Translocation of Group 1 Capsular Polysaccharide in Escherichia coli Serotype K30

STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE OUTER MEMBRANE LIPOPROTEIN Wza*

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The late steps in assembly of capsular polysaccharides (CPS) and their translocation to the bacterial cell surface are not well understood. The Wza protein was shown previously to be required for the formation of the prototype group 1 capsule structure on the surface of Escherichia coli serotype K30 (Drummelsmith, J., and Whitfield, C. (2000) EMBO J. 19, 57–66). Wza is a conserved outer membrane lipoprotein that forms multimers adopting a ringlike structure, and collective evidence suggests a role for these structures in the export of capsular polymer across the outer membrane. Wza was purified in the native form and with a C-terminal hexahistidine tag. WzaHis6 was acylated and functional in capsule assembly, although its efficiency was slightly reduced in comparison to the native Wza protein. Ordered two-dimensional crystals of WzaHis6 were obtained after reconstitution of purified multimers into lipids. Electron microscopy of negatively stained crystals and Fourier filtering revealed ringlike multimers with an average outer diameter of 8.84 nm and an average central cavity diameter of 2.28 nm. Single particle analysis yielded projection structures at an estimated resolution of 3 nm, favoring a structure for the WzaHis6 containing eight identical subunits. A derivative of Wza (Wza*) in which the original signal sequence was replaced with that from OmpF showed that the native acylated N terminus of Wza is critical for formation of normal multimeric structures and for their competence for CPS assembly, but not for targeting Wza to the outer membrane. In the presence of Wza*, CPS accumulated in the periplasm but was not detected on the cell surface. Chemical cross-linking of intact cells suggested formation of a transmembrane complex minimally containing Wza and the inner membrane tyrosine autokinase Wzx.

In Gram-negative bacteria, macromolecules destined for the cell surface or the extracellular environment must cross both the inner (IM)¹ and the outer membrane (OM). In protein export, where the processes are arguably best understood, secretion systems with varying complexity accomplish the translocation steps; all involve multi-enzyme complexes where an outer membrane protein (channel) is linked directly, or via helper proteins, to IM components. Cell-associated capsular polysaccharides (CPS) and their secreted (cell-free) counterparts, exopolysaccharides (EPS), represent another type of macromolecule that must be transported across the cell envelope. The early steps in assembly of these polymers are reasonably well established. However, there is little understanding of the terminal steps, including the mechanism by which they cross the bacterial cell envelope and the machinery involved.

In Escherichia coli, more than 80 antigenically distinct capsular (K) polysaccharide structures are recognized. They are divided into four groups based on the organization of their genetic loci, polymerization mechanisms, and regulation (1). Group 1 and 2 capsules have received the most attention, and they involve biosynthetic mechanisms that are conserved in other bacteria. Translocation of group 1 and 2 CPS occurs at specific sites where the IM and OM are in close apposition (so-called “Bayer junctions” or zones of adhesion) (2–4), providing indirect evidence for transmembrane complexes in capsule assembly. Translocation of group 2 capsules is proposed to involve coordinated synthesis and export (1, 5–7). In the current model, group 2 CPS is polymerized in the cytoplasm by a multi-enzyme membrane-bound complex and then modified with a phospholipid anchor. Next, the nascent CPS is exported across the IM by a member of the ABC-2 family of ATP-binding cassette transporters, before being transported across the OM by a currently unidentified channel protein. Our laboratory is interested in the biosynthesis of the E. coli K30 CPS, the established prototype for group 1 capsules. A model representing the current thinking about the assembly mechanism is presented in Fig. 1. Individual group 1 K-antigen repeat units are assembled on an undecaprenol phosphate carrier lipid at the cytoplasmic face of the IM and are then proposed to be transferred across the IM by a “flip-pass” Wzx. The majority of the repeating units serve as substrate for polymerization in a process minimally requiring the IM protein, Wzx, and resulting in high molecular weight CPS (8). However, a small fraction of the repeat units can also serve as substrate for the O-antigen

¹ The abbreviations used are: IM, inner membrane; OM, outer membrane; CPS, capsular polysaccharides; EPS, exopolysaccharides; K, capsular; LPS, lipopolysaccharide; NTA, nitritotriacetic acid; DSP, dithio-bisuccinimidyl propionate; DDM, n-dodecyl β-D-maltoside; DTT, dithiothreitol.

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accumulated high molecular weight periplasmic polymer in \(wza\) mutants make it difficult to assign a definitive role to this protein in the assembly pathway. An attractive explanation for the inability of \(wza\) and \(wzc\) mutants to form any high molecular weight polymer is that high level Wzy-dependent polymerization and translocation are linked processes in an enzyme complex; a defect in any component of the complex would impact preceding steps.

In this report we further characterize the structure and function of \(Wza\) in \(E. coli\) K30. The secondary structure of \(Wza\) was investigated, and electron crystallography revealed an octameric quaternary structure. A non-acylated \(Wza\) derivative (\(Wza^{*}\)) was found to target the OM but was unable to form stable multimers, leading to a capsule-assembly pathway defect in which CPS was retained in the periplasm. Chemical cross-linking was used to show an association between \(Wza\) and \(Wzc\). The collective data provide the first direct evidence for the involvement of \(Wza\) in translocation and for a transmembrane complex necessary for coordinated synthesis and surface assembly of group 1 capsules.

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**—\(E. coli\) K-12 strain LE392 (supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1) was used for all genetic manipulations and purification of proteins. A \(wza\)-deficient mutant (\(E. coli\) CWG281) derived from \(E. coli\) E69 serotype O9a: K30H12 (17) was used for complementation studies. \(E. coli\) E69 isolate contains two copies of the \(wzc-wzb-wza\) genes on its chromosome, and both encode proteins that can function to varying efficiency in K30 CPS assembly (12, 17). One copy (designated \(wza-wzb-wzc^{\text{core}}\)) resides in the caps (K30 biosynthesis gene cluster), and the other (\(wza-wzb-wzc_{22\text{min}}\)) is in a locus found in many \(E. coli\) isolates (18) at a position corresponding to 22 min. on the \(E. coli\) K-12 map. The \(wzc-wzb_{22\text{min}}\) genes have also been called \(etp-eth\) (18), and their products have broader physiological activities (19). Complementation experiments were performed in a background in which the 22-min locus has been inactivated by a polar insertion (\(wza_{22\text{min}}-aadA\); Ref. 17), and all functional and structural characterization reported here involves only the \(Wza\) (and \(Wzc\)) proteins encoded by the K30 biosynthesis \(cps\) locus. The \(E. coli\) \(wza\)-null mutant CWG281 (\(wza_{\text{core}}-aadC\), \(wza_{22\text{min}}-aadA\); \(G^{m}, S^{p}\)) was reported previously (17), as was the \(wzc\)-null strain, CWG285 (\(wza_{\text{core}}-aadC\), \(wza_{22\text{min}}-aadA\)); \(E. coli\) strains were grown at 37°C in Luria-Bertani medium supplemented with arabinose (0.02 or 0.006%), ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), or gentamicin (15 μg/ml) where appropriate.

**Plasmid Construction**—A hexahistidine (H₆tag) tag was added to the C terminus of \(Wza\). The sequence encoding the 6 histidine residues and the stop codon was provided by two complementary oligonucleotides (JDI35, 5’-CACCATCCACTCATCCTATGAAGCAGCGGTCGA-3’; and JDI36, 5’-TTAATTAGATCGAGGCACTTCGTCGTTTGGTAAA-3’). The an-nealed oligonucleotides possessing a PstI overhang were ligated to MscI-PstI-digested pQW126 (17). The resulting plasmid, pQW300, expresses \(Wza_{\text{His6}}\) under the control of the arabinose-inducible P₅₅ promoter in pBAD24 (20). To obtain a \(Wza\) derivative that is not acetylated but is processed, the lipoprotein consensus signal sequence and N-terminal 5 amino acid residues of mature \(Wza\) were replaced by the corresponding sequence from OmpF of \(E. coli\) K-12. Two complementary oligonucleotides provided the replacement sequence (OmpFSS85, 5’-ATTTCCGACATGTAGAAGGCGCAATATCTTCGGAGTAGTGCCTTGGCTGTAGGACGTACTGCAACAAGGCTCAGAAATCTATAAGC-3’; and OmpFSS82, 5’-TGACGCTTTAGAATTTCTTCGGAGTAGTGCCTTGGCTGTAGGACGTACTGCAACAAGGCTCAGAAATCTATAAGC-3’) were annealed giving terminal EcoRI and SalI overhangs. The fragment was ligated into pBAD24 (20) digested with EcoRI and SalI to obtain pQW301. The region of the \(Wza\) open reading frame corresponding to amino acids 8–359 of the mature \(Wza\) protein was amplified by PCR from \(E. coli\) E69 using primers wzasaasSalI (5’-ATACGCGTCGCACTCACAAGCAGCTGAAA-3’) and wzasaasHindIII (5’-CTCTCCGCGTCGCTTCT-3’) and wzasaasHindIII (5’-CTCTCCGCGTCGCTTCT-3’) and wzasaasSalI (5’-ATACGCGTGTTTACGTCATCTACCTGTTCCATCTATCG-3’) were used to amplify the coding sequences by PCR. The resulting plasmid, pQW302, expresses a chimeric mature \(Wza\) protein comprising the N-terminal 5 amino acid residues of OmpF plus 2 additional...
residues (derived from the introduced Sall site) and Wza beginning at amino acid residue 8. To obtain a Wza* derivative containing a C-terminal His6 tag, plasmid pWQ302 was digested with Ndel and EcoRI and the 750-bp fragment was ligated to the 4.9-kb NcoI-EcoRI fragment of pWQ300 to give pWQ303.

A C-terminal truncation derivative of Wza (WzaΔC85–14) was also constructed, in which the last 12 amino acid residues were deleted. Truncation of wza was achieved by PCR amplification of wza from E. coli E69 using primer pairs JD125 (17) and JN20 5′-AAGTCGACGTT-CATATATCATGAAACCTGTATG-3′ (PstI site is underlined, stop codon is in bold). The PCR fragment was cloned into pBAD24 using the PstI and EcoRI restriction sites to give pWQ304. Plasmid pWQ305 expresses an N-terminal hexahistidine-tagged Wza derivative under the control of the arabinose-inducible pBAD promoter.

To construct the plasmid, an Ncol-Sall fragment encoding WzaΔC85–14 was isolated from the plasmid-based plasmid pWQ141 (12) and cloned into pBAD24 (20) to generate pWQ305. In some experiments, native Wzx was used. This was expressed from plasmid pQ130, a pBAD18-Km derivative described previously (12).

To maintain the plasmids expressing Wza and WzxΔC85–14 together in E. coli LE392, the origin and antibiotic resistance cassette of the native Wza-expressing plasmid, pWQ126, was exchanged. Plasmid pWQ126 was digested with ClaI and ScaI, and the 3.3-kbp fragment containing araC and P ara promoter was ligated to pACYC184 previously digested with EcoRI and ClaI. This resulted in plasmid pWQ306 with a chloramphenicol resistance marker and Wza expression under control of the P ara promoter.

The nucleotide sequences of all PCR-amplified products were verified using the service at the University of Guelph Molecular Supercenter.

**Protein Purification**—Proteins were overexpressed by growing cells containing the arabinose-inducible pBAD24 derivatives to an A600 of 0.5 and inducing for 3 h at 37 °C following addition of 0.006% (final concentration) t-arabinose. Cells were harvested, resuspended in 20 mM sodium phosphate buffer, pH 7.0, and lysed by two passages through a French pressure cell. Cell envelopes were isolated in a pellet following centrifugation at 100,000g for 1 h. The residual pellet enriched in OM was then solubilized in 20 mM sodium phosphate buffer, pH 7.0, containing 0.5% SB–dodecyl β-maltoside as described above. Binding to Ni-NTA-agarose was allowed to proceed for 16 h at room temperature on a rotating mixer. The resin was then washed extensively with 10 mM Tris-HCl, pH 6.3, containing 100 mM sodium phosphate, 8 mM urea, and 0.5% SDS followed by a second wash step using 20 mM Tris-HCl, pH 6.0, containing 250 mM NaCl, 4 mM urea, 0.5% SDS, and 5 mM imidazole. Bound proteins were eluted from the resin with PAGE gel loading buffer (NuPAGE, Invitrogen, containing no reducing agents) supplemented with 0.4 mM imidazole and 50 mM Tris-HCl, pH 6.8 (final concentration), by incubating first at room temperature for 10 min, followed by 100 °C for 5 min. The samples were then split, and half received diithiothreitol (DTT) to give a final concentration of 1 mM DTT. Samples with or without DTT were incubated for 10 min at 100 °C prior to separation on an 8% PAGE gel.

**Affinity Purification of Anti-Wza Antiserum**—OM extracts containing overexpressed WzaΔC85–14 were solubilized overnight in 20 mM HEPES, pH 7.4, containing 300 mM NaCl and 0.5% SDS. The supernatant obtained after ultracentrifugation was mixed with Ni-NTA-agarose (Qiagen) and incubated for 3 h at room temperature, and then transferred into an Econo-Pac column. The column was extensively washed in sequence with 10 mM Tris-HCl, pH 6.3, containing 0.1% SDS and 500 mM NaCl followed by 10 mM Tris-HCl, pH 8, containing 0.1% SDS and 5% imidazole. Finally, the column was equilibrated with 10 bed volumes of 20 mM Tris-HCl, pH 8 containing 28 mM NaCl. To remove the non-cross-linked complexes, the samples were washed with 20 mM Tris-HCl, pH 8 containing 28 mM NaCl and 0.5% SDS, and then eluted with 4 M MgCl2.

**Protein Analysis**—Proteins were separated by SDS-PAGE and either stained with silver (24) or Coomassie Brilliant Blue. Wza and Wzx were detected by Western immunoblotting using anti-Wza polyclonal serum (12), or purified anti-Wza polyclonal antibodies. Immunocomplexes were detected by using either alkaline phosphatase-conjugated goat anti-rabbit antibodies (Caltag) and nitro blue tetrazolium/5-bromo-4-
chloro-3-indolyl phosphate (Sigma) as substrate, or horseradish-conju-
gated goat anti-rabbit antibodies (Sigma) and the ECL Plus Biosciences.

Purified Wza and WzaHis6 proteins were subjected to mass spectrom-
etry analysis at the University of Guelph biological mass spectrometry
facility. N-terminal sequencing of purified Wza protein was performed by
Edman degradation at the University of British Columbia, Biotechnol-
yogy Laboratory-Nucleic Acids Protein Service facility. Circular di-
ichroism (CD) spectra of purified Wza (0.5 mg/ml in 20 mM Tris-HCl, pH
7.5, containing 80 mM NaCl and 0.008% DDM) and WzaHis6 (1 mg/ml in
20 mM Tris-HCl, pH 7.5, containing 0.3% octyl-polyoxyethylene) were
performed at the Scottish circular dichroism facility at Glasgow (Scot-
land, United Kingdom).

Two-dimensional Crystallization of WzaHis6—Two-dimensional crys-
tallization of WzaHis6 was carried out according to the method of Levy
et al. (25). Briefly, 1 μg of purified WzaHis6, in 20 mM Tris-HCl, pH 8.0,
containing 625 mM NaCl, 0.4% SB, 0.04% Br-3, and 14 and 0.2 μg of E. coli
total lipids (Avanti Polar Lipids) was incubated under a functionalized lipid mono-
layer (1:1 E. coli total lipids/2,6-diolein-sn-glycero-3-[N-(5-amino-1-
carboxypentyl)iminodiacetic acid]succinyl] (nickel salt) (Avanti Polar
Lipids). Binding of the hexahistidine tags to the nickel ions presented by
these functionalized lipids leads to concentration of WzaHis6 at the
monolayer surface. Detergent was then removed by the addition of polystyrene BioBeads® (Bio-Rad). It is proposed that this results in the
replacement of detergent molecules with lipids from the aqueous phase, resti-
ilitating the protein within a bilayer matrix (25, 26). A range of
incubation times and temperatures was evaluated. The best planar
arrays were obtained after 24 h of incubation at room temperature.

Electron Microscopy and Processing of Two-dimensional Crystal Im-
ages—The two-dimensional crystals were transferred to fenestrated
cellulose acetate butyrate-coated copper grids and stained with 2% (w/v)
uranyl acetate. Electron microscopy was performed at the Univer-
sity of Guelph Natural Sciences and Engineering Council of Canada
Regional Scanning and Transmission Electron Microscopy facility using a
LEO912AB transmission electron microscope (LEO GmbH,
Oberkochen, Germany) at an accelerating voltage of 100 kV and nom-
inal magnifications of either ×20,000 or ×40,000. Digital image data
were collected using an EsVision CCD-EMLk SSSCCD camera. Imaging
was routinely performed under low electron dose conditions. Images
were analyzed by either Fourier filtering or single-particle analysis
(1591 individual particles) with the IMAGIC-V electron image processing
system (27, 28).

Examination of Capsular Phenotype by Electron Microscopy—The differ-
ent Wza variants were expressed in the wza mutant E. coli CWG281.
Overnight cultures were diluted and plated on Luria-Bertani agar plates
containing gentamicin, ampicillin, and 0.02% arabinose and
incubated for 18 h at 37 °C. Alternatively, the cultures were subcul-
tured, grown to an A600 = 0.5 and expression of the Wza derivatives
were induced by growth for 18 h following addition of 0.02% arabinose.
Capsular expression was examined by electron microscopy of thin sec-
tions. Cells were stained with cationized ferritin (11), or thin sections
were immunolabeled using a monoclonal anti-K30 antibody (29) and a
cold-conjugated anti-mouse IgG (Sigma). Preparation of thin sections
was immunolabeled using a monoclonal anti-K30 antibody (29) and a
cold-conjugated goat anti-rabbit antibodies (Sigma) and the ECL Plus Biosciences.

RESULTS

WzaHis6 Is Functional in Capsule Assembly—The OM li-
poprotein Wza is encoded by the K30 CPS biosynthesis (cps)
locus and exists as a multimer forming a ringlike shape with a
central cavity (17). To aid purification, a derivative with a
C-terminal hexahistidine tag was constructed. The WzaHis6
protein was expressed at a level comparable with native Wza
and complemented a wza mutation in E. coli CWG281 to re-
store expression of the K30 antigen (Fig. 2A). In Western
immunoblots, the amount of K30 antigen produced by the strain
complemented by WzaHis6 was slightly less than that seen with
the native Wza protein. This was consistent with thin section
electron micrographs that revealed a corresponding slight re-
duction in the amount of capsule on the cell surface (Fig. 2B).
However, WzaHis6 is clearly functional in CPS assembly.

The reduction in capsule assembly directed by WzaHis6 in
E. coli CWG281 could reflect either steric problems arising
from the C-terminal hexahistidine tag, or an essential require-
ment for specific C-terminal residues. To provide further in-
sight, a C-terminal deletion derivative (Wza348–359) was con-
bstructed. This derivative was expressed and generated near
wild-type levels of K30 CPS in E. coli CWG281 (Fig. 2A). The
capsule on the cell surface was indistinguishable from that
resulting from activity of the native Wza (Fig. 2B), indicating
that the terminal 12 residues of Wza are not essential for its
function.

Purification and Properties of Wza and WzaHis6—The Wza
and WzaHis6 proteins were purified from solubilized OM ex-
tracts by chromatography (Fig. 2). Both proteins were obtained with
>98% purity (based on Coomassie Blue-stained SDS-
PAGE gels). The purified proteins were analyzed by mass spec-
rometry (data not shown), and the observed molecular masses
were in the main peaks were 40,240.6 Da for WzaK30
and 41,077.1 Da for WzaHis6. These values are consistent with
the calculated theoretical molecular mass of the respective
mature lipoproteins, modified at the N-terminal cysteine with
diacylglycerol and palmitate, supporting previous labeling
studies (17). Wza and WzaHis6 both showed the typical SDS-
resistant multimers reported previously (17) (Fig. 3), suggest-
ing that the C-terminal hexahistidine tag did not influence
folding and assembly into multimers in any detectable way
and consistent with the functionality of WzaHis6 in CPS assembly.

Wza is a member of the outer membrane auxiliary protein
family (33). These proteins are proposed to be β-barrel channel
proteins that transport polysaccharides across the OM (1, 33).
To provide insight into the secondary structure of Wza, purified
Wza and WzaHis6 were subjected to CD analysis. The spectra
of the proteins were highly similar with a minimum at 208 nm
(data not shown), indicating that both proteins are folded and
that Wza does not comprise solely β-structure. Analysis of the
CD spectra for secondary structure using the SELCON proce-
dure (34) gave the following approximate values for Wza: 26%
α-helices, 14% antiparallel β-sheets, 4% parallel β-sheets, 22%
turns, and 33% other structures. The corresponding recorded
values for WzaHis6 were: 19%, 23%, 3%, 21%, and 34%, respec-
tively. The small differences in the deduced structures might be
because of the presence of hexahistidine tag in WzaHis6 or
differences in the concentration or buffer composition.

Electron Crystallography of WzaHis6 Reveals an Octameric
Structure—Initial electron microscopy of purified WzaHis6
revealed the same multimeric ringlike structures, mostly ar-
rangements in, as observed previously with native Wza (17).
Purified WzaHis6 produced ringlike structures indistinguish-
able from the native Wza and also formed higher order aggre-
gates that gave no significant structural information by elec-
tron microscopy (data not shown). The lack of any discernible
structural alterations in WzaHis6 allowed the exploitation of the
hexahistidine tag in electron crystallographic approaches to
gain more detailed two-dimensional information concerning
the quaternary structure of the multimer. In this approach, WzaHis6 was first bound to a lipid monolayer containing a mixture of nickel-chelating lipid and total E. coli lipids, and then reconstituted into lipid bilayers upon removal of the detergent by BioBeads (25, 26). The two-dimensional crystals were transferred from the air-water interface to a plastic-coated grid, negatively stained, and examined by transmission electron microscope. Although planar arrays of WzaHis6 complexes with varying degrees of regularity were routinely obtained, they frequently did not withstand transfer to the grid without some disruption. A representative array is shown in Fig. 4A (recorded at 20,000x magnification). This array diffracted to 2 orders with the outermost spot corresponding to a spatial resolution of 4.2 nm (Fig. 4B). Fourier filtering of a representative crystalline array yielded an image showing the square lattice more clearly (Fig. 4C). A segment of the filtered image is shown enlarged in Fig. 4D. The lattice spacing was 10 nm. The outer diameters of the individual ring structures were 8.84 ± 0.43 nm, and the central cavity had a diameter of 2.28 ± 0.28 nm. These crystals were reproducible, but better crystalline order could not be obtained under any of the various conditions evaluated.

To obtain structural detail to higher resolution, a series of planar arrays of WzaHis6 were imaged at higher magnification (40,000x), and 1591 individual unit cells were extracted from the micrographs for single particle analysis. The latter process comprised two cycles of unbiased multireference alignment, multivariate statistical analysis, and classification (35). This approach allows the decomposition of the image data set into groups (or classes) that are most similar to one another. Averaging the aligned images within each class yields a result with an enhanced signal-to-noise ratio. Here, the best six class averages (in terms of internal homogeneity) are shown in Fig. 4E with isodensity contour lines superimposed. The variability between classes is the result of conformational variability of individual unit cells, which explains the limited order proffered by the two-dimensional crystals. Although the class-averaged images in Fig. 4E are of better resolution than those in Fig. 4D, exceeding 3 nm, some major peaks were blurred. Nevertheless, the images, in conjunction with the rectangular symmetry of the planar crystals, are entirely consistent with a model in which each ring consists of 8 individual WzaHis6 subunits.

**Fig. 2.** Wza<sub>His<sub>6</sub></sub> and Wza<sub>348–359</sub> are functional in CPS assembly. Wza, Wza<sub>His6</sub>, and Wza<sub>348–359</sub> were expressed in the wza-null strain, E. coli CWG281. The Wza derivatives were present in cell lysates and detected in Western immunoblots using anti-Wza serum (panel A). Whole cell lysates of CWG281 expressing Wza<sub>His6</sub> synthesized K30 antigen, but the amount detected in Western immunoblots was less than that formed in the presence of native Wza and Wza<sub>348–359</sub> (panel A). The assembly of capsule on the surface of these bacteria was assessed by electron microscopy of thin sections of cells labeled with cationized ferritin (panel B). The capsular structures formed by E. coli CWG281 containing either Wza or Wza<sub>348–359</sub> were indistinguishable. In contrast, the capsule formed in E. coli CWG281 with WzaHis6 was reduced relative to that seen with native Wza, consistent with the Western blotting results. Bars on the micrographs represent 0.5 μm.

**Fig. 3.** Purification of Wza and Wza<sub>His6</sub>. The purification protocols are described under “Experimental Procedures,” and the figure shows a Coomassie Blue-stained SDS-PAGE gel of relevant fractions. Lanes 1–4 show the purification of Wza from SB 3–14-solubilized OM (lane 1) by sequential anion exchange chromatography (lane 2) and hydroxyapatite chromatography (lane 3). All three lanes show Wza migrating as a major band at ~40 kDa (i.e., Wza monomer) when the samples are heated in SDS-sample buffer at 100 °C prior to electrophoresis. The purified Wza sample from lane 3 shows the multimeric form when the samples are run in the same buffer without heating (lane 4). Lanes 5 and 6 show heated and unheated samples of purified Wza<sub>His6</sub>, respectively, confirming that Wza<sub>His6</sub> also forms SDS-stable multimers.
N-terminal Acylation Is Critical for the Formation of Functional Wza Multimers—The role of acylation of the N-terminal cysteine of Wza in multimer formation and CPS assembly was investigated. A modified derivative, Wza*, was constructed by replacing the signal sequence of Wza with that of the outer membrane porin protein, OmpF, from *E. coli.* To provide a signal sequence recognized by the signal peptidase I, the 5 N-terminal residues of Wza were also replaced by those from OmpF in mature Wza*. To assess expression of Wza*, whole cell lysates were probed in Western immunoblots using anti-Wza antibody (Fig. 5A) and the protein was expressed. The presence of two bands in the blot is indicative of both precursor and processed forms of Wza* present in whole cell lysates, as often is the case for native Wza (17). In qualitative evaluations of Western immunoblots, the amount of Wza* expressed in CWG281 was typically less than the amount of native Wza or WzaHis6 in the same background. However, Western immunoblot signal obtained with expression of all three derivatives exceeded that seen from chromosomal copy (data not shown).

Expression of Wza* in *E. coli* CWG281 led to restored synthesis of K30 antigen, evident in Western immunoblots of the appropriate whole cell lysates (Fig. 5A). However, the amount of immunoreactive K30 polymer was reduced and the polymer showed a reduction in apparent molecular mass in PAGE analysis, compared with the control. Surface expression of the K30 antigen is routinely assessed by examining the sensitivity of the organism to bacteriophages specific for the capsular K30 antigen and the LPS O9a antigen (8); the O9a receptor is the capsule is either reduced in amount or absent. Bacteria expressing Wza were only infected by bacteriophage K30, as anticipated from the data in Fig. 1. In contrast, *E. coli* CWG281 expressing WzaHis6, that produces a reduced amount of CPS is sensitive to both bacteriophages, indicative of the reduced barrier provided by the capsule in this strain. Surprisingly, *E. coli* CWG281 expressing Wza* was sensitive to phage O9a but resistant to phage K30. This phenotype is identical to defined acapsular mutants and suggested that no CPS was assembled on the cell surface of Wza* expressing cells. This conclusion was confirmed by electron micrographs of cationized ferritin-stained cells that showed no visible CPS on the surface of *E. coli* CWG281 cells expressing Wza* (Fig. 5B).

To further investigate the discrepancy between the Western immunoblotting data showing K30 polymer synthesis and the apparent absence of a capsule structure on the cell surface, the K30 antigen was localized by immunogold electron microscopy with anti-K30 monoclonal and anti-mouse-gold antibodies (Fig. 5C). Bacteria expressing native Wza exhibited the expected gold particles on the surface of the cell. No labeling was evident with *E. coli* CWG281 as a negative control (Fig. 5C). In contrast to the *E. coli* CWG281 cells expressing Wza, many of those expressing Wza* showed enlarged electron transparent domains within the periplasm. Immunogold labeling of these domains identified them as the site of accumulated K30 antigen, consistent with the interpretation that CPS is polymerized in strains expressing Wza* but not transported across the OM.

To determine whether the aberrant localization of K30 polymer in *E. coli* CWG281 expressing Wza* was the result of differences in location or structure of the protein, the IM and OM were separated by sucrose density gradient centrifugation. Wza* was clearly evident in the OM (Fig. 6A). To ensure that the protein was processed as expected, a C-terminal hexahistidine tag was added to Wza*, allowing it to be purified for N-terminal sequencing. The obtained N-terminal sequence, AEIYNVGLN, was that predicted from sequence data for native Wza (whose N-terminal sequence is CTIIPGQGLN). Despite its appropriate processing and localization, Wza* was unable to form SDS-stable multimers. When incubated at 22 °C in SDS-PAGE sample buffer, only monomers of Wza* were evident. As shown in Fig. 6A and reported previously (17), the majority of Wza exist as multimers under similar conditions. Wza*His6 was purified in the absence of SDS according to the protocol established for WzaHis6 (see above) and attempts were made at two-dimensional crystallization. Under these conditions, multimers were obtained and some ringlike structures were evident (Fig. 6B). However, the Wza*His6 multimers often showed irregular shapes and tended to form poorly resolved aggregates rather than the organized arrays typical of WzaHis6. From the limited well resolved ring structures, the outer diameters were measured to be 7.85 ± 0.70 nm and the central cavity diameter was 3.07 ± 0.45 nm. These results reflect a structure with a smaller overall size but containing a larger central cavity, in comparison to WzaHis6 multimers. In summary, the Wza* variant can be processed by the signal peptidase I and localized to the OM, suggesting that acylation is not a prerequisite for transport to the OM. However, the SDS instability of Wza* multimers at 22 °C and the altered size of the structures suggests aberrant folding and/or multimerization that leads to loss of function in CPS translocation.
Evidence That Wza Is Part of a Transmembrane Complex—We were interested in determining whether Wza K30 is part of a transmembrane complex acting in CPS translocation. One candidate for an IM interaction partner for Wza is Wzc. The Wzc protein is essential for capsule assembly and the close homolog Wzc from *E. coli* K-12 contains a large periplasmic loop (16); the corresponding region in Wzc from the K30 cps locus comprises 374 amino acids. The periplasmic loop is predicted to be mainly \( \alpha \)-helical and contains a predicted coiled-coil motif (36), a feature that could be involved in protein-protein interactions. To stabilize putative protein complexes, in vivo cross-linking experiments were performed with intact cells using DSP. Initial experiments were performed using the *wza* mutant *E. coli* CWG281 expressing Wza His6 and Wzc from arabinose-inducible expression vectors, to enrich the amount of both Wza His6 and Wzc in the cells. Cross-linked membrane complexes were purified by nickel chelation chromatography. After cross-linking, predominantly high molecular weight complexes were identified that barely migrated into the PAGE gel, although some non-cross-linked Wza His6 was still evident (Fig. 7A). The amount of the free Wza His6 varied from experiment to experiment and appears to be a common occurrence when this protein is overexpressed. On treatment with DTT, the complexes released Wza His6 in increased amounts, as well as Wzc.

Fig. 5. Functional analysis of the CPS-assembly capabilities of a non-acylated derivative of Wza (Wza*). Wza* was expressed in the *wza*-null strain, CWG281 and protein was detected by Western immunoblotting (panel A). Wza* was able to restore K30-antigen synthesis in CWG281, detected in Western immunoblots of whole cell lysates (panel A). Note the difference in migration and amount of K30 CPS of strains expressing Wza and Wza*. In panel B, E. coli CWG281 cells expressing Wza* were subjected to labeling with cationized ferritin and examined by electron microscopy. No capsule structure was formed. Panel C shows the results of K30 antigen localization in thin sections of bacteria, using a K30-specific monoclonal antibody and colloidal gold labeling. In CWG281 (control) no capsule is formed and there are only a few random gold particles from the nonspecific labeling typical in such experiments. A capsule is formed in *E. coli* CWG281 expressing Wza (see Fig. 1) and the amount of gold marker is increased and is confined mainly to the periphery of the cell. In contrast, cells expressing Wza* produce K30 antigen confined to the periplasm and often located in characteristic extended periplasmic domains. Bars on the micrographs represent 0.5 \( \mu \)m.

Fig. 6. Wza* forms OM multimers with altered stability. Panel A shows a Western immunoblot in which OM samples from *E. coli* CWG281 expressing either Wza or Wza* were probed with anti-Wza antibodies. Note that Wza* is unable to form multimers that are stable in SDS-containing sample buffer at 22 °C. Using the purification protocol described under “Experimental Procedures,” multimers of Wza His6 could be isolated. These were used in two-dimensional crystallography preparations and formed aggregates of ringlike structures rather than organized arrays when visualized by electron microscopy (panel B). The bar represents 0.5 \( \mu \)m.

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that is not present in samples without DTT. As a control for nonspecific binding to the Ni-NTA resin, parallel experiments were performed with E. coli CWG281 overexpressing native Wza and Wzc; neither protein was detected in samples processed with, or without, DTT (data not shown). These experiments provided direct evidence that Wzc is involved in a WzaHis6-containing complex. The results were confirmed by identification of interactions in an E. coli LE392 background, devoid of the remaining members of the K30 CPS assembly machinery. In these experiments, WzaHis6 was used to trap the complexes on Ni-NTA resin. After treatment with DTT, two predominant proteins were visible in a silver-stained gel, corresponding to WzaHis6 and Wza, and both were clearly identified in Western immunoblots (Fig. 7B). In a control experiment where Wza was expressed alone in LE392, no Wza was captured on Ni-NTA (data not shown).

In previous cross-linking experiments, Wzc was shown to oligomerize independent of Wza (13, 16). To determine whether the interaction between Wza and Wzc is essential for the formation of stable Wza multimers, Wza was expressed in the wzc-null strain, E. coli CWG285. Western immunoblot analysis of Wza in this background showed that SDS-stable multimers were still formed in the absence of Wzc (Fig. 8).

**DISCUSSION**

In Gram-negative bacteria, macromolecules such as CPS destined for the cell surface, or the extracellular environment, must cross both the IM and the OM. The Wza protein represents the best candidate for the OM channel protein for translocation of group 1 CPS in E. coli and K. pneumoniae. Furthermore, Wza from E. coli K30 also shares sequence similarity with a number of OM proteins associated with capsule and (secreted) extracellular polysaccharide production in other bacterial species (8, 17), suggesting a conserved function. The results presented here represent the first description of the secondary and quaternary structure of a member of this class of proposed OM polysaccharide export proteins (33).

The presence of significant α-helical domains in Wza indicates that it possesses a secondary structure distinct from OM porin proteins that have a predominantly β-barrel secondary structure (37). The multimeric structure formed by Wza is certainly more complex than porins. Single particle analysis of samples from two-dimensional crystals strongly suggests that WzaHis6 forms a multimer of eight identical monomers. The images of the Wza multimers resemble those obtained with members of the OM “secretin” family (38), involved in type II and III protein export, type IV pilus assembly, and filamentous phage assembly. Secretins are multimeric OM proteins in which a conserved C-terminal β-barrel rich domain is implicated in forming the multimeric OM channel (39–42). Typically, 6–14 identical subunits form the ringlike structure of the secretin (39, 42–52). Despite similarities in overall architecture of the secretin and Wza multimers, the respective monomers share no primary sequence similarity. With an average diameter of 8.84 ± 0.43 nm, WzaHis6 complexes are smaller than those observed for many secretins. For example, PilQ from Neisseria meningitidis has a diameter of 16.5 nm (43). The central cavity in the Wza multimer is also smaller than that of PilQ (2.28 ± 0.28 nm for WzaHis6 compared with 6.5 nm for PilQ). Such differences may reflect the types of substrates for these putative channels. The group 1 CPS polymeric product is large (>100,000 Da; Ref. 53), but polymers of this type tend to adopt a flexible random coil structure in solution (54) and it is certainly conceivable that the polymer could be “threaded” through the channel as a linear strand.

The channels formed by protein export secretins are gated (48, 55), and low resolution three-dimensional structures of three secretins suggest an open state for these channel proteins in the OM and a closed state in the periplasm entrance (42, 44, 49). Gated channels are also formed by TolC, an outer membrane protein involved in type I protein secretion and multidrug efflux (56). The solved crystal structure of the TolC trimer reveals a channel composed of a 40-Å β-barrel OM anchor domain and a 100-Å α-helical tunnel domain, long enough for a contiguous gated channel across the periplasm and interaction with the IM protein components (57). The TolC channel is proposed to be closed at the α-helical periplasmic domain by
intra- and intermolecular hydrogen bonds and salt bridges, and is thought to be opened by an "iris-like" action (58, 59). It remains to be determined whether Wza multimers form a gated channel and, if so, whether the α-helices detected in Wza play a similar role to those in TolC.

Wza is an OM lipoprotein but the function of the lipid moiety had not been investigated. The data for Wza* presented here suggest that the acylation is not required for OM targeting of Wza, but it is essential for assembly of a stable Wza multimer that can function in capsule assembly. Some protein export secretins are also lipoproteins (51, 60) but there is no information concerning the precise role of acylation in these examples. Non-acylated secretins typically require an additional OM lipoprotein ("secretin pilot") that associates with the secretin (61) and is required for formation of a stable multimer as well as, in some cases, localization of the multimer. Examples are found in type II (48, 50, 62, 63) and III (45, 46, 64, 65) protein secretion systems from various bacteria.

The assembly and localization of secretin multimers can also be influenced by other components of the protein secretin complex (65, 66). For example, export of filamentous phase φ1 requires only four proteins and cross-linking experiments suggest that the transmembrane complex is preformed in the absence of substrate by protein-protein interaction between the OM secretin pIV and the IM protein pl (67). This contrasts with the type I export hemolysin paradigm, where assembly of the functional complex and recruitment of TolC requires binding of the substrate to the IM components (22, 68, 69). The assembly and localization of Wza multimers shows no absolute requirement for Wzc. In fact, the observations that stable Wza multimers form in E. coli K-12 and that cross-linked Wza and Wzc could be isolated in the K-12 background both suggest that there is no absolute requirement for either the presence of substrate, or of other dedicated components of the capsule assembly machinery. However, as in all such cases, it is impossible to exclude the involvement of conserved "housekeeping" E. coli proteins in the formation of the multimeric complex. In type II protein export systems, the C-terminal domain of the secretin polypeptide contains the site of interaction with other proteins in the complex, including the pilot proteins (61, 64). The differences in sequence (and perhaps structure-function) between Wza and the protein secretins are emphasized by the observation that the elimination of the C terminus in Wza* does not abrogate its function in capsule assembly.

E. coli K30 mutants deficient in Wza are unable to form a capsule but also do not accumulate detectable amounts of polymer within the cell, despite the fact that the glycosyltransferases for K30 synthesis are still active and K₃₋₅ is formed (8, 17). A similar phenotype is observed with mutants defective in Wzb and Wzb (8, 12). One attractive explanation for these similar phenotypes is some type of feedback regulatory mechanism, potentially involving an enzyme complex. Wzc provides a good candidate for general interaction with the OM in a complex because of its large periplasmic domain (16) and the putative coiled-coil structures in this domain (36). The coiled-coil region is not involved in the known oligomerization of Wzc (14) but could certainly participate in interactions with other proteins. Although Wza shows no evidence of coiled-coil motifs itself, we have demonstrated here that Wza can be cross-linked in a complex that includes Wzc. It would be premature to take cross-linking data in isolation as definitive evidence of a direct interaction between Wza and Wzc, because other (unknown) proteins could serve as intermediaries in the interaction. However, the data are entirely consistent with the existence of a complex, minimally involving Wza and Wzc. These results differ from those obtained during attempts to cross-link Wzc and Wza in the E. coli K-12 c2 analonic acid (EPS) system using formaldehyde (16). The reason(s) for the difference in results are unknown. Possibilities include the use of experimental systems with different modes of detection (and presumably different sensitivities), and the study of enriched material captured by exploiting hexahistidines tags, rather than analyzing whole cell lysates.

The normal pathway for E. coli group 1 CPS assembly is uncoupled in strains expressing Wza*. Although the unstable multimers formed by Wza are unable to support CPS translocation to the cell surface, they are apparently sufficiently well recognized by the assembly system that any feedback regulation of early stages of synthesis is circumvented. As a result the strain with Wza* accumulates periplasmic K30 polymer in enlarged periplasmic bays. Periplasmic polymer has been observed in mutants affecting the translocation of group 2 CPS, and electron transparent domains are evident in mutants accumulating intracellular polymer (70–73). The periplasmic location of K30 polymer in strains expressing Wza* represents the first direct demonstration that Wza (and by implication, Wza homologs in other CPS systems) is indeed required for functional complex and recruitment of TolC requires binding of the assembly system that any feedback regulation...
