Green Fluorescent Chimeras Indicate Nonpolar Localization of Pullulanase Secreton Components PulL and PulM

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The *Klebsiella oxytoca* pullulanase secreton (type II secretion system) components PulM and PulL were tagged at their N termini with green fluorescent protein (GFP), and their subcellular location was examined by fluorescence microscopy and fractionation. When produced at moderate levels without other secreton components in *Escherichia coli*, both chimeras were envelope associated, as are the native proteins. Fluorescent GFP-PulM was evenly distributed over the cell envelope, with occasional brighter foci. Under the same conditions, GFP-PulL was barely detectable in the envelope by fluorescence microscopy. When produced together with all other secreton components, GFP-PulL exhibited circumferential fluorescence, with numerous brighter patches. The envelope-associated fluorescence of GFP-PulL was almost completely abolished when native PulL was also produced, suggesting that the chimera cannot compete with PulL for association with other secreton components. The patches of GFP-PulL might represent functional secretons, since GFP-PulM also appeared in similar patches. GFP-PulM and GFP-PulL both appeared in spherical polar foci when made at high levels. In *K. oxytoca*, GFP-PulM was evenly distributed over the cell envelope, with few patches, whereas GFP-PulL showed only weak envelope-associated fluorescence. These data suggest that, in contrast to their *Vibrio cholerae* Eps secreton counterparts (M. Scott, Z. Dossani, and M. Sandkvist, Proc. Natl. Acad. Sci. USA 98:13978–13983, 2001), PulM and PulL do not localize specifically to the cell poles and that the Pul secreton is distributed over the cell surface.

Many bacterial cell components are located at specific sites that are crucial for their function. For example, proteins involved in maintaining cell shape and chromosome segregation (22), as well as some outer membrane proteins and surface structures (15, 16), are organized in helical patterns, while the cell division machinery localizes to the mid-cell (52) and the Sec and Tat protein export complexes (4, 41), as well as many components of the type IV secretion machinery (23), are uniformly distributed in the cell envelope. Polar localization has been demonstrated for surface appendices, such as type IV pili (48) and proteins involved in their assembly and retraction (9), the chemoreceptor complex (25), and some components of the type I (47) and the type IV secretion machineries (19, 23). An intriguing question that remains is how the localization and assembly of different protein complexes at the correct site is achieved.

We approached this problem by studying the location and assembly of a prototypical type II secretion system (T2SS) machinery. These machineries (secretions) secrete hydrolytic enzymes and toxins that often play important roles in pathogenesis (42) in many gamma-proteobacteria (30, 34). Secretion via this pathway is a two-step process. Exoproteins are first translocated to the periplasm via the Sec (36) or Tat (51) pathways, whereafter they are secreted into the extracellular environment via the T2SS machinery. The latter is composed of at least 12 essential proteins that probably assemble into a large, envelope-spanning complex (13). Extensive studies on various secretons identified many protein-protein interactions and resolved structural aspects of some of the components, but the molecular mechanism of secretion is not clearly understood. Three cytoplasmic membrane proteins, generically referred to as GspL, GspM, and GspF, constitute a platform in the cytoplasmic membrane that forms the basis of the secreton (40). GspL and GspM interact directly and independently of other secreton components (32, 44) and protect each other from degradation (26, 32). The C-terminal periplasmic domain of GspL binds to GspM (45). The N terminus of GspE, an ATPase (5) that, together with the proton-motive force (24, 31), probably provides the energy for secretion, connects to the rest of the machinery via a direct interaction with the cytoplasmic N-terminal domain of GspL (1, 32, 39, 43). GspC probably forms a bridge between the inner membrane part of the secreton and the outer membrane channel formed by the secretin GspD (2, 7, 17, 33). Secretion might be promoted by extension and retraction of a type IV pilus-like structure (the pseudopilus), composed mainly of the pseudopilin GspG (11, 20, 50), through the secretin channel in a manner analogous to the extension and retraction of type IV pili. Minor pseudopilins contribute to pseudopilus formation and/or secretion, but their precise role is less clear (12, 45, 50). GspO is a cytoplasmic membrane-located pre pilin peptidase that cleaves and N-methylates the pseudopilins (29, 35).

Scott et al. reported that chimeras made by fusing green fluorescent protein (GFP) to GspL (EpsL) and GspM (EpsM) of the *Vibrio cholerae* Eps secreton are located at the older of the two cell poles, where secretion was shown to occur (47). EpsM was also polar when produced in *Escherichia coli* (28, 47). Interestingly, the Sec machinery is apparently evenly distributed in the cytoplasmic membrane of *Escherichia coli* (4), suggesting that polar targeting of the *V. cholerae* T2SS com-
ponents might occur in the periplasm. To corroborate and extend these observations, we examined the location of GspL and GspM (PulL and PulM) in the pullulanase (Pul) secretory of Klebsiella oxytoca, the first type II secretion machinery to be fully characterized (10), using the same gene fusion strategy as in the studies of EpsL and EpsM in E. coli.

Materials and Methods

Bacterial Strains and Growth Conditions. All E. coli K-12 strains used or constructed in this study were derived from strain MC4100 (Table 1). Luria-Bertani (LB) broth and LB agar were prepared as described previously (27).

Antibiotics were added as required at the following concentrations: ampicillin (Ap), 25 or 200 μg/ml; kanamycin (Km), 30 μg/ml; chloramphenicol (Cm), 34 μg/ml. Expression of genes under the promoter control was induced with 10 or 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Expression of genes from MalT-controlled promoters was induced with 0.4% maltose. Generalized transduction using P1 phage was performed as described previously (27). Gene fusions were stably integrated into the λ attachment site of the E. coli chromosome using the AcmCh integration vector (3).

Plasmid construction. PCR amplifications were carried out for 30 cycles (1 min at 94°C, 1.5 min at 52°C, and 1.5 min at 72°C) with Pfu polymerase (Invitrogen). Amplified DNA was purified using QIAGEN PCR purification columns and verified by sequencing. Amplified DNA was purified using QIAGEN PCR purification columns and verified by sequencing.

Plasmid pCHAP231, containing gfp-pulL and gfp-pulM, respectively, was constructed as follows. pulL and pulM fragments were generated by PCR, using primers Ngfp-pulL-5′-CCGTCGAAGCTTTTATGCATCGTTC-3′ and Ngfp-pulM-5′-CGAGAATTCAACAACAACAACAATAACCACC-3′. Gene fusions were stably integrated into the λ attachment site of the E. coli chromosome using the AcmCh integration vector (3).

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RESULTS

Cell Fractionation. Aliquots (100 ml) of cultures at an optical density at 600 nm of 0.8 to 1.0 were collected by centrifugation, and the pellet was resuspended in 10 ml of 25 mM HEPES (pH 7.4). Cells were disrupted by two passages through a French press (1,200 bar), and the lysate was mixed with 10 μg/ml each of DNase I, pancreatic RNase A, and Pefabloc protease inhibitor. The mixture was then centrifuged for 10 min at 50,000 × g to eliminate unbroken cells. Membranes were then collected by ultracentrifugation at 160,000 × g for 1 h at 4°C and resuspended in 1 ml of 25 mM HEPES (pH 7.4), and proteins were precipitated in 10% trichloroacetic acid. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with appropriate antibodies.

SDS-PAGE and Immunoblotting. Proteins were solubilized in loading buffer with 5% β-mercaptoethanol, heated at 100°C for 5 min, and separated by SDS-PAGE in gels containing 10% or 12% acrylamide. After transfer onto nitrocellulose membranes, proteins were detected by incubating the membranes with primary polyclonal rabbit antibodies raised against MalE-Pull, MalE-PulM, SecG, or GFP (Clontech) and then were incubated with horseradish peroxidase-conjugated secondary antibodies to rabbit immunoglobulin G (Amersham). Bound secondary antibodies were detected by enhanced chemiluminescence (Amersham).

Fluorescence Microscopy. Strains were grown overnight at 30°C, diluted 1:100 in fresh medium, and grown to an optical density at 600 nm of 0.2. IPTG was then added to a final concentration of 10 or 100 μM, and 0.4% maltose was added in strains carrying pul genes under MalT-dependent promoter control. The cells were incubated for 1 h at 30°C under continuous shaking. Live cells were immobilized on wet agarose-coated glass slides and analyzed using a Zeiss Axiosplan2 fluorescence microscope mounted with a Hamamatsu CCD camera. Images were collected using OpenLab and processed with Photoshop.

Table 1. Strains and plasmids

<table>
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<th>Strain or plasmid</th>
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<td>PAP105</td>
<td>Δ(lac-pro) pAAP501 [F′(lacI5 proAB lacI5ZM15 proAB 23 Tn10)] (Tc')</td>
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<tr>
<td>MC4100</td>
<td>F- araD139 ΔlacU169 relA1 rpsL150 thi mot floI-5301 deoC ptsF25 rbsR</td>
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<td>Klebsiella oxytoca</td>
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<td>This study</td>
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<tr>
<td>PAP9108</td>
<td>PAPI05Δ(kant-1::bla lacI5 pBS-gfp-pulM</td>
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<td>PAP9106 plus pCHAP1217</td>
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<td>PAP9176</td>
<td>PAP9108 plus pCHAP2235</td>
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* All strains are E. coli K-12, except for strain UNF5023.
of these two inner membrane proteins using fluorescent chimeras in which GFP moiety was fused to the cytoplasmic N termini, as in the studies of EpsL and EpsM (47), without interference from other secreton components. The gfp-pulL and gfp-pulM fusions were inserted into the E. coli chromosome at the /H9261 attachment site (/H9261 att), resulting in a homogeneous cell population in which their expression could be carefully controlled from a repressible trc promoter (53). Although maltose-induced expression of the pul genes in the E. coli or K. oxytoca chromosome does not lead to production of sufficient PulL or PulM to allow their detection by immunoblotting (32), both GFP-PulL and GFP-PulM were detectable by immunoblotting in the strains with chromosomal gene fusions after induction with small amounts (10 μM) of IPTG (data not shown; see Fig. 3). The protein levels are comparable to levels of PulL and PulM present in maltose-induced cells carrying a pBR322-derived plasmid bearing all of the pul genes (pCHAP231) (32). Thus, despite the fact that their genes were integrated into the chromosome and were induced to submaximal levels, both chimeras were more abundant than chromosome-encoded PulL or PulM.

Fluorescence microscopy revealed that GFP-PulM located circumferentially; i.e., in the envelope (Fig. 1C). A few brighter foci of GFP-PulM were also occasionally observed. GFP-PulL was also detected as circumferential membrane fluorescence, but the fluorescence intensity was lower than that of GFP-PulM (Fig. 1A). Note that GFP itself was detected as wholly cytoplasmic fluorescence and is clearly different from GFP-PulL (Fig. 1E), and that GFP-PulL is located in the membrane fraction of disrupted cells (see Fig. 3). These results show that GFP-PulM localizes over the entire E. coli surface rather than at the poles, like GFP-EpsM (47).

**GFP-PulM fluorescence and localization are unaffected by PulM.** A direct interaction between PulL and PulM was inferred from cross-linking experiments and from the fact that PulL is stabilized by PulM (32). Similarly, EpsL and EpsM in V. cholerae interact directly via their periplasmic domains (44), and GFP-EpsL is targeted to the V. cholerae and E. coli cell pole through a direct interaction with EpsM (47). To test whether PulM has any effect on the GFP-PulL chimera, the pulM gene was expressed from a multiple-copy plasmid in an E. coli strain with gfp-pulL integrated into its chromosome at

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**FIG. 1.** Cellular location of GFP-PulL and GFP-PulM in E. coli K-12. (A) Localization of GFP-PulL in wild-type E. coli and (B) in a recombinant E. coli strain in which all pul genes except pulL are expressed from plasmid pCHAP1217. (E) Localization of GFP alone in E. coli. (F) Localization of GFP-PulL in an E. coli strain carrying a plasmid encoding all pul genes including pulL (pCHAP10). (C) Localization of GFP-PulM in wild-type E. coli and (D) in E. coli harboring pCHAP1539 (lacking pulM). (G) Localization of GFP-PulM in an E. coli strain containing pCHAP710 (all pul genes, including pulM). The gfp gene and the gfp-pul chimeras were integrated into the chromosome at the katt site, and their expression was induced with 10 μM IPTG. Fluorescence images of live cells (right) were captured using a fluorescein isothiocyanate filter; corresponding phase-contrast images are presented in the panels on the left. The bar represents 2 μm.
the λatt site. The membrane fluorescence of GFP-PulL remained as low in this strain (Fig. 2A) as in the strain without PulM (Fig. 1A). Conversely, the membrane fluorescence of GFP-PulM was not affected by the presence of PulL (Fig. 2B).

**GFP-PulL and GFP-PulM assemble into patches in the E. coli envelope when other secreton components are present.** To study possible changes in the location of the fluorescent chimeras caused by other secreton components, we introduced pBR322-derived plasmids carrying all pul genes, except pulL or pulM (32), into *E. coli* with the chromosomal gfp-pulL or gfp-pulM gene fusion. Expression of the pul genes (induced by growth in medium containing maltose) resulted in slight elongation of the cells, as observed previously (10). The two gene fusions restored pullulanase secretion fully (data not shown) in these otherwise secretion-defective cells (32), indicating that they are functional. The location of GFP-PulL was unaltered by the presence of other Pul secreton components, except for the appearance of increased numbers of bright, mainly circumferential fluorescent foci (Fig. 1D). GFP-PulL showed increased membrane fluorescence and foci similar to that of GFP-PulM in the presence of the other Pul components (Fig. 1B). This observation suggests that GFP-PulL requires other secreton components besides or instead of PulM for its stability, membrane association, or assembly into the T2SS.

When all pul genes including pulL (encoded by pCHAP710 [21]) were expressed together with gfp-pulL, GFP-PulL was no longer found in envelope patches and the overall envelope fluorescence was reduced (Fig. 1F). This result suggests that endogenous PulL competes with GFP-PulL. Expression of extra copies of pulM from pCHAP710 did not affect the fluorescence pattern of GFP-PulM (Fig. 1G).

The GFP-PulL chimera might be stabilized by other Pul components, as suggested by previous studies with PulL (32). The GFP chimeras were therefore analyzed by immunoblotting after cell fractionation. Full-length GFP-PulL and GFP-PulM chimeras that reacted with antibodies against GFP and PulL or PulM were detected almost exclusively in the particulate (P; envelope) fraction, irrespective of the presence of other secreton components (Fig. 3). As expected (38), native PulM and PulL were also both exclusively present in the particulate fraction (lanes 6 and 8, respectively, in Fig. 3; note that PulL comigrates with MalE), as was the inner membrane protein SecG, whereas periplasmic MalE was almost totally in the soluble (S) fraction (Fig. 3).

Both GFP-PulL and GFP-PulM appeared as doublets in immunoblotting experiments (Fig. 3), probably due to proteolysis. The faster migrating band appeared in both the soluble and particulate fractions in the case of GFP-PulL and in the particulate fraction in the case of GFP-PulM. The expression of the pul genes did not affect the stability of either chimera or the amount of the chimera in the membrane fraction. Thus, the patches of envelope-associated GFP-PulL probably reflect its association with other secreton components, again suggesting that PulL depends on one or more Pul proteins for its folding and/or assembly.

**Overexpression of gfp-pulL or gfp-pulM results in polar localization of the encoded chimeras.** Scott et al. reported that GFP-EpsM localizes to the old cell pole in *E. coli* (as well as in...
V. cholerae) (47), which is contrary to what we report here for GFP-PulM. Shown are SDS-PAGE and immunoblot analysis with antisera against GFP, MalE-PulL, MalE-PulM, and SecG of soluble (S) and particulate (P) fractions of E. coli expressing gfp-pul chimeras. GFP-PulL was produced by strain PAP9106 with or without pCHAP1217 (encoding all Pul proteins except PulM). GFP-PulM was produced by strain PAP9108 with or without pCHAP1359 (encoding all Pul proteins except PulM). GFP chimeras are indicated on the right by asterisks. The prominent band around 43 kDa and indicated by a triangle is MalE (the antibodies were raised against chimeras similar to those when the fusions are expressed under the tcr promoter without induction from a high-copy plasmid. GFP-PulM was detected in the cell envelope (Fig. 5). Few clusters were observed. Faint envelope localization of GFP-PulL was also observed (compare the fluorescence pattern with that obtained with GFP alone; Fig. 5). These results suggest that K. oxytoca-specific factors do not play a role in the localization of PulL and PulM and that localization studies on the Pul secreton performed in reconstituted, secretion-proficient E. coli reflect the native situation in K. oxytoca. Here, however, the bacteria produced both the native protein (PulM or PulL) and the corresponding chimeras, which were probably much more abundant than the native proteins. Therefore, the fluorescence observed is mostly due to chimeras not associated with the secreton.

**DISCUSSION**

GFP and other fluorescent chimeras are simple experimental tools with which to examine membrane protein localization and assembly of protein complexes, but their analysis is not without potential pitfalls, such as overproduction artifacts and misfolding, altered membrane association, or altered affinity for other cellular components. For example, high-level production of GFP-PulL and GFP-PulM caused their substantial accumulation in bright polar foci. It is noteworthy that these foci were spherical rather than the crescent shape that one would expect for a membrane-associated fluorescent protein. We suspect, therefore, that these foci represent (micro)aggregates in which the GFP portion of the chimera has adopted its active (fluorescent) configuration, and the PulL or PulM portion has aggregated rather than inserted into the membrane. Their polar localization might result either from the fact that the nucleoid occupies most of the cell volume except the poles and excludes large complexes, such as these aggregates, or from reduced diffusion at the poles.

When produced at low levels, GFP-PulL and GFP-PulM were both envelope associated, indicating that GFP does not interfere with the ability of the single membrane-spanning hydrophobic segment in each protein to promote membrane insertion. In the case of GFP-PulM, the GFP tag is positioned immediately in front of the uncleaved signal sequence, but it is separated by over 240 amino acids from the PulM insertion-membrane anchor sequence (38). Although both gene fusions were functional in genetic complementation assays, one cannot exclude that degradation products, rather than the full-length chimeras, are the active form of the protein. The inability of E. coli chaperones to fold multidomain proteins efficiently (8) could be another factor that causes problems in the analysis of GFP chimeras, since one cannot assume that all chimeras in the cell have folded correctly and are fluorescent. The behavior of the membrane-associated GFP-PulL is an example. The intensity of the envelope fluorescence of this chimera increased dramatically when other secreton components were present, but the steady-state levels of the chimera, its stability, and the proportion of it that was in the envelope did not change dramatically under these conditions. Thus, we conclude
that other secreton factors might assist the folding of membrane-associated GFP-PulL, leading to increased fluorescence. The fact that production of PulL abolished the effect of the other secreton components on the fluorescence of the membrane-associated GFP-PulL corroborates this interpretation, since the native protein presumably prevented association of the chimera with other secreton components. We speculate that the GFP-PulL and GFP-PulM foci observed in E. coli expressing other pul genes might follow a specific pattern, such as a helix, as do other bacterial cell envelope protein complexes (6, 16), but the number of foci detected was insufficient to demonstrate such a pattern by deconvolution of stacked images (data not shown).

These considerations do not affect the main conclusions that we can draw from the analysis of the GFP-PulL and GFP-PulM chimeras, which are that they insert throughout the inner membrane in E. coli and K. oxytoca and that they form brighter fluorescent clusters in this membrane when other secreton components are present. These clusters could represent assembly intermediates or complete secretions. A similar reorganization and clustering was observed with components of the type IV secretion machinery of Agrobacterium tumefaciens (18, 19, 23). Secretion machineries, irrespective of their nature, might exist only transiently as active complexes. The clustered membrane fluorescence patterns of T2SS components might represent nucleation sites for the assembly of part of the secreton, while protein secretion occurs upon complete assembly with motor proteins, the outer membrane secretin, and the pilus at a specific site in the cell.

Our conclusion differs from that reached by Scott et al., who concluded that GFP-EpsM locates to the old pole after cell division, both in E. coli and in V. cholerae, and that GFP-EpsL associates with EpsM to achieve a similar polar localization, even in the absence of other Eps secreton components (47). These conclusions are particularly interesting in view of other evidence that type II secretion occurs at the pole in V. cholerae (47). We do not have any evidence that pullulanase is secreted at a specific site on the cell surface in E. coli or K. oxytoca. Pullulanase is an unusual type II exoprotein that remains cell surface anchored via its fatty acylated N terminus. Immunogold labeling of pullulanase revealed an even distribution over the entire cell surface without polar enrichment (10, 37). Initial studies of artificially elongated pseudopili produced by agar surface-grown E. coli overproducing the pseudopilin PulG or the entire secreton suggested that bundled pseudopili were located predominantly at the cell pole (46). However, examination of E. coli producing extended pseudopili that were unable to bundle due to a C-terminal histidine tag on PulG revealed an even surface distribution that presumably reflects the distribution of the secretin PulD (50). It will be of interest to determine whether components of other secretions are located at the poles.

A subcomplex of the Pul secreton composed of PulM, PulL, and PulE interacts with PulC, which itself interacts with secretin PulD (32, 33). Thus, PulC and PulD are likely to play an essential role in the assembly and localization of the secreton. Further studies will be directed at determining the spatial organization of stable Pul secreton complexes and the site of pullulanase secretion.
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REFERENCES


FIG. 5. Cellular location of GFP-PulL and GFP-PulM in Klebsiella oxytoca. Localization of GFP-PulL and GFP-PulM was determined by fluorescence microscopy. Genes were constitutively expressed from pCHAP7508 (gfp-pulL), pCHAP7509 (gfp-pulM), and pDSW207 (gfp) in K. oxytoca strain UNF5023. The bar represents 2 μm.


