Analysis of Eight out Genes in a Cluster Required for Pectic Enzyme Secretion by Erwinia chrysanthemi: Sequence Comparison with Secretion Genes from Other Gram-Negative Bacteria

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Received 13 July 1992/Accepted 9 September 1992

Many extracellular proteins produced by Erwinia chrysanthemi require the out gene products for transport across the outer membrane. In a previous report (S. Y. He, M. Lindenberg, A. K. Chatterjee, and A. Collmer, Proc. Natl. Acad. Sci. USA 88:1079–1083, 1991) cosmid pCPP2006, sufficient for secretion of Erwinia chrysanthemi extracellular proteins by Escherichia coli, was partially sequenced, revealing four out genes sharing high homology with pulH through pulK from Klebsiella oxytoca. The nucleotide sequence of six additional out genes reveals homology with pulC through pulG, pulL, pulM, pulO, and other genes involved in secretion by various gram-negative bacteria. Although signal sequences and hydrophobic regions are generally conserved between Pul and Out proteins, four out genes contain unique inserts, a pulN homolog is not present, and outO appears to be transcribed separately from outC through outM. The sequenced region was subcloned, and an additional 7.6-kb region upstream was identified as being required for secretion in E. coli. Out gene homologs were found on Erwinia carotovora cosmid clone pAKC651 but were not detected in E. coli. The outC-through-outM operon is weakly induced by polygalacturonic acid and strongly expressed in the early stationary phase. The out and pul genes are highly similar in sequence, hydrophilic properties, and overall arrangement but differ in both transcrip- tional organization and the nature of their induction.

Gram-negative bacteria employ several routes for the secretion of extracellular proteins (29). Among these, the Sec-dependent pathway appears to secrete the largest and most diverse group of proteins. This two-step pathway uses the Sec machinery for translocation across the inner membrane and an accessory secretion apparatus for translocation across the outer membrane. Secretion of the starch-degrad- ing enzyme pullulanase by Klebsiella oxytoca is the best studied example of the Sec-dependent route (34). Pugsley and coworkers have demonstrated that pulS and a 13-gene operon (pulC through pulO) are necessary and sufficient for secretion of the pullulanase enzyme across the outer mem- brane (34). Subsequent research has demonstrated that components of this secretion pathway are conserved among diverse gram-negative bacteria (29).

Erwinia chrysanthemi and Erwinia carotovora, like many other bacterial pathogens of plants and animals, depend on extracellular proteins for virulence. Both of the Erwinia species secrete a wide variety of cell wall-degrading en- zymes that macerate plant tissues and produce soft rot symptoms (27). Murata et al. (31) reported the cloning of a cluster of E. carotovora out genes that complemented all secretion mutants in that species. However, unlike the K. oxytoca pulS-pulC-through-pulO secretion cluster, which enables Escherichia coli to secrete pullulanase, the E. carotovora out cluster does not confer on E. coli the ability to secrete E. carotovora pectic enzymes.

The only other secretion genes known to confer on E. coli the ability to secrete heterologous proteins via a Sec-depen- dent pathway are the out genes of E. chrysanthemi EC16 (18). Cosmid pCPP2006 contains a 40-kb fragment of EC16 DNA and enables E. coli to secrete four pectate lyase isozymes, exo-poly-α-D-galacturonosidase, and pectin methylesterase. A 12.0-kb region of pCPP2006 was identified by TnphoA mutagenesis as containing out genes required for protein secretion by E. chrysanthemi and E. coli. Sequencing of a 2.4-kb region within the out cluster revealed two complete and two partial open reading frames (ORFs) sharing substantial homology with pulH through pulK (18).

Other reports of cloned pul homologs from Pseudomonas aeruginosa (4, 5, 15) Xanthomonas campestris pv. campestris (14, 19), and Aeromonas hydrophila (23) indicate that this secretion pathway is not limited to the family Enterobacteriaceae. Although substantial homology exists among secretion genes cloned from various gram-negative bacteria, none are able to secrete the others’ enzymes (10, 18). This observation holds true even for the two Erwinia species (18, 35).

Little is known of the signals in these exoproteins that target them to the secretion pathway or of pathway components that recognize these signals and thereby control protein traffic to the cell exterior. Two aspects of the Erwinia Out system make it particularly attractive for exploring targeting signals and recognition components. The E. chrysanthemi EC16 out cluster is unique in permitting E. coli to secrete multiple proteins by a Sec-dependent pathway, and E. chrysanthemi and E. carotovora are the most closely related bacteria from which secretion genes have been cloned. To potentiate the use of the Erwinia out system for exploring protein recognition processes in the Sec-dependent secretion pathway, we characterized the out genes of E. chrysanthemi and compared them with the secretion genes of other gram-negative bacteria. We report here (i) the DNA

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sequence and analysis of eight additional *E. chrysanthemi* out genes carried on pCPP2006, (ii) identification by subcloning of the minimum region in pCPP2006 sufficient for secretion in *E. coli*, (iii) Southern hybridization analysis of cloned *E. carotovora* out genes by using probes from *E. chrysanthemi*, (iv) the absence of highly conserved out homologs in *E. coli*, and (v) factors influencing out gene expression in *E. chrysanthemi* and *E. carotovora*.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *E. coli* DH5α (16) was used as the standard strain for propagating recombinant plasmids and assaying heterologous protein secretion. Marker-exchange mutagenesis of chromosomal out genes was done with *E. carotovora* subsp. *carotovora* EC71 and *E. chrysanthemi* AC4150 (Nal' derivative of EC16). *E. coli* strains were grown in Terrific broth (39, 42) at 37°C for isolation of plasmids and in King’s B medium (25) at 30°C for protein secretion assays. *Erwinia* strains were typically grown in King’s B medium, but strains tested for induction of out genes by polygalacturonate were grown at 30°C in M9 (39) plus 0.5% glycerol, with or without 0.2% (wt/vol) polygalacturonate (polygalacturonic acid from Pflantz and Bauer, Waterbury, Conn., was titrated to medium pH with NaOH). Strains tested for growth phase induction were grown in King’s B medium–0.1% (wt/vol) polygalacturonate–0.1 mM CaCl2. The following concentrations of antibiotics were used when appropriate: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 10 μg/ml.

**Recombinant DNA techniques.** General procedures for isolation, analysis, and manipulation of DNA fragments followed those described in Sambrook et al. (39). 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.

**Plasmids and subclones.** Regions within pCPP2006 were subcloned by exploiting a combination of intrinsic restriction sites and the NotI site present in two Tphoa insertions. Vectors used for subcloning were pSU20, a low-copy-number Cm' plasmid (6); pBluescript SK(−), a high-copy-number Ap' phagemid (Stratagene, La Jolla, Calif.); and pBCKS (Stratagene), a high-copy-number Cm' phagemid. The construction of pCPP2162, pCPP2163, and pCPP2164 involved several steps that are not evident from Fig. 1. By using the NotI site present on Tphoa insert 21, the BamHI-NotI fragment to the left of outC was first subcloned into pSU20 to create pCPP2170, and the BamHI-HindIII fragment was subsequently deleted. The Tphoa-derived NotI site in the resultant plasmid was cut and filled in by using the Klenow fragment of DNA polymerase I, enabling the fragment to be subcloned into HindIII-HincII-digested pBCKS. The resultant plasmid was then digested completely with XhoI and ApaI and partially with exonuclease III to produce pCPP2162 and pCPP2164. Plasmid pCPP2162 was cut with HindIII and EcoRI, after which the sites were filled in by using the Klenow fragment and ligated together to produce pCPP2163.

**DNA sequencing and data analysis.** Seven contiguous restriction fragments within the previously identified out region of *E. chrysanthemi* were individually cloned into pBluescript SK(−) (Stratagene). Sets of nested deletions were generated from each subclone by using the Erase-a-Base kit from Promega (Madison, Wis.). Both strands were sequenced by the dideoxy method (40) using the Sequenase version 2.0 kit from United States Biochemical (Cleveland, Ohio). Gaps between sequenced regions were filled by using primers synthesized at the Cornell Biotechnology Program Oligonucleotide Biosynthesis Facility. Sequence data were analyzed with the FASTA program (33) and the Genetics Computer Group Sequence Analysis Software Package (13).

**Expression and analysis of the OutC protein.** DH5α (pCPP2171) and DH5α[pBluescript SK(−)] were grown overnight in Terrific broth containing 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Saturated culture (30 μl) was centrifuged and resuspended in 15 μl of Terrific broth. These samples were boiled for 3 min in 4× sample buffer (39) and analyzed by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) using a vertical slab unit (model SE 280; Hoefer Scientific Instruments) (39).

**Southern hybridization.** pAKC651 was digested with SalI and BamHI, HindIII and PstI, and HindIII alone. Fragments were separated on a 0.7% agarose gel and transferred to Immobilon membranes (Millipore, Bedford, Mass.) by capillary action. The membranes were prehybridized at 45°C for 2 h, pCPP2162, pCPP2160, pCPP2059, and pCPP2058 were labeled with 32P by using the Prime-It random primer kit from Stratagene. Following addition of probes, the blots were hybridized at 45°C overnight. Each was washed for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.5% SDS, 15 min in 1× SSC with 0.5% SDS at 45°C, and 30 min in 0.5× SSC with 0.5% SDS at 65°C (39).

The genome of *E. coli* K-12 was screened for the presence of out homologs by probing the Kohara *E. coli* DNA library (26) with pCPP2159. Two microcultures of each of 680 λ clones, representing the entire genome of *E. coli* K-12 (26), were spotted onto a GeneScreen membrane (Du Pont, Wilmington, Del.). The DNA was denatured and blot-digested according to the GeneScreen protocol. Following addition of 32P-labeled pCPP2159, the blot was hybridized at 45°C overnight. The blot was washed as described above. Clones hybridizing to the probe were amplified, and their DNA was extracted (3). DNA was digested with PstI or KpnI, run on a 0.7% agarose gel, transferred to Immobilon membranes, and probed with labeled pCPP2159 as described for the Immobilon blot of pAKC651.

**Tns-gusA1 mutagenesis.** Tns-gusA1 carried on a λ vector was used to mutagenize *E. coli* DH5α(pCPP2159) and *E. coli* DH5α(pAKC651) (41). To permit marker exchange of the Tns-gusA1 insertions, plasmids were isolated from Km' Tc' transformsants, electroporated into *E. chrysanthemi* AC4150 and *E. carotovora* EC71 (17), and cultured in low-phosphate media supplemented with kanamycin (38).

**Assays for β-glucuronidase and pectate lyase activity and pectate lyase secretion.** Strains tested for induction by polygalacturonate were grown to late logarithmic phase. β-Glucuronidase activity was assayed fluorometrically as described by Jefferson et al. (21). Sonicated culture (20 μl) was added to an equal volume of 2× assay buffer (100 mM sodium phosphate buffer [pH 7.0], 0.2% Triton X-100, 20 mM β-mercaptoethanol, 2 mM 4-methylumbelliferyl β-glucuronide [Sigma catalog no. M-9130]) and incubated at 37°C for 20 h. The reaction was terminated by 50-fold dilution in 0.2 M Na2CO3. Fluorescence was measured in 0.2 M Na2CO3 by excitation at 365 nm and emission at 455 nm with a spectrophotometer (model TKO 100; Hoefer Scientific Instruments) and expressed in units of methylumbelliferone fluorescence per hour. Pectate lyase activity of sonicated cultures was
assayed with the \( A_{323} \) assay (8) and expressed in micromoles of unsaturated product liberated per minute per milliliter of culture.

Secretion of pectate lyase was assayed by fractionating 1 ml of culture at late logarithmic phase by centrifugation. The cell pellets were washed once in cold fresh media and sonicated in 1 ml of cold media. Culture supernatants and sonicated cell pellets were assayed by the \( A_{323} \) assay described above. Distribution of the plasmid marker \( \beta \)-lactamase encoded by pPL7421 (24) was assayed as a control for nonspecific leakage.

**Nucleotide sequence accession number.** The nucleotide sequence presented in this paper has been assigned GenBank accession number L02214.

**RESULTS**

DNA sequencing reveals eight additional \( E. \) chrysanthemi EC16 \( out \) genes. We had previously reported the DNA sequence of a 2.4-kb EcoRI fragment which contains two complete and two incomplete ORFs with substantial homology to \( pulH, pulI, \) and \( pulK \) in the pullulanase operon of \( K. \) oxytoca (18). It was therefore not unexpected when sequencing of the DNA 3.2 kb downstream and 7.2 kb upstream of this EcoRI fragment revealed the presence of eight additional ORFs sharing substantial homology with other genes in the \( pulC \)-through-\( pulO \) operon. The location of these ORFs on pCPP2006 is shown in Fig. 1. The region upstream of the 2.4-kb EcoRI fragment encoded proteins having similarity with PulC, PulD, PulE, PulF, and PulG, whereas the region downstream encoded proteins similar to PulL, PulM, and PulO. Each \( out \) gene has been designated with the same letter as its \( pul \) homolog. No homolog of \( pulN \) was present in the sequenced \( out \) region. Important features of the nucleotide sequence are shown in Fig. 2 and are discussed in later sections.

Amino acid identity between predicted \( Out \) proteins and their \( Pul \) homologs is 38% for C, 67% for D, 73% for E, 67% for F, 77% for G, 34% for H, 50% for I, 52% for J, 44% for K, 38% for L, 37% for M, and 62% for O. These percentages were calculated for overlapping DNA only and do not include nonhomologous inserts. Pul homologs in \( P. \) aeruginosa (4, 5, 15, 32, 43), \( X. \) campestris (14, 19), \( A. \) hydrophila (23), and \( B. \) subtilis (1, 30) have been reported. As expected, the \( Out \) proteins had various degrees of homology with the other \( Pul \) homologs; however, in sequence and overall arrangement, \( Out \) and \( Pul \) are most similar to one another. Comparisons of \( Pul \), \( Out \), \( Xcp \), and \( Xps \) homologs are shown in Fig. 3.

\( outC \) encodes a polypeptide with an apparent molecular mass of 30 kDa. We could not determine the start site of the
outC ORF from the DNA sequence because homology between the N terminus of OutC and PulC is low and none of the candidate methionine codons are preceded by an obvious ribosome binding site. The three candidate Met residues predict polypeptides of 26.6, 27.6, and 30.03 kDa. When total protein of DH5α(pCPP2171) induced with IPTG was compared with total protein of similarly treated DH5α [pBluescript SK(−)], a polypeptide of approximately 30 kDa was found in the cells carrying pCPP2171 (Fig. 4). We concluded from these data that the Met codon predicting a 30-kDa protein is the actual start site. Although the N terminus of this predicted protein diverges substantially from the N terminus of PulC, the two proteins are similar in length and contain a hydrophobic region at approximately the same location.

Signal peptides and hydrophobic regions are conserved between Pul and Out proteins. Signal sequences in the Pul proteins include a signal peptidase I signal peptide at the N terminus of PulD and type 4 fimbrial subunit-like signal peptides at the N termini of PulG, PulH, PulI, and PulJ. Each of the five signal peptides is highly conserved in the corresponding Out homolog. Although cleavage of a PulK homolog by a PulO homolog has not been demonstrated, the N terminus of PulK and OutK also have potentially significant homology with the type 4 fimbrial subunit-like signal peptides. An R-Q-R-G motif immediately preceding the cleavage site in PulG and OutG and in PulH and OutH is present near the N terminus of PulK and OutK. This motif in PulK and OutK is immediately followed by a hydrophobic stretch characteristic of those seen in other proteins with this type 4 fimbrial subunit-like signal peptides. However, the highly conserved 5-residue sequence that generally separates R-Q-R-G from the hydrophobic stretch is not present in either PulK or OutK.

Hydrophobic sequences long enough to qualify as membrane-spanning regions are conserved between the Pul pro-
teins and their Out homologs. These regions are highlighted in Fig. 3. Included are the hydrophobic regions in OutG, OutH, OutI, and OutJ that follow the cleavage site.

Four Out proteins contain regions not shared by their Pel homologs. Four Out proteins have inserts of various lengths that are not shared by their Pel counterparts (Fig. 1 and 3).

The largest of these inserts extends from residue 295 to residue 357 in OutD and is noteworthy for being more than half serine. Other unique regions in the Out proteins include an asparagine-rich insert in OutK extending from residue 110 to residue 152, a 20-amino-acid insert extending from residue 146 to residue 165 of OutF, and a 17-amino-acid insert.

FIG. 3. Comparison of the deduced amino acid sequences for genes involved in Sec-dependent secretion from K. oxytoca (pel), E. chrysanthemi (out), P. aeruginosa (cep), and X. campesiris (xop). Shared residues are boxed, and hydrophobic regions are shaded. The signal peptidase I cleavage site in PelD and OutD is indicated by an open arrowhead. Cleavage sites for the type 4 pilus subunit-like leader sequence peptidase are indicated with dark arrowheads.
The DNA sequence of the gene cluster encoding these three enzymes, which is located immediately downstream of the pulS region, was analyzed. These enzymes include pullulanase, pullulase, and pullulanase, which extend from residues 44 to 86 of one gene, respectively. 

The gene cluster consists of four genes, *pulS*, *pulU*, *pulV*, and *pulW*. The *pulS* gene is located immediately downstream of the *pulU* gene, and *pulV* and *pulW* are located immediately downstream of the *pulS* gene. These genes are expressed as an operon, *pulU-pulS-pulV-pulW*, which is transcribed as a single transcript. The transcript is terminated by a Shine-Dalgarno sequence and a ribosome binding site, and the translation initiation codon is located at position 38 of the transcript.

The DNA sequence of the gene cluster was analyzed to determine whether the region required for the Out phenotype is encoded by the *pulS* gene. This was done by searching for the presence of two transcriptional units, the *pulU-pulS* and *pulV-pulW* units, in the DNA sequence of the gene cluster. The *pulS* gene was found to be located immediately downstream of the *pulU* gene, and both genes are transcribed as a single transcript. The transcript is terminated by a Shine-Dalgarno sequence and a ribosome binding site, and the translation initiation codon is located at position 38 of the transcript.

The presence of the *pulU-pulS* and *pulV-pulW* units was also analyzed in the DNA sequence of the gene cluster. The *pulU* gene was found to be located immediately downstream of the *pulS* gene, and both genes are transcribed as a single transcript. The transcript is terminated by a Shine-Dalgarno sequence and a ribosome binding site, and the translation initiation codon is located at position 38 of the transcript.

The gene cluster was also analyzed to determine whether the region required for the Out phenotype is encoded by the *pulU* gene. This was done by searching for the presence of two transcriptional units, the *pulS-pulU* and *pulV-pulW* units, in the DNA sequence of the gene cluster. The *pulU* gene was found to be located immediately downstream of the *pulS* gene, and both genes are transcribed as a single transcript. The transcript is terminated by a Shine-Dalgarno sequence and a ribosome binding site, and the translation initiation codon is located at position 38 of the transcript.

The presence of the *pulS-pulU* and *pulV-pulW* units was also analyzed in the DNA sequence of the gene cluster. The *pulU* gene was found to be located immediately downstream of the *pulS* gene, and both genes are transcribed as a single transcript. The transcript is terminated by a Shine-Dalgarno sequence and a ribosome binding site, and the translation initiation codon is located at position 38 of the transcript.
sufficient to confer the secretion phenotype to *E. coli*, the sequenced out cluster was subcloned into pWSK129 by using a NorI site present on TnphoA insertion 14.3 kb upstream of the outC start site and a Bsu361 site 0.8 kb downstream of outO (Fig. 1). This subclone, designated pCPP2159, was not sufficient to confer the Out phenotype to *E. coli* DH5a(pPL7421) encoding the *E. chrysanthemi* pelE gene (24). When pCPP2159 was complemented in trans with pCPP2161, containing the *BamH* fragment upstream of outO subcloned into pCPP2162, the ability of *E. coli* to secrete PelE was restored. The specific region required for complementation was localized to a 7.6-kb fragment beginning 0.7 kb upstream of outC and designated pCPP2162.

Deletions extending 0.5 kb into the 5' end as in pCPP2163 and 1 kb into the 3' end of pCPP2162 as in pCPP2164 resulted in a loss of complementation. Whether the entire internal region is required is not known.

*E. chrysanthemi* out gene homologs are present in *E. carotovora* but were not detected in *E. coli*. The SalI fragment from pAKC601 was recloned into pSU20. Restriction digests of this construct, designated pAKC651, were probed by Southern blot hybridization with pCPP2162 and three subclones of pCPP2159 spanning outD through outO. The three subclones from pCPP2159 hybridized to the restriction fragments of pAKC651 in a pattern indicating that the outC through outO genes in the two *Erwinia* spp. are arranged in collinear
clusters (Fig. 5). pCPP2162 did not hybridize to any part of pAKC651 and consequently was tested for its ability to confer secretion proficiency to *E. coli* DH5α(pAKC651). However, cells carrying both plasmids failed to secrete the products of the cloned *E. carotovora* pell (44) or the *E. chrysanthemi* pell.

When a homology search of the GenBank database was conducted with the OutO sequence, a putative protein from *E. coli* with 52% similarity to OutO was identified (2, 43). Two putative proteins with type 4 fimbrial subunit-like leader sequences have been identified in *E. coli* (43); however, similarity between these two proteins and OutG, OutH, OutI, and OutJ does not extend beyond the signal sequence. The region upstream of the *E. coli* outO homolog does not contain genes similar to other out genes. To investigate whether out homologs other than outO might be
present elsewhere on the *E. coli* chromosome, we probed the Kohara *E. coli* DNA library with pCPP2159. pCPP2159 hybridized weakly to several nonoverlapping clones but not at all to those carrying the *outO* homolog. DNA was extracted from the seven clones to which pCPP2159 hybridized most strongly and reprobed with pCPP2159. No hybridization was evident, suggesting that positive clones in the initial dot blot resulted from nonspecific binding. The lack of hybridization suggests that the *outO* homolog and any other *out* homologs that might be present in *E. coli* are not conserved sufficiently to be detectable by these probe procedures.

*E. chrysanthemi* and *E. carotovora* *out* genes are weakly induced by polygalacturonic acid and strongly regulated by growth phase. Several of the *E. chrysanthemi* pectic enzymes are inducible by polygalacturionate degradation products. This regulation is mediated, in part, by the KdgR repressor protein, which binds to a consensus sequence located upstream of pectic enzyme structural genes (36, 37). A candidate KdgR binding box was found 158 bp upstream of the *outC* start site (Fig. 2). To test whether *E. chrysanthemi* *out* genes are induced by polygalacturonate, *out::Tn5-gusA* fusions were constructed in the *outC*-through-*outM* region of both pCPP2159 and pAKC651 and marker exchanged into the chromosomes of *E. chrysanthemi* AC4159 and *E. carotovora* EC71 (Fig. 1 and 5). The resultant Erwinia mutants, *E. chrysanthemi* CUCPB5042, *E. caroto-
FIG. 4. SDS-PAGE analysis of total proteins from DH5α (pCPP2171) (lane 1) and DH5α(Bluescript SK(−)) (lane 2), showing the polypeptide encoded by *outC*. The location of the OutC polypeptide is indicated by the arrowhead; sizes are indicated on the left in kilodaltons.

**DISCUSSION**

The discovery of sequence similarity among secretion genes from *K. oxytoca, E. chrysanthemi, E. carotovora, P. aeruginosa*, and *X. campestris* has revealed the presence of a conserved pathway for Sec-dependent secretion in gram-
TABLE 1. Polyalacturonic acid induction of pectate lyase synthesis and β-glucuronidase activity in chromosomal out::Tn5-gusA1 insertion mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polyalacturonic acid</th>
<th>Pectate lyase (μmol/min/mg)</th>
<th>β-Glucuronidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. chrysanthemi</td>
<td>No</td>
<td>0.028</td>
<td>13</td>
</tr>
<tr>
<td>AC4150-GUS3</td>
<td>Yes</td>
<td>0.121</td>
<td>22</td>
</tr>
<tr>
<td>E. carotovora</td>
<td>No</td>
<td>0.011</td>
<td>25</td>
</tr>
<tr>
<td>61-GUS29</td>
<td>Yes</td>
<td>0.058</td>
<td>34</td>
</tr>
<tr>
<td>E. carotovora</td>
<td>No</td>
<td>0.008</td>
<td>55</td>
</tr>
<tr>
<td>61-GUS36</td>
<td>Yes</td>
<td>0.058</td>
<td>66</td>
</tr>
</tbody>
</table>

* Cultures were grown to late logarithmic phase in M9 supplemented with 0.5% glycerol ± 0.2% polyalacturonic acid. Data represent average results of eight different trials for each insertion mutant. The differences in pectate lyase and β-glucuronidase activity ± polyalacturonic acid were statistically significant at the 0.010 level for all three mutants.

* Measured in units of unsaturated product liberated per minute per milliliter of culture.

Variable levels of transcription for different pulO homologs may reflect variations in the range of functions played by each in different bacteria.

The degree of similarity between the pul genes 5′ to pulA and the out genes carried on pCPP2162 remains to be determined. Of the two pul genes 5′ to pulA, only pulS is required for secretion of pullulanase (11). It is quite possible that a pulS homolog is carried on pCPP2162.

Regulation of Sec-dependent secretion genes has been studied in *K. oxytoca*. pulC through pulO and pulA are induced by maltose, starch, and pullulan (12). As with pulA, the genes encoding several *E. chrysanthemi* pectic enzymes are induced by the enzyme substrate. Previous work with Mu-lac insertion mutations in *E. chrysanthemi* 3937 indicated that expression of secretion-related genes is constitutive (22). The relationship of the mutated strain 3937 out genes to the out genes studied here is unclear. Furthermore, the identification of a candidate KdgR box upstream of outC suggests that the outC-through-outM operon of *E. chrysanthemi* EC16 may be inducible by pectic compounds. Differences in β-glucuronidase activity detected from out::Tn5-gusA1 fusions in the presence and absence of polyalacturonate indicated that out genes in *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* are induced by polyalacturonate (Table 1). However, the degree of out gene induction is far lower than the degree of polyalacturonate induction of pectic enzymes or Pul induced by maltose. The candidate KdgR box identified upstream of outC diverges in several places from the consensus sequence. This may result in low binding efficiency of KdgR and suboptimal repression under noninducing conditions. Impairment of pectate lyase induction in out-*Erwinia* strains is possible because extracellular degradation of the polymeric substrate to assimilable oligogalacturonates is required for the formation of intracellular induc-

![FIG. 6. Pectate lyase production and expression of the outC-through-outM operon during the growth cycle of *E. chrysanthemi* CUCPBS042. Mutant CUCPBS042 carries the promoterless uidA transposon Tn5-gusA1 in the outC-through-outM operon. β-Glucuronidase (GUS) production resulting from the transcription of the operon was assayed fluorometrically with methylumbelliferone-β-D-glucuronic acid as a substrate and is expressed in units of fluorescence produced per hour. Pectate lyase (PL) activity is expressed in micromoles of unsaturated product liberated per minute per milliliter of culture. OD600, optical density at 600 nm.](http://jb.asm.org/)
ers (7, 9). However, the high level of pectate lyase induction observed here suggests that sufficient exo-poly-
α-β-galacturonosidase escapes from these out mutants to initiate the induction process. We do not know whether outO is regulated
differently from outC through outM, although the absence of any potential KdGR boxes upstream suggests that it may be. Apparently, Sec-dependent secretion systems vary not only in the type and number of proteins secreted but also in their regulation.

Previous studies indicate that E. chrysanthemi extracellular enzymes are also regulated by growth phase (7, 20). In contrast to this work which suggested that expression of Mu-lac out insertion mutants in E. chrysanthemi 3937 is independent of growth phase (22), our results indicate that out gene regulation by growth phase parallels the regulation of E. chrysanthemi extracellular enzymes (Fig. 6). Increased expression of the out genes in parallel with increased pectic enzyme production is not surprising.

The E. carotovora out genes carried on pAKC651 were expressed at levels similar to those of E. chrysanthemi outC through outO (Table 1), indicating that their inability to secrete E. carotovora pelI from E. coli does not result from a lack of transcription. This suggests that pAKC651 cannot function in E. coli because essential out genes are missing. The Southern hybridization data indicate that the missing genes may be homologs of those encoded by pCPP2162.

The Sec-dependent secretion pathway is now known to be widely conserved among gram-negative bacteria, but little is known of the interactions between pathway components and exoproteins that lead to secretion. One overriding question concerns identification of targeting signals on the exoproteins and recognition components of the secretion apparatus that control entry of proteins into the secretion pathway. Comparison of protein sequences has revealed that membrane-spanning regions are highly conserved between Pst and Out proteins, suggesting that protein localization is generally similar for both systems. Differences between Pst and Out proteins included unique inserts in four Out proteins encoding flexible inserts with high surface probability. These inserts may form loops extending away from the membrane and be involved in recognition of E. chrysanthemi exoproteins. Knowledge of these differences may be useful in guiding systematic analysis of the factors which control recognition of exoproteins and specificity among the various Sec-dependent secretion systems.

ACKNOWLEDGMENTS

We thank F. de la Cruz for providing pSU20, Sidney Kushner for pWSK129, J. M. Calvo for use of the Kohara E. coli DNA library, and Arun Chatterjee for pAKC601 and his thoughtful discussion. This work was supported by NSF grant DCB-9106431.

REFERENCES

17. He, S. Y., and A. Collmer. 1990. Molecular cloning, nucleotide sequence, and marker exchange mutagenesis of the exo-poly-