A Novel Outer Membrane Protein, Wzi, Is Involved in Surface Assembly of the *Escherichia coli* K30 Group 1 Capsule

Andrea Rahn, Kostantinos Beis, James H. Naismith, and Chris Whitfield

*Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1, and Centre for Biomolecular Sciences, North Haugh, The University, St. Andrews, Fife KY16 9ST, Scotland*

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*Escherichia coli* group 1 K antigens form a tightly associated capsule structure on the cell surface. Although the general features of the early steps in capsular polysaccharide biosynthesis have been described, little is known about the later stages that culminate in assembly of a capsular structure on the cell surface. Group 1 capsule biosynthesis gene clusters (cps) in *E. coli* and Klebsiella pneumoniae include a conserved open reading frame, *wzi*. The *wzi* gene is the first of a block of four conserved genes (*wzi-wza-wzb-wzc*) found in all group 1 K-antigen serotypes. Unlike *wza*, *wzb*, and *wzc* homologs that are found in gene clusters responsible for production of exopolysaccharides (i.e., predominantly cell-free polymer) in a range of bacteria, *wzi* is found only in systems that assemble capsular polysaccharides. The predicted Wzi protein shows no similarity to any other known proteins in the databases, but computer analysis of Wzi predicted a cleavable signal sequence. Wzi was expressed with a C-terminal hexahistidine tag, purified, and used for the production of specific antibodies that facilitated localization of Wzi to the outer membrane. Circular dichroism spectroscopy indicates that Wzi consists primarily of a β-barrel structure, and dynamic light scattering studies established that the protein behaves as a monomer in solution. A nonpolar *wzi* chromosomal mutant retained a mucoid phenotype and remained sensitive to lysozyme. However, the mutant showed a significant reduction in cell-bound polymer, with a corresponding increase in cell-free material. Furthermore, examination of the mutant by electron microscopy showed that it lacked a coherent capsule structure. It is proposed that the Wzi protein plays a late role in capsule assembly, perhaps in the process that links high-molecular-weight capsule to the cell surface.

Capsular polysaccharides (CPSs) are high-molecular-weight (HMW) acidic polysaccharides found on the cell surfaces of many bacteria. In *Escherichia coli*, there are approximately 80 different capsular (K) serotypes, reflecting differences in the structure and immunochemistry of the capsular polysaccharide repeat units. *E. coli* capsules have been divided into four groups on the basis of features of the capsule synthesis systems, including genetics, polymerization mechanisms, and regulation (reviewed in reference 54). The majority of the capsular serotypes belong to either group 1 or group 2. While capsules from either group can function as virulence determinants, their modes of synthesis are quite distinct (reviewed in references 10 and 54).

Our laboratory is interested in the biosynthesis of group 1 capsules, which are manufactured via the Wzy-dependent pathway (15); *E. coli* serotype K30 is the prototype. Group 1 K-antigen biosynthesis involves the production of undecaprenol pyrophosphate-linked repeat units by glycosyltransferases acting at the cytoplasmic face of the inner membrane. The repeat units are thought to be exported across the inner membrane by a process involving a putative flippase (Wzx), where they are polymerized by a reaction requiring the *wzy* gene product (reviewed in reference 54). At this point, short oligosaccharides consisting of one or a few K-repeat units can be ligated to lipid A core, resulting in a form of lipopolysaccharide (LPS), termed K<sub>LPS</sub> (28). This reaction has been identified only in *E. coli*, and the significance of K<sub>LPS</sub> in the biology of *E. coli* is unknown. The majority of the undecaprenol pyrophosphate-linked repeat units are used for polymerization of HMW capsular polysaccharide whose assembly on the cell surface is independent of lipid A core (28). This high-level polymerization and surface expression of K30 antigen requires the participation of a tyrosine autokinase, Wzc, and its cognate phosphatase, Wzb (15, 56). The level of phosphorylation of Wzc is known to affect the production of K30 polymer, and insertional mutations in either *wzc* or *wzb* abolish the ability of the mutant strain to make HMW K30 polymer without impeding K<sub>LPS</sub> production (15, 36, 56). After polymerization, the capsule is transported to the cell surface in a process that involves an outer membrane lipoprotein, Wza. Purified Wza forms multimeric structures resembling secretins for type II and type III protein secretion (16). These structures are believed to act as channels in the outer membrane, allowing the HMW polymer to reach the cell surface. It is not clear how the polymer is moved through the periplasm to these channels, or how it is assembled on the cell surface to form the capsular structure evident in electron micrographs. In fact, the manner in which capsules are organized and linked to the surface is poorly understood in most systems.

The sequence of the 16-kb capsule biosynthesis cluster (cps) has been determined for the group 1 prototype isolate, *E. coli* E69 (O9a:K30) (15, 39). The operon consists of 12 open reading frames, which can be divided into two regions by a transcriptional attenuator (40). Proteins encoded by genes located downstream of the attenuator are involved in synthesis and
low-level polymerization of the K30 repeat units. These proteins include the serotype-specific glycosyltransferases and components of the Wzx- and Wzy-dependent polymerization pathway. This region of the operon is sufficient for the production of K_{LPS} but not for synthesis of the HMW capsule. Three of the four genes found upstream of the attenuator (wza, wzb, and wzc) are required for high-level polymerization and surface assembly of the HMW capsule polysaccharide. This region contains an additional open reading frame, termed wzl, whose role in capsule assembly has not been established. Homologs of wzl-wza-wzb-wzc are found in the cps loci from Klebsiella pneumoniae (4, 39), and the conservation of gene content, organization, and sequence in E. coli and K. pneumoniae cps loci are indicative of past lateral gene transfer events between these species (39). Other bacteria make exopolysaccharides that are similar in both structure and mode of biosynthesis to the group 1 capsules. However, the exopolysaccharides are distinguished by having a large amount of secreted (cell-free) polymer or by being loosely associated with the cell surface. Examples where the CPS biosynthesis genetic loci have been studied include amylovoran from Erwinia amylovora (11) and colanic acid from E. coli (46). Interestingly, the respective exopolysaccharide biosynthesis loci contain homologs of wzl-wza-wzb-wzc but lack wzl.

Initial studies showed that Wzi was not essential for capsule synthesis in E. coli K30 (15). A K. pneumoniae wzi deletion mutant was also isolated by screening a random transposon insertion library for serum-sensitive mutants (1). The mutant was also isolated by screening a random transposon insertion library for serum-sensitive mutants (1). The mutation described elsewhere (8).

### MATERIALS AND METHODS

**Growth conditions.** Bacteria were grown at 37°C in Luria-Bertani (LB) medium (30) (Invitrogen, Burlington, Ontario, Canada) or in M9 minimal medium with 0.2% glucose (31). Gene expression from pBAD vectors was induced by growth in the presence of 0.002 to 0.2% arabinose. The media were supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), or gentamicin (30 μg/ml) as required.

**Construction of Wzi and Wzi-His<sub>6</sub> overexpression plasmids.** For expression of Wzi, the gene was cloned into pBAD24, placing expression under the control of an arabinose-induced promoter (21). The wzl gene was amplified from E. coli B44 (O9:K30:H<sup>+</sup>) chromosomal DNA template by PCR using primers AR115 (5′-GGCTGAAATGGATCTCAGTTAAGC [the EcoRI restriction site is underlined]) and AR119 (5′-CAGCAATTCACTGATCTGAAATACAAAGC [the BamHI restriction site is underlined]). The 1.5-kb PCR product was digested with EcoRI and BamHI and ligated to the corresponding sites in the vector. The resulting plasmid was pWQ192. For ease of purification, Wzi was also expressed with a C-terminal hexahistidine (His<sub>6</sub>) tag (Wzi-His<sub>6</sub>) from plasmid pWQ193. A linker containing an in-frame His<sub>6</sub> tag was made by annealing oligonucleotides AR117 (5′-GGCCGGATCCACCCACCAACACACCCACCAACACAC [the BamHI restriction site is underlined]) and AR118 (5′-GGCCAGCTTCTAGCTGTTGTTGG TGGTGTGT [the HindIII restriction site is underlined]). The linker was digested with BamHI and HindIII and ligated to the corresponding sites in pWQ193. The cloned genes were confirmed to be error free by sequencing. Bacteria were transformed with plasmid constructs using electroporation as described elsewhere (8).

**Generation of wzi chromosomal insertion mutants.** Chromosomal wzi mutations were made in E. coli E69 (serotype O9a:K30:H12) and B44 (O9:K30:H<sup>+</sup>) using allelic exchange methods described elsewhere (15, 39). The construction of E. coli CWG314 (E69 wzl:wzc::CJ) has been reported elsewhere (15). To make E. coli CWG44 (B44 wzl:wzc::CJ), the wzl gene was subcloned from pWQ193 as a BamHI fragment and cloned into the suicide delivery vector, pWQ173. This vector is a derivative of pMAK705 (23) with a different counterselectable marker (40). A Smal fragment carrying the nonpolar gentamicin resistance cassette from pUCGM (42) was inserted into the unique EcoRV site within wzi in the same transcriptional orientation. The mutation was confirmed by PCR and by sequence analysis.

**Subcellular localization of Wzi.** One milliliter of a culture (grown overnight) of either E. coli DH5α [6800 deor lacZΔM15 endA1 recA1 hsdR17(rk− m<sup>−</sup>) supE44 thi−1 proA65 relA1 Δ(lacZΔM15)U169 F−] (41) or E. coli B44 harboring pWQ192 or pWQ193 was used to inoculate 250 ml of fresh medium. After 1 h, the culture was induced with 0.002% arabinose. Once the cultures reached an optical density of 0.6 (OD<sub>600</sub>) of ~1.0, the cells were collected by centrifugation and resuspended in 25 ml of 10 mM HEPES, pH 7.4. Inner and outer membranes were separated by sucrose density gradient centrifugation, following methods described elsewhere (2). Briefly, cells were lysed by using a French pressure cell, and cell envelopes were isolated as a pellet after ultracentrifugation (100,000 × g, 1 h). The pellet was resuspended in 0.5 ml of TE (10 mM Tris, 1 mM EDTA [pH 8.0]) and layered on top of a step gradient consisting of 1.6 ml of 20% (wt/vol) sucrose, 5.5 ml of 49% (wt/vol) sucrose, and 3.9 ml of 26% (wt/vol) sucrose. All sucrose gradient were made using TE. The gradient was centrifuged for 18 h at 100,000 × g in a swinging bucket rotor. The inner and outer membrane generated visible bands in the gradients and were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western immunoblotting.

**Purification of Wzi-His<sub>6</sub> using affinity chromatography.** A culture (grown overnight) of E. coli DH5α containing pWQ193 was used to inoculate fresh medium. In typical experiments, 6 liters of culture was used. The culture was incubated at 37°C for 2 h, and arabinose was then added to a final concentration of 0.02% to induce protein expression. The culture was then grown for a further 4 h at 37°C, at which point the cells were collected by centrifugation and stored as a paste at −20°C overnight. The cells were thawed in 20 mM sodium phosphate buffer (pH 7.0) and lysed by passage through a French pressure cell. Unbroken cells were removed from the suspension by low-speed centrifugation (5,000 × g, 10 min), followed by ultracentrifugation at 100,000 × g for 1 h to isolate the cell envelope fraction. The inner membranes were selectively solubilized by resuspending the cell envelope pellet in 20 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 2% N-laurylsarcosine (18). Solubilization was performed for 1 h at room temperature with gentle rotation. The outer membranes were then isolated as a pellet after ultracentrifugation (100,000 × g for 1 h) and resuspended in 20 ml of 20 mM sodium phosphate (pH 7.0) containing 500 mM NaCl and 0.5% SB3-14 detergent (Sigma, St. Louis, Mo.). Solubilization was performed with gentle rotation at room temperature for 16 h. Particulate material was removed by centrifugation at 20,000 × g for 20 min at 20°C. The soluble fraction was applied to a 1-ml HiTrap chelating column (Pharmacia), and elution was performed with a step gradient with 5 column volumes of eluant per step. The elution buffers were composed of 20 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl and 0.05% SB3-14, and increasing amounts of imidazole (0, 12.5, 25, 50, and 500 mM). The Wzi-His<sub>6</sub> protein routinely eluted in the step containing 50 mM imidazole. For biophysical measurements, the buffer was exchanged to 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl and 0.008% n-dodecyl-β-D-maltoside. All fast-perfor-
mass of Wzi-His6 was calculated using the algorithm of Garcia de la Torre et al. (19).

Production of anti-Wzi antibodies. All procedures were performed in the University of Guelph Animal Care Facility. A female New Zealand White rabbit was immunized with 100-μg aliquots of purified Wzi-His6, mixed 1:1 with Freund’s incomplete adjuvant (Sigma). Following a subcutaneous injection on day 1, intramuscular injections were given on days 4, 7, 35, and 49. The serum was collected on day 63 and adsorbed against E. coli DH5α whole cells before use. Briefly, cells from a 50-ml culture of DH5α grown overnight were washed in phosphate-buffered saline (PBS) and then resuspended in 5 ml of PBS and separated into 1 ml aliquots. Each aliquot was centrifuged, and the cell pellets were placed on ice. One milliliter of serum was mixed with a single pellet and incubated at 37°C for 1 h. The sample was then centrifuged, and the serum was transferred to the next pellet. This procedure was repeated until the serum was mixed with the fifth pellet, at which point the suspension was incubated at 4°C overnight. The serum was collected by centrifugation and stored at −20°C.

Protein SDS-PAGE and Western immunoblotting. Proteins were separated using standard 12% resolving gels by the method of Laemmli (27). The gels were stained with Coomassie brilliant blue, or the proteins were transferred to a BioTrace membrane (Pall Gelman Laboratory Inc., Ann Arbor, Mich.) by electrophoresis in carbonate buffer (10 mM sodium bicarbonate, 3 mM sodium carbonate, 20% [vol/vol] methanol). The anti-Wzi antibodies were used at a 1:1,000 dilution, and goat anti-rabbit secondary antibodies (CalTag Laboratories, Burlingame, Calif.) were used in detection. Wzi-His6 was detected using an anti-His antibody (QIAGEN Inc., Mississauga, Ontario, Canada) with a goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.). The secondary antibody was conjugated to alkaline phosphatase.

Cell surface polysaccharide analysis. Total polysaccharides were examined in whole-cell lysates prepared by the method of Hitchock and Brown (24). Samples were analyzed using 4 to 12% gradient NuPAGE gels (Novex, San Diego, Calif.), followed by Western immunoblotting using anti-K30 antibodies (14) or silver staining (50). For structural analyses, larger amounts of K30 polymer were isolated by a modified version of the hot water-phenol method (52) at 65°C. Total polysaccharide was extracted with an equal volume of 90% phenol at 65°C for 20 min. The phases were separated by centrifugation (14,000 × g for 20 min), and the aqueous phase was retained. The phenol phase was reextracted with another 15 ml of PBS, and the aqueous phases were combined, dialyzed for 3 days to remove residual phenol, and then treated with DNase (Roche, Laval, Quebec, Canada), RNase A (Roche), and proteinase K (Sigma). LPS was removed as a pellet after centrifugation at 105,000 × g for 16 h at 15°C. The supernatants were then lyophilized.

NMR spectroscopy. Proton nuclear magnetic resonance (NMR) analysis of K30 polysaccharide samples was performed using a Bruker AX500 spectrometer operated at 300 K using standard Bruker software. The samples were prepared in D2O, and the internal reference was the HOD peak.

Distribution of K30 antigen in cell-associated capsule and cell-free exopolysaccharide. In order to quantitate the amount of cell-bound CPS and cell-free material, bacteria were grown in M9 minimal medium with 0.2% glucose. Complementation was restored when these mutants were transformed with pWQ192, a pBAD derivative expressing Wzi with a cleavable signal sequence (SignalP 2.0 [http://www.cbs.dtu.dk/services/SignalP-2.0/]) (34). Assuming the signal sequence recognition site AQA, removal of the putative signal sequence would result in a protein (from E. coli E69) with a predicted molecular mass of 50,762 Da (52,871 Da for the precursor form) and a pI of 5.18. The corresponding protein from E. coli B44 has a predicted molecular mass of 50,854 Da (52,977-Da precursor) and a pI of 5.31. No additional homologs were identified by searches of the databases.

To verify the identity of Wzi, the ATG initiation codon was used and the gene was placed downstream of an optimized ribosome-binding site in pBAD24 in a construct that would encode a fusion protein with a C-terminal His6 tag. This construct (pWQ193) was expressed in E. coli DH5α (Fig. 1A), and the recombinant Wzi-His6 was identified by Western immunoblotting using commercially available monoclonal antibodies directed against the His tag. The cell lysates were separated into soluble (cytoplasmic and periplasmic components) and inner and outer membranes. A unique protein with an apparent molecular mass of 52 kDa was detected in the outer membranes of E. coli DH5α(pWQ193), consistent with the size predicted from sequence data (51,784 Da for mature Wzi-His6) and the relevance of a cleavable signal sequence.

The Wzi-His6 protein was purified by nickel affinity chromatography (Fig. 1B) and used to raise anti-Wzi serum in rabbits. This serum was used to examine Western immunoblots of whole-cell lysates of E. coli B44 and E69 (serotype K30 isolates) to verify the size of the native Wzi protein expressed from the chromosomal gene. As seen in Fig. 2A, the antibodies detected a protein of approximately 52 kDa in both strains. The protein was absent in lysates from the corresponding wzi:aacC1 mutants, E. coli CWG480 and CWG314, but expression was restored when these mutants were transformed with either pWQ192, a pBAD derivative expressing Wzi with a
out the C-terminal His\(_6\) tag, or pWQ193 expressing Wzi-His\(_6\). The differences in the size of Wzi and Wzi-His\(_6\) (approximately 930 Da) could not be distinguished by SDS-PAGE due to the interference of LPS molecules in the lysates and the resulting "wavy" gel bands. The subcellular localization of Wzi was confirmed with the native protein in \(E. coli\) B44, using inner and outer membrane proteins purified by sucrose gradient centrifugation (Fig. 2B). As with the Wzi-His\(_6\) protein in \(E. coli\) DH5\(_\alpha\)/H9251, the native Wzi protein was confined to the outer membrane of \(E. coli\) B44. The protein profiles of outer membranes from \(wzi\) mutants showed no defects other than the expected loss of the Wzi protein (data not shown).

Wzi is a monomeric \(\beta\)-barrel protein. Bacterial outer membrane proteins are often stable in SDS unless incubated at high temperatures. Upon denaturation by heating at 100°C, Wzi-His\(_6\) runs on SDS-polyacrylamide gels as a single band that corresponds to an apparent molecular mass of 52 kDa (Fig. 1B, lane 3), which is in accordance with the theoretical calculated mass. The same preparation gives a smaller apparent molecular mass of approximately 36 kDa when samples were not heated prior to electrophoresis (Fig. 1B, lane 4). This type of behavior has been reported for folded \(\beta\)-barrel proteins like OmpA that exist as monomers (6, 12). Since the predicted size of Wzi-His\(_6\) from \(E. coli\) B44 is 51,784 Da, the smaller size seen in unheated samples probably results from a folded monomer state. The molecular mass was determined by dynamic light scattering (19), which identified the major species as having an average molecular mass of 52.5 kDa (data not shown), which is consistent with a monomeric form. Minor contaminants were detected in the sample with molecular masses of 80.5, 191.0, and 175.2 kDa. These minor species were seen on SDS-polyacrylamide gels (Fig. 1B) and varied in amounts from preparation to preparation, but they did not react with antibodies against either Wzi or the His\(_6\) tag (data not shown).

To examine the secondary structure of Wzi, CD spectra were recorded. Wzi displays a CD spectrum with a single minimum at 219 nm (data not shown), which is indicative of a \(\beta\)-sheet-rich secondary structure (25). The relative contributions of \(\alpha\)-helix, \(\beta\)-sheet, coil, and turn were calculated by computer analysis of the CD spectrum using different methods (38, 45). These analyses indicated that Wzi-His\(_6\) has a \(\beta\)-sheet content of 65% and a \(\beta\)-turn content of 35%. Collectively, this data confirmed that Wzi-His\(_6\) is a monomeric \(\beta\)-barrel protein that remains folded after extraction from the membranes with 0.5% SB3-14 zwittergent and subsequent buffer exchange into 0.008% \(n\)-dodecyl-\(\beta\)-D-maltoside.

**Capsule phenotypes of \(wzi\) mutants.** We previously constructed an \(wzi::aacC1\) insertion mutant in \(E. coli\) E69 (O9a:...
K30) (15). The mucoid phenotype of this mutant (CWG314) indicated that it still produced K30 antigen, leading to the conclusion that \textit{wzi} was not essential for capsule synthesis. The subsequent report that a \textit{wzi} mutant of \textit{K. pneumoniae} caused a significant reduction in CPS production (1) led us to reevaluate the role of \textit{wzi}. Since \textit{E. coli} E69 makes a small amount of group 1 CPS relative to \textit{K. pneumoniae}, we constructed an additional \textit{wzi:}aacC1 mutation in \textit{E. coli} B44 (O9:K30), a virulent isolate (22, 32, 43, 44) that is visibly more mucoid than \textit{E. coli} E69. The B44 \textit{wzi} mutant (CWG480) retained its mucoid colony morphology and its sensitivity to K30-specific bacteriophage (data not shown). The \textit{wzi} mutations in either background (E69 or B44) had no effect on the expression of serotype O9/O9a smooth LPS or KLPS (Fig. 3). However, when whole-cell lysates of these \textit{wzi} mutants were examined for K30 antigen production by Western immunoblotting, there was only a barely detectable trace amount of reactivity in the region of the immunoblot corresponding to HMW capsular K30 antigen in our standard whole-cell lysate protocol (Fig. 3). The K30 defect was corrected by introducing either plasmid-encoded Wzi (pWQ192) or Wzi-His6 (pWQ193), indicating that the C-terminal His6 tag did not compromise function. The HMW CPS profiles of the \textit{wzi} mutations were essentially similar to capsule-deficient mutants, but this was inconsistent with the mucoid appearance of colonies of the mutants, as well as their wild-type level of sensitivity to K30-specific bacteriophage that requires HMW CPS as a receptor (data not shown).

In the standard whole-cell lysate preparations used for detection of HMW CPS in the Western immunoblot analysis, broth-grown bacteria are prepared by centrifugation and washing steps, prior to treatment with SDS and enzymes. These steps could potentially remove any loosely cell-associated polymer. Therefore, an increased amount of lysate sample was loaded for \textit{E. coli} CWG480. As shown in Fig. 3, these samples did show the presence of a much reduced amount of immunoreactive HMW CPS. To ensure that the relatively poor signal was not due to a difference in the structure of the HMW K30 antigen, polymer was extracted from \textit{E. coli} B44 and
CWG480 after growth on solid media. Proton NMR spectra were identical for the two samples (not shown), indicating that the low signals for the mutant in anti-K30 Western immunoblots did not reflect any differences in capsular polysaccharide structure or immunogenicity.

To examine the capsule morphology of the mutants, cells were labeled with cationized ferritin (Fig. 4). This electron-dense marker nonspecifically labels anionic molecules on the cell surface and has been used extensively to examine capsular layers in bacteria, including *E. coli* K30 (26). Capsule-deficient mutants of *E. coli* O9/O9α:K30 (such as CWG28) bind no cationized ferritin due to the structure of the (neutral) polymannan O antigens, leaving the K30 capsule as the sole product labeled by the marker. The wzi mutation led to a significant reduction in the amount of cell surface-associated capsule and loss of a coherent capsule structure covering the cell surface (Fig. 4). Analysis of several preparations (with sections through the cells in various planes) showed no differences in cell size and shape between the wild type and wzi mutants. The wzi mutation was complemented in trans by introduction of a plasmid expressing Wzi-His6 (pWQ193). These results confirmed that the capsule phenotype was due only to the loss of wzi and not due to any unanticipated polar effects on downstream genes. The results for the wzi mutants were essentially the same, regardless of the parental background, and the capsule phenotypes were not influenced by growth in broth or on plates. Therefore, only the results for broth-grown *E. coli* B44 and CWG480 are presented.

To determine whether the wzi mutant affected only the cell-associated capsular polysaccharide, the distribution of cell-bound K30 polysaccharide to cell-free K30 polysaccharide was determined from *E. coli* B44 cells grown in broth. As shown in Fig. 5, approximately 80% of the K30 antigen was in the cell-associated form. The wzi mutation resulted in a 2.1-fold reduction of cell-associated CPS, but the amount of cell-free polymer increased by 3.4-fold. Introduction of pWQ193 into the mutant caused the values to return to those seen for the parent, *E. coli* B44, although complementation was incomplete and the parental values were not fully restored. This incomplete complementation was anticipated, because the use of M9 minimal medium containing glucose precludes full induction of wzi expression in pBAD constructs by added arabinose.

**DISCUSSION**

The conservation of wzi in all of the *E. coli* and *Klebsiella* group 1 capsule clusters examined thus far suggested that it might play an important role in group 1 capsule biosynthesis. In some related gene clusters, such as that for Vi antigen biosynthesis in *Salmonella enterica* serovar Typhi, the cluster begins with a gene whose product is not involved in biosynthesis per se. In this case, TviA is a positive transcriptional activator that is required for Vi antigen production (51). The continued formation of K30 antigen in wzi mutants and the lack of any effect of the mutation on expression of the wza and wzc gene products (data not shown) ruled out any regulatory role for Wzi. This was further supported by the subsequent localization of Wzi to the outer membrane.

The wzi gene is located in the region of the *cps* cluster responsible for high-level polymerization and surface assembly of the capsular K antigen. The wzi mutants were visibly mucoid, but other parameters routinely used to follow capsule expression gave contradictory data. For example, Western immunoblotting implied a defect in the production of HMW capsular polymer in the wzi mutants. However, tests for susceptibility to LPS-specific bacteriophages showed that sufficient quantities of K30 antigen were made to mask the underlying phage receptor. The polymeric product made by the wzi mutants was confirmed to be K30 antigen by proton NMR. The characteristics of the wzi mutants were resolved by the finding that they contained a marked reduction in capsular (cell-associated) polymer and a corresponding increase in cell-free K30 antigen. The reason(s) for the inability of the anti-K30 antibody to detect CPS at the level found in lysates of the wzi mutant is unclear. It is possible that the reactivity is influenced by differences in conformation (rather than primary structure) of the epitope in the CPS of the mutant. However, this interpretation is limited by a lack of detailed knowledge of the exact specificity of the antibody. Alternatively, it is possible that the CPS from the wzi mutant is altered in some way (e.g., size) such that it no longer enters the SDS-polyacrylamide gel.

Although LPS O-side chains are recognized determinants of resistance to complement-mediated killing in *K. pneumoniae*, there exist some clinical isolates that are deficient in O-side
chains. In these naturally occurring rough LPS mutants, the capsule plays a critical role in protecting the cell from complement and opsonophagocytosis (1). A \textit{wzi} mutant was isolated in one of these \textit{K. pneumoniae} rough mutants using random transposon mutagenesis and screening for susceptibility to serum killing (1). The mutant made fourfold less capsule than the isogenic parent strain. However, it is not clear whether the capsule quantitated in this study was in the cell-associated or cell-free form or both. If only the cell-associated polymer was measured, the phenotype is comparable to that of \textit{E. coli} CWG480 (\textit{wzi::aacC1}). Certainly, the lack of a coherent capsular structure in \textit{E. coli} CWG480 might be expected to influence access of cellular host defense components to the bacterial surface.

While the role of a protective capsule is well established in \textit{K. pneumoniae}, the distribution of cell-associated and cell-free capsular antigen may also play an important role in the pathogenesis of this organism. Cell-free polysaccharide is implicated in the neutralization of circulating anticapsular antibody (37), induction of immune tolerance (7, 33, 35), and impairment of macrophage maturation (57, 58). Furthermore, an extracellular toxic complex containing capsular polysaccharide, LPS, and protein is implicated in the lung tissue damage that characterizes \textit{K. pneumoniae}-induced pneumonia (47, 48).

\textit{Wzi} is the second outer membrane component in the capsule assembly pathway. \textit{Wza} forms a multimeric secretin-like structure that is proposed to be involved in translocation of capsular polysaccharide across the outer membrane (16). The conservation of this function in different exopolysaccharide-synthesizing systems is evident in the growing number of homologs of \textit{Wza} found in the databases. Interestingly, \textit{wza} mutants do not secrete polymer but also do not accumulate HMW intermediates within the periplasm, as might be expected if only the late processes are affected. Since \textit{wza} mutants can still

**FIG. 4.** Capsule morphology of \textit{E. coli} B44 and its \textit{wzi::aacC1} mutant. Capsular polysaccharides were labeled by using cationized ferritin, and thin sections were prepared for electron microscopy. \textit{E. coli} CWG480 is the \textit{wzi::aacC1} mutant derived from B44 (O9:K30). The capsule structure was restored by complementation of CWG480 using pWQ193, expressing Wzi-His. \textit{E. coli} CWG28 (O9a:K-1) is a spontaneous capsule-deficient mutant (55) used as a control to show the lack of labeling of unencapsulated cells. Bars, 0.5 μm.
determining the distribution of cell-associated and cell-free K antigen. A simple explanation would involve Wzi being required for attachment of capsular polymer to the cell surface. Wzi could potentially fulfill this role by providing an anchor itself or, indirectly, by providing an assembly system for a different molecule that serves as the anchor. Despite the fact that *E. coli* group 1 capsules are firmly cell associated (and typically withstand washing and repeated centrifugation), the mechanism by which the capsule structure is attached is unknown. If cell association requires covalent linkage, the actual linker molecule has yet to be identified. This situation exists for most capsules and is related to the technical difficulties in identifying covalently linked molecules at the termini of long polymer chains. Extraction procedures for capsule favor the use of phenol extractions, and any linkage would have to withstand such treatments. It is particularly interesting that the *E. coli* colanic acid biosynthesis locus has no homolog of Wzi (46), in striking contrast to the corresponding group 1 capsule loci. The locus contains homologs of *wza*, *wcb*, and *wzc* and encodes a Wzy-dependent polymerization system comparable to that for the K30 antigen. Significantly, colanic acid is loosely associated with the cell surface and much of the product is secreted as an extracellular polysaccharide traditionally referred to as slime (3, 20). Microscopy studies of *E. coli* K-12 cells producing colanic acid yielded no evidence of a capsule structure (49). While the exact role of Wzi is not resolved by these studies, characterization of Wzi and the wzi mutant identifies an additional novel component in the capsule assembly system and may provide an initial insight into the capsule anchoring mechanism in *E. coli* and *K. pneumoniae*.

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