The C-terminal Domain of the Nucleotide-binding Domain Protein Wzt Determines Substrate Specificity in the ATP-binding Cassette Transporter for the Lipopolysaccharide O-antigens in Escherichia coli Serotypes O8 and O9a*§

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The polymannan O-antigenic polysaccharides (O-PSs) of Escherichia coli O8 and O9a are synthesized via an ATP-binding cassette (ABC) transporter-dependent pathway. The group 2 capsular polysaccharides of E. coli serve as prototypes for polysaccharide synthesis and export via this pathway. Here, we show that there are some fundamental differences between the ABC transporter-dependent pathway for O-PS biosynthesis and the capsular polysaccharide paradigm. In the capsule system, mutants lacking the ABC transporter are viable, and membranes isolated from these strains are no longer able to synthesize polymer using an endogenous acceptor. In contrast, E. coli strains carrying mutations in the membrane component (Wzm) and/or the nucleotide-binding component (Wzt) of the O8 and O9a polymannan transporters are nonviable under conditions permissive to O-PS biosynthesis and take on an aberrant elongated cell morphology. Whereas the ABC transporters for capsular polysaccharides with different structures are functionally interchangeable, the O8 and O9a exporters are specific for their cognate polymannan substrates. The E. coli O8 and O9a Wzt proteins contain a C-terminal domain not present in the corresponding nucleotide-binding protein (KpsT) from the capsule exporter. Whereas the Wzm components are functionally interchangeable, albeit with reduced efficiency, the Wzt components are not, indicating a specific role for Wzt in substrate specificity. Chimeric Wzt proteins were constructed in order to localize the region involved in substrate specificity to the C-terminal domain.

ATP-binding cassette (ABC)1 transporters, or traffic ATPases, are responsible for the import and export of a variety of molecules across membranes. In the Escherichia coli K-12 genome, 79 known or putative ABC transporters have been identified indicating the importance of this protein superfamily in cellular physiology (1). The prototypical transporter consists of four domains (two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs)), which may be organized in a variety of ways (2). The TMD components between different systems share low sequence similarity and contain a variable number of transmembrane segments, whereas the NBD proteins of different systems share a higher overall sequence similarity as a result of the conserved sequence motifs required for ATP hydrolysis (3). X-ray structures are available for a number of NBD proteins as well as four complete transporters (reviewed in Refs. 4–7). The structures reveal a common organization of the NBD monomers into two subdomains: a RecA-like domain and a helical domain not found in other ATP-hydrolyzing proteins (4). The ATP-binding site is found along the NBD dimer interface and is made up of conserved residues from each monomer, namely the Walker A motif from one monomer and the ABC signature motif from the other monomer (8). Because the ABC transporter TMD components differ greatly between systems, whereas the NBD components remain highly similar, ABC transporters have been said to consist of a substrate-specific membrane channel powered by a common ATP-hydrolyzing engine (3, 9).

ABC transporter substrate specificity in periplasmic solute-binding protein-dependent systems involves the solute-binding protein. This protein acts as a receptor and is required for high affinity substrate import (10). In mutant ABC systems, which no longer require the solute-binding protein for substrate import, substrate specificity is maintained due to binding sites located in the TMD component of the transporter (11–13). The solute-binding proteins are believed to play an additional role in transport by transmitting a signal across the membrane, thus stimulating ATP hydrolysis by the NBD subunits (14, 15). Substrate-binding sites in many ABC efflux systems are also located in the TMD component of the transporter, with the eukaryotic multidrug transporter P-glycoprotein and its wide range of hydrophobic substrates, serving as a well studied example (16, 17). However, in the secretion of the soluble protein hemolysin in E. coli, there is evidence for an interaction between the substrate and the NBD portion of the transporter (18), as might be anticipated by the direction of transport.

The group 2 capsular polysaccharides of E. coli serve as the prototype system for polysaccharide export via an ABC transporter. In this system, the TMD component is designated KpsM, and the NBD component is designated KpsT. Initial evidence for the involvement of this ABC transporter in the export of capsule came from the observation that mutants in

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§ The on-line version of this article (available at http://www.jbc.org) contains Tables 2 and 3.

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either kpsM or kpsT accumulated cytoplasmic polysaccharide (19–21). In the capsule system, all available evidence suggests that the proteins involved in capsule export operate independent of the repeat unit structure of the polymeric substrate (22–27). The export apparatus is functionally interchangeable between different capsule serotypes, even between different bacterial species, indicating that a conserved motif is recognized. Group 2 capsules are modified at the reducing terminus with a lipid moiety prior to export from the cytoplasm (28). However, the specific gene products responsible for this modification are as yet unidentified and remain an area of controversy (29–31). In the current model for export of group 2 capsules, the reducing terminal lipid modifications are believed to provide the conserved moiety recognized by the export apparatus (32).

The O-antigenic polysaccharide (O-PS) portions of the lipopolysaccharide molecules in E. coli serotypes O8 and O9a (and its minor variant O9a) serve as the prototypes for O-PS synthesis via an ABC transporter-dependent pathway (reviewed in Ref. 33). Both antigens are homopolymers of mannose and are distinguished by the number of mannose residues and linkages in the repeat units (Fig. 1A). The genetic loci responsible for O-PS synthesis in these serotypes encode proteins required for GDP-mannose synthesis, polymer synthesis, termination, and export (Fig. 1B). The current working model for this system is outlined in Fig. 2. The product of the wecA gene primes synthesis through the transfer of a GlcNAc-1-phosphate to the undecaprenol phosphate carrier lipid. The wecA gene is located outside of the O-PS biosynthesis locus and is involved in synthesis of other polysaccharides (34). The products of the wbdA, wbdB, and wbdC genes, located in the O-PS biosynthesis cluster, are mannoyltransferases responsible for O-PS polymerization (35). The WbdD enzymes of the E. coli O8 and O9a biosynthesis systems are required for the addition of the nonreducing terminal modifications to the nascent polymer (36). In the O8 serotype, this modification is a 3-O-methyl group, whereas in O9a, WbdD acts as both a kinase and a methyltransferase. The addition of these terminal residues is required not only for polymerization termination and chain-length regulation but also for the export of O-PS from the cytoplasm (36). The ABC transporter required in this system consists of Wam (TMD; similar to KpsM) and Wat (NBD; similar to KpsT), consistent with the domain architecture of other ABC transporters (35).

Here we provide evidence that, unlike the capsule exporter prototype, O-PS exporters are not functionally interchangeable, and we demonstrate that Wzt, the NBD portion of the ABC transporter, imparts substrate specificity.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are described in Table I. Strains and plasmids used only in cloning and mutagenesis are described in supplementary materials. Bacteria were grown at 37 °C in either LB medium (37) or M9 minimal medium (38). Media were supplemented with glucose (0.4% w/v), mannose (0.2% w/v), sucrose (5% w/v), glycerol (0.2% v/v), arabinose (0.4%), histidine (22 μg/ml), tryptophan (20 μg/ml), or thiamine (1 μg/ml), as required. The antibiotics ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), gentamycin (15 μg/ml), kanamycin (50 μg/ml), and tetracycline (15 μg/ml) were added where appropriate. All mutant strains carried a defect in β-mannose-6-phosphate aldolase-ketose-isomerase (EC 5.3.1.8), making GDP-mannose (and polymannan) synthesis dependent on the addition of mannose to the growth medium (36). Strains containing wzm and/or wzt mutations were maintained on medium lacking mannose, because secondary mutations were selected at high frequency when polymannan synthesis occurred in the absence of transport.

**DNA Methods**—Custom oligonucleotide primers were obtained for use in PCR from Sigma. Template DNA for use in PCR was purified using the DNAzol reagent (Invitrogen) and a modified protocol for purification of DNA fragments from agarose gels was carried out using the Ultraceulate15 DNA purification kit (MOBIO Laboratories). Alternatively, the Qiaquick PCR purification kit (Qiagen) was used to purify DNA fragments directly from PCR reactions or restriction digestions. Plasmid DNA was purified using the GeneElute plasmid purification kit (Sigma). Restriction digestions and DNA ligation reactions were performed as per the manufacturer’s instructions. DNA sequencing was performed by the Guelph Molecular Supercenter (University of Guelph, Guelph, Canada).

**Cloning of wzm and wzt from E. coli O8 and O9a**—The genes encoding the Wzm and Wzt proteins from both E. coli O8 and O9a were cloned separately and in tandem for use in complementation experiments. The relevant PCR products were amplified from the chromosomes of E. coli 2775 (O8), E69 (O9a). E. coli B38337–41 (K5) was the source of kpsM and kpsT. Restriction sites were designed into the PCR primers to facilitate cloning behind the arabinose-inducible promoter of either pBAD24 or pBADHisA (40) (Invitrogen). Primer sequences and features are described in supplementary material.

**Construction of Plasmids Encoding Chimeric Wzt Proteins**—Chimeric Wzt proteins were generated using an overlapping PCR protocol (41, 42). In the creation of pWQ337, primers LC33 and LC37 were used...
**TABLE I**

**Bacterial strains and plasmids**

| Strain/Plasmid | Description or genotype | Reference or source |
|----------------|-------------------------|---------------------|
| **Strains**    |                         |                     |
| E69            | *E. coli* O9a:K30       | F. Orskov           |
| CWG2084       | E69 derivative; O9a:K30 F. Orskov |
| CWG672        | CWG2084 derivative; manA; Sm'; Te' |
| CWG638        | CWG672 derivative; manA; aacC1; Sm', Gm' |
| CWG708        | CWG638 derivative; manA; aacC1; Sm', Te', Kmr |
| CWG709        | CWG638 derivative; manA; aacC1; Sm', Te', Kmr |
| 2775          | *E. coli* O8:K40        | E. Jann             |
| CWG291        | 2775 derivative; O8:K40 F. Orskov |
| CWG636        | CWG291 derivative; manA; Gm' |
| CWG710        | CWG636 derivative; manA; aacC1; Gm', Sm', Te', Kmr |
| CWG711        | CWG636 derivative; manA; aacC1; Gm', Sm', Te', Kmr |
| RS218         | E. coli O18ac-K1        | R. P. Silver       |
| EV36          | K-12-K1 hybrid strain; galP23 rpsL9 argA' rha' kpsM |
| RS2604        | EV36 derivative; kpsM |
| RS2436        | EV36 derivative; kpsT |
| Bi8337-41     | *E. coli* O18ac-K5     | I. Orskov           |
| **Plasmids**  |                         |                     |
| pWQ331        | pBAD24 derivative containing an XbaI/PstI fragment encoding Wzm-Wzt (O8): Ap' |
| pWQ332        | pBAD24 derivative containing an EcoRI/KpnI fragment encoding Wzm-Wzt (O9a): Wzt |
| pWQ333        | pBADHisA derivative containing an Xhol/KpnI fragment encoding Wzt (O9a): Ap' |
| pWQ334        | pBADHisA derivative containing an Xhol/KpnI fragment encoding Wzt (O9a): Ap' |
| pWQ335        | pBAD18-Cm derivative containing an XbaI/HindIII fragment encoding Wzm (O8): Cm' |
| pWQ336        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding Wzm (O8): aCm |
| pWQ337        | pBAD24 derivative containing an XbaI/Sphl fragment encoding a chimeric Wzt |
| pWQ338        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
| pWQ339        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
| pWQ340        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
| pWQ341        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
| pWQ342        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
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| pWQ344        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |
| pWQ345        | pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt |

**FIG. 2. Working model for O8 and O9a biosynthesis.** Synthesis begins on the cytoplasmic face of the inner membrane on the carrier lipid undecaprenol phosphate and is initiated by the transfer of a GlcNAc-1-phosphate by WecA. The WbdABC mannosyltransferases then synthesize the polymannan O-PS through the sequential addition of mannose residues, from the GDP-mannose donor to the nonreducing terminus (step 1). WbdD action causes termination of polymannan growth through the addition of a methyl group in the *E. coli* O8 or a phosphate and a methyl group in the O9a serotype (step 2). The completed undecaprenol pyrophosphate-linked polymer is transported to the periplasmic face of the inner membrane by an ABC transporter consisting of Wzm and Wzt (step 3). The O-PS is then transferred to a preformed molecule of lipid A core in a reaction involving WaaL (step 4).
to amplify the 5' region of wzto8 (encoding amino acids 1–61). LC33, used in initial amplification, was designed to anneal upstream of wzt in order to reduce the size of the amplified fragment. Using this primers, the LC33 fragment was found to be manageable for purification. LC37 was designed to include a 25-bp overhang containing sequence corresponding to wzto8. Primers LC17 and LC35 were then used to amplify DNA encoding amino acids 61–431 of WztO9a. Primer LC35 was designed to include 25 bp of sequence corresponding to wzto8. The resulting PCR fragments were annealed and used as template in a third PCR. The full-length chimeric open reading frame was then further amplified with primers LC40 and LC17, designed to contain EcoRI and SphI sites, respectively, to facilitate cloning in pBADC4. Additional chimeras (Table I) were made using a similar strategy. The primers used are described in supplementary material. Plasmid pWQ432 was created using a different approach. Primer LC62 (encoding amino acids 394–404 of WztO8) was used in conjunction with LC42 to amplify the mutated WztO9a for ligation into pBAD24.

Construction of Chromosomal Insertion Mutations—Chromosomal insertion mutants E. coli CWG713 (wzm-wztO8), CWG710 (wzmO8), CWG711 (wzmO9a), CWG709 (wzm-wztO8) and CWG708 (wzm-wztO9a) were created through allelic replacement using the suicide delivery vector pRE112 (43). Insertion mutants were selected on M9 (glucose, sucrose, kanamycin, tetracycline) agar at room temperature and confirmed by PCR using primers DNA polymerase (Invitrogen). Plasmid pWQ432 was used in allelic replacement of wzm-wztO8 to create CWG713. Primer pairs LC30/LC32 (MluI) and LC6 (XhoI)/LC4 were used to amplify the first 295 bp of wzmO9a. Primer LC35 was designed to include 25 bp of sequence found in all ABC transporters (4). As expected, these primers used were described in supplemental material. Plasmid pWQ432 was constructed using a different approach. Primer LC62 (encoding amino acids 394–404 of WztO8) was used in conjunction with LC42 to amplify the mutated WztO9a for ligation into pBADC4.

Substrate Specificity of O-antigen Export

The wzt homologues from the O8 and O9a serotypes are predicted to encode proteins of 404 and 431 amino acids, respectively, and share 63% identity (83% similarity). An alignment of the E. coli O8 and O9a Wzm proteins with KpsM from E. coli K1 (Fig. 3) shows that the O-PS exporter homologues share only limited primary sequence similarity with the capsule exporter. However, hydropathy plots for the three ABC transporter TMDs are very similar, with all three proteins predicted to contain six membrane-spanning segments at similar positions (data not shown).

The wzto8 and wzto9a contain a C-terminal region not present in either KpsT1K1 or KpsT5K5. The wzto genes from E. coli O8 and O9a are predicted to encode protein homologues of 264 and 261 amino acids, respectively, and share 63% identity (83% similarity). An alignment of the E. coli O8 and O9a Wzm proteins with KpsM from E. coli K1 (Fig. 3) shows that the O-PS exporter homologues share only limited primary sequence similarity with the capsule exporter. However, hydropathy plots for the three ABC transporter TMDs are very similar, with all three proteins predicted to contain six membrane-spanning segments at similar positions (data not shown).

The wzto genes from the O8 and O9a serotypes are predicted to encode proteins of 404 and 431 amino acids, respectively. Over their entire length, the two proteins share 43% identity (62% similarity). Examination of an alignment (Fig. 4) shows higher similarity in the N-terminal region of the proteins (80% similarity) than in the C-terminal region (40% similarity). The N-terminal region contains sequence motifs, including the Walker A and B motifs and the ABC signature sequence found in all ABC transporters (4). As expected, these sequence motifs are also conserved in KpsT1K1. Comparison of the amino acid sequences of WztO8, WztO9a, and KpsT5K5 reveals that the variable C-terminal domain present in the O8 and O9a Wzt homologues is absent from KpsT5K5.
The E. coli O8 and O9a Transporters Are Not Able to Function in the Export of E. coli K1 Capsular Polysaccharide—Previous data suggest that the E. coli K1 and K5 export machinery are functionally interchangeable, despite differences in the structure of their polymeric substrates (24). This observation has since been extended to cross-species complementation (22, 23, 27). The KpsM and KpsT homologues that make up the ABC transporter in the K1 and K5 systems are highly similar (47, 48). The KpsM homologues from the two systems share 98% identity (100% similarity), and the KpsT homologues share 71% identity (88% similarity). Some of the published complementation experiments with group 2 capsule ABC exporters were done with DNA fragments carrying additional genes. To confirm the results and test whether the individual KpsM and KpsT homologues (rather than the entire export apparatus) were functionally exchangeable, kpsM<sub>K5</sub> and kpsT<sub>K5</sub> were cloned behind an arabinose-inducible promoter and expressed in E. coli RS2604 (kpsM<sub>K1</sub>) and RS2436 (kpsT<sub>K1</sub>), respectively. Transformed cells were then examined for sensitivity to the K1F phage. K1F is a lytic phage that requires a polysialic acid capsule receptor for infection (49, 50). Sensitivity to K1F is therefore indicative of surface expression of the E. coli K1 polysaccharide. The K5 KpsM and KpsT homologues were able to function in the export of K1 polysaccharide as determined by the restoration of K1F sensitivity in the K1 mutants (Fig. 5).

The ability of the E. coli O8 and O9a transporter homologues to function in place of KpsM<sub>K1</sub> and KpsT<sub>K1</sub> was tested by expression of the cloned genes in RS2604 and RS2436. Neither the O8 nor the O9a wzm and wzt genes were able to function in the export of the K1 polysaccharide (Fig. 5), providing the first direct evidence for possible differences in the mechanism of polysaccharide recognition and/or export between the O-PS and capsule transporters.

Mutations in wzm and wzt Result in Growth Inhibition in both E. coli O8 and O9a—In order to examine whether the E. coli O8 and O9a ABC transporters were functionally interchangeable, chromosomal deletion mutants were made in wzm<sub>wtz</sub>, wzm, and wzt in both E. coli serotypes by allelic exchange. Since mutations in wbdD, which prevent polymer export from the cytoplasm, resulted in impaired cell growth, chromosomal mutations in wzm and wzt were made in strains deficient in D-mannose-6-phosphate aldose-ketose-isomerase activity, making O-PS synthesis conditional on the inclusion of mannose in the growth medium (36). Examination of growth in M9 medium shows a reduction in viability of CWG638 (wzm-wzt<sub>O9a</sub>) following the addition of mannose (Fig. 6A). A similar reduction was seen in CWG713 (wzm-wzt<sub>O8</sub>) (data not shown). Differential interference contrast images of CWG638 grown in the presence and absence of mannose show that cells adopt an elongated cell morphology when grown under conditions that were permissive for O-PS biosynthesis (i.e., presence of mannose) (Fig. 6B). Such mutants rapidly accumulate secondary (alleviating) mutations in polymannan synthesis genes (data not shown). Incorporation of radioactivity from GDP-[<sup>14</sup>C]mannose into isolated membrane fractions shows only a slight reduction in the E. coli O9a transporter knockouts relative to the parent strain (Fig. 6C). CWG672 (wbdA<sub>O9a</sub>) contains a mutation in one of the methyltransferases required for O9a polymer synthesis and does not incorporate radiolabel. The
radioactive incorporation seen in the wild type and ABC transporter mutants is therefore due only to incorporation of radiolabel into O9a polymer. A similar trend was seen for radioactive incorporation from GDP-[14C]mannose into membrane fractions for the corresponding E. coli O8 transporter knockouts (data not shown).

The ABC Transporters Required for E. coli O8 and O9a Antigen Export Are Serotype-specific—To determine whether the O8 and O9a transporters were functionally interchangeable, Wzm and Wzt were expressed from plasmid-encoded genes in trans in the E. coli O8 and O9a wzm-wzt backgrounds. O-PS synthesis was examined by SDS-PAGE and immunoblotting of LPS in whole-cell lysates (Fig. 7). Only data for the O9a complementation are presented; the results for the inverse complementation in O8 led to the same conclusion. A typical ladder pattern of high molecular weight smooth LPS in SDS-PAGE and immunoblotting requires ligation to lipid A core (51, 52). However, in the absence of export and ligation, intracellular O-PS can be identified by comparing immunofluorescence (IF) images of intact and permeabilized bacteria (Fig. 8). O9a polysaccharide could be detected by IF to be on the cell surface (Fig. 8A). In an O9a wzm-wzt chromosomal mutant (CWG638), polymer was not detected by SDS-PAGE, immunoblotting, or IF to intact cells (Figs. 7A and 8B). However, when CWG638 (wzm-wzt) cells were permeabilized, intracellular O9a polysaccharide could be detected by IF (Fig. 8C). When wzm-wzt was supplied in trans on pWQ335, O9a polysaccharide could again be detected on the cell surface, and normal cell morphology and growth were restored (Fig. 8F), indicating that no secondary mutations affecting O-PS synthesis were present in CWG638 (wzm-wzt). When wzm-wzt was supplied in trans on pWQ331, cell surface polymer could not be detected (Fig. 8D), but intracellular polymer remained (Fig. 8E). These results are consistent with the O8 and O9a transporters being O-antigen-specific. To test whether substrate specificity was a function of the TMD component of the transporter (Wzt), or both, plasmid-encoded copies of the genes were expressed in the O8 and O9a single mutant wzm and wzt backgrounds. As with exchange of the completed transporter, Wzt O9a was only able to function in the export of its cognate polymer (Figs. 7B and 8G–K). A wzm O9a mutant (CWG709) showed the same phenotype as the wzm-wzt O9a mutant; no O-PS was detected in SDS-PAGE, and intracellular polymer was detected by IF in permeabilized cells. However, exchange of the Wzm components resulted in the expression of cell surface polysaccharide. Surface expression of O-PS and normal cell growth were restored when wzm (pWQ336) was supplied in trans in CWG709 (wzm) (Fig. 8D). In contrast, expression of wzm (pWQ335) in trans restored surface expression of O-PS in CWG709 (wzm), but the cells remained elongated (Fig. 8N). Taken together, these results are consistent with the Wzm proteins being able to function (albeit with reduced activity) in the heterologous serotype and the NBD component of the transporter, Wzt, dictating O-PS specificity.

Chimeric Proteins Implicate the C-terminal Domain of Wzt in O-PS Recognition in E. coli O8 and O9a—To further localize
regions involved in substrate specificity in the *E. coli* O8 and O9a Wzt homologues, a series of plasmids expressing chimeric Wzt proteins were created for use in further complementation experiments (Fig. 9). The N-terminal 61 and 105 amino acids from WztO8 were exchanged with the corresponding amino acids from WztO9a in pWQ337 and pWQ338, respectively. In both cases, the chimeric open reading frame fully complemented an O9a *wzt* mutation (CWG708), indicating that this N-terminal portion of Wzt is not involved in serotype specificity. Exchange of the N-terminal 149 amino acids of WztO9a with the corresponding amino acids from WztO8 generated a construct (pWQ339) that was only able to partially restore an O9a *wzt* mutation (CWG708). In this case, O9a antigen was surface-expressed, but the bacteria retained the elongated cell morphology seen in the transport-deficient mutants (data not shown). The efficiency of complementation is therefore compromised. One interpretation of this result is that beyond a requirement for the C-terminal domain in substrate recognition, subtle differences in the more conserved N-terminal domain of Wzt could be required for specific interactions with the cognate Wzm. To test interactions between the cognate Wzm and Wzt proteins, the chimeric Wzt constructs were co-expressed in an O9a *wzm-wzt* mutant (CWG638) with WzmO8 (expressed from pWQ335). Expression of a chimeric Wzt protein containing the first 61 amino acids from WztO8 and the remainder of WztO9a (pWQ337) was able to only partially complement an O9a *wzm-wzt* mutation when co-expressed with WzmO8. Exchange of the first 105 amino acids of WztO9a with those from WztO8 (pWQ338) was, however, able to fully complement an O9a *wzm-wzt* mutation when co-expressed with WzmO9a, consistent with a region from the N-terminal portion of Wzt being involved in interactions with Wzm.

To examine the requirement of the extreme C-terminal domain of Wzt in substrate specificity, the last 14 and 60 amino acids of WztO9a were exchanged with the corresponding amino acids from WzmO8 (pWQ342 and pWQ343, respectively). Neither construct was able to function in the export of O9a antigen when expressed with either WzmO8 or WzmO9a, indicating a requirement for the larger C-terminal domain of Wzt in determining serotype specificity.

**DISCUSSION**

An ABC transporter-dependent pathway is responsible for the synthesis of many bacterial cell surface polysaccharides. Both capsular polysaccharides and O-PSs are synthesized by this pathway, with the two types of polysaccharides being distinguished by the involvement of lipid A core as the acceptor for O-PS and the processes involved in the later steps of translocation of polysaccharide from the periplasm to the cell surface (33, 53, 54). However, the essential features of the capsular polysaccharide and O-PS synthesis systems are conserved. The mechanism of polymer export across the inner membrane is relatively poorly understood in both pathways, although hypothetical models have been proposed (33, 55). In the case of group 2 capsules (*E. coli* K1 and K5), the current paradigm for polysaccharide transport via an ABC transporter, the transporter does not distinguish between different capsular polysaccharide substrates. In contrast, we provide evidence that the ABC transporter, specifically the NBD component (Wzt), required for O-PS export, is involved in substrate specificity in *E. coli* O8 and O9a. Thus, the two export systems are fundamentally different.

In biosynthesis of the *E. coli* K1 and K5 antigens, the apparatus required for capsule export is functionally interchangeable. The same is true for group 2 polysaccharide systems in different species (22–27, 57). All available evidence suggests that the exporter function is not influenced by differences in polymer structure. Here we extend the information by confirming that the individual components of the capsule ABC transporter (KpsM and KpsT) can also be exchanged between *E. coli* K1 and K5. The molecular basis for the conserved export signal in capsular polysaccharides has not been resolved. However, group 2 capsules are modified at the reducing terminus by phosphatidylinositol moieties prior to export from the cytoplasm. It is conceivable that this modification may represent a conserved moiety recognized by the ABC transporter (55).
Fig. 8. Immunofluorescence images using anti-O9a antibody of *E. coli* O9a transporter knockouts. Intact cells and cells permeabilized with lysozyme and Triton treatment (36) were examined. For each set of images, a differential interference contrast image is shown on the left, and the corresponding fluorescence image is shown on the right.

Fig. 9. Complementation of CWG708 (wzt*O9a*) and CWG638 (wzm-wzt*O9a*) + pWQ335 (wzm*O8) with chimeric Wzt proteins. A schematic diagram showing the key features of each chimeric construct is shown. Coding sequence from *E. coli* WztO8 is shown in gray, and sequence from WztO9a is shown in black. A plus sign indicates full complementation (i.e., surface-expressed O9a antigen in SDS-PAGE and IF experiments). A minus sign indicates that no cell surface polymer could be detected, O9a antigen remained intracellular, and the cells were elongated. A plus sign indicates full complementation (i.e., surface-expressed O9a antigen in SDS-PAGE and IF experiments). A minus sign indicates that no cell surface polymer could be detected, O9a antigen remained intracellular, and the cells were elongated.

Substrate Specificity of O-antigen Export
The fundamental differences in the capsule and O-PS systems are exemplified by the inability of Wzt-Wzt or the individual proteins to complement the analogous mutations in *E. coli* K1. In the converse experiments, defects in *E. coli* O8 and O9a could not be complemented with the *E. coli* K5 KpsM-KpsT, KpsM, or KpsT proteins (data not shown). The mechanisms of export for the group 2 capsules and the polymannan O-PSs, specifically the process of substrate recognition, are therefore quite different. The data presented here clearly demonstrate that the C-terminal domain of Wzt is essential for substrate specificity. Previous work from this laboratory has shown that the nonreducing terminal modifications of the *E. coli* O8 and O9a antigens are essential for both chain termination and export (36). It is tempting to speculate that the C-terminal domain of Wzt recognizes these nonreducing terminal residues. Unfortunately, the *E. coli* O8 and O9a Wbd enzymes, responsible for the addition of the terminal residues, are specific for a given polysaccharide repeat unit and not interchangeable between the two serotypes (36). As a result, testing this hypothesis will prove challenging and may require *in vitro* rather than *in vivo* approaches.

In *E. coli* K5, membrane targeting for two of the K5-specific glycosyltransferases is lost in the absence of either KpsM or KpsT, and as a result it was postulated that the capsular polysaccharide export apparatus forms the basis for assembly of a multienzyme biosynthesis complex (58). Mutants in kpsT in both *E. coli* K1 and K5 are unable to incorporate sialic acid onto endogenous polysialosyl acceptor in vitro (50, 60), providing further evidence for a biosynthesis complex. It is interesting to note that whereas mutants in kpsM or kpsT are viable and maintain normal cellular morphology in the K1 and K5 systems (20), introduction of a dominant negative allele of kpsT causes cells to take on an elongated morphology (61) similar to that observed in the *E. coli* O8 and O9a wzm and wzt mutants. In contrast to the capsule system, the polymannan O-PS biosynthesis mechanism is not significantly affected by a transport defect. In the *E. coli* O8 and O9a wzm and wzt mutants, only a slight reduction of *in vitro* mannose incorporation into polymer was seen. Ligation of undecaprenyl pyrophosphate-linked polymer from ABC transporter-dependent systems onto the lipid A core (and therefore recycling of undecaprenyl phosphate) does not occur in vitro (62, 63). As a result, the incorporation of radiolabeled mannose observed in membrane preparations represents one round of polymer extension, and differences between the mutants and wild type would only be detected if multiple rounds were possible. The data presented here indicate that a mutation in the ABC transporter required for O-PS export does not affect O-PS biosynthesis, suggesting that any essential targeting of the O8- and O9a-specific mannosyltransferases is not affected.

It has previously been suggested that the ABC transporters required for the export of the *Klebsiella pneumoniae* O12 and *Serratia marcescens* O4 antigens were not functionally interchangeable despite quite similar repeat unit structures shared by the two antigens (64). Functional complementation could be achieved when the gene just downstream of wzm and wzt (wbbB in the case of *K. pneumoniae* O12 or wbbA in the case of *S. marcescens* O4) was exchanged along with the transporter. This complementation, however, resulted in exchange of O-PS repeat unit structure as determined by antibody recognition. The wbbB<sub>Kp</sub>O12 and wbbA<sub>Smar</sub>O4 are predicted to encode glycosyltransferases (65). The O-PS of *K. pneumoniae* O12 contains a terminal β-3-deoxy-α-manno-oct-2ulosonic acid residue (66), whereas the possibility of a terminating residue on the *S. marc-
Some or all of the NBD proteins of the LPSE, LOSE, TAE, and DrugE1 exporter families also contain a C-terminal domain. The E. coli K1 and K5 transporters belong to the CPSE family and lack an extended C terminus. However, it is interesting that some of the NBD proteins of the LPSE family (containing O-PS exporters) lack a C-terminal domain comparable with Wzx_{OP} and Wzx_{OmpC}. One example is Wzt of K. pneumoniae O1. This raises the fascinating possibility that more than one mechanism may be used for export in the ABC transporter systems. Investigations on WztO8 and WztO9a and this will only be resolved by detailed functional analysis of additional systems.

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