

# Multiple Interactions between Pullulanase Secretion Components Involved in Stabilization and Cytoplasmic Membrane Association of PulE

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**We report attempts to analyze interactions between components of the pullulanase (Pul) secretion (type II secretion machinery) from *Klebsiella oxytoca* encoded by a multiple-copy-number plasmid in *Escherichia coli*. Three of the 15 Pul proteins (B, H, and N) were found to be dispensable for pullulanase secretion. The following evidence leads us to propose that PulE, PulL, and PulM form a subcomplex with which PulC and PulG interact. The integral cytoplasmic membrane protein PulL prevented proteolysis and/or aggregation of PulE and mediated its association with the cytoplasmic membrane. The cytoplasmic, N-terminal domain of PulL interacted directly with PulE, and both PulC and PulM were required to prevent proteolysis of PulL. PulM and PulL could be cross-linked as a heterodimer whose formation in a strain producing the secretion required PulG. However, PulL and PulM produced alone could also be cross-linked in a 52-kDa complex, indicating that the secretion exerts subtle effects on the interaction between PulE and PulL. Antibodies against PulM coimmunoprecipitated PulL, PulC, and PulE from detergent-solubilized cell extracts, confirming the existence of a complex containing these four proteins. Overproduction of PulG, which blocks secretion, drastically reduced the cellular levels of PulC, PulE, PulL, and PulM as well as PulD (secretin), which probably interacts with PulC. The Pul secretion components E, F, G, I, J, K, L, and M could all be replaced by the corresponding components of the Out secretions of *Erwinia chrysanthemi* and *Erwinia carotovora*, showing that they do not play a role in secretory protein recognition and secretion specificity.**

Gram-negative bacteria use several different pathways for protein secretion. In the two-step general secretory pathway, proteins are first translocated across the cytoplasmic membrane via the Sec machinery. Selected secretory proteins are subsequently transported across the outer membrane by one of several branch pathways (27). In *Klebsiella oxytoca*, pullulanase secretion by the main terminal branch of the general secretory pathway requires a putative multiprotein, envelope-associated complex, the Pul secretion (also called type II secretion machinery), encoded by 15 *pul* genes (the 13-gene *pulC* to *-O* operon, *pulB*, and *pulS*) that flank the pullulanase structural gene *pulA* (30). The organization of secretion genes is conserved in different gram-negative bacteria (27), most of which use the same secretion to secrete several secretory proteins lacking sequence-conserved segments that could correspond to common secretion signals. The identity of the secretion component that recognizes secretion signals (40) in secretory proteins has not been firmly established (8, 41).

Here, we report attempts to study interactions between components of the Pul secretion. Previously, we demonstrated multimerization of the type IV pilin-like (pseudopilin) product of the *pulG* gene (28, 32), processing of pre-PulG by the *pulO* gene product (prepilin peptidase) (29), intimate association of PulD with PulS (11, 20), and a possible interaction between PulC and PulD (secretin) (26). Studies reported here center

around interactions involving PulE protein, which is associated with the cytoplasmic membrane (25) but does not have hydrophobic transmembrane segments. The membrane association of PulE homologues has already been studied in other systems. For example, the PulE homologue EpsE of *Vibrio cholerae* is anchored to the cytoplasmic membrane via the PulL homologue EpsL (36). Analysis of a chimera between the PulE homologue ExeE from *Aeromonas hydrophila* and EpsE led Sandkvist et al. to conclude that membrane association occurred via the amino-terminal part of EpsE (36). Similar results were observed in *Pseudomonas aeruginosa*, in which XcpR (XcpE) associates with the cytoplasmic membrane via XcpY (XcpL) (1). Although the XcpR (XcpE) component of *Pseudomonas putida* could not substitute for the corresponding *P. aeruginosa* protein, secretion could be promoted by a chimera composed of the N-terminal region of the E protein from *P. aeruginosa* and the C-terminal region from the E protein of *P. putida* (5). Further studies of both Xcp and Eps secretions revealed that protein L is unstable in the absence of the M protein (EpsM in *Vibrio cholerae* and XcpZ in *P. aeruginosa*) (18, 37). Furthermore, coimmunoprecipitation revealed that EpsL interacts directly with EpsM (37). Recent two-hybrid and protease accessibility studies of secretion components from *Erwinia chrysanthemi* also indicated that the E, L, and M components interact (34).

The reconstitution and amplification of the complete Pul secretion in *Escherichia coli* (7) provide a unique opportunity to examine interactions between secretion components in a readily accessible system. We began by creating nonpolar mutations in each of the *pul* genes on a plasmid carrying the complete Pul secretion gene cluster. The effects of the muta-

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TABLE 1. *E. coli* K-12 strains

Strain	Relevant characteristics	Reference or source
MC4100	$\Delta(lac-argF)U169 araD139 relA1 respL150$	Lab collection
PAP105	$\Delta(lac-pro)$ pAPIP501 [F' ( <i>lacI</i> <sup>q</sup> $\Delta lacZM15 pro^+$ Tn10)] (Tc <sup>r</sup> )	Lab collection
PAP7232	MC4100 <i>malP::(pulS pulA-B pulC to -O)</i> F' ( <i>lacI</i> <sup>q</sup> $pro^+$ Tn10) (Tc <sup>r</sup> )	29
PAP3175	PAP7232:: $\Delta pulE3$	22
PAP3389	PAP7232:: $\Delta pulF$	This study
PAP3390	PAP7232:: $\Delta pulL$	This study
PAP7452	PAP7232 <i>pulB::kan1</i>	26
HS2019	MC4100 $\Delta malE444 malG501$	H. Shuman
PAP7460	HS2019 pAPIP501 [F' ( <i>lacI</i> <sup>q</sup> $\Delta lacZM15 pro^+$ Tn10)] (Tc <sup>r</sup> )	This study
PAP7500B	PAP7460 <i>malP::(pulS pulA pulB::kan1 pulC to -O)</i>	This study

tions on the stability, multimerization (as determined by chemical cross-linking), and localization of PulE were also examined. These studies pointed to a specific role for PulL in stabilizing and anchoring PulE to the cytoplasmic membrane.

Therefore, we examined the role of different domains of PulE and PulL in the interaction between the two proteins. The mutants were also used in complementation experiments with the *out* secretion genes from *E. chrysanthemi* and *Erwinia carotovora* (17) to identify components that were specific to the Pul secretion.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, culture conditions, and pullulanase assay.** *E. coli* K-12 strains are listed in Table 1, and plasmids are listed in Table 2. Strains HS2019, PAP7460, and PAP7500B (Table 1) were employed in many of the experiments using antibodies raised against MalE hybrid proteins. These strains do not produce MalE protein (which reacts with these antibodies) and carry a *malG* mutation that allows MalE-independent uptake of maltose (44) in order to activate MalT, the positive activator of *pulA* and *pulC* (7). Details of the construction of other clones and mutants not previously reported are given below. Plasmids carrying cloned *out* genes under *lacZ* control used here (Table 3) were described by Lindeberg et al. (17).

Bacteria were grown at 30°C in Luria-Bertani medium buffered at pH 7.2 with 10 mM phosphate or with 10% M63 medium (19) and supplemented, where appropriate, with 0.4% maltose to induce expression of *pulA* and the *pulC to -O* operon or 1 mM isopropyl thiogalactoside (IPTG) to induce expression of genes under *lacZ* promoter control. Expression of the *lexA-pulE'* fusion was induced with 200  $\mu$ g of anhydrotetracycline (Acros Organics) per ml. Antibiotics were added at the following concentrations: ampicillin, 100  $\mu$ g/ml; kanamycin, 50  $\mu$ g/ml; chloramphenicol, 25  $\mu$ g/ml; spectinomycin, 50  $\mu$ g/ml; and tetracycline, 16  $\mu$ g/ml.

TABLE 2. Plasmids carrying cloned *pul* genes

Plasmid	Vector/origin/resistance	Cloned genes and/or mutations	Reference and/or source of cloned fragment
pCHAP231	pBR322/ColE1/Ap	pBR322:: <i>(pulS pulA-B pulC to -O)</i>	7
pCHAP1219	pBR322/ColE1/Ap	pCHAP231 <i>pulS::Tn5</i>	6
pCHAP1201	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1</i>	This study; 26
pCHAP1218	pBR322/ColE1/Ap	pCHAP231 <i>pulA::Tn5</i>	7
pCHAP1229	pBR322/ColE1/Ap	pCHAP231 <i>pulC::kan2</i>	26
pCHAP1226	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1 <math>\Delta pulD</math></i>	26
pCHAP1230	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1 <math>\Delta pulE3</math></i>	This study
pCHAP1227	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1 <math>\Delta pulF</math></i>	This study
pCHAP1216	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1 <math>\Delta pulG1</math></i>	This study
pCHAP1324	pBR322/ColE1/Ap	pCHAP231 <i>pulH::kan2</i>	This study
pCHAP1357	pBR322/ColE1/Ap	pCHAP231 <i>pull::kan2</i>	This study
pCHAP1323	pBR322/ColE1/Ap	pCHAP231 <i>pull::kan2</i>	This study
pCHAP1325	pBR322/ColE1/Ap	pCHAP231 <i>pulK::kan2</i>	This study
pCHAP1217	pBR322/ColE1/Ap	pCHAP231 <i>pulB::kan1 <math>\Delta pulL</math></i>	This study
pCHAP1359	pBR322/ColE1/Ap	pCHAP231 <i>pulM::kan2</i>	This study
pCHAP1350	pBR322/ColE1/Ap	pCHAP231 <i>pulN::kan2</i>	This study
pCHAP1225	pBR322/ColE1/Ap	pCHAP231 <i>pulO::Mud</i>	7
pCHAP580	pSU18/p15/Cm	<i>lacZp-pulS</i>	This study; 6
pCHAP1228	pSU18/p15/Cm	<i>lacZp-pulA</i>	14
pCHAP5001	pSU18/p15/Cm	<i>lacZp-pulC</i>	26
pCHAP3635	pSU18/p15/Cm	<i>lacZp-pulD</i>	8
pCHAP2273	pSU18/p15/Cm	<i>lacZp-pulE</i>	This study; 25
pCHAP1206	pSU18/p15/Cm	<i>lacZp-pulF</i>	This study
pCHAP1205	pSU18/p15/Cm	<i>lacZp-pulG</i>	This study; 29
pCHAP1321	pSU18/p15/Cm	<i>lacZp-pulH</i>	This study
pCHAP1352	pSU18/p15/Cm	<i>lacZp-pulI</i>	This study
pCHAP1329	pSU18/p15/Cm	<i>lacZp-pulJ</i>	This study
pCHAP1271	pSU18/p15/Cm	<i>lacZp-pulK</i>	This study
pCHAP2245	pSU18/p15/Cm	<i>lacZp-pulL</i>	This study
pCHAP1354	pSU18/p15/Cm	<i>lacZp-pulM</i>	This study
pCHAP1356	pSU18/p15/Cm	<i>lacZp-pulN</i>	This study
pCHAP1224	pSU18/p15/Cm	<i>lacZp-pulO</i>	This study
pCHAP2014	pBGS18/ColE1/Km	<i>lacZp-pulE pulF'</i>	22
pCHAP2045	pBGS18/ColE1/Km	<i>lacZp-pulE pulF pulG'</i>	22
pCHAP2230	pBGS18/ColE1/Km	<i>lacZp-pulL</i>	This study
pCHAP2235	pHSG575/pSC101/Cm	<i>lacZp-pulL</i>	This study
pCHAP5107	p733/ColE1/Ap	<i>tetp-lexA-pulE'</i>	This study
pCHAP5112	pSU18/p15/Cm	<i>lacZp-pulL'</i>	This study
pCHAP125	pHSG576/pSC101/Cm	<i>lacZp-'pulK-pulL-pulM-pulN-pulO</i>	33

TABLE 3. Complementation of single-locus *pul* mutations by corresponding *pul* genes and by *out* genes from *E. chrysanthemi* and *E. carotovora*<sup>a</sup>

Mutation	Secretion (%)	Complementation (% secretion) after incubation with (+) or without (-) IPTG					
		<i>pul</i> gene		<i>E. ch</i> <i>out</i> gene		<i>E. ca</i> <i>out</i> gene	
		-	+	-	+	-	+
<i>pulS</i>	<10	>80	>80	>80*	>80*	NT	NT
<i>pulB</i>	>80	NT	NT	NT	NT	NT	NT
<i>pulC</i>	<10	>80	>80	<10	<10	<10	<10
<i>pulD</i>	<10	>80	>80	<10	<10	<10	<10
<i>pulE</i>	<10	>80	>80	30	>80	>80	50
<i>pulF</i>	<10	>80	<10	40	<10	>80	50
<i>pulG</i>	<10	>80	>80	<10	>80	<10	>80
<i>pulH</i>	>80	NT	NT	NT	NT	NT	NT
<i>pulI</i>	<10	>80	>80	>80	>80	>80	>80
<i>pulJ</i>	<10	>80	>80	>80	>80	>80	>80
<i>pulK</i>	<10	>80	>80	<10	>80	<10	>80
<i>pulL</i>	<10	>80	>80	>80	60	>80	70
<i>pulM</i>	20	>80	>80	>80	>80	70	60
<i>pulN</i>	>80	NT	NT	NA	NA	NT	NT
<i>pulO</i>	<10	>80	>80	NT	NT	NT	NT

<sup>a</sup> Assays were performed with derivatives of strain PAP105. The *pul* mutations were present on pCHAP231 derivatives (Table 2). Strains carrying pCHAP231 secreted >80% of the pullulanase produced. All data are averages from three or more independent assays. All complementing plasmids were derivatives of pSU vectors carrying cloned *pul* genes (Table 2) or cloned *out* genes (17). NT, not tested; NA, not applicable; *E. ch.*, *E. chrysanthemi*; *E. ca.*, *E. carotovora*; \*, see reference 11.

Pullulanase secretion was measured as the proportion of the enzyme accessible to its substrate (pullulan) in whole cells compared to that in cells lysed with 0.5% octylpolyoxyethylene (22).

**Construction of *pulL* and *pulF* gene deletions in the chromosomal *pul* locus.** In order to create an in-frame deletion in *pulL*, a 2.0-kb *EcoRI*-*MscI* fragment of pCHAP125 bearing the full-length *pulL* flanked by the 3' end of *pulK* and the 5' end of *pulM* (33) was cloned into pBGS18 (42). The resulting plasmid (pCHAP2230) was then cleaved at the two *EcoRV* sites in *pulL* and religated with an 8-bp *SacII* adapter, generating a 384-bp deletion between codons 201 and 355 of the 398-codon *pulL* gene (pCHAP2236). The entire cloned fragment in pCHAP2236 was then subcloned into M13mp11 and used to create a homologous exchange (3) with the chromosomal *pul* region of PAP7232 (29). The resulting strain, PAP3390, was shown to have a single, internally deleted copy of *pulL* by Southern hybridization.

To create an in-frame deletion in *pulF*, two site-directed mutagenesis reactions (16) were performed with pCHAP2014 and pCHAP2045 (25), generating *Bam*HI sites at codons 195 and 275 of *pulF* (401 codons). After *Bam*HI and *EcoRI* cleavage of both plasmids, a recombinant plasmid carrying a 237-bp *pulF* deletion (pCHAP2238) was reconstituted by ligating the purified DNA fragments. The mutated *pulF* allele was introduced in place of wild-type *pulF* in PAP7232 as described above to give strain PAP3389 ( $\Delta$ *pulF*), which was shown by Southern blotting to carry a single, internally deleted copy of *pulF*.

**Construction of pCHAP231 derivatives carrying mutations in specific *pul* genes.** The strategy used to transfer the chromosomal deletions in *pulE* (25), *pulF*, *pulG* (29), and *pulL* onto pCHAP231 (pBR322 with the full set of *pul* genes [6]) was the same as that described previously for *pulD* carried by pCHAP1226 (26), resulting in plasmids pCHAP1230, pCHAP1227, pCHAP1216, and pCHAP1217, respectively (Table 2).

All other mutations in pCHAP231 were created by a strategy similar to that used previously to create the *pulC* mutation carried by pCHAP1229 (26). DNA fragments carrying the complete *pulH*, *pulI*, *pulJ*, *pulK*, *pulM*, or *pulN* gene together with approximately 1 kb of flanking DNA were amplified by PCR and cloned into pUC18Cm (S. Lory). The resulting plasmids were linearized using restriction enzymes that cleaved only within the gene of interest as follows: *pulH*, *Mlu*I site at codon 93 of the 160-codon gene; *pulI*, *Nhe*I site at codon 47 of the 121-codon gene; *pulJ*, *Msc*I site at codon 18 of the 198-codon gene; *pulK*, *Bgl*II site at codon 93 of the 326-codon gene; *pulM*, *Bst*EII site at codon 138 of the 161-codon gene; *pulN*, *Sac*II site at codon 125 of the 252-codon gene. The linearized DNA was then ligated with compatible *Sna*BI adapters. The *kan2* cassette was inserted at these sites as previously described (26). (The *kan2* cassette was positioned so that the ATG codon immediately after the kanamycin resistance gene is fused to the reading frame of the gene into which it is inserted.)

The resulting plasmids were then linearized and electroporated into *recD* or *recB sbcA* strains carrying pCHAP231, with selection for the homologous exchange reaction that replaced the wild-type gene on pCHAP231 by the mutated copy as described previously (26). The presence of the desired mutation on the resulting plasmids (Table 2) was verified by PCR using primers hybridizing to sites in pCHAP231 and in the *kan2* cassette.

**Construction of *pulE* and *pulL* gene fusions.** The 5' end of *pulE*, encoding the first 177 amino acids of the protein, was amplified by PCR using pCHAP231 as template. The resulting DNA fragment was flanked by *Bam*HI and *Xho*I sites at the 5' and 3' ends, respectively, and was subcloned into p733 carrying the full-length *lexA* gene (15) to give plasmid pCHAP5107. Expression of the resulting in-frame *lexA-pulE* fusion was induced with anhydrotetracycline; the stability of the hybrid protein was confirmed by immunodetection using LexA antibodies (Santa Cruz Biotechnology).

The 5' end of *pulL*, encoding the cytoplasmic domain of the protein (amino acids 1 to 247), was amplified by PCR using a 5' oligonucleotide that introduced a DNA sequence encoding a hexahistidine tag. The amplified fragment was flanked by *Eco*RI and *Hind*III sites at its 5' and 3' ends, respectively. The PCR product was subsequently subcloned into pSU18, giving pCHAP5112.

**Cloning of *pul* genes for complementation assays.** *pul* genes that were not previously cloned separately were amplified by PCR using pCHAP231 as a template and cloned into pSU18 using restriction sites introduced by the primers. The 5' primer was engineered with an *Eco*RI site in the same reading frame as the *Eco*RI site in the vector so that the cloned gene was fused directly to the *lacZ* open reading frame. A second set of plasmids in which a 60- to 80-bp segment of the upstream *pul* gene was cloned in frame with the *lacZ* reading frame of the vector gave identical results.

**Antibodies.** DNA corresponding to the predicted periplasmic domain of PulL was excised from pCHAP231, and DNA corresponding to the predicted periplasmic domain of PulM was amplified by PCR. Both fragments were cloned into pMAL-p2 (New England Biolabs) to generate *malE-pulL* and *malE-pulM* gene fusions coding for periplasmic hybrids. DNA coding for the entire sequence of PulE was PCR amplified and cloned into pMAL-c2 to create a *malE-pulE* gene fusion coding for a cytoplasmic hybrid protein. All three hybrid proteins were purified from total soluble protein extracts of IPTG-induced cells by amylose chromatography (New England Biolabs) and injected into rabbits to obtain antibodies.

**Gel electrophoresis and immunoblotting.** After solubilization in sample buffer containing 2% sodium dodecyl sulfate (SDS) and heating at 100°C, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose. Ponceau red staining allowed visualization of porins (outer membrane markers) in membrane fractions. Pul proteins and DjIA were detected by incubation with specific primary antisera diluted to 1:2,000 to 1:10,000, followed by secondary horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) (Amersham-Pharmacia; 1:10,000) and enhanced chemiluminescence (ECL kit; Amersham-Pharmacia). The LexA-PulE' hybrid was detected with anti-LexA primary antibodies (Santa Cruz Biotechnology) diluted 1:2,000 and horseradish peroxidase-linked anti-goat IgG diluted 1:10,000 (Santa Cruz Biotechnology). The His-tagged truncated PulL protein was detected using monoclonal (His)<sub>6</sub> antibodies (Clontech; 1:1,000) and horseradish peroxidase-linked anti-mouse IgG (1:10,000; Amersham-Pharmacia). Before the membrane was reprobed, bound antibodies were stripped off as described by the manufacturer (Amersham-Pharmacia).

**Immunoprecipitation.** The MalE-PulM antibodies were affinity purified using the MalE-PulM hybrid protein covalently linked to CNBr-activated Sepharose 4B (Amersham-Pharmacia). Protein A Dynabeads (Dyna) were washed three times with phosphate-buffered saline containing 1% bovine serum albumin and incubated overnight with the affinity-purified anti-PulM antibodies. Cross-linking of antibodies to the beads was performed according to the instructions of the manufacturer using dimethyl pimelimidate (Sigma). Whole-cell extracts of *E. coli* K-12 harboring pCHAP231 were generated as described previously (37). Two hundred microliters of bacterial extract was incubated with different volumes of antibody-loaded beads at 4°C for 2 h on a rotating device. After being washed three times with TBST buffer (10 mM Tris-Cl, 150 mM NaCl, 0.05% Tween 20, pH 8.0), the beads were resuspended in SDS sample buffer and heated for 5 min at 100°C. Proteins in the loaded material and in the nonadsorbed and immunoadsorbed fractions were separated on a 12% polyacrylamide gel in SDS. After they were blotted onto a nitrocellulose membrane, different Pul proteins were visualized using specific monoclonal antibodies.

**Cross-linking and proteolysis.** Four cross-linkers [ethylene glycol bis(succinimidylsuccinate) (EGS; spacer length, 16.1 Å; Pierce), dithiobis(succinimidyl propionate) (DSP; 12.0 Å; Sigma), disuccinimidyl tartrate (6.4 Å; Pierce), and formaldehyde] were used essentially as described previously (26, 28). In some cases, one of each pair of samples prepared in parallel was treated with phenol to dissociate PulD multimers prior to analysis by SDS-PAGE and immunoblotting (9, 26). Nitrocellulose membranes onto which the proteins had been blotted were probed successively with antibodies recognizing different Pul secretion components. All DSP-induced cross-links described here could be reversed by treatment with 10 mM dithiothreitol.

To analyze the stability and protease accessibility of the Pul secretion components, bacteria that were washed and resuspended in 25 mM Tris (pH 7.5) to an

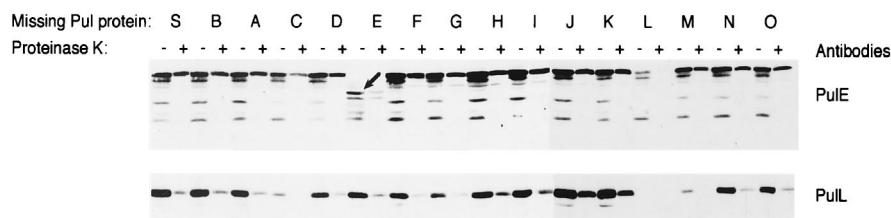


FIG. 1. Immunodetection of PulE and PulL in sonicated, untreated, or proteinase K-treated cells of strain HS2019 carrying pCHAP231 with mutations in specific *pul* genes. The sample buffer contained a 10 mM concentration of the reducing agent dithiothreitol. The double band of PulE is due to incomplete reduction of the intramolecular disulfide bond that forms when the cells are disrupted by sonication (24). The arrow indicates the internally truncated form of PulE produced in the *pulE* mutant.

optical density at 600 nm of 1.0 were disrupted by sonication and then incubated at 37°C with or without 100 µg of proteinase K per ml for 30 min. Samples were then chilled on ice, and proteins were precipitated with 12.5% trichloroacetic acid. Precipitated proteins were examined by SDS-PAGE and immunoblotting.

**Cellular fractionation and flotation sucrose gradients.** Two hundred milliliters of *E. coli* cultures was grown to an optical density at 600 nm of 1.0 to 1.2 before being lysed with or without prior conversion to spheroplasts (4) by passage through a French press at  $5 \times 10^7$  or  $1.2 \times 10^8$  Pa, respectively. DNase, RNase, and Pefabloc (Interchim) were added to a final concentration of 0.1 mg/ml. Cell debris was removed by low-speed centrifugation. The clarified lysate was then fractionated by ultracentrifugation at  $180,000 \times g$  for 45 min at 4°C, resulting in separation of soluble material (cytoplasmic and periplasmic contents) from particulate material (aggregated and membrane fractions).

For flotation gradients, the particulate fraction was first washed with 25 mM HEPES (pH 7.4) and then resuspended in the same buffer adjusted at 60% (wt/wt) in sucrose. Membranes were then loaded at the bottom of Beckman SW55 centrifuge tubes and successively overlaid with 56 to 32% (wt/vol) sucrose solutions (steps of 3%). The gradients were centrifuged at  $220,000 \times g$  for 36 h at 10°C. Fractions of 250 µl were collected from the top of the tubes.

## RESULTS

**PulB, PulH, and PulN are nonessential.** Nonpolar mutations in each of the 15 *pul* genes (*pulA*, *pulB*, *pulC* to *pulO*, and *pulS*) carried by pCHAP231 (Table 2) were tested for effects on pullulanase secretion in *E. coli* K-12 (Table 3). Three of the mutations, *pulB*, *pulH*, and *pulN*, had no effect on pullulanase secretion. The absence of a requirement for *pulB* was observed previously in studies of Tn5 insertions and deletions in pCHAP231 and other multiple-copy-number plasmids (7, 14). To determine whether *pulB* is required for secretion when the secretion genes are integrated in the *E. coli* K-12 chromosome, we constructed strain PAP7452 (26), a derivative of PAP7232 [*malP::(pulS pulAB pulC -O)*] (7, 28) in which *pulB* was disrupted by a kanamycin resistance cassette (Table 1). Pullulanase secretion levels in PAP7452 and PAP7232 were indistinguishable (data not shown). Thus, *pulB* is not required for pullulanase secretion, at least in *E. coli*.

Previous studies indicated that both *pulH* and *pulN* were required for pullulanase secretion (33, 35). However, the transposon insertion mutations analyzed previously might have had a polar effect on the expression of downstream genes. *pulN* homologues are frequently absent from secretion gene clusters in other bacteria (27). Furthermore, a mutation in the *E. chrysanthemi pulH* homologue *outH* did not abolish pectate lyase secretion promoted by the reconstituted Out secretion on a plasmid in *E. coli*, although the chromosomal *outH* mutation in *Erwinia* did block secretion (17).

All other mutations except *pulA* (which abolished pullulanase production) and *pulM* (see below) abolished pullulanase secretion (<10% of wild-type levels). The *pulM* mutation reduced secretion only to 20%. The failure of polyclonal PulM-specific antiserum to detect a truncated form of the protein in

extracts of the *pulM* mutant (data not shown) suggested that the PulM was not synthesized by the mutant, although the antibodies might not recognize epitopes present in the remaining, N-terminal part of the truncated protein. In all cases, pullulanase secretion was fully restored by complementation by a cloned copy of the wild-type gene in *trans* on a compatible plasmid (Table 3), indicating that the mutations are nonpolar.

**Effects of *pul* mutations on Pul protein stability.** We next examined the possibility that some Pul proteins might be destabilized by the absence of another protein with which they would normally interact. The only reproducible effect observed by immunoblotting of total protein extracts from the mutant strains was a modest reduction in the level of PulE in the *pulL* mutant (data not shown). The instability of PulE in the absence of PulL was amplified by sonicating the bacteria and incubating them with or without proteinase K (Fig. 1). However, no other *pul* mutation affected the stability of PulE. The *pulE* mutation did not exert a reciprocal effect on the level of PulL (Fig. 1), possibly because a truncated PulE protein is produced (25). The absence of PulM also destabilized PulL (Fig. 1), indicating a possible interaction between these two proteins. Finally, PulL was also destabilized in the *pulC* mutant (Fig. 1), a novel finding that suggests a previously unsuspected link between PulC and PulL. The levels of PulC and PulM were unaffected by the *pulL* mutation (data not shown).

**Overproduction of PulG destabilizes several Pul proteins.** Overproduction of certain secretion components has been reported to diminish secretion, possibly because stoichiometric imbalance disrupts interactions between secretion components and causes loss of secretion function (2, 29). Since such an approach could indicate interactions within the secretion, we examined the effects of increased expression of individual *pul* genes in strain PAP7500B on pullulanase secretion and the levels of other Pul proteins. Overexpression of *pul* genes *S*, *C*, *D*, *E*, *H*, *I*, *J*, *K*, *M*, and *O* had no effect on pullulanase secretion. However, PulG overproduction blocked secretion, as observed previously (29), and reduced PulC, PulD, PulE, PulL, and PulM to low or barely detectable levels (Fig. 2). The level of coregulated pullulanase produced by the cells (as measured by specific enzymatic activity) was unaffected by high-level production of PulG (data not shown), indicating that expression of the *pul* gene cluster is unaffected. Thus, overproduction of PulG seems to destabilize Pul proteins C, D, E, L, and M.

Increased expression of *pul* genes *F* and *L* also reduced secretion to <30% (*pulL*) or abolished it completely (*pulF*). In none of these cases did we observe a dramatic effect on the levels of other Pul proteins similar to that caused by PulG overproduction (data not shown). The inhibitory effects of increased *pulL* expression could not be suppressed by increasing *pulE* expression (data not shown), which is in contrast to

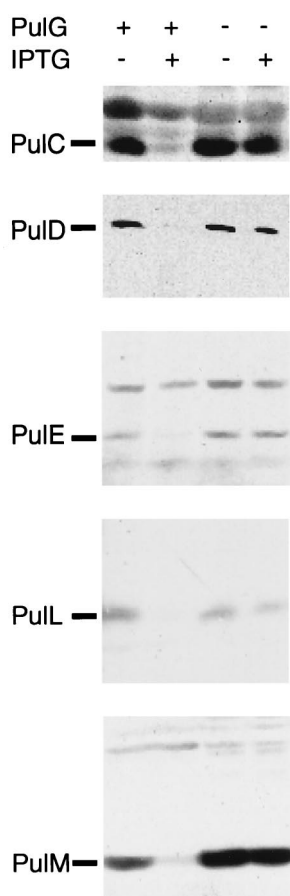


FIG. 2. Effect of increased expression of *pulG* carried by pCHAP1205 on the levels of PulC, PulD, PulE, PulL, and PulM proteins in strain PAP7500B grown in maltose to induce expression of the chromosomal *pul* genes. Paired samples from the same number of bacteria from noninduced and IPTG-induced cultures are shown. Control cells (PulG  $-$ ) carried the vector pSU18. Protein extracts were blotted onto nitrocellulose and detected with antibodies against the indicated Pul proteins. In some panels, bands that are recognized nonspecifically by the antibodies serve as internal controls.

results reported recently in similar studies with the E and L components of the Eps, Xcp, and Out secretons (1, 34, 36, 37). The negative interference phenotype caused by increased *pulL* expression could be due to interactions involving the cytoplasmic N-terminal domain of PulL, because high-level production of this domain (in strains carrying pCHAP5112) also caused a 90% drop in pullulanase secretion. Once again, however, the negative interference effect was not overcome by simultaneously increasing PulE levels.

**Cross-linking and immunoprecipitation to probe interactions involving PulC, PulE, PulG, PulL, and PulM.** The data presented so far suggest possible interactions involving Pul secreton components C, E, G, L, and M. In an attempt to obtain more direct evidence for such interactions, we studied the effects of the different *pul* gene mutations on the formation of multimers that could be stabilized by cross-linking. The mutants were treated with four different amine-specific cross-linkers with different spacer lengths (EGS, DSP, disuccinimidyl tartrate, and formaldehyde) under optimized conditions, and proteins were blotted onto nitrocellulose sheets that were probed with antibodies to specific Pul proteins.

PulC formed the previously observed 110-kDa complex (26)

after treatment with all four cross-linkers except when PulS or PulD was absent (Fig. 3; note that variations in the level of the 110-kDa band detected in other strains were not reproducible). Since PulS is required for PulD assembly into the outer membrane (9), these results indicate that formation of the 110-kDa complex requires PulC and correctly assembled PulD and does not involve any other Pul protein. As reported previously (26), we conclude that the 110-kDa band is either a PulC trimer or, less likely, a PulC-PulD heterodimer.

A 52-kDa cross-linked product that reacted with PulL antibodies was observed after treatment with EGS (Fig. 4) and DSP but not with the other cross-linkers (data not shown). Formation of this band did not occur in mutants lacking PulG or PulM and was increased in mutants devoid of PulI, PulJ, and PulK (Fig. 4). The size of this band is close to that expected for PulL-PulG or PulL-PulM heterodimers. Both PulG and PulM produced cross-linked bands (possibly homomultimers) of ca. 52 kDa even in the absence of PulL, making it impossible to determine whether the 52-kDa band detected by the PulL antibodies band contained one or another of these proteins. However, three lines of evidence suggest that it is a heterodimer of PulL and PulM. First, the size of the band was unaffected by a mutation in *pulO*, which abolishes processing of PulG and thereby causes it to migrate more slowly during SDS-PAGE (reference 29 and data not shown). Second, a 52-kDa band was also observed after DSP (Fig. 5) or EGS (data not shown) cross-linking of cells carrying pCHAP2230 (*pulL*) plus pCHAP1354 (*pulM*). Third, purified antibodies coupled to magnetic beads could immunoprecipitate not only PulM but also PulL and, to a lesser extent, PulC and PulE from detergent (Triton X-100)-solubilized extracts of *E. coli* K-12(pCHAP231) (Fig. 6). We could not apply this assay to other Pul proteins because they were either insoluble in the detergent used or bound nonspecifically to the magnetic beads.

In no case did we find any evidence for the presence of cross-linked PulL or PulE homomultimers. However, small amounts of what appeared to be PulM homodimers were observed after cross-linking of *E. coli* K-12(pCHAP231) with DSP and EGS. Formation of these homodimers was also observed in cells expressing only PulM, where they could be detected even in samples that had not been treated with cross-linkers (data not shown).

**Factors required for cytoplasmic membrane association of PulE.** The locations of PulE, PulL, and PulM were determined by floating membranes through centrifuged sucrose gradients. PulE, PulL, and PulM were all found exclusively in the membrane (pellet) fraction after ultracentrifugation of French press-disrupted *E. coli* producing the full set of Pul proteins (pCHAP231) and were recovered almost exclusively from the cytoplasmic membrane fraction near the top of the sucrose gradients (Fig. 7). The deletion in *pulM* (pCHAP1359) reduced the amounts of PulE and especially PulL recovered after fractionation, but they remained cytoplasmic membrane associated (Fig. 7). Deletion of *pulF* (pCHAP1227) had no effect whatsoever on the location of PulE, PulL, or PulM (data not shown), while deletion of *pulL* (pCHAP1217) resulted in failure to detect PulE in membranes after fractionation (see above) without affecting the localization of PulM (data not shown).

The instability of PulE provoked by the *pulL* deletion (pCHAP1217) (Fig. 8b) was corrected by introducing pCHAP2245 (*pulL*) (Fig. 8c). PulE was also stabilized in *E. coli* (pCHAP1217) by pCHAP5112, which encodes only the N-terminal, cytoplasmic region of PulL (PulL' [Fig. 8d]), but pullulanase secretion was not restored (data not shown). In

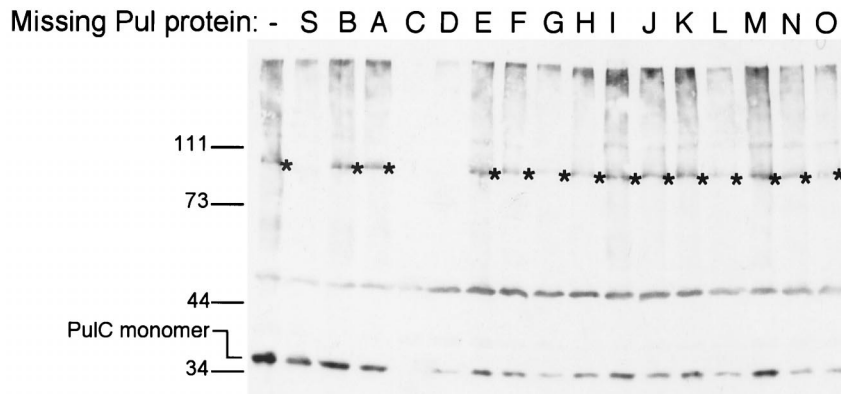


FIG. 3. Formation of the 110-kDa multimer containing PulC after cross-linking with DSP requires PulS and PulD but not any of the other components of the Pul secretin. The bacteria were maltose-induced HS2019 carrying pCHAP231 derivatives lacking different *pul* genes (Table 2). The position of the ca.-110-kDa cross-linked PulC multimer is indicated by an asterisk. Differences in yields of PulC monomer and multimer in different strains were not reproducible. Size markers (kilodaltons) and PulC monomers are indicated on the left.

this strain, approximately 50% of PulE was in the soluble fraction (Fig. 8d). This is the first time that we have observed a soluble form of PulE that might be amenable to biochemical analysis.

To investigate whether the PulE in the pellet fraction was membrane associated or aggregated, it was subjected to flotation through sucrose gradients. In control membranes from strains carrying both pCHAP1217 (pCHAP231  $\Delta$ *pulL*) and pCHAP2245 (*pulL*), PulE was found mainly in the cytoplasmic membrane (Fig. 9a), as expected, whereas some PulL was found in intermediate-density and outer membrane fractions as well as in the cytoplasmic membrane (Fig. 9b). In contrast, in cells carrying both pCHAP1217 and pCHAP5112 (*pulL'*), the carboxy-terminally truncated form of PulL that was recovered in the pellet (membrane fraction) remained at the bottom of the gradients, indicating that it is not membrane associated (Fig. 9d). Nevertheless, PulE was membrane associated in these cells, forming a broad peak that spanned from the cytoplasmic membrane through the outer membrane to the soluble-aggregated fraction at the bottom of the gradient (Fig. 9c). Thus, PulE is stable and associates to at least some extent with

the cytoplasmic membrane even when PulL itself is unable to associate with this membrane.

Previous studies suggested that the amino-terminal region of PulE and its homologue EpsE might be involved in their association with the cytoplasmic membrane (25, 36). Therefore, we examined the location of the amino-terminal region of PulE (amino acids 1 to 177) by expressing a *lexA-pulE'* fusion in a strain carrying the full set of *pul* genes with the  $\Delta$ *pulE3* deletion in the chromosome [PAP3175(pCHAP5107)]. The hybrid protein was recovered in both cytoplasmic membrane and intermediate fractions (Fig. 9e), indicating that it is able to associate with the membrane (presumably via PulL). Some of the LexA-PulE' formed aggregates that appeared in higher-density fractions.

To determine whether PulL alone is sufficient for the association of PulE with the cytoplasmic membrane, we examined the location of these proteins in the absence of all other secretion components. For these experiments, we used pCHAP2273 coding for wild-type PulE lacking the 38-amino-acid N-terminal extension resulting from the fusion of *lacZ* and *pulE* coding regions in previously used plasmids (25). As re-

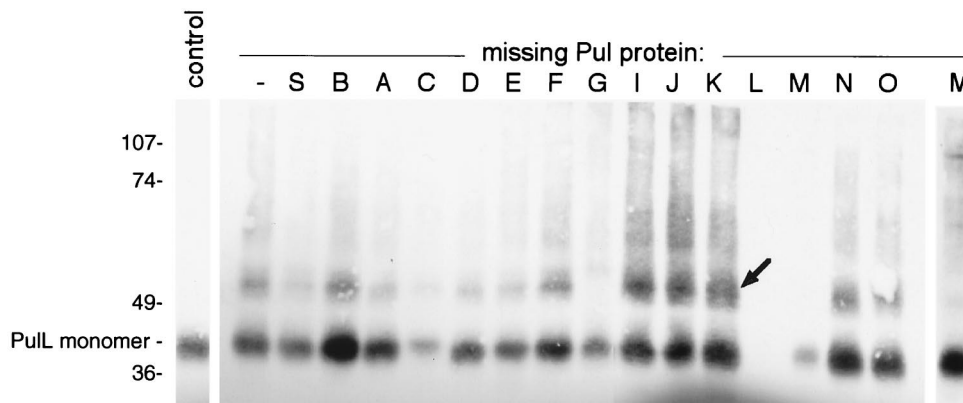


FIG. 4. Formation of a 52-kDa band that reacts with PulL antibodies after treatment of cells producing secretin components with EGS. The bacteria were maltose-induced HS2019 carrying pCHAP231 derivatives lacking different *pul* genes (Table 2). The control sample was HS2019(pCHAP231) that was not treated with EGS. The lane at the extreme right (labeled M) shows a prolonged chemiluminescence exposure of the sample from the strain lacking PulM. Proteins were separated by SDS-PAGE on an 8% acrylamide gel. Size markers (kilodaltons) and the position of the PulL monomer are indicated on the left, and the position of the 52-kDa band is indicated by an arrow. The higher yields of cross-linked product in strains lacking PulI, PulJ, or PulK were reproducible.

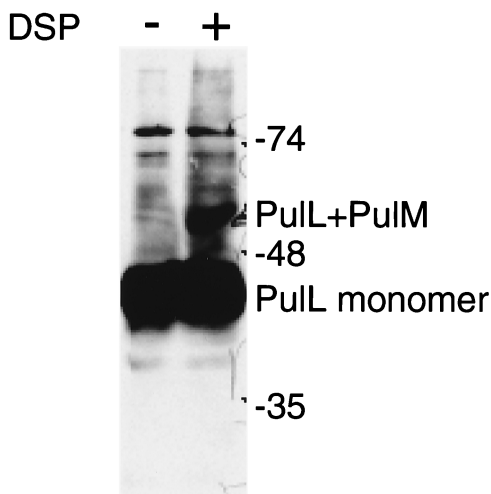


FIG. 5. Formation of a cross-linked multimer of ca. 52 kDa in IPTG-induced cells of PAP7500B carrying pCHAP2230 (*pulL*) and pCHAP1354 (*pulM*), as detected by SDS-PAGE and immunoblotting with PulL antibodies. The cross-linker was DSP. The positions of the PulL monomer, the presumed PulL-PulM heterodimer, and molecular size markers (in kilodaltons) are indicated at the right.

ported previously (25), PulE aggregated when produced alone (Fig. 9f), but when *pulE* and *pulL* were coexpressed [PAP105(pCHAP2273, pCHAP2230)], PulE was evenly distributed between the cytoplasmic membrane and high-density (aggregated) fractions (Fig. 9g). PulL alone was found in the cytoplasmic membrane (data not shown; note that PulL syn-

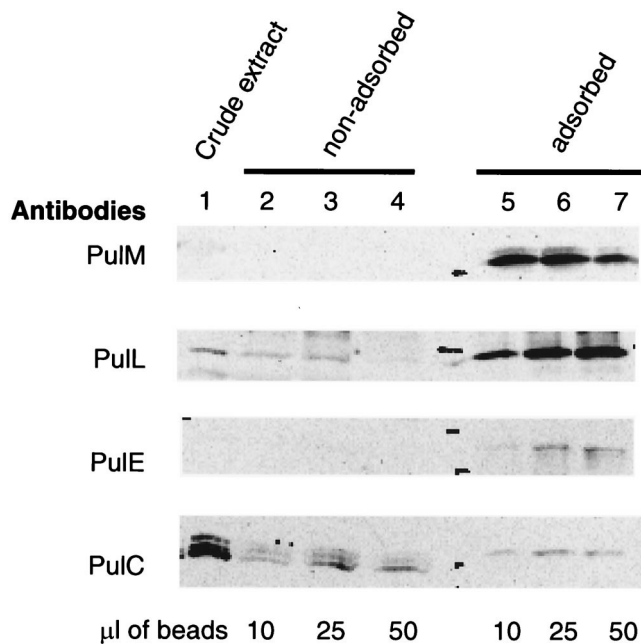


FIG. 6. Coimmunoprecipitation of Pul proteins L, E, and C together with PulM by purified antibodies directed against PulM. Lane 1 shows the relative abundance of the proteins in the crude extracts. Lanes 2 to 4 show the unadsorbed proteins after incubation with different volumes of magnetic beads coated with the antibodies. Lanes 5 to 7 show the proteins adsorbed onto the beads. Note that the PulC antiserum detected three bands, only one of which (the upper band) corresponds to PulC and was immunoprecipitated.

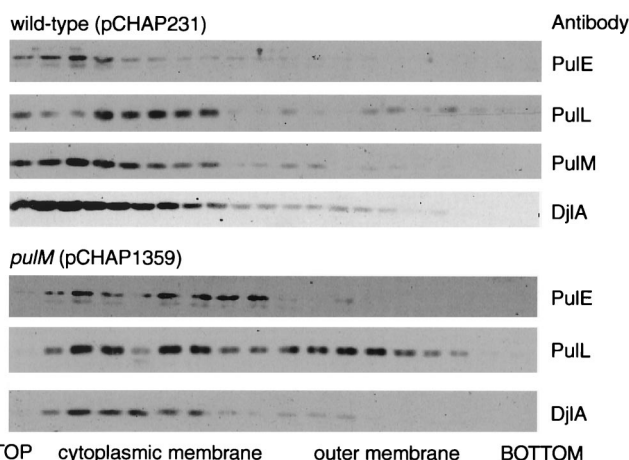


FIG. 7. Localization of PulE, Pull, and PulM proteins in HS2019(pCHAP231) (wild type) and effect of  $\Delta pulM$  mutation introduced into pCHAP231 (to give pCHAP1359) on the localization of PulE and Pull. Membranes were separated by flotation through centrifuged sucrose gradients, and proteins were detected by immunoblotting. Antibodies against the cytoplasmic membrane protein DjIA were used as a control. The outer membrane fraction was identified by the presence of porins and OmpA protein. The PulE and Pull immunoblots in the  $\Delta pulM$  strain were developed for longer than were the immunoblots of membranes from the wild-type strain to compensate for the presence of lower amounts of these proteins.

thesized alone is apparently stable in the absence of PulM). Thus, PulL prevents PulE aggregation and colocalizes with PulE in the cytoplasmic membrane. This result confirms the direct interaction between PulE and Pull.

**Erwinia out genes can complement pul mutations.** The data presented above suggest the existence of a tight interaction between PulE and Pull that is modulated by PulM and possibly by PulC and PulG. Heterologous complementation can provide valuable information on secretion specificity and interactions between different secretion components (5, 17, 36). Here, we extended previous studies (8, 11, 26) by analyzing the ability of *E. chrysanthemi* and *E. carotovora out* genes to substitute for their *pul* homologues. The results (Table 3) show that all *pul* genes except *pulC* and *pulD* but including *pulE*, *pulG*, *pulL*, and *pulM* could be replaced by the corresponding *out* genes, although full complementation was sometimes obtained only at high levels of (IPTG-induced) expression.

DISCUSSION

We report several different approaches (protease protection, cross-linking, immunoprecipitation, and subcellular fractionation) to probe protein-protein interactions involving components of the pullulanase secretion complex, the secretion. The main objective was to study the pivotal PulE protein and its interactions with the cytoplasmic membrane. Before discussing the broader implications and specific questions raised by our work, we will summarize the data and indicate the limitations of the different techniques.

The protease protection data provide indirect evidence of protein-protein interactions. PulE is destabilized in the absence of Pull, and Pull is destabilized in the absence of PulC and PulM. Therefore, one can conclude that these four proteins probably form a complex, but one cannot determine which proteins interact directly, nor can one exclude the involvement of other proteins in the complex. No other new interactions were revealed by these assays.

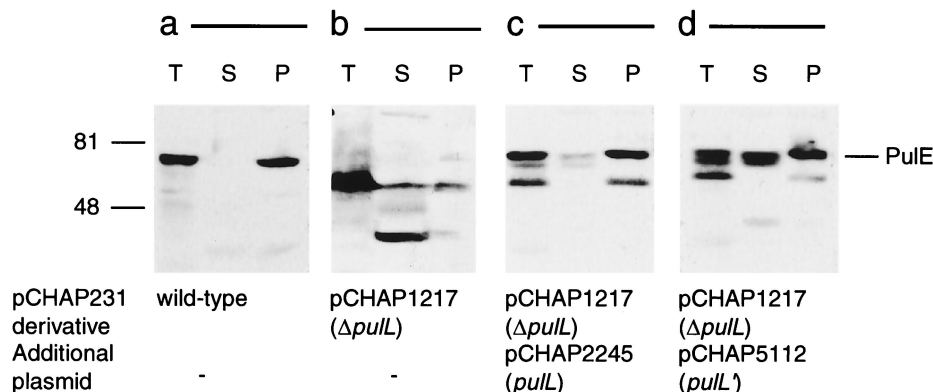


FIG. 8. Protection of PuE by PuL and its sedimentation in total membrane fraction. French press lysates from spheroplasts of *E. coli* strain PAP105 carrying the complete *pul* gene cluster in pCHAP231 (a), pCHAP231  $\Delta pulL$  (pCHAP1217) (b), pCHAP231  $\Delta pulL$  (pCHAP1217) and pCHAP2245 in which *pulL* is under IPTG-inducible *lacZp* control (c), or pCHAP231  $\Delta pulL$  (pCHAP1217) and pCHAP5112 in which the 5' end (247 codons) of *pulL* is under IPTG-inducible *lacZp* control (d) were fractionated by ultracentrifugation. PuE was detected by immunoblotting. Molecular size markers (kilodaltons) are indicated on the left. T, whole bacteria; S, soluble fraction; P, total membrane (pellet) fraction.

Cross-linking data provide more direct information on protein-protein interactions. However, the fact that the absence of one protein from the secreton changes the ability of a particular cross-linker to stabilize multimers formed by another does not necessarily indicate that the two proteins interact directly, although it is probably safe to assume that they are in the same multiprotein complex. For the four proteins mentioned above, PulM formed homodimers, even in the absence of all other secreton components (data not shown), and PuL and PulM probably form a heterodimer (Fig. 4 and 5). PuL seems to influence the formation of this heterodimer when all other secreton components are present (see below). The cross-linking data do not provide any evidence of PuE or PuL homodimers or of an interaction between PuE and PuL. Formation of the previously described 110-kDa cross-linked product detected by antibodies against PuL is shown here to be unaffected by mutations in any of the other *pul* genes except *D* and *S*. Therefore, we can extend the previous interpretation

that the 110-kDa product could represent a PuL homotrimer by adding that its detection requires the presence of PuD that is correctly localized and stabilized by the previously established interaction with PuS. Formation of this 110-kDa complex is independent of any interaction between PuL and PuE, PuL, or PuM.

The immunoprecipitation data show unambiguously that PuL, PuE, and PuL interact (directly or indirectly) with PuM but do not exclude the possibility that other proteins are present in the complex. The fractionation data, which confirm the intimate association between PuE and PuL, reveal several different states of PuE, depending on which other secreton components are present. PuE alone forms aggregates that float up the sucrose gradient to a position near that of the outer membrane fragments. When the rest of the secreton is present, aggregation is prevented and PuE associates with the cytoplasmic membrane. Removal of PuL from the secreton causes degradation of PuE. Finally, PuL alone can anchor

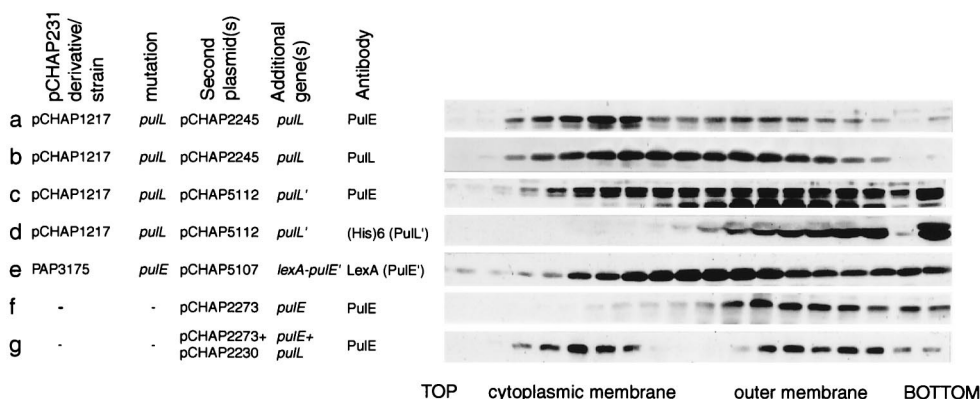


FIG. 9. Association of PuE and PuL and a LexA-PuE' hybrid protein with the cytoplasmic membrane in strains with different combinations of secreton components. Membranes prepared by disrupting whole bacteria or spheroplasts in a French press were separated by flotation through density sucrose gradients. (a and b) *E. coli* PAP105 carrying pCHAP1217 bearing the full set of *pul* genes except *pulL* and with *pulL* provided in *trans* on pCHAP2245; (c and d) like gradients a and b except that the in *trans* plasmid was pCHAP5112 coding for truncated PuL (PuL'); (e) PAP3175 with the complete *pul* gene cluster in the chromosome but with the  $\Delta pulE$  mutation and bearing pCHAP5107 (*lexA-pulE'*); (f) *E. coli* PAP105 expressing *pulE* alone (pCHAP2273); (g) *E. coli* PAP105 expressing *pulE* (pCHAP2273) and *pulL* (pCHAP2230) from compatible plasmids (note that, for gradients f and g, secreton components other than PuE and PuL are absent). Proteins were detected by immunoblotting with antibodies specific for PuE, PuL, LexA (for LexA-PuE' hybrid), or (His)<sub>6</sub> (for PuL'). Only those parts of the immunoblots displaying PuE, LexA-PuE', PuL, or PuL' are shown.



PulE to the cytoplasmic membrane. The N-terminal, cytoplasmic domain of PulL alone did not promote association of PulE with the cytoplasmic membrane, but it could do so if all other secretion components (except full-length PulL) were present. This observation might indicate that, although PulE can associate with the membrane via PulL, it can also do so through another component of the secretion, provided it is protected from proteolysis and aggregation by interacting transiently with the cytoplasmic domain of PulL. Finally, the fractionation data show that association of PulE with the cytoplasmic membrane involves the N-terminal region of the protein.

These data allow us to build up a first picture of the interactions between some of the Pul secretion components and lead us to propose that Pul proteins E, L, and M form a core subcomplex. Recent studies in several other laboratories have begun to reveal similar interactions between secretion components E, L, and M (1, 18, 34, 36, 37, 43). Taken together, the data indicate protein L-mediated cytoplasmic membrane association of protein E via the N-terminal regions of the two proteins and indicate that interaction between the L and M proteins probably occurs through their periplasmic domains (34). In addition, dimerization of XcpR (XcpE) via its amino-terminal domain was also reported (45), OutE and OutL both form homodimers in a two-hybrid system (34), PulE forms disulfide-bonded dimers when cells are disrupted (24, 25), and PulM forms homodimers (data not shown). Thus, the E-L-M subcomplex probably contains two copies of each of its components.

There is also increasing evidence that the putative E-L-M subcomplex interacts with other secretion components. For example, we show for the first time that PulC might interact with the PulELM subcomplex, as indicated by the fact that its absence destabilizes PulL and that it can be immunoprecipitated by antibodies against PulM. It will be recalled that PulC also interacts (directly or indirectly) with PulD (Fig. 3 and reference 26). PulG protein also seems to interact with the ELM complex. Kagami et al. reported a conditional mutation in the *P. aeruginosa* secretion gene encoding the pseudopilin XcpT (XcpG) that was suppressed by a second mutation in *xcpR* (*xcpE*) (13). Here, we extend this observation by reporting that PulG is required to form the cross-linked PulL-PulM heterodimer when the rest of the secretion is present. Interestingly, the effect of inactivating *pulG* (failure to form the PulL-M heterodimer) was the opposite of that observed when three other pseudopilin genes (*pull*, *pull*, and *pulK*) were inactivated (consistently increased levels of this heterodimer) (Fig. 4). Presumably, the pseudopilins interact with the PulELM complex but in ways that differentially affect the contact between proteins L and M. Surprisingly, PulL and PulM can be cross-linked to form a heterodimer in the complete absence of pseudopilins and all other secretion components (Fig. 5), which suggests that the pseudopilins affect this heterodimer only when the rest of the secretion is present. One possible interpretation of these results is that the PulELM complex is involved in the assembly of pseudopilins into a putative supramolecular structure that we have tentatively called the pseudopilus (28, 29, 32). The interaction between PulL and PulM or between PulM monomers might occur when only these two proteins are present or when the pseudopilins are being assembled. However, the absence of PulG might prevent pseudopilus assembly, causing a change in PulL-PulM interactions such that cross-linking can no longer stabilize them. The absence of the other pseudopilins might provoke the opposite effect, causing increased assembly of PulG into pseudopili and increasing the interactions between PulL and PulM.

It is interesting that overproduction of PulG reduced the cellular levels of at least five Pul proteins (C, D, E, L, and M), causing a total block in pullulanase secretion (29). Previously, we reported that the secretion defect resulting from overproduction of PulG could not be suppressed by increasing the expression of other, individual *pul* genes (29), implying that no particular Pul protein was titrated by high levels of PulG. The fact that increasing the level of PulG causes a decrease in the levels of several secretion components provides an explanation for this observation.

The Pul secretion cannot secrete the cellulase CelZ, which is a substrate for the *E. chrysanthemi* Out secretion (39). However, the corresponding *out* genes from either *E. chrysanthemi* or *E. carotovora* can complement mutations in all but two of the *pul* genes (C and D). These results have three important implications. First, the only secretion components that can be responsible for substrate specificity are C and D, as suggested previously by Lindeberg et al. (17). Second, the proposed PulELM subcomplex can form even when one of the partners is replaced by the homologous Out protein. Third, Out pseudopilins can substitute for their Pul counterparts and be assembled in functional form by the PulELM subcomplex. It is well established that type IV pilins from one species of bacterium can be assembled into pili by the type IV pilus assembly machinery of other species (38). It will be interesting to determine whether homologous components of the type IV pilus machinery and the secretion are interchangeable, as is the case for prepilin peptidase (31), and whether the secretion can be used to assemble type IV pili.

Secretion components E and L are not interchangeable between the closely related species *V. cholerae* and *A. hydrophila* (36), despite the fact that they can secrete proteins normally secreted by the other (10, 12, 21). This situation is different from that described here for the Pul and Out secretions, presumably reflecting the independent evolution of substrate-specific determinants (proteins C and D [17]) and other, structural or mechanical components of the secretion (E and L). From this, we predict that secretion components C and D might be interchangeable between *V. cholerae* and *A. hydrophila* and that the incompatibility between the E and L proteins of *Vibrio* and *Aeromonas* probably represents divergent coevolution of these two components in the different species.

The data do not provide any evidence for a link between PulF and the PulELM complex, which is surprising in view of the fact that genes encoding PulF homologues are invariably adjacent to a *pulE* homologue (27). The role of PulF in pullulanase secretion remains to be clarified, but it is the most likely candidate for the protein that anchors PulE to the cytoplasmic membrane when PulL is synthesized without its periplasmic domain and its transmembrane anchor.

On the basis of the data presented here and elsewhere, we can begin to construct a conceptual model of the secretion. We propose that the ELM subcomplex in the cytoplasmic membrane is involved in the export of pseudopilins and/or their assembly into a pilus-like structure. This process could be driven by ATP hydrolysis via PulE. The pilus-like structure could act as a scaffold for the assembly of other secretion components or could play an active role in secretion, for example, by providing a conduit linking the periplasm to the outside of the cell. The fact that two lines of evidence show a link between the cytoplasmic membrane ELM subcomplex and the C protein is particularly intriguing because it has already been proposed that this protein could form a bridge between the two membranes (2, 26). Furthermore, PulC probably interacts with PulD (secretin) (26), the protein that probably forms the channel via which pullulanase crosses the outer

membrane (20). Thus, we propose that PulC could control opening and closing of the secretin channel via specific recognition of translocation substrates (in this case, pullulanase) and transduction of energy from the proton gradient across the cytoplasmic membrane (23). This model for secretin function can now be tested by purification and analysis of its component parts and by isolation of the complete machinery.

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