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Functional Analysis of Conserved Gene Products Involved in Assembly of *Escherichia coli* Capsules and Exopolysaccharides: Evidence for Molecular Recognition between Wza and Wzc for Colanic Acid Biosynthesis

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Group 1 capsular polysaccharides (CPSs) of *Escherichia coli* and some loosely cell-associated exopolysaccharides (EPSs), such as colanic acid, are assembled by a Wzy-dependent polymerization system. In this biosynthesis pathway, Wza, Wzb, and Wzc homologues are required for surface expression of wild-type CPS or EPS. Multimeric complexes of Wza in the outer membrane are believed to provide a channel for polymer export; Wzc is an inner membrane tyrosine autokinase and Wzb is its cognate phosphatase. This study was performed to determine whether the Wza, Wzb, and Wzc proteins for colanic acid expression in *E. coli* K-12 could function in the *E. coli* K30 prototype group 1 capsule system. When expressed together, colanic acid Wza, Wzb, and Wzc could complement a *wza-wzb-wzc* defect in *E. coli* K30, suggesting conservation in their collective function in Wzy-dependent CPS and EPS systems. Expressed individually, colanic acid Wza and Wzb could also function in K30 CPS expression. In contrast, the structural requirements for Wzc function were more stringent because colanic acid Wzc could restore translocation of K30 CPS to the cell surface only when expressed with its cognate Wza protein. Chimeric colanic acid-K30 Wzc proteins were constructed to further study this interaction. These proteins could restore K30 biosynthesis but were unable to couple synthesis to export. The chimeric protein comprising the periplasmic domain of colanic acid Wzc was functional for effective K30 CPS surface expression only when coexpressed with colanic acid Wza. These data highlight the importance of Wza-Wzc interactions in group 1 CPS assembly.

Escherichia coli capsules (comprising capsular polysaccharides [CPSs]) are acidic polysaccharide layers surrounding and tightly associated with the surface of the cell. They are recognized virulence determinants and act by increasing adherence to host tissues and conferring resistance to phagocytosis. In some cases, the host immune response against the CPS is impaired because the capsule structure mimics host cell components. More than 80 distinct capsular or K antigens in *E. coli* have been described, and these are classified into four groups on the basis of genetic and biosynthetic criteria (61). These capsule groups provide prototypes for CPSs and the loosely associated exopolysaccharides (EPSs) produced by many other bacteria.

Group 1 capsules and related polysaccharides are found in pathogens of humans and animals and in plant-associated bacteria, where they enhance virulence or mediate symbiosis. Group 1 CPSs and related EPSs are assembled by a Wzy-dependent polymerization system. The early stages of synthesis are identical to those of lipopolysaccharide biosynthesis, where the action of the Wzy-dependent system is best understood (reviewed in reference 48). In the present model, lipid-linked repeat units are built up on undecaprenol diphosphate at the cytoplasmic face of the inner membrane by the sequential activity of glycosyltransferases. The lipid-linked repeat units are then flipped to the periplasmic face of the inner membrane by the putative flippase (Wzx), where the putative polymerase, Wzy, generates a long-chain polymer. The products of three conserved genes (*wza*, *wzb*, and *wzc*) are required for late steps in the expression of group 1 CPSs and related EPSs, including *E. coli* colanic acid (reviewed in reference 60). In the absence of Wza and Wzc in the prototype *E. coli* K30 system, oligosaccharides with low degrees of polymerization are made and ligated to lipid A-core in a glycoform known as K_{LPS} (17, 37, 62).

The functions of Wza, Wzb, and Wzc in *E. coli* K30 have been studied in detail. Wza is an outer membrane lipoprotein that is essential for the assembly of high-molecular-weight (HMW) CPS on the surfaces of *E. coli* K30 cells. Wza forms ring-like multimers composed of eight subunits arranged as a tetramer of dimers (3, 4, 17, 43). These complexes are believed to provide a channel through which K30 CPS crosses the outer membrane. Wzc is a tyrosine autokinase that is dephosphorylated by its cognate phosphatase, Wzb (62). Wzc is also essential for HMW-polysaccharide expression in all systems examined to date. The precise role of Wzc is not known, but the presence of homologues in CPS systems in gram-positive bacteria suggests a role in a conserved step of CPS/EPS assembly, perhaps in determining polymer chain length or in coordinating a putative multienzyme complex for CPS/EPS biosynthesis and export (60). While *wzb* mutants allow formation of some K30 CPS on the cell surface, the Wzb phosphatase is required

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for a wild-type capsular phenotype (62). The finding that Wzc and Wza interact (43) suggests that the homologues in gram-negative bacteria may have additional roles that are not conserved in the Wzc proteins in gram-positive bacteria.

Wzc homologues are integral membrane proteins harboring two transmembrane domains flanking a large periplasmic loop and have a cytoplasmic C-terminal region with a Walker A ATP-binding motif and a tyrosine-rich C terminus (2, 15, 16, 20, 40, 58, 62). Phosphorylation of Wzc occurs at the tyrosine-rich C terminus, at the expense of ATP (5, 20, 39, 40, 42, 44, 58, 62). It appears that phosphorylation of Wzc is essential for capsule expression in *E. coli* K30 (62) and that the level of Wzc phosphorylation rather than the phosphorylation of specific residues is important in this system (46). Interestingly, in the colanic acid system (59) and in *Streptococcus pneumoniae* type 19F CPS (40), phosphorylation of Wzc is reported to be a negative regulator of EPS/CPS expression. However, recent data revealed contrasting phenotypes for phosphatase mutants of two different *S. pneumoniae* strains, suggesting that discrepancies in this example potentially reflect the involvement of other unidentified cellular factors (5). Thus, it is unclear whether Wzc proteins have identical functions in all systems.

In *E. coli*, colanic acid can be coexpressed with the prevalent group 2 capsules but not with group 1 capsules (30). This is because the group 1 *cps* cluster and the colanic acid *cps* cluster occupy the same genetic location, reflecting genetic insertions and rearrangements that have resulted in the acquisition of the group 1 *cps* cluster and the loss of the colanic acid cluster (50). Despite the similar arrangements in the group 1 and colanic acid *cps* operons and the shared biosynthetic mechanisms of these systems, important differences exist in the regulation of colanic acid and K30 CPS expression. Colanic acid is not produced in significant amounts at physiological temperatures and apparently plays no role in virulence (1, 25, 35, 52). However, this polymer provides protection against desiccation (45) and heat and acid (33, 38), as well as osmotic and oxidative stress (10), and has been implicated in biofilm formation by *E. coli* K-12 (13, 18, 47). Colanic acid expression is transcriptionally regulated by the RcsC-YojN-RcsB phosphorelay system (11, 19, 55, and 56 and references therein), a system now known to regulate a range of functions (18, 24). In contrast, *E. coli* group 1 capsule *cps* loci are constitutively expressed (51) and the polymers produced are important virulence determinants (8, 9, 23, 28, 41, 53, 54).

An important distinction between CPS and EPS polymers is their degrees of attachment to the cell surface. While EPSs are generally loosely associated with the cells, CPSs tend to be more firmly anchored. The method of attachment of the group 1 capsule to the surface is not known, but surface distribution is influenced by the outer membrane protein Wzi, which is confined to group 1 CPS systems (49). The absence of Wzi from EPS systems (including the colanic acid system) may be a factor in the loose arrangement of most EPS polymers. However, it is unclear whether Wza, Wzb, and Wzc also contribute to the features that distinguish CPS and EPS expression. The predicted protein sequences for Wza, Wzb, and Wzc from *E. coli* K30 and *E. coli* K-12 (strain MG1655) are well conserved (Fig. 1). While some homologues of Wza, Wzb, and Wzc have been identified and some of the Wzb and Wzc homologues have had phosphatase and kinase activities confirmed in vitro, it has not been determined whether they perform identical functions in CPS and EPS synthesis. The goal of this study was to address these questions by examining the function of *E. coli* K-12 colanic acid biosynthesis proteins in the prototype *E. coli* K30 group 1 CPS system.

MATERIALS AND METHODS

Bacterial 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 Luria-Bertani (LB) medium (Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), or chloramphenicol (30 μg ml^{-1}) where appropriate. Complementation experiments were carried out with derivatives of *E. coli* E69 (O9a:K30:H12), the prototype group 1 capsule strain. Most *E. coli* isolates (like E69) contain two chromosomal copies of the *wza-wzb-wzc* cluster, one in the *cps* locus and the other at a position corresponding to 22 min on the *E. coli* K-12 linkage map (17, 29, 31). To simplify the analyses, all mutants studied here were constructed by using strain CWG258 as a background. CWG258 is an E69 derivative with a polar *aadA* insertion in the 22-min copy of *wza* that eliminates expression of the 22-min copy of the *wza-wzb-wzc* cluster (17). The polarity of this insertion and the phenotypes resulting from individual chromosomal mutations in the *cps* locus versions of *wza*, *wzb*, and *wzc* in this background are well established (17, 62). For induction of gene expression from pBAD (22) derivatives, cells were grown in LB to mid-exponential phase prior to the addition of a final concentration of L-arabinose of 0.002% (for expression of Wza derivatives) or 0.02% (for expression of Wzb and Wzc derivatives). The cultures were then grown for an additional 2.5 h prior to preparation of cell membranes or lysates.

Cloning of *wza*, *wzb*, and *wzc* homologues. The *wza*, *wzb*, and *wzc* genes were amplified from chromosomal DNA of either *E. coli* E69 (K30 antigen biosynthesis locus, hereafter designated with the subscript K30) or *E. coli* DH5α (colanic acid biosynthesis locus, hereafter designated with the subscript CA). Amplification reactions were carried out with primers engineered to include desired restriction sites for cloning into arabinose-inducible pBAD vectors (22). The primers used for PCR amplifications are listed in Table 2. The *wza*, *wzb*, and *wzc* genes were cloned individually and in combination to evaluate the need for interacting partners. Plasmid sequences were verified and inserts deemed to be free of PCR-induced errors by sequencing at the Guelph Molecular Supercenter (University of Guelph, Guelph, Ontario, Canada).

Construction of a chromosomal deletion in the $(wza\text{-}wzb\text{-}wzc)_{K30}$ cluster. Strain CWG655 [$wza_{22\ min}$::*aadA* Δ$(wza\text{-}wzb\text{-}wzc)_{K30}$::*aphA3*] was constructed by chromosomal deletion in strain CWG258. Briefly, the region spanning the $(wza\text{-}wzb\text{-}wzc)_{K30}$ cluster was amplified using primers EB6/EB7 (each containing a BamHI site to facilitate cloning). The PCR product was cloned into the BamHI-digested suicide vector pWQ173 (51). An internal fragment of about 1,800 bp was removed by digestion of the plasmid with BglII/BclI. This removed most of the *wza* and *wzc* genes and all of *wzb*. The BamHI-digested nonpolar *aphA3* kanamycin resistance cassette was ligated into BglII/BclI-digested plasmid (these are compatible ends), generating pWQ96. *E. coli* CWG258 was electrotransformed (6) with pWQ96, and gene replacement occurred through homologous recombination, as described elsewhere (51). Replacement of the wild-type genes by allelic exchange was confirmed by PCR using primers internal and external to the mutation site.

Construction of chimeric Wzc proteins. Chimeric Wzc proteins were constructed by using a gene splicing overlap procedure (27, 34). Construction of pWQ87 involved PCR amplification of the 5′ portion (corresponding to amino acids 1 to 455) of wzc_{K30} by using a forward primer (AP66) designed to introduce an EcoRI site and a reverse primer (AP67) that yields a product with a 25-bp overhang of the wzc_{CA} sequence. The 3′ portion (corresponding to amino acids 454 to 721) of wzc_{CA} was then amplified using a forward primer (AP68) that generates a 25-bp wzc_{K30} overhang in the product and a reverse primer (AP35) designed to introduce a PstI site. The products of the first two PCRs were used as templates for a third PCR that involved extension of the partly complementary templates and amplification using the AP66/AP35 primer pair. This PCR product was digested with EcoRI/PstI and ligated into pBAD24. Plasmid pWQ88 (N-terminal amino acids 1 to 453 of Wzc_{CA}) was constructed using a similar approach.

SDS-PAGE and Western immunoblotting. Whole-cell lysates were prepared in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (32) and heated to 100°C for 10 min. Proteins from whole-cell lysates were separated on 14% resolving SDS-PAGE gels and transferred onto BioTrace NT nitrocellulose membranes (Gelman Laboratory) for Wza expres-

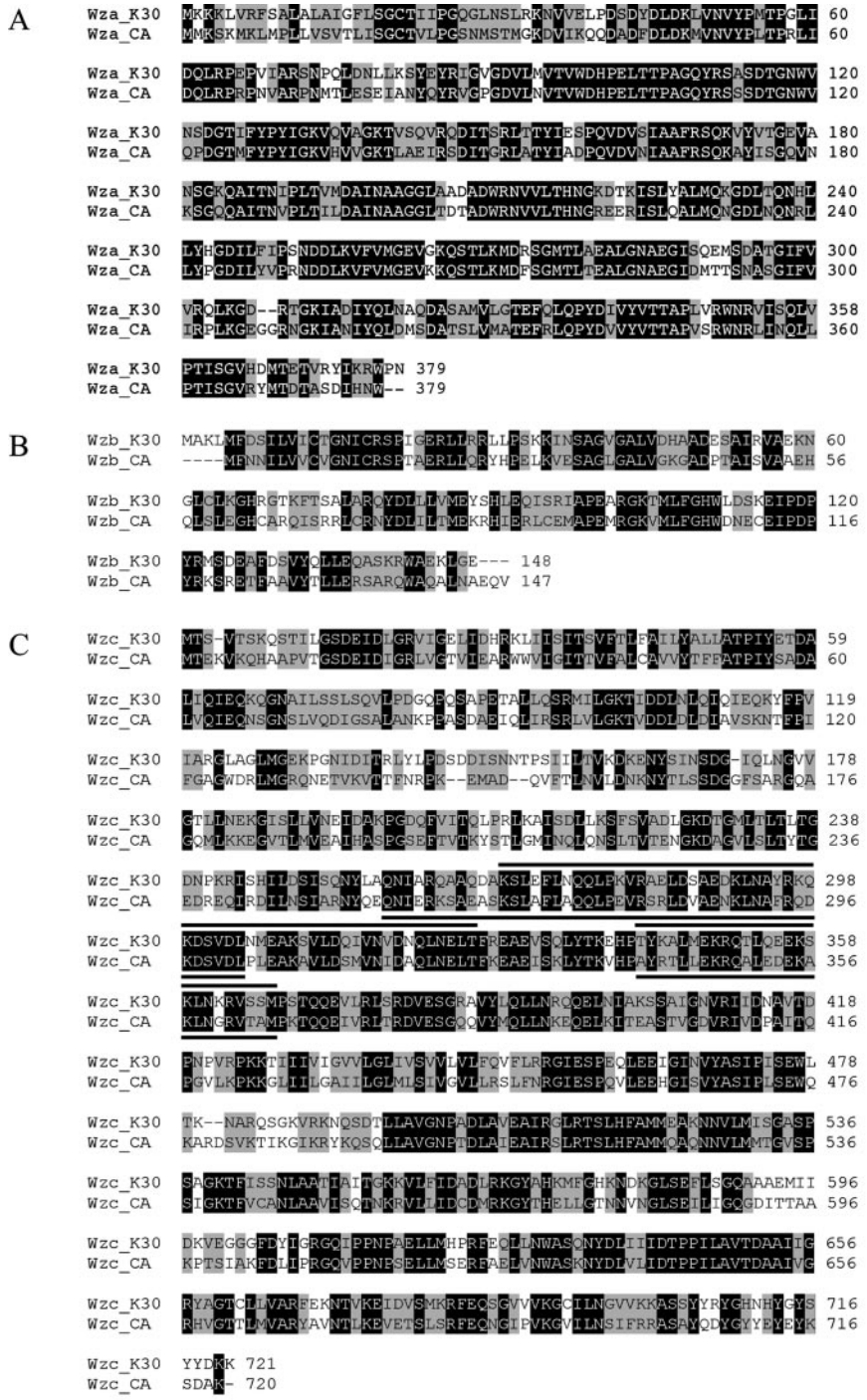

FIG. 1. Alignment of amino acid sequences of Wza, Wzb, and Wzc from *E. coli* K30 and K-12. Wza_{K30} and Wza_{CA} share 65% identity and 90% similarity at the amino acid level (A), the respective Wzb proteins are 51% identical and 80% similar (B), and the Wzc proteins share 51% identity and 85% similarity (C). Identical amino acids are highlighted in black and similar amino acids are highlighted in gray. In panel C, the putative coiled-coil domains are indicated by black bars above or below the sequences. This alignment was generated using Clustal W (http://npsa-pbil.ibcp.fr) (57). Coiled-coil predictions were generated using the COILS program (http://www.ch.embnet.org) with an unweighted matrix and a 21-residue window (36).

sion analysis. Wzc expression and phosphorylation were assessed by Western immunoblotting of isolated cell envelopes in order to detect the low signal intensities that were difficult to visualize in whole-cell lysates. Briefly, cells were grown in 200-ml cultures, and expression of plasmid-carried proteins was induced as described above. Cells were harvested, resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0, and lysed by ultrasonication. Following a low-speed centrifugation step to remove unbroken cells and cell debris, cell envelopes were collected by ultracentrifugation (100,000 × *g*; 1 h) and resuspended in 2 ml of 10 mM Tris-HCl, pH 8.0. A sample of isolated cell membranes corresponding to 5 μg of total protein was subjected to electrophoresis using 10% resolving SDS-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype, serotype, or description[a]	Reference or source
E. coli strains		
DH5α	K-12 ϕ80d*lacZ*Δ*M15 endA1 recA1 hsdR17*($r_K^-m_K$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* F^-	Clontech
E69	Prototroph, serotype O9a:K30:H12	F. Ørskov[b]
CWG258	E69 with $wza_{22\ min}$::*aadA* insertion (polar on $wzb_{22\ min}$ and $wzc_{22\ min}$); Sp^r	17
CWG281	CWG258 with wza_{K30}::*aacC1* insertion; Gm^r Sp^r	17
CWG285	CWG258 with wzc_{K30}::*aacC1* insertion; Gm^r Sp^r	62
CWG343	CWG258 with wzb_{K30}::*aphA3* insertion; Km^r Sp^r	62
CWG655	CWG258 with Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3*; Km^r Sp^r	This study
Plasmids		
pBAD18-Km	Arabinose-inducible expression vector; Km^r	22
pBAD24	Arabinose-inducible expression vector; Ap^r	22
pWQ81	pBAD24 derivative carrying wza_{CA} on an KpnI/PstI fragment; Ap^r	This study
pWQ83	pBAD24 derivative carrying wzb_{CA} on an EcoRI/PstI fragment; Ap^r	This study
pWQ85	pBAD24 derivative carrying wzc_{CA} on an EcoRI/PstI fragment; Ap^r	This study
pWQ87	pBAD24 derivative carrying $wzc_{K30(1–455)CA}$ fusion on an EcoRI/PstI fragment; Ap^r	This study
pWQ88	pBAD24 derivative carrying $wzc_{CA(1–453)K30}$ fusion on an EcoRI/PstI fragment; Ap^r	This study
pWQ89	pBAD24 derivative carrying (*wzb-wzc*)$_{K30}$ on an EcoRI/SalI fragment; Ap^r	This study
pWQ90	pBAD24 derivative carrying (*wzb-wzc*)$_{CA}$ on an EcoRI/PstI fragment; Ap^r	This study
pWQ91	pBAD24 derivative carrying (*wza-wzb-wzc*)$_{K30}$ on an EcoRI/PstI fragment; Ap^r	This study
pWQ92	pBAD24 derivative carrying (*wza-wzb-wzc*)$_{CA}$ on a KpnI/PstI fragment; Ap^r	This study
pWQ96	pWQ173 derivative carrying (5′ *wza-aphA3-wzc*3′)$_{K30}$ on a BamHI fragment; Cm^r	This study
pWQ98	pBAD18-Km derivative carrying wza_{CA} on an NheI/PstI fragment; Km^r	This study
pWQ99	pBAD18-Km derivative carrying wza_{K30} on an NheI/PstI fragment; Km^r	This study
pWQ126	pBAD24 derivative carrying wza_{K30} on an EcoRI/PstI fragment; Ap^r	17
pWQ130	pBAD18-Km derivative carrying wzc_{K30} on an EcoRI/PstI fragment; Km^r	62
pWQ147	pBAD24 derivative carrying wzb_{K30} on an EcoRI/blunt-ended fragment; Ap^r	62
pWQ173	Counterselectable, temperature-sensitive suicide vector; Cm^r	51

[a] Sp, spectinomycin; Gm, gentamicin; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol.
[b] International *Escherichia* and *Klebsiella* Center, Statens Seruminstitut, Copenhagen, Denmark.

PAGE gels. Proteins were either stained with Coomassie brilliant blue or transferred onto Westran polyvinylidene difluoride membranes (Schleicher and Schuell) or nitrocellulose membranes for Western immunoblot detection of tyrosine-phosphorylated proteins or Wzc, respectively. The transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol. Wzc and Wza were detected by using polyclonal antisera from rabbits immunized with the *E. coli* K30 proteins (17, 62). Detection was achieved by using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Caltag, Burlingame, CA). Phosphorylation of Wzc was detected using the PY20 antiphosphotyrosine monoclonal antibody (Transduction Laboratories, Lexington, KY) and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were used for detection.

SDS-PAGE analysis of K30 polysaccharide. Total cellular polysaccharide samples were prepared by the method of Hitchcock and Brown (26). Cells were grown as described above, harvested, and washed prior to preparation of whole-cell lysates in 1× SDS-PAGE sample buffer without β-mercaptoethanol. Proteins in the lysate were digested with proteinase K (0.5 mg ml^{-1}) overnight at 55°C. Polysaccharide preparations were separated by electrophoresis on NuPAGE 4 to 12% bis-Tris gels (Invitrogen Life Technologies Inc., Burlington, Ontario) and subsequently transferred onto nitrocellulose membranes. A rabbit polyclonal anti-K30 polysaccharide antiserum (14) and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody were used to detect K30 polysaccharide.

Visualization of CPS by electron microscopy. Cells were grown to mid-exponential phase in 5 ml of LB broth containing the appropriate antibiotics. Where appropriate, L-arabinose was added to induce expression of plasmid-carried proteins. Cultures were standardized such that the optical densities at 600 nm were 0.5, and cells from 0.5 ml of culture were collected by centrifugation. All centrifugation steps were carried out at 5,600 × *g* for 5 min to minimize adverse effects on the integrity of the capsule. Cells were washed twice with 0.5 ml of 100 mM HEPES, pH 7.5. The cell pellets were then incubated with 20 μl of cationized ferritin solution (Sigma, St. Louis, MO) for 30 min at room temperature with gentle agitation. Cationized ferritin binds to the negatively charged capsule, stabilizing it against dehydration for analysis by electron microscopy. Cells were collected by centrifugation and washed twice with 0.5 ml of 100 mM HEPES, pH 7.5, to remove unbound ferritin. The cells were then processed for electron microscopy (Natural Sciences and Engineering Research Council Guelph Regional Scanning Transmission Electron Microscopy Facility, Guelph, Ontario, Canada).

Visualization of K30 CPS by immunofluorescence microscopy. Cells were grown overnight in 5 ml of LB broth in the presence of antibiotics and 0.02% L-arabinose (final concentration) and collected by centrifugation. All centrifugation steps were carried out at 5,600 × *g*. Cell pellets were washed with phosphate-buffered saline (PBS) and incubated on circular glass coverslips coated with poly-L-lysine for 5 min at room temperature. Cells were fixed to the coverslips by overnight incubation in 3.7% (vol/vol) formaldehyde. Following two PBS wash steps, free aldehydes were quenched with 50 mM NH_4Cl. Where indicated, cells were then permeabilized by incubation with EDTA, lysozyme, and Triton X-100, as described previously (12). Coverslips were blocked in 1% (wt/vol) bovine serum albumin in PBS for 30 min prior to overnight incubation with polyclonal anti-K30 polysaccharide antiserum (14), used at a 1/500 dilution. Coverslips were washed by three successive transfers into PBS before incubation with rhodamine red-conjugated goat anti-rabbit antibodies (1/50; Jackson Immunoresearch) for 60 min at 37°C in the dark. Coverslips were mounted with Vectashield fluorescence mounting medium (Vector Laboratories) and examined using a Zeiss Axiovert 200 microscope at a total magnification of ×1,000. OpenLab software (Improvision) was used for image processing.

RESULTS

Wza, Wzb, and Wzc from the colanic acid system can complement a *wza-wzb-wzc* defect in *E. coli* K30. Initial experiments examined the ability of the *wza*, *wzb*, and *wzc* genes from the colanic acid locus to complement the corresponding defect in *E. coli* CWG655 [$wza_{22\ min}$::*aadA* Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3*]. For all experiments reported in this study, the restoration of K30 polysaccharide synthesis was assessed by Western immunoblotting of cellular polysaccharides probed with anti-K30

TABLE 2. Primers used in this study

Primer	Sequence[a]	Application and feature
EB6	CCTGGTCAGGGATccAACAGTCTG	Amplification of $(wza\text{-}wzb\text{-}wzc\text{-}wbaP)_{K30}$ for pWQ96; BamHI site
EB7	TCGCGgAtCCAATTGTTACGA	Amplification of $(wza\text{-}wzb\text{-}wzc\text{-}wbaP)_{K30}$ for pWQ96; BamHI site
JD125	TGATGATGTGAatTcATGAAGAAAA	Amplification of $(wza\text{-}wzb\text{-}wzc)_{K30}$ for pWQ91; EcoRI site
JD102	ACCATACCAAGCAACACAACAG	Amplification of $(wza\text{-}wzb\text{-}wzc)_{K30}$ for pWQ91
JD152	GGAAACAGTACGATgaATTcAGAGATG	Amplification of $(wzb\text{-}wzc)_{K30}$ for pWQ89; EcoRI site
AP06	CATTAAGTAGAGTCgAcGGTTTTGTCAGTC	Amplification of $(wzb\text{-}wzc)_{K30}$ for pWQ89; SalI site
AP34	GGGGTACCTATGATGAAATCCAAAATGAAATTG	Amplification of $(wza\text{-}wzb\text{-}wzc)_{CA}$ for pWQ92 and wza_{CA} for pWQ81; KpnI site
AP35	AACTGCAGTTATTTCGCATCCGACTTATATTCG	Amplification of $(wza\text{-}wzb\text{-}wzc)_{CA}$ for pWQ92, wzc_{CA} for pWQ85 and pWQ87, and $(wzb\text{-}wzc)_{CA}$ for pWQ90; PstI site
AP49	ggaattcGTCATGTTTAACAACATCTTAGTTGTCTGTGTC	Amplification of wzb_{CA} for pWQ83 and $(wzb\text{-}wzc)_{CA}$ for pWQ90; EcoRI site
AP50	aactgcagTTATACCTGCTCTGCGTTCAATGC	Amplification of wzb_{CA} for pWQ83; PstI site
AP51	aactgcagTTACCAGTTATGAATGTCGCTGG	Amplification of wza_{CA} for pWQ81; PstI site
AP54	ggaattcaccATGACAGAAAAAGTAAAACAACATGCC	Amplification of wzc_{CA} for pWQ85 and pWQ88; EcoRI site
AP66	gGAATTCACCATGACTTCAGTCACATCTAAGCAATCAACG	Amplification of N-terminal region of wzc_{K30} for pWQ87; EcoRI site
AP67	gttcttccagtacttgcgggcttteGATACCACGGCGTAGAAACACTTGGAACAATAC	Amplification of N-terminal region of wzc_{K30} for pWQ87; overhang from wzc_{CA}
AP68	ccaagtgtttctacgccgtggtatcGAAAGCCCGCAAGTACTGGAAGAACACGG	Amplification of C-terminal region of wzc_{CA} for pWQ87; overhang from wzc_{K30}
AP69	tttcttcgagttgctctgggaattcAATGCCGCGATTAAACAACGAGCGCAGCAG	Amplification of N-terminal region of wzc_{CA} for pWQ88; overhang from wzc_{K30}
AP70	gcgctcgttgtttaatcgcggcattGAATCCCCAGAGCAACTCGAAGAAATCGG	Amplification of C-terminal region of wzc_{K30} for pWQ88; overhang from wzc_{CA}
AP71	aactgcagCTATTTTTTATCATAATAAGAATAGCCATAATG	Amplification of wzc_{K30} for pWQ88; PstI site

[a] Bases altered from those in the chromosome or plasmid template are in lowercase letters, and restriction sites are underlined and listed in the right-hand column.

FIG. 2. Wza_{CA}, Wzb_{CA}, and Wzc_{CA} can restore K30 CPS synthesis and surface expression in an *E. coli* K30 $\Delta(wza\text{-}wzb\text{-}wzc)_{K30}$ strain. Synthesis of K30 polysaccharide was detected by Western immunoblotting of polysaccharide preparations probed with a polyclonal anti-K30 polysaccharide antiserum (anti-K30 PS), and surface-expressed K30 CPS was evident as electron-dense material on the surface of cationized ferritin-stained whole cells examined by electron microscopy. Size bars = 0.5 μm.

polysaccharide antiserum. As polysaccharide detected in this manner can be either intracellular or surface expressed, cell surface polysaccharides were either stabilized with cationized ferritin and visualized using transmission electron microscopy or labeled with anti-K30 polysaccharide antiserum for analysis by immunofluorescence microscopy. As expected based on the phenotypes of *wzc* and *wza* mutants (see below), CWG655 was acapsular and produced no immunoreactive K30 polysaccharide (Fig. 2). The defect in CWG655 was complemented with the respective K30 genes (pWQ91) in *trans*, restoring K30 polymer synthesis and surface expression of CPS in a phenotype indistinguishable from that of the parent (Fig. 2). This defect was also complemented by the $(wza\text{-}wzb\text{-}wzc)_{CA}$ cluster (pWQ92), with the restoration of synthesis and surface expression of HMW K30 CPS (Fig. 2). There was a noticeable difference in the migration patterns of the HMW polysaccharides in the gel when the two complemented strains were compared, suggesting that the average size distribution of the product resulting from expression of the colanic acid proteins was significantly larger (Fig. 2). This could also reflect a difference in the amounts of material produced. Given the complex nature of the complementation, it is impossible to establish the pre-

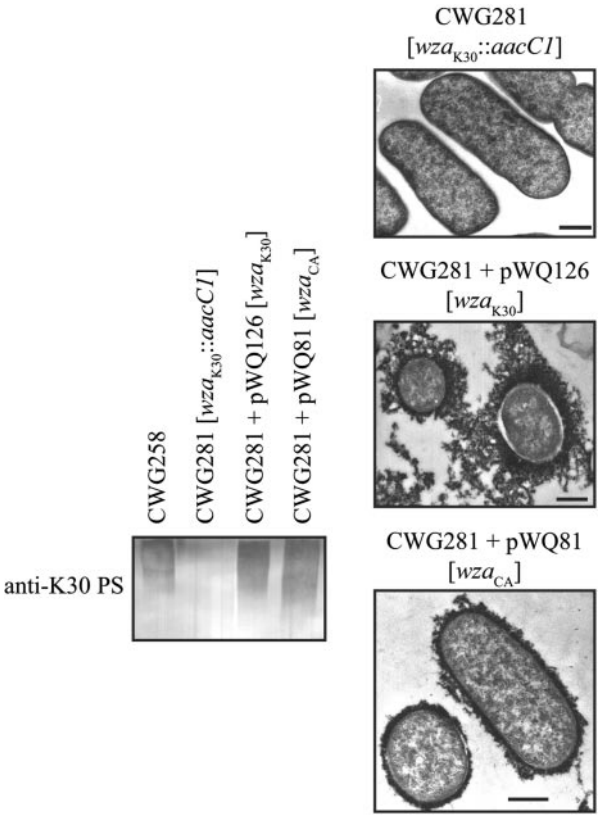

FIG. 3. Wza_{CA} can functionally replace Wza_{K30}. Plasmids carrying either wza_{K30} or wza_{CA} were used to transform CWG281 ($wza_{22\ min}$::*aadA* wza_{K30}::*aacC1*). K30 polysaccharide synthesis was assessed by Western immunoblotting of polysaccharide preparations probed with anti-K30 polysaccharide antiserum (anti-K30 PS), and surface expression of K30 CPS was determined by examination of cationized ferritin-stained whole cells by electron microscopy. Size bars = 0.5 μm.

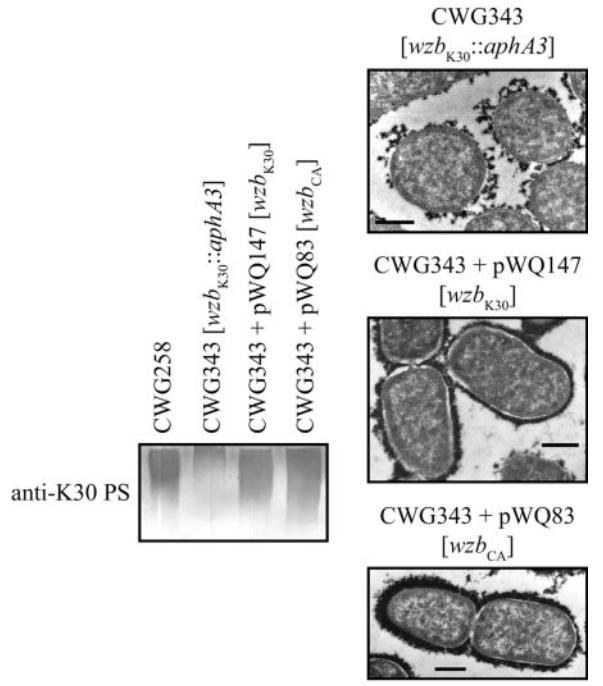

FIG. 4. Wzb_{CA} is a functional homologue of Wzb_{K30}. Complementation of CWG343 ($wza_{22\ min}$::*aadA* wzb_{K30}::*aphA3*) was achieved using plasmids carrying either wzb_{K30} or wzb_{CA}. A Western immunoblot of polysaccharide preparations was used to assess K30 polysaccharide synthesis, while K30 CPS surface expression was visualized by electron microscopy. Size bars = 0.5 μm. Anti-K30 PS, anti-K30 polysaccharide antiserum.

cise reason for the difference in PAGE migration patterns. However, despite this difference in phenotype, the Wza-Wzb-Wzc proteins for colanic acid expression can effectively interface with the remaining K30 polymer synthesis and export machinery in CWG655 to produce a capsule.

Wza shows no specificity towards the translocated polymer. Wza proteins involved in the assembly of group 1 CPSs are highly conserved among different serotypes (50). This conservation makes it unlikely that Wza proteins recognize the polymer being transported but does not preclude the possibility that differences between a CPS and an EPS polymer could affect Wza function or that Wza function is influenced by the presence or absence of Wzi. To begin to address these questions, Wza_{K30} and Wza_{CA} were expressed in an *E. coli* K30 *wza* mutant (CWG281), and complementation of the defect was assessed. Complementation with either Wza_{K30} or Wza_{CA} restored K30 CPS synthesis and surface expression (Fig. 3). The higher apparent M_rs of the polysaccharide products seen upon complementation of the CWG655 [Δ(*wza-wzb-wzc*)$_{K30}$] mutant with the colanic acid genes (Fig. 2) were not evident in complementation of the *wza* single mutant (Fig. 3).

A Wzb phosphatase for EPS synthesis functions in a CPS system. To determine whether the Wzb phosphatase involved in colanic acid assembly could functionally replace that of *E. coli* K30, Wzb_{K30} and Wzb_{CA} were individually expressed in CWG343 ($wza_{22\ min}$::*aadA* wzb_{K30}::*aphA3*). In CWG343, residual HMW K30 polysaccharide of higher apparent M_r than the wild type was detected in Western immunoblots probed with anti-K30 polysaccharide antiserum, and a small amount of electron-dense material was seen on the surfaces of cells in micrographs (Fig. 4). Expression of either Wzb_{K30} or Wzb_{CA} in CWG343 restored synthesis of K30 CPS (with comparable apparent M_rs in PAGE analysis) and surface expression to a phenotype indistinguishable from that of the parent strain (Fig. 4).

Wzc_{CA} must be coexpressed with Wza_{CA} to function in K30 CPS assembly. In *E. coli* K30, complementation of CWG285 ($wza_{22\ min}$::*aadA* wzc_{K30}::*aacC1*) could be achieved by using plasmids expressing either Wzc_{K30} alone (62), Wza_{K30}-Wzb_{K30}-Wzc_{K30} (Fig. 5), or Wzb_{K30}-Wzc_{K30} (Fig. 6). In all cases, the polymer was detectable by probing Western immunoblots with anti-K30 polysaccharide antiserum and formed an electron-dense layer on the cell surface reminiscent of the wild-type capsule. In contrast, expression of Wzc_{CA} alone in this background did not restore K30 CPS synthesis, despite detectable, albeit low, levels of Wzc_{CA} expression and phosphorylation (Fig. 5). The low level of Wzc_{CA} produced may reflect proteolysis, as a smaller tyrosine-phosphorylated polypeptide was evident in this sample. Analysis of culture supernatants revealed that no polysaccharide was secreted from the cell and released into the growth medium (data not shown), consistent with the inability of Wzc_{CA} to function in K30 CPS synthesis

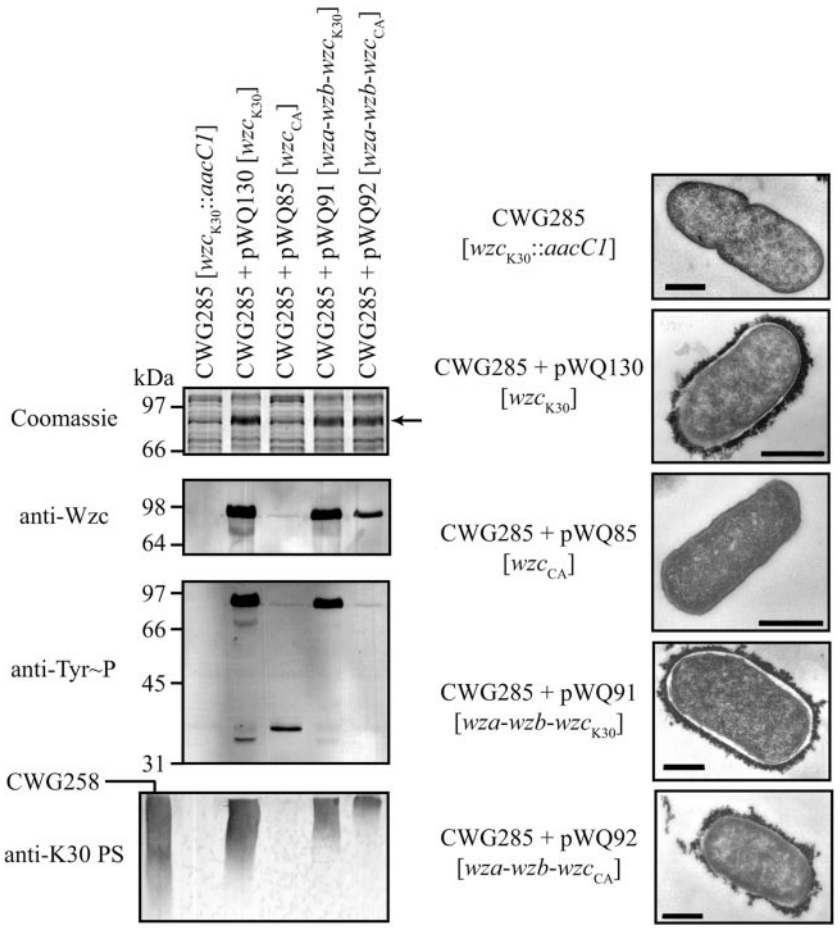

FIG. 5. Coexpression of Wza_{CA}, Wzb_{CA}, and Wzc_{CA} is required to restore K30 CPS synthesis and surface expression in a *wzc* mutant strain. Wzc_{K30} or Wzc_{CA} was expressed alone and in combination with its cognate Wza and Wzb proteins in CWG285 ($wza_{22\ min}$::*aadA* wzc_{K30}::*aacC1*). Expression of Wzc and Wzc~P in membrane fractions was detected by using Western immunoblots probed with anti-Wzc and antiphosphotyrosine (anti-Tyr~P) antibodies, respectively. Synthesis of K30 polysaccharide was assessed by Western immunoblotting of polysaccharide preparations probed with anti-K30 polysaccharide antiserum (anti-K30 PS), and surface-expressed K30 CPS was visualized by electron microscopy. Size bars = 0.5 μm.

rather than with a loss of the polymer's surface association. Simultaneous expression of Wza_{CA}, Wzb_{CA}, and Wzc_{CA} restored K30 CPS synthesis and surface expression in CWG285(pWQ92) (Fig. 5). As seen when *E. coli* CWG655 [Δ(*wza-wzb-wzc*)$_{K30}$] was complemented with the colanic acid derivatives (Fig. 2), the polymer had an altered migration pattern in SDS-PAGE, suggesting an increased average size or a reduction in the amount of K30 polysaccharide produced (Fig. 5). The amount of Wzc_{CA} detected was also greatly increased upon coexpression with Wza_{CA} and Wzb_{CA}, although the level of phosphorylation was low (Fig. 5). This increase in Wzc_{CA} could reflect altered transcript stability in the constructs or protection from the proteolytic degradation of Wzc_{CA} observed when it was expressed alone. While the former cannot be ruled out, it is certainly not the only explanation since the phosphorylated Wzc degradation product was no longer detected in CWG285(pWQ92) (Fig. 5).

The failure of Wzc_{CA} to restore CPS expression in CWG285 unless expressed with its cognate Wza and Wzb proteins raised the question of whether both the cognate outer membrane protein and the phosphatase were required for Wzc_{CA} activity or whether the requirement was specifically for one of these components. To address this, wzb_{CA} and wzc_{CA} were cloned into pBAD24 (pWQ90) and expressed in CWG285. The resulting strain produced significant amounts of K30 antigen, but this material was not present on the cell surface when the cells were examined by electron microscopy (Fig. 6). The accumulation of intracellular K30 polysaccharide in CWG285(pWQ90) represents only the second situation where polymer synthesis occurs in the absence of its surface assembly. While Wza and Wzc are thought to act late in the capsule assembly pathway, a characteristic feature of *wza* and *wzc* mutants is the absence of extensive polymer synthesis. In the case of a *wza* mutation, this is proposed to result from a feedback mechanism whereby cells unable to export polymers turn down polymer synthesis. A nonacylated Wza derivative that forms unstable outer membrane multimers lacks this regulatory control and produces a periplasmic polymer (43).

To examine the influence of Wza_{CA} coexpression on Wzc_{CA} function, CWG285 was transformed with pWQ98 (Wza_{CA}) and pWQ85 (Wzc_{CA}); this restored both K30 CPS synthesis and surface expression (Fig. 6). While the amounts of full-length Wzc and Wzc~P (Fig. 6) and the susceptibility to deg-

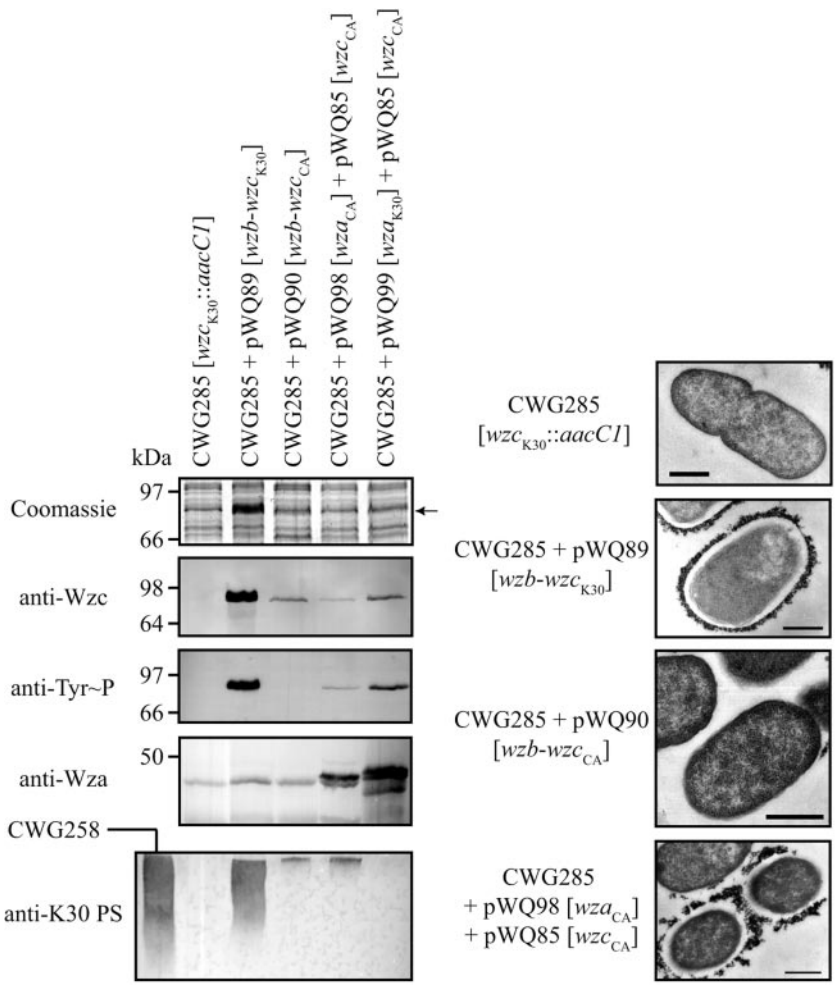

FIG. 6. Coexpression of Wza_{CA} and Wzc_{CA} is required to restore K30 CPS synthesis and surface expression in a *wzc*$_{K30}$::*aacC1* strain. Wzc_{K30} or Wzc_{CA} was expressed with its cognate Wza or Wzb protein in CWG285 (*wza*$_{22\ min}$::*aadA wzc*$_{K30}$::*aacC1*). Expression of Wza in cell lysates and Wzc and Wzc~P in membrane fractions was assessed by using Western immunoblots probed with the relevant antibodies. The two anti-Wza immunoreactive bands apparent upon overexpression of Wza result from inefficient processing of the precursor lipoprotein (17). In addition, degradation products of Wza_{K30} are often detected under high-level-expression conditions (unpublished results). K30 polysaccharide synthesis was assessed by Western immunoblotting of polysaccharides probed with anti-K30 polysaccharide antiserum (anti-K30 PS), and K30 CPS surface expression was detected by electron microscopy. Size bars = 0.5 μm. Anti-Tyr~P, antiphosphotyrosine.

radation (data not shown) were not altered drastically by coexpression with Wza_{CA}, synthesis (albeit in small amounts with higher apparent M_rs) and surface expression of K30 CPS were restored. To test whether Wzc_{CA} specifically required its cognate Wza protein, Wza_{K30} was coexpressed with Wzc_{CA}. K30 CPS synthesis was not restored in this strain (Fig. 6). In summary, while coexpression of Wzc_{CA} with either Wzb_{CA} or Wza_{CA} was sufficient to restore K30 polysaccharide synthesis, only coexpression with Wza_{CA} allowed expression of detectable levels of K30 CPS on the cell surface (Fig. 6).

Activity of chimeric Wzc proteins in K30 antigen synthesis and surface expression. Interactions between Wza_{K30} and Wzc_{K30} have been previously shown by using chemical cross-linking (43). Thus, the requirement for coexpression of Wza_{CA} and Wzc_{CA} to complement a *wzc* mutation could reflect specificity in interacting domains of these colanic acid biosynthesis proteins.

In an attempt to determine which domains of Wzc_{CA} were responsible for the specific requirement for Wza_{CA}, chimeric Wzc proteins were constructed that expressed portions of both the K30 and CA proteins. The goal was to generate chimeric Wzc proteins that could interface productively with Wza_{K30}. A region of six identical residues located immediately C-terminal to the predicted end of the second transmembrane domain of Wzc was chosen as the breakpoint in order to isolate the functions of the periplasmic domain from those of the C-terminal kinase domain. A derivative [$Wzc_{K30(1–455)CA}$ (pWQ87)] was constructed that expressed the N-terminal 455 amino acids of Wzc_{K30} (this includes both transmembrane domains) and the C-terminal 268 amino acids of Wzc_{CA} (including Walker motifs A and B and the tyrosine-rich C terminus) (Fig. 7). The second derivative [$Wzc_{CA(1–453)K30}$ (pWQ88)] was the reciprocal chimera, containing the N-terminal portion of the colanic acid protein and the C-terminal portion of the K30 protein (Fig. 7).

The amounts of the Wzc chimeras produced in CWG285 (*wza*$_{22\ min}$::*aadA wzc*$_{K30}$::*aacC1*) differed dramatically. While the $Wzc_{K30(1–455)CA}$ (pWQ87) derivative was evident in significant

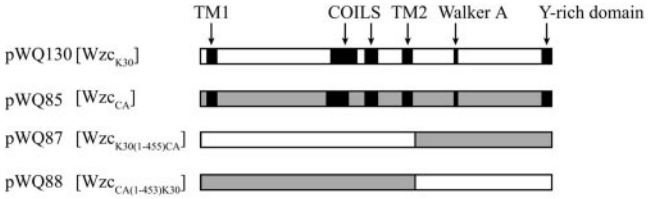

FIG. 7. Composition of chimeric Wzc proteins. Full-length Wzc_{K30} (white) and Wzc_{CA} (gray) are illustrated, with the positions of the transmembrane domains (TM), coiled-coil motifs (COILS), and Walker A and tyrosine (Y)-rich motifs indicated. The fusion points of the chimeric Wzc proteins are indicated, with gray shading to indicate portions of the colanic acid derivative and white to indicate regions of Wzc_{K30}.

amounts in both Coomassie-stained gels and Western immunoblots, the corresponding amount of $Wzc_{CA(1–453)K30}$ (pWQ88) was much lower (Fig. 8). This reflected the extensive degradation of $Wzc_{CA(1–453)K30}$, consistent with the proteolytic susceptibility of Wzc_{CA} described above. The phosphorylation levels of these chimeric proteins appeared to correlate with amounts of full-length Wzc (Fig. 8). While both chimeric Wzc proteins were able to restore polymer synthesis in CWG285, K30 CPS was not detected on the cell surface (Fig. 8), suggesting that K30 polysaccharide was accumulating within the cells. To confirm this, cells of CWG285 expressing the chimeric proteins were permeabilized prior to antibody labeling for immunofluorescence microscopy. Unlike the intact cells, the permeabilized cells showed a strong fluorescent signal (Fig. 8). Cells expressing $Wzc_{CA(1–453)K30}$ (pWQ88) were also elongated, suggesting that expression of this chimeric protein had additional detrimental effects on cellular physiology. Given that intracellular polysaccharide was detected in strains expressing either chimera, the altered cellular morphology observed upon expression of $Wzc_{CA(1–453)K30}$ (pWQ88) did not appear to be a simple consequence of intracellular polymer accumulation. Derivatives with the elongated cell shapes still produced smooth serotype O9a lipopolysaccharide (data not shown).

Chimeric Wzc proteins also require coexpression with Wza for proper function. The failure of the $Wzc_{K30(1–455)CA}$ and $Wzc_{CA(1–453)K30}$ chimeras to complement a *wzc* defect may indicate a requirement for features of both the N- and C-terminal domains for function in a given system or be indicative of improper protein folding. However, given that coexpression of Wzc_{CA} with Wza_{CA} was necessary to restore CPS expression in CWG285, it was of interest to determine if coexpression of the chimeras with elevated levels of their cognate Wza protein could restore surface expression of K30 CPS. Coexpression of $Wzc_{K30(1–455)CA}$ (pWQ87) with either of the Wza proteins restored surface expression of K30 CPS (Fig. 9 and 10). It is unclear why this chimera was active only when Wza was overexpressed since the wild-type Wzc_{K30} functions effectively in the presence of chromosomal levels of Wza expression (Fig. 5). One possible explanation is that this Wzc chimera has lower affinity for Wza_{K30}, which could be overcome by higher-level expression of the outer membrane component. This was in contrast to the results obtained with $Wzc_{CA(1–453)K30}$ (pWQ88). Strong fluorescent signals for surface-expressed CPS were detected only when the chimera was coexpressed with Wza_{CA} (Fig. 9 and 10). These complemented cells had a morphology that more closely resembled that of the parent strain. In contrast, the cells expressing $Wzc_{CA(1–453)K30}$ (pWQ88) and Wza_{K30} still appeared elongated and indistinguishable from cells expressing $Wzc_{CA(1–453)K30}$ alone (Fig. 10). Small amounts of very faint and poorly distributed fluorescence were detected on the surfaces of some of the elongated cells. Given that the chimera alone did not yield any surface fluorescence, this is not likely a result of "leaky" cells but rather of an extremely low efficiency of export.

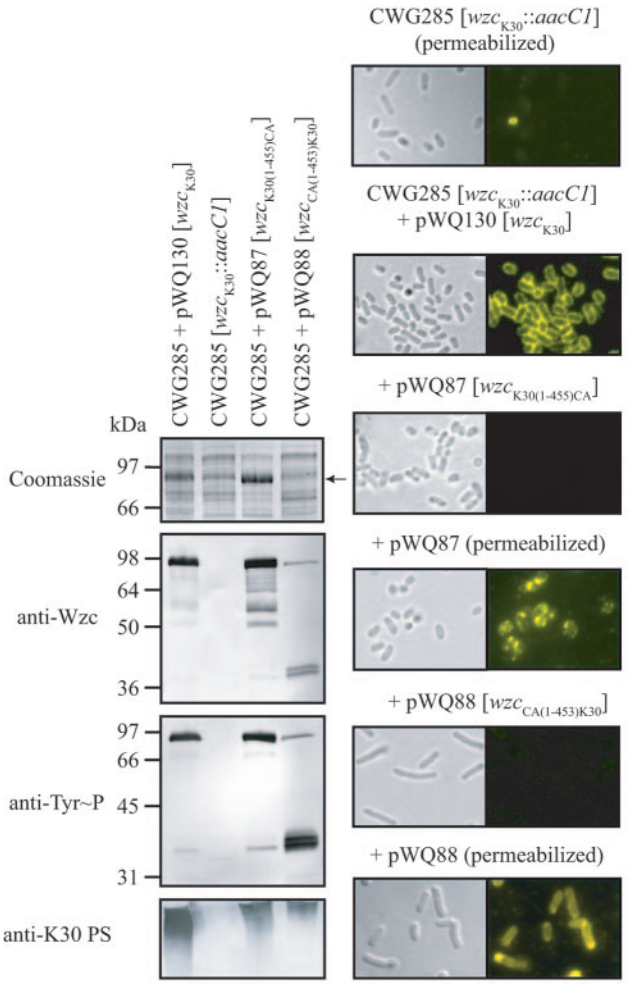

FIG. 8. Chimeric Wzc proteins cannot restore K30 CPS surface expression in *E. coli* CWG285. Chimeric Wzc proteins from pWQ87 and pWQ88 were expressed in CWG285 ($wza_{22\ min}$::*aadA* wzc_{K30}::*aacC1*). The expression levels of Wzc and Wzc~P in membrane fractions were assessed by using anti-Wzc and antiphosphotyrosine (anti-Tyr~P) antibody-probed Western immunoblots, respectively. The synthesis of K30 polysaccharide was assessed by Western immunoblotting of polysaccharide samples probed with anti-K30 polysaccharide antiserum (anti-K30 PS), and the presence of polymers on the surface was assessed by immunofluorescence microscopy (where indicated, cells were permeabilized prior to antibody labeling).

DISCUSSION

The presence of homologues of Wza, Wzb, and Wzc in assembly systems for group 1 CPSs and group 1-like EPSs, as well as homologues of Wzc in CPS systems in gram-positive bacteria, raises the question of whether these proteins are functionally conserved. Wza_{CA}, Wzb_{CA}, and Wzc_{CA} could collectively function to restore K30 CPS synthesis and surface

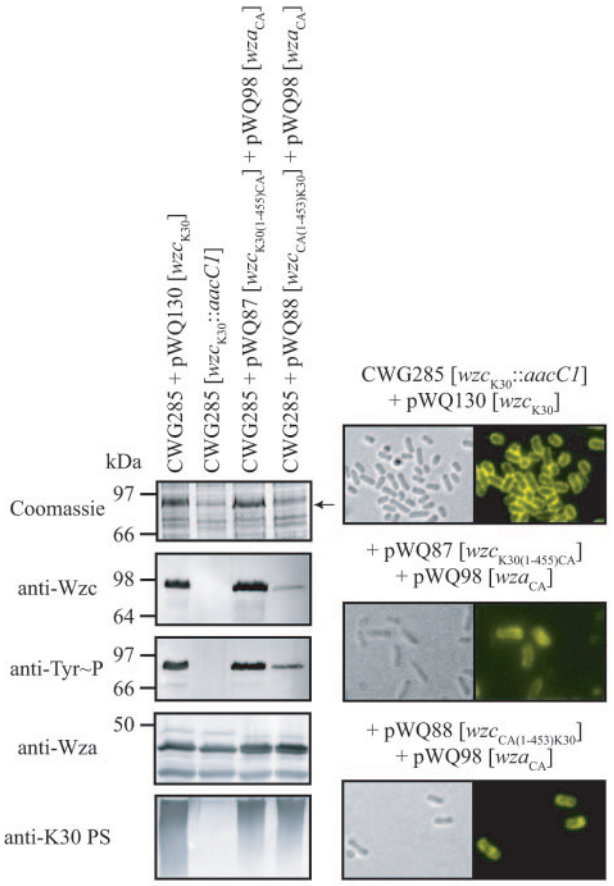

FIG. 9. Coexpression of chimeric Wzc proteins with Wza_{CA} restores surface expression of K30 CPS. Wza_{CA} (from pWQ98) was coexpressed in CWG285 with chimeric Wzc proteins expressed from pWQ87 and pWQ88. Expression of Wza in cell lysates and Wzc and Wzc~P in membrane fractions was assessed by using Western immunoblots probed with the relevant antibodies. Synthesis of K30 polysaccharide was determined by Western immunoblotting of polysaccharide preparations probed with anti-K30 polysaccharide antiserum (anti-K30 PS), and surface-expressed K30 CPS was detected by immunofluorescence microscopy. Anti-Tyr~P, antiphosphotyrosine.

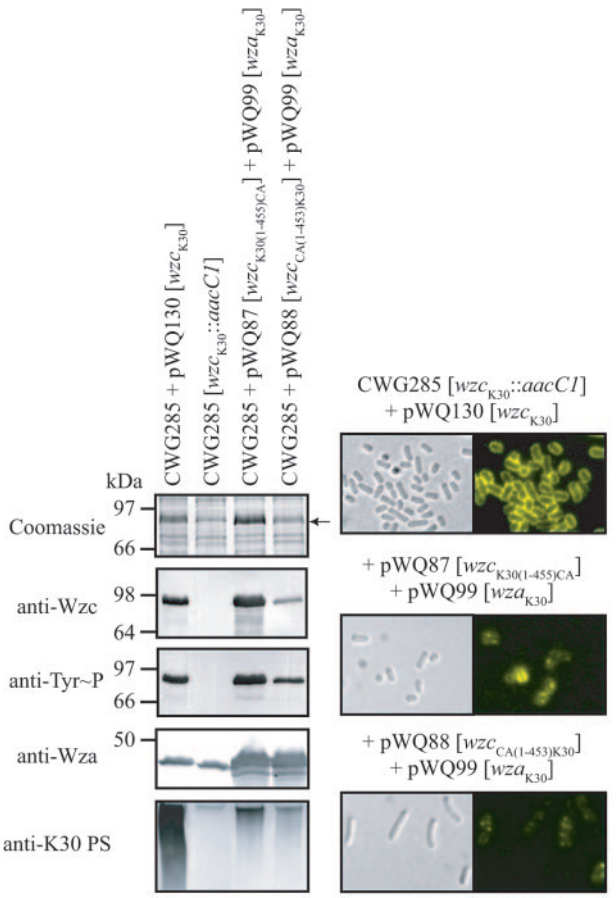

FIG. 10. Coexpression of $Wzc_{K30(1–455)CA}$ with high levels of Wza_{K30} restores surface expression of K30 CPS. The amount of Wza_{K30} in CWG285 was increased through the introduction of plasmid-expressed Wza_{K30} from pWQ99. Chimeric Wzc proteins were introduced into this strain, and Western immunoblots were used to determine the levels of Wza in cell lysates and Wzc and Wzc~P in membrane fractions. A Western immunoblot of polysaccharides was used to assess K30 polysaccharide synthesis, and immunofluorescence microscopy was used to detect K30 CPS on the cell surface. Anti-Tyr~P, antiphosphotyrosine; anti-K30 PS, anti-K30 polysaccharide antiserum.

expression in a $\Delta(wza\text{-}wzb\text{-}wzc)_{K30}$ strain, suggesting that the overall function of these proteins is conserved between the K30 CPS and the colanic acid EPS systems.

The ability of Wza_{CA} and Wzb_{CA} to restore K30 CPS synthesis and surface expression in *E. coli* K30 mutant strains is consistent with a shared function of these proteins in CPS and EPS systems. Thus, any essential protein-protein interactions involving components of the K30 assembly system were not compromised by the involvement of the colanic acid homologues. Restoration of K30 CPS surface expression in the *wza* mutant supports the idea that the outer membrane channel does not recognize the polymer being transported and is consistent with the high conservation of *wza* sequence in isolates with different group 1 serotypes (50). To the extent that the phenotypes can be examined with the available methods, the Wza homologues have indistinguishable functions in assembling capsules comprising CPSs with similar size distributions. The finding that Wzb_{CA} could function in the K30 system was also not surprising, given that Wzb proteins from several organisms have been shown to dephosphorylate a number of exogenous substrates (7, 21, 42, 58).

Vincent and coworkers have proposed that Wzc_{CA}~P is a negative regulator of colanic acid expression in *E. coli* K-12 (59). This conclusion is based on decreased colanic acid production in a *wzb* mutant strain. Given that colanic acid-producing strains previously used to study Wzc_{CA} function (59) overexpressed RcsA (a positive regulator of the colanic acid *cps* locus), these results could reflect the involvement of other unidentified cellular components. In fact, the Rcs system is now known to have many effects on cellular physiology (18, 24). However, in *E. coli* K30, both phosphorylation of Wzc_{K30} and expression of Wzb_{K30} are essential for wild-type capsule formation, suggesting that the relationships are more complex and that Wzc_{K30} may need to cycle between phosphorylated and dephosphorylated forms (62). The results presented here suggest that Wzc_{K30} and Wzc_{CA} generally function in the same manner within the context of the K30 CPS biosynthesis system,

given that at least some phosphorylation of Wzc_{CA} was detected in strains competent for K30 CPS synthesis and surface expression. The complex nature of this system and the use of complementation experiments that disturb the natural stoichiometry of the proteins preclude conclusions regarding the "active" form of Wzc or interpretations of differences in the amounts and apparent M_rs of the products formed.

Wzc_{CA} showed a specific requirement for its cognate Wza_{CA} protein. In the absence of Wza_{CA}, Wzc_{CA} was unable to support surface expression of K30 CPS. These results are consistent with specific interactions between Wzc_{CA} and Wza_{CA}; such interactions have been established by chemical cross-linking in the K30 system (43). Coupled with the outer membrane location of Wza, the data from chimeric Wzc proteins implicate the periplasmic domain of Wzc_{CA} in specific interactions with Wza_{CA}. Neither Wzc_{CA} nor the chimeric Wzc protein harboring the periplasmic domain of Wzc_{CA} [$Wzc_{CA(1–453)K30}$] was able to restore surface expression of K30 CPS in CWG285 in the absence of overexpressed Wza_{CA}. Coexpression of $Wzc_{CA(1–453)K30}$ with Wza_{CA} significantly reversed the aberrant cellular morphology (elongation) evident upon expression of this chimera in CWG285. Although it is possible that this reversal in cell morphology reflects in part the relief of intracellular polymer accumulation, it is important that cells showed normal morphology when the reciprocal chimera was expressed alone, despite also producing intracellular K30 polysaccharide. The complexity of cell wall growth and cell division makes it difficult to unequivocally explain the elongation defect. However, the inability to provide a definitive explanation for the elongated shape does not detract from the central conclusion that Wzc_{CA} must be coexpressed with its cognate Wza protein in order to function within the K30 CPS biosynthesis and assembly system.

In the absence of additional Wza, the chimeric Wzc proteins both restored K30 polysaccharide biosynthesis but could not restore the surface expression of K30 polysaccharide. Significantly, the expression of Wzc_{CA} in CWG285 did not result in detectable polymer synthesis. The collective data therefore suggest that the chimeras are able to uncouple the apparent feedback that turns off polymer synthesis when the final product cannot be translocated to the cell surface. It is known from previous studies that in the absence of Wza, the CPS biosynthesis machinery exhibits reduced output similar to that of the *wzc* mutant and that some Wza derivatives can overcome this feedback process without facilitating export and capsule assembly on the cell surface (43). The partnership between Wza and Wzc therefore appears to be important in maintaining the feedback control, although the precise mechanism is still unknown. These observations might indicate two roles for Wzc, one involved in interaction with the K30 biosynthesis machinery and the other coupled to the export pathway. Whether phosphorylation of Wzc would be important in both stages remains to be determined.

The large periplasmic loop of Wzc is the logical site of interaction of Wzc with Wza. In Wzc proteins from *E. coli* K30 and K-12, this region is 374 amino acids long. Interestingly, homologues of Wzc in gram-positive bacteria have much shorter periplasmic loops (e.g., *S. pneumoniae* CpsC has a predicted 135-amino-acid loop). Given that homologues in gram-positive bacteria do not have to interact with an outer membrane protein, the longer periplasmic loop in gram-negative bacteria may have functional significance. However, it may not be possible to define interacting domains using primary sequence information alone, since Wzc proteins can oligomerize (15, 46) and such higher-order structures may create new biologically relevant domains. Conclusive mapping of the interacting domains of these proteins may require structural elucidation.

Overall, the data presented here reveal a conserved mechanism in the terminal steps of group 1 CPS and EPS expression and reflect the complexity of the system responsible for biosynthesis and assembly of K30 CPS. Furthermore, these data lend support to the existence of essential interactions in a multienzyme complex coordinating polymer biosynthesis and surface expression.

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