior to secretion, we suggest that external loops of the protein are more likely to be involved in targeting than regions of the β-helical core. Examination of structural elements in regions B, D, and E reveals two places where loops extend away from the body of the protein. One is composed of the three loops at the C terminus in region E, and the other consists of a loop in region B which forms part of the active site. Neither of these loop regions is completely conserved at the sequence level between PelC and Pel1, but without structural information on Pel1 it is difficult to predict how they structurally differ. However, comparison of PelC with the known structure for *E. chrysanthemi* PelE can be used to identify which of these regions are most similar between the cosecreted Pels. Although PelC and PelE differ substantially in sequence, the comparison done by Lietzke et al. (27) reveals that the β-helical core and several elements in the C-terminal loops are structurally conserved between the two proteins. In contrast, the loop in the active site is highly divergent in both size and folding, making it an unlikely candidate for a conserved targeting signal among the *E. chrysanthemi* Pels. We therefore propose that the primary signal for species-specific targeting is contained within the C-terminal loops in region E

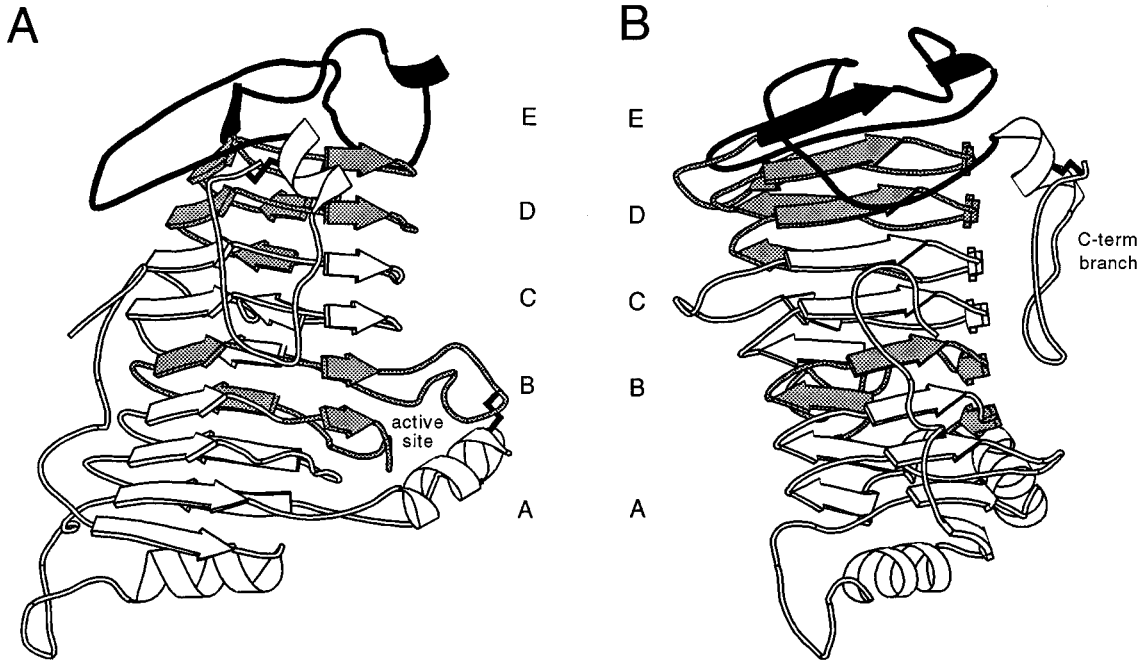

FIG. 4. Two views of a ribbon diagram of PelC with regions required for species-specific secretion shaded. Regions A and C and the C-terminal (C-term) branch of region E, none of which are required for species-specific secretion, are indicated in white. Regions B and D, required for targeting, are lightly shaded. Region E, excluding the C-terminal branch, is shaded darkly because the external loops in this region are considered the best candidates for a primary targeting signal. Disulfide bonds linking C72 to C155 and C329 to 352, where visible, are indicated in black.

whereas regions D and B play a secondary role in the structural positioning of these residues.

In an attempt to better define the putative targeting signal contained within the three C-terminal loops, all divergent residues in the third loop, or C-terminal branch, of PelC were mutagenized to the corresponding sequence in Pel1. It was hypothesized that if the C-terminal branch plays a key role in species-specific targeting, changing the divergent sequences should disrupt secretion by the *E. chrysanthemi* type II pathway. However, the resulting protein, encoded by pPELC/1-335-351, was secreted as efficiently as wild-type PelC, indicating that the signal for species-specific targeting is most likely located in the other loops of the C-terminal cap.

Mutagenesis of the these other loops is a major undertaking, as the number of divergent residues is substantial and the structural implications of changes difficult to predict without knowledge of the Pel1 structure. Structural determination of additional Pels from both *E. chrysanthemi* and *E. carotovora* is anticipated to yield further clues regarding structurally conserved regions. Comparison of the location of putative targeting regions on PelC with the targeting regions identified in pullulanase and exotoxin A indicates that the location of these signals among proteins using the type II pathway is not conserved at the level of primary sequence. However, the pullulanase and exotoxin A fusions to β-lactamase were made with the goal of identifying general targeting signals that could confer secretion on a normally periplasmic enzyme, while the PelC-Pel1 hybrid strategy was directed at identification of those signals involved in species-specific recognition by the Out pathway. Given their overall structural similarity, it is highly possible that additional regions in the Pels play a role in targeting but, being conserved between the two proteins, are not revealed by this approach.

As a corollary to our experiments involving hybrid *E. chrysanthemi-E. carotovora* Pel proteins, we have used pCPP2006 as the basis for constructing hybrid *E. chrysanthemi-E. carotovora* type II secretion systems. This has revealed OutD, an outer membrane protein, as a candidate gatekeeper for the species-specific secretion of *E. chrysanthemi* Pels (29). Furthermore, the *E. chrysanthemi* OutD protein interacts with *E. chrysanthemi*, but not *E. carotovora*, Pel proteins (51). The species-specific interactions of cognate gatekeepers and structurally defined, secreted proteins could provide a new avenue for determining the structural features that control the secretion of proteins through the type II pathway.

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