Functional and Structural Characterization of Polysaccharide Co-polymerase Proteins Required for Polymer Export in ATP-binding Cassette transporter-dependent Capsule Biosynthesis Pathways*

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Neisseria meningitidis serogroup B and Escherichia coli K1 bacteria produce a capsular polysaccharide (CPS) that is composed of α2,8-linked polysialic acid (PSA). Biosynthesis of PSA in these bacteria occurs via an ABC (ATP-binding cassette) transporter-dependent pathway. In N. meningitidis, export of PSA to the surface of the bacterium requires two proteins that form an ABC transporter (CtrC and CtrD) and two additional proteins, CtrA and CtrB, that are proposed to form a cell envelope-spanning export complex. CtrA is a member of the outer membrane polysaccharide export (OPX) family of proteins, which are proposed to form a pore to mediate export of CPSs across the outer membrane. CtrB is an inner membrane protein belonging to the polysaccharide co-polymerase (PCP) family. PCP proteins involved in other bacterial polysaccharide assembly systems form structures that extend into the periplasm from the inner membrane. There is currently no structural information available for PCP or OPX proteins involved in an ABC transporter-dependent CPS biosynthesis pathway to support their proposed roles in polysaccharide export. Here, we report cryo-EM images of purified CtrB reconstituted into lipid bilayers. These images contain molecular top and side views of CtrB and showed that it formed a conical oligomer that extended ∼125 Å from the membrane. This structure is consistent with CtrB functioning as a component of an envelope-spanning complex. Cross-complementation of CtrA and CtrB in E. coli mutants with defects in genes encoding the corresponding PCP and OPX proteins show that PCP-OPX pairs require interactions with their cognate partners to export polysaccharide. These experiments add further support for the model of an ABC transporter-PCP-OPX multiprotein complex that functions to export CPS across the cell envelope.

Capsular polysaccharides (CPSs)3 are produced by many bacteria and form a layer (the capsule) that coats the surface of the cell. The capsule helps prevent bacteria from desiccation and is important in infection because it provides protection from the host immune response by interfering with both complement-mediated killing (1–3) and the adhesion of monocytoid and neutrophils to the bacteria (4, 5). Consequently, capsules are an important virulence factor for numerous pathogenic bacteria including those that cause disease in livestock, like Pasteurella multocida, and bacteria such as Escherichia coli and Neisseria meningitidis, which cause bacterial meningitis and sepsis in humans (6).

CPSs vary significantly in carbohydrate composition and glycosidic linkages. Despite the diversity of structures, the majority of CPSs produced by Gram-negative bacteria are assembled via either an ATP-binding cassette (ABC) transporter-dependent biosynthesis pathway or a Wzy-dependent biosynthesis pathway (for review, see Refs. 7, 8). These pathways are fundamentally different in the topology of the polysaccharide polymerization reaction at the inner membrane. In Wzy-dependent pathways, polymer repeat units are assembled at the cytoplasmic face of the inner membrane on undecaprenyl diphosphate and flipped to the periplasmic face of the inner membrane in a process that requires a putative flippase protein, Wzx. Polymerization occurs on the periplasmic face of the inner membrane in a process that is dependent on the polymerase Wzy. In contrast, CPSs assembled via ABC transporter-dependent pathways are polymerized entirely at the cytoplasmic face of the inner membrane. The complete polysaccharide is exported across the inner membrane in a process that requires an ABC transporter that is composed of two nucleotide binding domain subunits and two transmembrane domain subunits.

Translocation of full-length CPS to the surface of the cell requires a polysaccharide co-polymerase (PCP) protein and an outer membrane polysaccharide export (OPX) protein in both the Wzy-dependent pathway and the ABC transporter-dependent pathway (for review, see Refs. 7, 8). PCP proteins share a conserved membrane topology that consists of N- and C-terminal membrane-spanning regions (or a C-terminal membrane-associated region) that flank a predominantly α-helical periplasmic domain (for review, see 7, 9, 10). Most

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PCP proteins possess a proline-containing consensus motif (P₈ₓPₓₓSPₓₓ₁₁GMGMXGAG) that is located at the interface between the periplasmic domain and the C-terminal membrane-spanning region, as well as amino acid sequences that are predicted to form periplasmic coiled-coils (7, 9–11). Three PCP subfamilies have been established based on their roles in different polysaccharide biosynthesis pathways (9), and their phylogenetic relationships have been investigated (7). PCP-1 proteins (Wzz homologs) determine LPS O antigen chain length in a Wzy-dependent biosynthesis pathway and represent the best characterized family members. PCP-2a proteins are involved in Wzy-dependent CPS biosynthesis and possess a C-terminal cytoplasmic domain with tyrosine kinase activity; this domain is absent in PCP-1 and PCP-3 proteins. Certain PCP-2 proteins (including proteins from prominent Gram-positive bacteria) lack the C-terminal cytoplasmic domain but function in conjunction with a separate cognate kinase polypeptide. PCP-3 proteins are confined to the ABC transporter-dependent CPS biosynthesis. Structural information is available for PCP-1 (12, 13) and PCP-2a (14) proteins and shows that they form conical oligomers that extend ~100 Å from the inner membrane into the periplasm.

OPX proteins have limited sequence similarity and are identified by a polysaccharide export sequence motif (7). Wza from E. coli K30 is the prototype OPX protein and operates in a Wzy-dependent assembly system. The crystal structure of Wza shows that it is an octamer that forms a large (140 × 105 Å) barrel with eight amphipathic α-helices that insert into the outer membrane to form a channel (15). The majority of Wza is located in the periplasm, where it encloses a central water-filled cavity. A cryo-EM structure of a complex of Wza and the PCP-2a protein Wzc shows that PCP-OPX proteins are capable of assembling into cell envelope-spanning structures that are consistent with their proposed roles in polysaccharide export (16). PCP-3 proteins and their partner OPX proteins are thought to form a similar polysaccharide export complex in ABC transporter-dependent assembly systems, but there are currently no structural data available supporting this hypothesis.

E. coli K1 and N. meningitidis group B both make ABC transporter-dependent capsules that are polymers of α2,8-linked polysialic acid (PSA) (17). The biosynthesis of PSA in E. coli serves as an influential model for ABC transporter-dependent CPS assembly and export (reviewed in (18, 19). The E. coli PSA export apparatus consists of two proteins providing the transmembrane domains and nucleotide binding domains of the ABC transporter (KpsM and KpsT, respectively), the PCP-3 protein (KpsE) and the OPX protein (KpsD). Homologous proteins in other bacteria are proposed to function in similar CPS exporters. As expected, given the shared mode of assembly, conserved components are encoded by the capsule loci from E. coli and N. meningitidis (7, 18, 20). PSA export in N. meningitidis requires two proteins that form an ABC transporter (CtrC and CtrD), a PCP-3 protein (CtrB), and an OPX protein (CtrA). In this study, we provide evidence that the PCP-3-OPX pairs from E. coli and N. meningitidis are functionally interchangeable, but they require cognate interactions between partner proteins for CPS export. In addition, cryo-EM is used to examine the PCP-3 protein CtrB from N. meningitidis reconstituted in lipid bilayers and shows that it forms a structure resembling PCP-1 and PCP-2 family members. These data support the model for a cell envelope-spanning CPS export system in ABC transporter-dependent biosynthesis pathways.

Experimental Procedures

Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C in LB medium supplemented with ampicillin (100 µg/ml), chloramphenicol (34 µg/ml), anhydrotetracycline (10 ng/ml), and arabinose (0.1% w/v) as required. K1F phage (21) was propagated using E. coli EV36 and stored at a titer of 10¹² pfu/ml in LB medium containing chloroform.

DNA Methods—Genes were amplified by PCR using Pwo polymerase (Roche Applied Science) with custom oligonucleotides (Sigma) designed to introduce appropriate restriction sites and N-terminal polyhistidine tags when required. PCR products were purified by gel extraction using the PureLink Gel Extraction kit (Invitrogen). Plasmid DNA was purified using the PureLink Plasmid Miniprep kit (Invitrogen). Purified PCR products and plasmid DNA were digested using restriction endonucleases and ligated according to individual manufacturer’s specifications. pWQ557, pWQ565, pWQ566, pWQ567, and pWQ568 were generated by site-directed mutagenesis according to the Stratagene QuikChange Site-directed Mutagenesis procedure using PfuUltra polymerase (Stratagene) and DpnI restriction endonuclease. Gene constructs were transformed into E. coli TOP10 by electroporation or by heat shock and verified by sequencing (University of Guelph Laboratory Services).

Phage Sensitivity Assays—10 µl of overnight culture from appropriate E. coli strains was cross-streaked on LB agar plates prepared with 50 µl of K1F bacteriophage (10¹¹ pfu/ml) spread over half of the surface of the media. Plates were photographed after an incubation period of 4–6 h at 37 °C.

Site-directed Fluorescence Labeling—CtrB proteins with N-terminal decahistidine tags were expressed from pWQ555, pWQ556, pWQ557, and pWQ558 in E. coli BL21. Labeling of membrane-embedded proteins with Oregon Green 488 maleimide carboxylic acid (OGM) was based on the protocol described by Culham et al. (22). Cells expressing each CtrB variant were grown in 200 ml of broth inoculated with an overnight culture (1% v/v) and incubated at 37 °C with shaking for 2 h. Cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C, resuspended in 10 ml of buffer A (50 mM Tris-Cl, pH 8.0, containing 100 mM NaCl), and divided into three aliquots. OGM (40 µM) was added to aliquot 1, labeling Cys residues accessible to the periplasm. The sample was incubated for 15 min at room temperature and quenched with 1 mM β-mercaptoethanol. In aliquot 2, periplasmic Cys residues were blocked with methanethiosulfonate ethyltrithymethylammonium (MTSET) (2 mM) for 15 min at room temperature. Aliquots 1 and 2 were washed twice by centrifugation using buffer A to resuspend the cell pellets. All three aliquots were pelleted by centrifugation and resuspended in 2 ml of buffer A containing 5 mM EDTA, 100 µg/ml lysozyme, and 20% (w/v) sucrose. After a 15-min incubation at room temperature, 18 ml of cold water...
was added to each aliquot to disrupt the cells. OGM (10 μM) was then added to aliquots 2 and 3, and all three aliquots were subjected to 30 s of sonication (10 s on, 10 s off), followed by a 15-min incubation at room temperature. This labeled any available and unblocked cysteine (Cys) residues in aliquot 2 and labeled all of the accessible Cys residues in aliquot 3. The labeling reaction was quenched in aliquots 2 and 3 with 1 mM β-mercaptoethanol, and the unbroken cells and cell debris were collected from all three aliquots by centrifugation at 15,000 × g for 15 min at 4 °C. Membranes were collected from the cell-free lysates by centrifugation at 100,000 × g for 30 min at 4 °C and solubilized overnight at 4 °C in 1 ml of buffer A containing 1% (w/v) dimethylmethylammonio propanesulfonate (SB3-14) (Sigma-Aldrich). Insoluble material was removed by centrifugation at 16,000 × g for 10 min at room temperature, and the supernatant was mixed with 100 μl of nickel affinity resin (Qiagen) for 1 h at room temperature. The solubilized membrane-resin mixture was then loaded into 1-ml minicolumns (Bio-Rad) and washed twice with 1.5 ml of buffer A containing 0.05% (w/v) SDS and 500 mM imidazole into 200 μl of SDS-PAGE loading buffer. 15-μl samples were analyzed by SDS-PAGE using 12% (w/v) gels, and fluorescence was recorded with exposure of the gels to UV light using a Bio-Rad Gel Doc equipped with a CCD camera. Gels were stained with Coomassie Blue following UV exposure to visualize protein bands. The experiment was conducted in duplicate and produced the same results.

To confirm the interpretation of SDS-PAGE results from the OGM-labeling experiments described above, purified CtrB variants were labeled with OGM in an SDS solution and analyzed by SDS-PAGE followed by Western blotting. The CtrB variants were expressed from their respective plasmids in 200-ml cultures and purified according to the procedure described above but omitting OGM labeling and MTSET blocking. The solubilized membrane-resin mixture was washed with 2 ml of buffer A containing 0.05% (w/v) SB3-14 and 20 mM imidazole followed by 0.5 ml of buffer A containing 0.05% (w/v) SB3-14 and 50 mM imidazole. Proteins were eluted with 2 ml of buffer A containing 1% (v/v) SDS and 500 mM imidazole. A 50-μl aliquot from each CtrB sample was incubated with OGM (40 μM) for 15 min at room temperature and quenched with ~1 mM β-mercaptoethanol. Untreated and OGM-treated samples were analyzed by SDS-PAGE using 12% (w/v) gels and transferred to nitrocellulose membranes. The nitrocellulose membranes were exposed to UV light, and fluorescence was recorded as described above. After exposure to UV light, the nitrocellulose membranes were probed with anti-His6 antibodies (Qiagen) using an alkaline phosphatase-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Separate gels were also stained with Coomassie Blue to visualize protein bands (data not shown).

Purification of CtrB for EM Studies—His6-CtrB was overexpressed from pWQ555 in E. coli TOP10. One liter of LB containing ampicillin was inoculated with 10 ml of overnight culture and grown with shaking at 37 °C. When an A600 nm of 0.6 was reached, expression of CtrB was induced by adding 0.1% (w/v) arabinose, and growth was continued for an additional 2 h. Cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C. Cell pellets from 1-liter cultures were resuspended.
in 25 ml of 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl and 5 mM EDTA. Cells were disrupted by sonication, and the cell-free lysate was collected by centrifugation at 15,000 × g for 20 min at 4 °C. Membranes were collected from the cell-free lysate by centrifugation at 100,000 × g at 4 °C. The membrane pellet was solubilized overnight in 10 ml of 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl and 1% (w/v) decyl β-D-maltopyranoside (DM) (Fluka). Insoluble material was removed by centrifugation at 100,000 × g for 1 h at 4 °C, and 2 ml of His-Select Nickel Affinity Gel (Sigma-Aldrich) was added to the supernatant. The mixture of resin and solubilized membranes was incubated for 1 h at 4 °C with rocking and loaded into a 20-ml Bio-Rad chromatography column. The resin was washed with 20 ml of 20 mM sodium phosphate, pH 7.0, containing 300 mM NaCl, 0.1% (w/v) DM, and 20 mM imidazole, followed by 5 ml of the same buffer containing 75 mM imidazole. His$_{10}$ CtrB was eluted with a 5-ml fraction of 20 mM sodium phosphate, pH 7.0, containing 300 mM NaCl, 0.1% (w/v) DM, and 300 mM imidazole, followed by 5 ml of the same buffer containing 500 mM imidazole. Fractions collected throughout the purification were analyzed by SDS-PAGE. The fraction containing purified CtrB was diluted to 50 ml using 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl and 0.1% (w/v) DM and concentrated by centrifugation using a 100,000-Da cutoff filter to 1/100 of the original volume. Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay kit with BSA as a standard.

Reconstitution of CtrB in Proteoliposomes—Reconstitution experiments were performed using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleyl-sn-glycero-3-phospho-1-serine (DOPS), 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleyl-sn-glycero-3-phosphoethanolamine (DOPE), DMPC-DOPS mixtures (3:1, 1:1, and 1:3 (w/w)), and DOPC-DOPE mixtures (3:1, 1:1, and 1:3 (w/w)) (Avanti Polar Lipids). Lipids and lipid mixtures were prepared at a concentration of 2.5 mg/ml and solubilized with 1% (w/v) octyl β-D-octylthioglycolpyranoside (Sigma-Aldrich) in a 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. 50-µl reconstitution reactions were prepared at lipid-to-protein ratios of 0.25:1, 0.5:1, and 0.75:1 (w/w) at a protein concentration of 0.5 mg/ml. Reconstitution was initiated by the addition of ~ 10 hydrophobic beads (Bio-Rad) that had been equilibrated in water. The beads were replaced at ~12-h intervals for 2–4 days. The formation of proteoliposomes and membrane sheets was screened by transmission electron microscopy of negatively stained specimens, as described previously (12).

Cryo-EM and Image Processing—3-µl samples from reconstitution experiments were applied undiluted to Quantifoil R 2/2 holey carbon-coated EM grids and plunged frozen in liquid ethane using a Vitrobot plunge freezing system (FEI, Hillsboro, OR) using 2 × 1-s blots and an offset setting of ~1 mm. Cryo-EM was performed using a Tecnai F20 200kV EM operating in low dose mode at 200 kV at the University of Guelph Microscopy Imaging Facility. Micrographs were recorded using a Gatan 4K CCD at defocus values of 2–8 µm with a calibrated magnification corresponding to 3.88 Å/pixel. Images were recorded with a 1-s exposure time corresponding to an overall electron dose of ~2500 electrons/nm$^2$. Images were manipulated using the EMAN software package (23). Molecular side views and small membrane patches containing top views of CtrB were selected using BOXER. The degree of underfocus for each micrograph was determined, and the CTF correction was performed using the CTFIT program within the EMAN package. Classification of the particles (after CTF correction) and averaging of translationally and rotationally aligned particles within each class was also carried out using the EMAN package. The contour-delineated CtrB projection map shown in Fig. 5E was generated using the SPIDER software contouring function (24).

Analysis of PSA—To examine total cellular PSA, E. coli strains were grown overnight on LB agar at 37 °C and whole cell lysates were prepared for SDS-PAGE and Western blotting. Cells were harvested from the surface of the LB agar and resuspended in water. The equivalent of 1 ml of cells with an $A_{500\ nm}$ = 3.0 was collected by centrifugation and resuspended in 150 µl of 70% (v/v) ethanol. Samples were incubated for 15 min at room temperature, harvested by centrifugation, resuspended in 150 µl of 100% ethanol, and incubated for 15 min at room temperature. Cells were collected again by centrifugation, and the supernatant from each sample was discarded. After allowing the cell pellet to dry completely, it was resuspended in a 50-µl solution containing 10 µg/ml DNase I, 10 µg/ml RNaseA, 100 µg/ml lysozyme, and 10 mM MgCl$_2$. The samples were incubated for 90 min at room temperature and mixed with 50 µl of 2× sample loading buffer (100 mM MOPS, 100 mM Tris, 2% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 250 µg/ml proteinase K, pH 7.7). The cell lysates were incubated at 37 °C for 2 h, and 15-µl samples were loaded into 5% (w/v) gels prepared with a 3% (w/v) stacking layer. The gel buffer and running buffer contained 50 mM MOPS, 100 mM Tris, 0.1% (w/v) SDS, and 1 mM EDTA. Samples were separated by electrophoresis for 45 min at 200 V. After SDS-PAGE, samples were transferred to nitrocellulose membranes in a buffer containing 50 mM MOPS, 50 mM Tris, 1 mM EDTA, and 20% (v/v) methanol at 200 mA for 1 h. Western blots were probed with a monoclonal anti-PSA (mAb 2.2.B, obtained from Dr. Michael Apicella, University of Iowa), using an alkaline phosphatase-conjugated secondary antibody to visualize the PSA.

RESULTS

Functional Exchangeability of CtrAB and KpsDE—KpsE and CtrB, the PCP-3 proteins from E. coli K1 and N. meningitidis serogroup B, are phylogenetically related (7). They share 28% identity (50% similarity), and their predicted secondary structures are almost identical (Fig. 1). In contrast, the corresponding OPX proteins are significantly different in both size and sequence, particularly in the C-terminal portion of the protein (Fig. 1), and they are well separated in a dendrogram of OPX homologs (7). To examine the specificity of PCP-OPX pairs required for surface expression of PSA, we performed cross-complementation experiments with CtrA and CtrB in EV36-derivative strains carrying mutations in kpsD and kpsE (Fig. 2). Complementation was assessed using the K1F phage sensitivity assay; K1F is a lytic phage that requires capsular PSA for infection in E. coli and is used to detect assembly and export of the
polymer (21, 25–27). Expression in trans of the ctrAB genes in a ΔkpsED strain restored K1F phage sensitivity, confirming that the PCP-OPX pairs from E. coli and N. meningitidis are functionally equivalent. However, when expressed individually, ctrB did not complement the PSA export defect in EV36 ΔkpsE. To verify that CtrB was functional when expressed from pBAD24, a compatible plasmid expressing ctrA (pWQ570) was transformed into EV36 ΔkpsE harboring pWQ561 (ctrB). The co-expression of ctrA and ctrB from separate plasmids restored phage sensitivity. The same results were obtained from reciprocal experiments performed in EV36 ΔkpsD transformed with plasmids carrying ctrA and ctrB, where ctrA could not restore polymer export when expressed alone. These experiments suggest that cognate interactions between PCP and OPX pairs are required for polymers export but that either PCP protein can form a productive interface with the PSA ABC transporter in E. coli EV36.

Membrane Topology and Orientation of CtrB—Studies with KpsE have predicted that this PCP-3 prototype contains an N-terminal transmembrane helix and a membrane-associated C-terminal amphipathic helix (28, 29). This differs from the canonical PCP structure, which contains two transmembrane helices (7, 9, 10). The membrane topology was therefore reinvestigated using a cysteine-labeling strategy with CtrB.
Three single-Cys His10-CtrB variants were constructed, expressed in *E. coli*, and subjected to site-directed fluorescence labeling experiments using the membrane-impermeant reagents OGM and MTSET. This approach has been validated to establish the membrane topology and orientation of several polytopic inner membrane proteins (22, 30). The location of each introduced Cys residue in the three single-Cys variants was selected to test the general orientation and topology of CtrB. His10-CtrB provided the negative control in labeling experiments. CtrB-N-Cys has a Cys residue added immediately following the His10-tag and is predicted to be on the cytoplasmic side of TM1. The Cys residue in CtrB-R192C is predicted to be periplasmic, whereas CtrB-C-Cys has a C-terminal cysteine that is predicted to reside on the cytoplasmic end of TM2. To confirm that each variant was functionally active, His10-CtrB, CtrB-N-Cys, CtrB-C-Cys, and CtrB-R192C were co-expressed with CtrA in *E. coli* RS3175 (ΔkpsDE) and tested for K1F phage sensitivity. The genes encoding all four CtrB variants restored K1F sensitivity in RS3175 when co-expressed with *ctrA*. To confirm their reactivity with the dye, the CtrB variants were purified from membrane preparations and incubated with OGM in an SDS solution and analyzed by SDS-PAGE followed by immunoblotting (Fig. 3A). Exposure of the nitrocellulose membrane to UV light confirmed that the three His10-tagged CtrB Cys variants fluoresced after incubation with OGM, whereas His10-CtrB remained unlabeled. Immunoblotting showed that CtrB-N-Cys, CtrB-R192C, and His10-CtrB migrated as a doublet on SDS-PAGE, and the same was true in a Coomassie-stained gel (data not shown). The band with decreased mobility corresponding to CtrB-N-Cys fluoresced after labeling with OGM, and the band with increased mobility corresponding to CtrB-R192C fluoresced after labeling with OGM.

To distinguish between Cys residues with a periplasmic versus cytoplasmic localization, intact cells expressing the CtrB proteins were either labeled with membrane-impermeable OGM (targeting periplasmic cysteine residues) or blocked with MTSET and subsequently lysed by sonication and then labeled with OGM (targeting unblocked cytoplasmic cysteine residues). Fig. 3B shows the fluorescence analysis of an SDS-polyacrylamide gel containing the four CtrB variants, purified following whole cell OGM-MTSET labeling experiments. These data show that the N and C termini of CtrB are exposed and the absence of OGM labeling in intact cell is indicative of a cytoplasmic location. As expected, the central portion of the protein is periplasmic. As with the labeling of purified CtrB variants with OGM, CtrB-N-Cys migrated on SDS-PAGE as a doublet with only the upper band fluorescing upon exposure to UV light. Likewise, only the lower band of the CtrB-R192C doublet fluoresced upon exposure to UV light.

Purification of His10-CtrB from *E. coli* TOP10—Fig. 4 shows the SDS-PAGE analysis of fractions from a representative CtrB

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**FIGURE 2. Complementation of PCP-3 and OPX proteins from *N. meningitidis* serogroup B and *E. coli* K1.** *E. coli* EV36 is a K-12/K1 hybrid strain that harbors the K1 CPS biosynthesis gene locus (26, 59). Infection and lysis of the identified *E. coli* strains by the K1F phage are dependent on the surface expression of PSA.
purification using DM-solubilized *E. coli* membranes obtained from 2 liters of culture. The 300 mM imidazole fraction contained a protein that is consistent with the predicted molecular mass of His<sub>10</sub>-CtrB (44.7 kDa). After exchanging the sample into a 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, it was concentrated to 2.75 mg/ml protein. Purifications consistently yielded ~0.75 mg of protein/liter of culture.

**EM of CtrB**—EM of negatively stained samples showed that CtrB reconstitution reactions produced small proteoliposomes that were densely packed with protein particles (data not shown). Occasionally, samples produced larger proteoliposomes and membrane sheets that contained visible protein particles. Of the conditions investigated, reconstitution reactions at a lipid-to-protein ratio of 0.25:1 using DOPC or a 1:1 DOPC-DOPE mixture produced the largest proteoliposomes and membrane sheets. Samples generated under these conditions are optimal for structural studies and were selected for cryo-EM. Fig. 5A illustrates a frozen-hydrated DOPC membrane sheet containing CtrB particles, and Fig. 5B shows the edges of three frozen hydrated ~0.2–0.3-μm diameter DOPC-DOPE CtrB proteoliposomes. The membrane sheet shown in Fig. 5A contains protein particles that appeared to form a two-dimensional crystal. However, crystallographic image analysis showed that this area contained a mosaic crystalline array with only short range ordered patches that was unsuitable for further processing using standard crystallographic image processing (31). To obtain low resolution structural data, we therefore employed an alternative patch-averaging approach where small patches from the mosaic crystal are selected and averaged after translational and rotational orientation. 252 patches of 96 × 96 pixels (corresponding to 372.48 × 372.48 Å) that were centered on an individual CtrB particle were selected using BOXER. Classification of the patches revealed no major differences in the patches, and therefore an averaged top view was generated with 212 of the patches using EMAN. Fig. 5D shows the averaged top view containing six ring-shaped CtrB particles packed around the central CtrB. The approximate diameter of a single CtrB particle is 100 Å.

Liposomes with sizes of ~0.2–0.5-μm diameter produced distinct bands of density corresponding to the lipid bilayer at the edge of the flattened vesicles. 93 particles extending from the folded edge of proteoliposomes were selected using

**FIGURE 4. Purification of His<sub>10</sub>-CtrB.** CtrB was purified from solubilized *E. coli* membranes as described under “Experimental Procedures.” The DM-solubilized protein fraction was applied to nickel-nitrilotriacetic acid resin and eluted with an imidazole step gradient. Samples were analyzed by SDS-PAGE and visualized by staining with Coomassie Blue.

**FIGURE 5. Cryo-EM of CtrB reconstituted in lipid bilayers and frozen in vitreous buffer.** A, membrane sheet containing CtrB particles. B, edges of three flattened vesicles with side views of CtrB extending distally from the membrane (arrows). C, magnified view of the CtrB particles from A. D, averaged top view containing seven CtrB molecules. The corresponding projection map shown in E. F, averaged side view of CtrB, with the lipid bilayer and the proposed periplasmic region emerging from the membrane highlighted in G.
BOXER, and an averaged side view of CtrB was generated using EMAN (Fig. 5F). The dimensions of the averaged side view (Fig. 5G) are consistent with the top view showing that CtrB forms a ring-shape structure with a diameter of \( \sim 100 \text{Å} \) and extends \( \sim 125 \text{Å} \) from the lipid bilayer.

**Mutational Analysis of the Proline Consensus Motif in KpsE**—PCP proteins lack sequence identity between subfamilies except for a Pro-containing motif that overlaps the C terminus of the periplasmic domain and TM2 (Fig. 1) (32). Mutational analysis in PCP-1 (Wzz) and PCP-2a (ExoP) proteins indicates that the second and third Pro residues in the motif are important for function (33, 34). PCP-3 proteins possess the least conserved Pro-containing motif in the family; KpsE has only the three conserved proline residues from the motif (Fig. 6A). Constructs encoding KpsE with single Pro to Ala substitutions (KpsE-P341A, KpsE-P344A, and KpsE-P350A) and a triple Pro→Ala variant (KpsE-AAA) were generated to investigate whether these residues are required for PSA polymerization, whole cell lysates were analyzed by SDS-PAGE. PSA from E. coli whole cell lysates, separated by SDS-PAGE, and probed with an anti-PSA monoclonal antibody.

**DISCUSSION**

PCP proteins are involved in several fundamentally different bacterial polysaccharide assembly pathways, and they play roles in polymer chain-length regulation and/or export, depending...
on the system (for review, see Ref. 7). PCP-1 (Wzz) proteins were the first family members identified (35–37). PCP-1 proteins determine the chain length of O antigens in the Wzy-dependent LPS assembly pathway. In their absence, cells can only synthesize short undecaprenol-linked oligosaccharides containing a limited number of O-repeat units. It is not understood how PCP-1 proteins interact with an outer membrane component. PCP-2a proteins (like Wzlc from *E. coli* K30) participate in the regulation of high level polymerization (39) of CPS assembled via the Wzy-dependent pathway, and in this respect they parallel the role of PCP-1 proteins. However, the interaction between the PCP-2a protein and its cognate OPX representative (16, 40) strongly suggests that these proteins also form part of the trans-envelope export machinery. The size difference in the periplasmic domains of the PCP-1 and PCP-2 proteins is proposed to reflect the interaction of PCP-2a proteins with OPX partners (7). The PCP-2a protein (Wzlc) forms an oligomeric structure whose periplasmic domain is superficially similar to those observed for PCP-1 proteins (12–14), although there is some debate about the precise oligomeric states. Crystal structures for several PCP-1 proteins suggest varied numbers of monomers in the oligomer (13), but a consistent hexameric structure was found when those proteins were examined by cryo-EM of proteoliposomes (12). Single-particle EM studies of detergent-solubilized Wzlc showed that it is a tetramer (14, 16). However, members of this subfamily carry an extended cytoplasmic kinase domain (not found in other PCP proteins) that, in isolated form, is an octamer in crystal structures (41, 42). It is unclear whether the difference in oligomeric states reflects the presence of constraining transmembrane and periplasmic domains in the crystal structure.

In contrast to the relatively well studied PCP-1 and PCP-2a proteins, PCP-3 proteins participate in ABC transporter-dependent pathways. In these systems, polymerization is completed by cytoplasmic or peripheral membrane proteins prior to export mediated by the ABC transporter (8, 19). The PCP-3 proteins therefore cannot play a role similar to that of their PCP-1 and PCP-2 counterparts in polymerization. In comparable ABC transporter-dependent LPS O antigen biosynthesis, polymer chain-length regulation is achieved either by the addition of unusual nonreducing terminal residues (43, 44) (which are not evident in PSA) or by coordinating the activities of the ABC transporter and biosynthesis enzymes (45). The latter may be a candidate for ABC transporter-dependent CPSs (7, 19). In the context of the ABC transporter-dependent systems, the PCP “polysaccharide co-polymerase” name may therefore prove to be a misnomer. However, the PCP-3 (KpsE) protein from *E. coli* is required for export of the polymer from the periplasm to the cell surface (46, 47). KpsE co-localizes with its OPX partner (KpsD) and forms part of a protein complex identified by chemical cross-linking (48). It is therefore proposed that PCP-3 proteins act in export, via interactions with the OPX proteins, in a manner analogous to their PCP-2a counterparts.

The cross-complementation data presented here provide further supporting evidence for a functional interaction between PCP and OPX proteins by showing that only cognate protein pairs are active. The export machineries from CPS systems have no specificity for a particular repeat-unit structure; that feature is established by the biosynthesis machinery before the product is exported. *E. coli* provides the best example, where the genes encoding the ABC transporter, PCP, and OPX proteins are identical in serotypes producing radically different CPS structures (47, 49). Functional hybrid CPS exporters composed of the *E. coli* ABC transporter proteins paired with *Actinobacillus pleuropneumoniae* PCP-3-OPX proteins (CpxC-CpxD) and the reciprocal exporter composed of *A. pleuropneumoniae* ABC transporter proteins (CpxBA) paired with *E. coli* PCP-3-OPX proteins have been described in a review, but the experimental data are not available (50). It has also been shown that the ABC transporter proteins encoded by the *Mannheimia hemolytica* CPS biosynthesis locus can restore PSA export in an *E. coli ΔkpsMT* mutant strain (51). The ability to transfer the OPX-PCP-3 pairs between *E. coli* and *N. meningitidis* supports the concept of a generic polysaccharide export conduit. Although no structural data are available for OPX proteins from ABC transporter-dependent CPS systems, secondary structure analysis predicts that they share general features of the family (including the defining polysaccharide export sequence motif) (7). Interestingly, KpsD and CtrA are significantly different in size and possess different export signal sequences (KpsD: 58.2 kDa, signal peptidase I cleavage site; CtrA: 38.4 kDa, signal peptidase II cleavage site) and cluster in separate phylogenetic groups (7). The PCP-3 proteins from *E. coli* (KpsE) and *N. meningitidis* B (CtrB) share nearly identical predicted secondary structures, are the same size (KpsE, 43.1 kDa; CtrB, 43.4 kDa), and cluster in the same group in phylogenetic analyses (7) (Fig. 1). This may explain the requirement for a cognate interaction between PCP-3 and OPX proteins, whereas PCP-3 proteins are suited to form a generic interface with the ABC transporter proteins. These observations reinforce the analogies made between the structure and functions of PCP-2a and PCP-3 proteins and the adapter proteins that link outer membrane channels to inner membrane exporters in tripartite efflux pumps (7).

This work provides the first evidence that a PCP-3 protein forms an oligomeric periplasmic structure with a central cavity, similar to PCP-1 and PCP-2a proteins. Cryo-EM of CtrB reconstituted into lipid bilayers provided top and side views of a particle that were used to generate averaged images. These low resolution images of CtrB are roughly similar in shape and size to those that were observed for the PCP-1 protein from *Salmonella enterica* serovar Typhimurium (12). Cryo-EM structures of PCP-1 proteins in proteoliposomes revealed hexameric structure and the current data suggest that this is the most likely organization of the CtrB oligomer. However, other oligomeric possibilities cannot be ruled out unequivocally without a higher resolution dataset. This is dependent on the protein forming significant areas of better ordered two-dimensional arrays. Although such arrays have been obtained for the PCP-1 proteins, this has not been possible with CtrB, despite the application of an extensive range of conditions. The averaged top view
of CtrB was generated using small membrane patches that contained several particles that were organized comparably to the two-dimensional crystals obtained with PCP-1 hexamers. The EM images of CtrB are consistent with the structures of PCP-2a proteins and suggest that PCP-3 proteins may also assemble into structures that are capable of forming the interface between the cytoplasmic membrane and the periplasm in a cell envelope-spanning complex required for CPS export.

A defining characteristic of the PCP family of proteins is a conserved predicted membrane topology (7, 9, 10). Hydropathy analysis yields nearly identical results for representative proteins from all three PCP subfamilies, where transmembrane-spanning regions are predicted at each end of the polypeptide. Experimental data for the membrane topology and orientation of PCP-2a (Wzc) and PCP-1 (Wzz) proteins confirm the presence of N- and C-terminal transmembrane helices that flank a periplasmic region (11, 52). To investigate whether this is a conserved theme among PCP-3 proteins, site-directed fluorescence labeling experiments were used to analyze the membrane topology of CtrB and showed that the N and C termini are located in the cytoplasm, whereas the central portion of the protein is located in the periplasm. These results show that CtrB is a bitopic membrane protein, and the shared functions of CtrB and KpsE, as well as the conservation of predicted secondary sequences, suggest that other members of the PCP-3 family may also be bitopic. Previous studies with KpsE predicted a C-terminal amphipathic helix that interacts with, rather than spans, the membrane (28, 29). It is difficult to reconcile the two sets of data. They could reflect either the different methods of analysis or subtle differences between KpsE and CtrB that have no significant impact on function.

The PCP Pro-containing motif has been proposed to mediate protein-protein interactions that play a role in determining polymer chain length. Site-directed mutagenesis of the PCP-1 (Wzz) protein from Shigella flexneri suggested that the second Pro in the motif is critical for LPS O antigen chain-length regulation (33). A similar analysis of the PCP-2a (ExoP) protein from Rhizobium meliloti showed that the Pro in the conserved SPK region of the motif was required for a wild-type ratio of high molecular mass to low molecular mass exopolysaccharide (34). KpsE from E. coli K1 has a minimally conserved Pro-containing consensus motif that is well suited to probe the role of these residues in a PCP-3 homolog. Complementation experiments were performed using KpsE variants with specific amino acid substitutions at the three conserved Pro residues. These experiments showed that none of the Pro residues was required for polymer export, and all three residues could be mutated without effect. PSA was also analyzed by SDS-PAGE to determine whether the KpsE variants affected chain-length regulation. We were unable to detect any significant alteration in the molecular mass profile of PSA produced by these strains compared with the strain expressing wild-type KpsE. The ΔkpsE mutant does accumulate CPS chains with higher average chain lengths than the wild type, based on SDS-PAGE analysis. This effect (increased size) is the opposite of the phenotype of PCP-1 and PCP-2a mutants, and there are several potential explanations for this phenotype. The assembly process dictates that CPS polymerization is established before export, but communication between the PCP-3 protein and the ABC transporter, or other assembly components on the cytoplasmic face of the membrane, could alter normal chain-length regulation. PAGE analysis of PSA from an EV36 ΔkpsT mutant also showed the production of high molecular mass polymer (data not shown) that is consistent with the ΔkpsE mutant phenotype and suggests an interaction between the assembly and export systems. Communication between the export and biosynthesis machinery is evident in the Wzy-dependent CPS systems although, in this case, the absence of the PCP-2a or OPX proteins also inactivates high level polymerization (53). Alternatively, the biosynthesis system might always produce PSA with a homogeneous size range similar to the ΔkpsE mutant, but exposure of the PSA on the cell surface of the wild type may lead to physical shearing and/or partial hydrolysis, resulting in a wider range of sizes. The apparent expendability of residues in the Pro-containing motif of PCP-3 proteins points to important functional differences between PCP-1/2a and PCP-3 proteins. We conclude that this motif is important for regulating polymerization (an activity missing in the PCP-3 proteins) but not for export.

There is a growing body of data from structural biology and biochemical initiatives targeted toward proteins in the PCP family. Interestingly, a phylogenetic analysis of the PCP family showed that PCP-1 and PCP-3 proteins are more closely related than the PCP-2 family is to either subfamily 1 or 3 (7). The designation of PCP is consistent with the data available for PCP-1 and PCP-2 family members, and there is a clear relationship in export between PCP-2 and PCP-3 families. Although the general roles in regulating polymerization and export are known, the next challenge is to define the structural features and mechanisms that are important for each of these processes. The functional link among all three PCP subfamilies will likely only be resolved by establishing how polysaccharides and other secreted macromolecules interact with assembly and export proteins at the inner membrane.

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