Structural Insights into the Secretin PulD and Its Trypsin-resistant Core*  

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Limited proteolysis, secondary structure and biochemical analyses, mass spectrometry, and mass measurements by scanning transmission electron microscopy were combined with cryo-electron microscopy to generate a three-dimensional model of the homotrimeric complex formed by the outer membrane secretin PulD, an essential channel-forming component of the type II secretion system from *Klebsiella oxytoca*. The complex is a dodecameric structure composed of two rings that sandwich a closed disc. The two rings form chambers on either side of a central plug that is part of the middle disc. The PulD polypeptide comprises two major, structurally quite distinct domains; an N domain, which forms the walls of one of the chambers, and a trypsin-resistant C domain, which contributes to the outer chamber, the central disc, and the plug. The C domain contains a lower proportion of potentially transmembrane β-structure than classical outer membrane proteins, suggesting that only a small part of it is embedded within the outer membrane. Indeed, the C domain probably extends well beyond the confines of the outer membrane bilayer, forming a centrally plugged channel that penetrates both the peptidoglycan on the periplasmic side and the lipopolysaccharide and capsule layers on the cell surface. The inner chamber is proposed to constitute a docking site for the secreted exoprotein pullulanase, whereas the outer chamber could allow displacement of the plug to open the channel and permit the exoprotein to escape.

The widespread type II secretion systems (T2SS)1 of Gram-negative bacteria allow the secretion of hydrolytic enzymes (lipases, amylases) or virulence factors, collectively referred to as exoproteins, to the external medium (1, 2). These exoproteins are first translocated by the Sec (3) machinery visible in the reconstituted three-dimensional image (28, 30), and from regular patterns of stain accumulation visible in the reconstituted three-dimensional image (28, 30), respectively.

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§ The abbreviations used are: T2SS, type II secretion systems; HPLC, high performance liquid chromatography; STEM, scanning transmission electron microscope; Tricine, N-[2-hydroxy-1,1,1-triethylglycyl]glycine.
The pullulanase T2SS of *K. oxytoca* is one of the most extensively studied secretions and has been completely reconstituted in *E. coli*. A low resolution three-dimensional structure of a purified complex of the pullulanase T2SS secretion PulD and its pilotin, PulS, revealed a cylindrical complex with 12-fold symmetry and a central open channel of about 7 nm encircled by radial spokes that we originally presumed to be the pilotin, which is absent from pIV and PilQ (16). Here, we report refined biochemical and structural analyses of intact and proteolyzed PulD multimers.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The *E. coli* strains PAP105 (Δ(lac-pro) F(lacUV5 proAB+ Tn10)) and XL1-Blue (ΔlacZAM15 recA1 endA1 gyrA96 thi hsdR17 galV44 relA1 F-lacP+proAB+ Tn10) were used for recombinant DNA work. *E. coli* strain BL21 (DE3) (F-*ompT hsdS*2 gal dcm) was used to produce the PulD N domain. The principal characteristics of the plasmids used in this study are summarized in TABLE ONE. Bacteria were grown at 30 °C in Luria-Bertani (LB) medium (31) containing chloramphenicol (25 μg/ml, except where noted), or kanamycin (50 μg/ml).  

**Construction and Purification of PulD-N**—See the supplemental material.

**Purification of Secretins**—Outer membranes were isolated from PAP105 (pCHAP3516/pCHAP5506) and PAP105 (pCHAP3678/pCHAP585) producing PulD and PulShis or PulDhis and PulS, respectively, as described previously (16). Membrane proteins were solubilized in 50 mM Tris-HCl (pH 7.5) containing 3% n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (ZW3–14), 250 mM NaCl, and 0.1 mg/ml protease inhibitor Pefabloc (Pierce) and incubated for 1 h at room temperature. Solubilized proteins were recovered after ultracentrifugation at 185,000 × g and mixed with covalent agarose resin (Talon, Clontech) previously equilibrated in 50 mM Tris-HCl (pH 7.5), 0.6% ZW3–14, 250 mM NaCl (TZN buffer) containing 5 mM imidazole for 1 h at 20 °C. The resin was washed with 10 volumes of TZN buffer plus 5 mM imidazole and then poured into a column. Bound proteins were eluted with 5 column volumes of TZN buffer containing 5 mM EDTA and immediately loaded onto a HiTrap Q HP column (1 ml) (Amersham Biosciences) connected to an AKTAprime system (Amersham Biosciences) at a flow rate of 0.1 ml/min. After extensive washing, secretin complexes were eluted using a linear gradient of 250 mM to 1.25 M NaCl. To completely dissociate PulS from the complex, the fractions containing PulD (eluted at 700–750 mM NaCl) were incubated with 50 mM reducing agent Tris-[2-carboxyethyl]phosphine] hydrochloride (Pierce) for 2 h at 20 °C. PulS was separated from the PulDhis complex by gel filtration on Sephacryl S300HR. The absence of PulS in the PulDhis fractions was confirmed by immunoblotting using antibodies directed against MalE-PulS. The fractions were concentrated and stored in 100-μl aliquots at −80 °C.

**Trypsin Proteolysis of PulDhis Secretin**—100 μg of purified PulDhis complex (0.1 mg/ml) was incubated with 10 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin (Sigma) for 2 h at 18 °C. Pefabloc was then added to 0.1 mg/ml, and the sample was immediately injected onto a Sephacryl S300HR column. A control experiment was performed under the same conditions except that the trypsin was omitted. In both cases aliquots from all fractions were treated with phenol to dissociate the PulD complexes (8) and then analyzed by SDS-PAGE. The Sephacryl S300HR column was calibrated using thyroglobulin, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase. PulD-containing fractions were concentrated and stored in 50-μl aliquots at −80 °C for electron microscopy.

**Electrophoresis, Immunoblotting, and Protein Sequencing**—See supplemental material.

**Circular Dichroism Analysis**—Circular dichroism (CD) spectra were recorded from 190 to 250 nm on a Jasco dichrograph (J-710) equipped with a Jasco J-370 spectropolarimeter. CD spectra were collected at 20 °C below and above the temperature of α-helix dissociation (17) and at 80 °C for electron microscopy.
with a thermostatically controlled cell holder and connected to a computer for data acquisition. Purified PulD-N at 100 μg/ml was dialyzed against 1 mM Tris-HCl (pH 8) containing 250 mM NaF and submitted to CD analysis in a 1-mm quartz cell (3 data sets were acquired). The PulD-C domain was dialyzed against 1 mM Tris-HCl (pH 7.5) containing 250 mM NaCl and 0.6% ZW3–14 immediately before analysis in a 5-mm quartz cell at a final concentration of 10 μg/ml (5 data sets were acquired). Purified PulDhis at 60 μg/ml in 50 mM Tris-HCl (pH 7.5) containing 250 mM NaF and 0.6% ZW3–14 was diluted twice in 500 mM NaF containing 0.6% ZW3–14 immediately before CD analysis in a 1-mm quartz cell (5 data sets were acquired).

Peptide Analysis by HPLC, Sequencing, and Mass Spectrometry—See the supplemental material.

Scanning Transmission Electron Microscopy—A Vacuum Generators (East Grinstead) HB-5 STEM interfaced to a modular computer system (Tietz Video and Image Processing Systems) was used. Samples were prepared on 200-mesh-per-inch, gold-plated copper grids as previously (35). Grids were washed on 5–7 droplets of quartz double-distilled water to remove detergent. Isolated tobacco mosaic virus particles (kindly supplied by R. Diaz-Avalos) adsorbed to a separate grid and air-dried served as the mass standard.

For structural examination, digital 512 × 512 pixel dark-field images were recorded from negatively stained (2% uranyl acetate) PulD at an acceleration voltage of 100 kV and a nominal magnification of 500,000× using doses between 3450 and 10250 electrons/nm. The pixel size (around 0.33 nm) depended on the focus conditions (35).

For mass determination, digital 512 × 512 pixel dark-field images were recorded from unstained freeze-dried samples of the PulD complexes at an acceleration voltage of 80 kV and a nominal magnification of 200,000×. The recording dose used varied between 295 and 640 electrons/nm² for these main data sets. In addition, repeated low dose scans were recorded from some grid regions to assess beam-induced mass loss (35). The images were evaluated with the IMPSYS program package as described previously (35). Complexes were delimited by circles, the total mass was calculated, and the background was subtracted. The beam-induced mass loss was assessed as previously (35). The experimental mass data were multiplied by the correction factors calculated for the recording doses available was used to measure the dimensions of the fragment to obtain a clean separation of the two, thereby eliminating symmetrization artifacts at the interface. The contrast-transfer function was corrected as above.

RESULTS

Purification and Proteolysis of Intact His-tagged Secretin Multimers—The PulD-PulS complex reported previously by Nouwen et al. (16) precipitated from ZW3–14 solutions at low temperature and at low salt concentrations, which hampered high resolution cryo-electron microscopy analysis. To circumvent this problem, we dissociated PulS from PulD without denaturing the latter. To facilitate the purification of PulD alone, a hexahistidine tag was inserted in the S domain of PulD at position 635 (i.e. in the S domain; see Fig. 3). The tagged protein, PulDHis, restored pullulanase secretion in a strain lacking PulD but producing all other secreton components ("Experimental Procedures"). When produced at high levels together with PulS, PulDHis was readily solubilized from membranes in ZW3–14 and was purified as a high molecular mass complex by cobalt and anion exchange chromatography ("Experimental Procedures"). PulS co-eluted with PulDhis but at a stoichiometry of only 0.1:1 (Fig. 1A), compared with ~1:1 for PulS+PulD (16) or PulShis+PulD (Fig. 1A), showing that the His tag in the S domain PulD did not prevent it from inserting into the outer membrane but might cause PulS to dissociate. Reduction of the disulfide bridge in PulS (42) by Tris[2-carboxyethylphosphine] hydrochloride...
completely dissociated the remaining PulS from the purified PulDhis complex (Fig. 1A).

The purified PulD complex and the complex remaining after limited trypsin proteolysis (“Experimental Procedures”) were subjected to size exclusion chromatography (Fig. 1B). The intact complex eluted at 46 ml, corresponding to a Stokes radius of \( \pm 12 \) nm. This is exactly the same elution volume as that of PulD-PulShis or PulD-PulS complexes (data not shown), indicating that the removal of PulS does not drastically modify the shape of the complex. In contrast, the protease-resistant complex eluted at 52 ml, indicating a Stokes radius of \( \sim 9 \) nm.

STEM mass analysis (Fig. 1C, left histogram) showed the intact PulDhis complex to have a mass of 919 kDa \((\pm 109 \) kDa). Previous studies of PulD-PulS revealed a stoichiometry of 12 (17). Assuming that this stoichiometry is unaltered by the removal of PulS and the insertion of a His tag (see the next paragraph), the PulDhis complex comprises 12 of the 68.745-kDa monomers with a total mass of 825 kDa, the remaining 94 kDa being bound detergent. This is in good agreement with the earlier experiments on purified PulD-PulS complexes, in which the detergent contribution was estimated to be 56 kDa (17), and with the amount of detergent \((75 \pm 20 \) kDa) expected to bind to the hydrophobic surface of the PulD homomultimer \((112 \) nm\(^2\) for a 12-nm diameter (see below), 3-nm thick \((43 \) hydrophobic belt). STEM analysis showed the mass of the proteolyzed complex to be 537 kDa \((\pm 64 \) kDa; Fig. 1C, right histogram; see the next section).

To verify the stoichiometry, both complexes were freeze-etched, metal-shadowed, and observed by transmission electron microscopy. As expected, the complexes appeared as ring-like structures when viewed end-on (Fig. 2A). Individual protomers were sometimes visible for the proteolyzed complex (Fig. 2B) but not for the intact complex (data not shown), possibly because flexible loops averaged out the signal in the latter. The projection average obtained upon single particle analysis of the metal-shadowed proteolyzed complex has a pronounced 12-fold symmetry (Fig. 2C), confirming that the stoichiometry of the complex is not modified by removal of PulS and proteolysis.

**Polypeptide Composition of Proteolyzed PulDhis**—The polypeptide composition of the trypsin-resistant PulDhis multimers was determined by SDS-PAGE, mass spectrometry, and Edman degradation analyses. A single band with an apparent molecular size of 40 kDa was observed after Tris-Tricine SDS-PAGE (Fig. 3A). Successive cycles of Edman degradation indicated that this polypeptide started with QAAK, corresponding to residues 298–301 of PulDhis.

Mass spectrometry of proteolyzed PulDhis complexes revealed the presence of molecular species with masses of 3,950 and 33,797 Da (F1 and F2, respectively) (Fig. 3A). No other peaks were detected, indicating complete homogeneity. F2 corresponds to the 40-kDa band seen on SDS-PAGE and, thus, comprises residues 298–617 of PulDhis (calculated mass, 33,783 Da). A genetically engineered truncated PulD protein corresponding to residues 298–616 of PulDhis also migrated more slowly than expected during SDS-PAGE (data not shown). To identify F1, which was not detected by Tris-Tricine SDS-PAGE, the proteolyzed complex was dissociated with phenol (8) and analyzed by HPLC on a C18 column. Several peptides were detected, recovered, and submitted to Edman degradation and mass spectrometry. All had a mass of 3950 Da and began with QQAT, corresponding to residues 262–265 of PulDhis. The reason why these identical peptides eluted in several peaks from the C18 column is unknown. From the precise mass of the fragment, we unambiguously identified F1 as residues 262–265 of PulDhis. The protease-resistant region, which is called the C domain, is highly conserved in all secretins and comprises the third of three CD1 modules and the CD2 module (see Fig. 3B for the explanation). The C domain corresponds to the \( \beta \) domain defined previously (17) plus peptide F1.

According to mass spectrometry, only F1 among the peptides cleaved off by trypsin remained associated with the trypsin-resistant PulDhis complex. Therefore, the latter has a mass of 452.6 kDa \((12 \times (33,783 + 3,936))\), indicating that 46% of the mass of the secretin is removed by

**FIGURE 2. Single particle analysis of top views imaged from rotary metal-shadowed trypsin-resistant PulDhis complexes.** A, overview of rotary metal-shadowed sample. The scale bar corresponds to 40 nm. B, average of metal-shadowed top views. The scale bar corresponds to 10 nm. C, the rotational power spectrum of a characteristic average indicates the prevailing 12-fold symmetry. The strong 4th harmonic supports an oligomeric state of 12.

**FIGURE 1. Mass measurement of intact and proteolyzed PulDhis.** A, Coomassie Blue-stained SDS-PAGE of purified secretin complexes. PulD-PulShis or PulDhis/PulS were purified from \( E. coli \) outer membranes. Tris-[2-carboxyethylphosphine] hydrochloride \((TCEP)\) was added before size exclusion chromatography on S300HR and concentrated on Q-Sepharose. Samples were treated with phenol before solubilization in sample buffer to dissociate completely the complex. B, intact or trypsin-proteolyzed PulDhis complexes were subjected to size exclusion chromatography on a calibrated Sephacryl S300HR column in 50 mM Tris-HCl, 250 mM NaCl, and 0.6% ZW3–14. C, STEM mass analysis. The histograms show the mass distributions for the intact and proteolyzed PulDhis complexes. The Gaussian is at 919 ± 109 kDa \((n = 1872, S.E. = 2.5 \) kDa, overall uncertainty = 46 kDa) for intact PulDhis (left) and at 537 ± 64 kDa \((n = 3906, S.E. = 1.0 \) kDa, overall uncertainty = 27 kDa) after trypsin treatment (right). The higher masses on the latter histogram arise from the occasional association of two complexes (see Fig. 3B).
proteolysis. Likewise, the STEM data indicate a mass of 537 kDa for the proteolyzed complex (Fig. 1C, right histogram). The difference of 84 kDa between the value determined by STEM and that calculated from the mass spectrometry data represents the amount of bound detergent, which is very close to that associated with the intact complex (see the previous paragraph). This is entirely consistent with the fact that the C domain includes the membrane-integrated part of the complex, which is presumably where the majority of the detergent binds (see “Discussion”).

Secondary Structure of PulDhis—Circular dichroism was used to determine the secondary structure of the trypsin resistant C domain of PulDhis (262 to 617). The proteolyzed complex was dialyzed to replace NaCl by NaF so that a reliable signal could be recorded down to a wavelength of 190 nm. As shown in Fig. 4, the spectrum displays a single minimum at 218 nm. Deconvolution of this spectrum using the CON- TIN program on the Dichroweb site (www.cryst.bbk.ac.uk/cdweb/html/home.html) (44, 45) indicated that 27% of the polypeptide is α helixes (Fig. 4). The observed difference in the CD spectra of the isolated N and C domains of PulD clearly indicates the radically different organization of the polypeptide chain in these two regions, confirming that they are two structurally distinct domains.

As noted above, intact PulDhis complexes are very sensitive to NaCl concentration, and we could not completely replace NaCl by NaF without precipitating the protein. Thus, we could only record a reliable CD spectrum between 200 and 250 nm, and it was not possible to deconvolute this spectrum. However, the spectrum of PulD secretin and the calculated sum of the isolated N and C domain spectra could be almost perfectly superimposed. Accordingly, the spectra of the isolated N and C domains reflect their secondary structure in the native protein.

Cryo-electron Microscopy of Intact and Proteolyzed PulDhis—When frozen for cryo-electron microscopy, membrane proteins, and in particular secretin complexes, tend to cluster at the edge of the grid holes. To avoid this problem, intact and trypsin-proteolyzed PulDhis complexes were adsorbed onto thin carbon films before freezing. Both PulDhis and the trypsin-resistant complexes mainly oriented end-on, appearing as ring-like structures with a large central density (Fig. 5). PulDhis also sometimes oriented on its side, revealing a “cup and saucer” structure (Fig. 5A) similar to that observed previously upon negative staining (17). Occasional side-view dimeric complexes interacting via their saucer side were also observed. Trypsin-proteolyzed PulDhis complexes were rarely seen in side views unless the sample had been left at room temperature before analysis. These side views were almost always dimers in which the two complexes were associated on their cup side (Fig. 5B), indicating that the physico-chemical properties of this part of the complex are modified by proteolysis.
A striking common feature of native and trypsin-resistant PulDhis top views is a sharp ring-shaped density at the edge of the projected cylinder (arrowheads in Fig. 5 insets). The diameter of this sharp ring of density, 11.9 nm, is the same as the distance between the fine structures that connect the cup and saucer (marked by arrowheads in the side-view averages shown in Fig. 5 insets). In addition, native PulDhis complexes possess distinct peripheral densities with a 12-fold rotational symmetry (Fig. 5A inset). These peripheral densities are missing from proteolyzed PulDhis complexes (Fig. 5B inset). In agreement with these observations, peripheral structures were clearly visible on some of the negatively stained PulDhis complexes imaged by STEM but were not detected on proteolyzed samples (Supplemental Figs. S1 and S2).

More than 6000 intact PulDhis complexes were selected interactively from the micrographs recorded by cryo-electron microscopy, reference-free-aligned, classified, and averaged (see “Experimental Procedures”). Characteristic top- and side-view class averages are shown in the insets of Fig. 5A. All class averages were employed to generate an initial three-dimensional volume, imposing C12 symmetry. Refinement cycles using projection matching of an increasing number of particles with calculated back-projections were repeated until the three-dimensional map did not improve further (Fig. 6, A and B). The resolution of the generated map was estimated to be 1.7 nm from the Fourier shell correlation function using the 50% criterion.

Class averages of a total of nearly 3300 top views and 1500 side views of the complex dimer were similarly calculated for the proteolyzed PulDhis complex. The inset of Fig. 5B displays a typical top- and side-view class average. Because only ~100 side views of the complex monomer could be found, the three-dimensional map of the proteolyzed secretin was computed as the volume of the digested complex, only class averages of dimers are shown. The scale bar in the overview corresponds to 50 nm, and the scale bar in the insets corresponds to 5 nm.
The diameter of the saucer also decreases from that the major contribution to the mass of the plug and the saucer is the larger of the two domains that are cleaved (Fig. 2). Because the saucer has a central hole, a plug occludes the saucer end of the chamber formed by the cup. Almost all of these features are smaller after trypsin treatment (TABLE TWO), the outer contours give the structure the appearance of a cup and saucer. The essential features of the model are two rings that form large chambers on either side of the complex and a central disc sandwiched between, including the plug that occludes the channel. This is in good agreement with the model proposed earlier based on images recorded from negatively stained PulD-PulS complexes (17). The changes caused by proteolysis indicate that the N-terminal domain of PulDhis forms the outer ring of the cup, whereas the C domain forms most of the plug and the saucer with its outer chamber. The overall features of the model are remarkably similar to those proposed for the quite distantly related bacteriophage f1-encoded secretin plV in the non-ionic detergent Triton X-100 (18) and for Salmonella enterica secretin InvG in purified type III secretion needle complexes (46). The major difference between the models proposed for PulD and plV is that the former is clearly dodecameric, whereas plV is a tetradecamer (15, 18). STEM analysis gave a mass of 919 kDa for the native PulDhis complex, including the detergent present, thereby excluding the presence of 14 subunits for which the calculated mass is 962 kDa. Moreover, the PulD complex is highly unlikely to contain 13 subunits like YscC (13), since the detergent contribution would then only be about 25 kDa, which is incompatible with the dimensions of the hydrophobic belt (43). Changes in stoichiometry might have occurred during the evolution of the secretin superfamily to permit the transport of differently structured macromolecules (bacteriophage, pseudopili, or folded exoproteins). Only relatively small changes in subunit dimensions and packing would be required to accommodate a change from 12 to 14 secretin subunits (or vice versa). A similar scenario was recently proposed to explain the variations in dimensions of TatA complexes isolated from the E. coli (47). These complexes apparently form the channel through which specific folded proteins of varying sizes are translocated across the inner membrane. The TatA complexes might acquire or release protomers to adapt to particular substrates or might exist as a mixed population to enable the entire range of substrates to be accommodated. As with secretins, the number of protomers in the TatA complex is probably so high that the addition or subtraction of a small number of TatA monomers would involve minimal distortion of the entire complex.

The apparent structural similarity of the InvG, plV, and PulD complexes makes it all the more remarkable that the structure proposed for N. meningitidis PilQ secretin complex is so different (29). Initial electron microscopy of negatively stained PilQ extracted in deoxycholate that was then replaced by SDS (12) or zwitterionic detergent 3–10 (28) indicated a ring-like structure similar to that of other secretins in SDS (12) and with peripheral densities similar to those of PulD in zwitterionic detergent (16, 17). However, class averages of side views of deoxycholate-extracted PilQ complexes dissolved in zwitterionic detergent 3–10 and examined by cryo-electron microscopy after negative staining with ammonium molybdate revealed that the central density corresponds to the closed aperture at the tip of a cone-like structure (29) rather than the central plug observed for plV (18) and PulD (this study).
or the septum of InvG (46). The closed aperture in PilQ appears to be the site to which the type IV pilus binds (48). Although it is not inconceivable that different secretins might assemble into different structures, we note that the primary sequences of PulD and PilQ are more closely related than are those of PulD and pIV. In addition, although normally dedicated to pullulanase secretion, PulD can perform the same function related than are those of PulD and pIV. In addition, although normally dedicated to pullulanase secretion, PulD can perform the same function related than are those of PulD and pIV.

Table Two: Dimensions of native and trypsin-resistant PulD complexes

| Domain   | Feature measured                          | Native (nm) | Trypsin-resistant (nm) |
|----------|-------------------------------------------|-------------|------------------------|
| Saucer   | Outer diameter including spokes           | 17.9        |                        |
|          | Outer diameter excluding spokes           | 13.7        | 14.3                   |
|          | Inner diameter                            | 5.9         | 6.9                    |
|          | Height                                    | 3.0         | 2.5                    |
| Cup      | Outer diameter including satellites       | 18.3        |                        |
|          | Outer diameter excluding satellites       | 12.8        | 12.2                   |
|          | Outer diameter at periplasmic end         | 11.1        |                        |
|          | Inner diameter cavity max.                | 8.9         | 8.9                    |
|          | Inner diameter at periplasmic end         | 5.4         | 7.7                    |
|          | Height at side                            | 6.4         | 4.0                    |
|          | Height center including plug              | 7.1         |                        |
| Plug     | Width                                     | 6.5         | 4.9                    |
|          | Height                                    | 2.6         | 2.3                    |
| Satellites | Width                                 | 1.4         |                        |
|          | Height                                    | 2.9         |                        |
| Complex  | Total height                              | 10.9        | 8.0                    |
|          | Space between cup and saucer              | 1.1         | 1.8                    |

The central plug is a newly defined feature of the PulD complex (Fig. 6). The plug was not apparent in the previous three-dimensional model (16) but was observed on later, negatively stained samples (17). Its presence is consistent with the failure of PulD to form constitutively open channels (16, 19). The fact the major contribution to the mass of the plug is made by the C domain and not by the N domain as originally proposed (17) explains why the low electrical conductance of the PulD homologue XcpQ in artificial planar bilayers does not increase when the N domain is removed by proteolysis (14). In addition, mutations in secretin structural genes that alter the permeability of the outer membrane invariably map to the region encoding the C domain (20, 21, 51, 52). Fine connections presumably anchor the central plug to the perimeter of the cup in proteolyzed PulD complex. Our failure to visualize the plug in the previous model (16) might be explained by differences in preparation and analytical methods. It is worth noting that the mechanism by which the secretin channel is occluded is completely different from that used to prevent the uncontrolled movement of solutes through TonB-dependent outer membrane transporters, where the N-domain constitutes the plug (53).

The structural changes caused by proteolysis allow the membrane orientation of the PulD complex to be predicted. The N domain, corresponding mostly to the rim of the cup that disappears upon proteolysis, must face the periplasm to perform its proposed function in exoprotein recognition (23). The location of this domain at one end of the secretin is in line with gold labeling experiments performed with pIV (18). The secretin N domain forms the outer part of a large chamber into which macromolecules (e.g. exoproteins or filamentous bacteriophages) might insert before they are transported across the outer membrane. Exoprotein docking to this site might cause displacement of the plug to create a continuous channel that would remain blocked by the exoprotein during its translocation. Assembly of pseudopilins into a pseudopilus that reaches the cup of the secretin might provide the driving force to expel the exoprotein from the inner chamber (7, 50). Exoprotein release and pseudopilus retraction would allow the plug to reform and the channel to close. A similar model explains the opening of invG secretin channel to allow the assembly of the type III secretion system needle, except that the septum does not close again once it is opened (46).

Another striking feature of both PulD complexes that is clearly seen on specific class averages of top views is a sharp but faint ring-shaped density at the periphery of the cylinder (marked by arrowheads in Fig. 5 insets). This corresponds to the fine connections present between cup and saucer in side views but is not seen in the final three-dimensional map (Fig. 6). Their absence in the latter is explained by the limited resolution of the three-dimensional map (1.7 nm), which results from using all projections to reconstruct the volume rather than considering specific top and side views only. Bound detergent, not visible on the images (the Zw3–14 detergent used has a density close to 1), could explain why this region was not proteolyzed. Accordingly, these fine connections could reflect the β-barrel inferred from CD spectra and indicate the position of the lipid bilayer. Indeed, a small number of β strands from each monomer would suffice to form a large ring structure, as in the integral outer membrane region of the E. coli protein TolC trimers (54) or the Mycobacterium tuberculosis outer membrane porin MspA (55).

If the β strands are indeed located in between the cup and saucer parts of the structure, some of the saucer (∼1.5 nm, see Fig. 6A) would be on the outside of the outer membrane, possibly even reaching beyond the lipopolysaccharide and other outer layers of the cell. The disc and outer ring of the cup would extend ∼5 nm into the periplasm, which would leave sufficient space for exoproteins to enter its inner chamber. An alternative model where the ring corresponding to the saucer is fully embedded in the outer membrane with little structure exposed on the cell surface as proposed for pIV (18) is inconsistent with the relatively

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4 A. Lusting, personal communication.
low amount of β strand secondary structure in the C domain of PulD (27%). It would also be inconsistent with the irregular outer wall of the saucer (note the lateral projections in Fig. 6), around which the outer membrane lipids would not fit snugly. Furthermore, this model would imply that the rest of the structure extends through the periplasm almost to the inner membrane, which might make it difficult for exoproteins to access the inner chamber.

Peripheral protein mass first observed in PulD-PulS complexes (16, 17) imaged by negative staining and by cryo-electron microscopy was proposed to represent PulS bound to the periphery of the PulD complex because it was almost completely absent from top views of negatively stained, trypsin-digested PulD-PulS particles (which lack PulS and the S domain of PulD as well as the N domain) (17). However, as shown by Figs. 5 and 6, similar peripheral objects (termed peripheral densities) are also associated with native PulDhIs complexes without PulS. Thus, the earlier assignment was not quite correct; the peripheral densities do not correspond solely to PulS, as proposed, but must also contain a small domain of PulD as well as the N domain) (17). However, as shown by stained, trypsin-digested PulD-PulS particles (which lack PulS and the S domain) (17) imaged by negative staining and by cryo-electron microscopy was almost to the inner membrane, which might make it difficult for exo-
saucer (note the lateral projections in Fig. 6), around which the outer membrane.

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