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*

Leslie Cuthbertson‡, Jacqueline Powers§, and Chris Whitfield¶

From the Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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 capsular 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) 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...
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 O9) serve as the prototypes for O-PS synthesis. Genes encoding proteins with the same (or comparable) function in the biosynthesis of each O-PS are shown only once.

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% w/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), gentamicin (15 μg/ml), kanamycin (50 μg/ml), and tetracycline (15 μg/ml) were added where appropriate. All mutant strains carried a defect in D-mannose-6-phosphate aldose-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 bacterial DNA (39). Pwo polymerase (Roche Applied Science) was used in PCR amplification when the desired product was obtained. Restriction digestions and DNA ligation reactions were performed as per the manufacturer’s instructions. Plasmid DNA was purified using the GeneElute plasmid purification kit (Sigma). Template DNA for use in PCR was purified in the absence of transport.

**Figure 1.** Structure and biosynthesis of the polymannan O-PSs of E. coli O8, O9, and O9a. A, structure and organization of the polymers (66). Polysaccharide repeating units are enclosed by square brackets. B, organization of the gene clusters responsible for O-PS biosynthesis. Genes encoding proteins with the same (or comparable) function in the biosynthesis of each O-PS are shown only once.
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. 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. The completed undecaprenol phosphate-linked polymer is transported to the periplasmic face of the inner membrane by an ABC transporter consisting of Wzm and Wzt. The O-PS is then transferred to a preformed molecule of lipid A core in a reaction involving WaaL.

### Strains

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description or genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E69</td>
<td>E. coli O9a:K30</td>
<td>F. Orskov</td>
</tr>
<tr>
<td>CWG28</td>
<td>E69 derivative; O9a:K30</td>
<td>Ref. 79</td>
</tr>
<tr>
<td>CWG634</td>
<td>CWG28 derivative; manA; Sm' Te'</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>CWG672</td>
<td>CWG634 derivative; manA wbdA::aacC1; Sm', Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>CWG638</td>
<td>CWG634 derivative; manA wzm-wzt::aphA-3; Sm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>CWG709</td>
<td>CWG634 derivative; manA wzm::aphA-3; Sm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>CWG710</td>
<td>CWG634 derivative; manA wzt::aphA-3; Sm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>CWG708</td>
<td>CWG634 derivative; manA wzt::aphA-3; Sm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>RS218</td>
<td>E. coli O8:K40</td>
<td>B. Jann</td>
</tr>
<tr>
<td>CWG291</td>
<td>2775 derivative; O8:K40</td>
<td>Ref. 59</td>
</tr>
<tr>
<td>CWG636</td>
<td>CWG291 derivative; manA; Gm' Te'</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>CWG711</td>
<td>CWG636 derivative; manA wzm-wzt::aphA-3; Gm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>CWG712</td>
<td>CWG636 derivative; manA wzt::aphA-3; Gm', Te', K' r</td>
<td>This study</td>
</tr>
<tr>
<td>RS218</td>
<td>E. coli O18ac:K1</td>
<td>R. P. Silver</td>
</tr>
<tr>
<td>EV36</td>
<td>K-12-K1 hybrid strain; galP23 rpsL9 argA' rha' kpsM'</td>
<td>Ref. 56</td>
</tr>
<tr>
<td>RS2604</td>
<td>EV36 derivative; kpsM</td>
<td>Ref. 21</td>
</tr>
<tr>
<td>RS2436</td>
<td>EV36 derivative; kpsT</td>
<td>Ref. 20</td>
</tr>
<tr>
<td>Bi8337–41</td>
<td>E. coli O10:K5</td>
<td>I. Orskov</td>
</tr>
</tbody>
</table>

### Plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description or genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWQ331</td>
<td>pBAD24 derivative containing an Xbal/PstI fragment encoding Wzm-WztO8: Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ332</td>
<td>pBAD24 derivative containing an EcoRI/KpnI fragment encoding Wzm-WztO9a; Wzt contains a C-terminal His6-tag; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ333</td>
<td>pBADHIsA derivative containing an Xhol/KpnI fragment encoding WztO9a; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ334</td>
<td>pBADHIsA derivative containing an Xhol/KpnI fragment encoding WztO9a; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ335</td>
<td>pBAD18-Cm derivative containing an XbaI/HindIII fragment encoding WzmO9a; Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ336</td>
<td>pBAD24 derivative containing an EcoRI/Sphl fragment encoding WzmO9a; a Cm cassette was inserted into the Scrl site; Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ337</td>
<td>pBAD24 derivative containing an Xbal/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–61 from WztO8, and 61–431 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ338</td>
<td>pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–105 from WztO8, and 105–431 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ339</td>
<td>pBAD24 derivative containing an Xbal/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–149 from WztO8, and 149–431 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ340</td>
<td>pBAD24 derivative containing an Xbal/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–202 from WztO8, and 202–431 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ342</td>
<td>pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–417 from WztO8, and 394–404 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ343</td>
<td>pBAD24 derivative containing an EcoRI/Sphl fragment encoding a chimeric Wzt protein encoding amino acids 1–371 from WztO8, and 345–404 from WztO9a</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ344</td>
<td>pBAD24 derivative containing an Ncol/Sphl fragment encoding KpsMogra; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ345</td>
<td>pBAD24 derivative containing an Ncol/Sphl fragment encoding KpsTogra; Ap'</td>
<td>This study</td>
</tr>
</tbody>
</table>
to amplify the 5' region of wzt\textsubscript{O8} (encoding amino acids 1–61). LC33, used in initial amplification, was designed to anneal upstream of wzt\textsubscript{O8} in order to take full advantage of the amplification yield, which was manageable for purification. LC37 was designed to include a 25-bp overhang containing sequence corresponding to wzt\textsubscript{O8}, in order to increase the size of the amplified fragment to make the product suitable for cloning in pBAD24. Additional chimeras (Table I) were made using a similar strategy. The primers used are described in supplementary material. Plasmid pWQ43 was created using a different approach. Primer LC62 (encoding amino acids 394–404 of Wzt\textsubscript{O8}) was used in conjunction with LC4 to amplify the mutated Wzt\textsubscript{O8}, for ligation into pBAD24.

Construction of Chromosomal Insertion Mutations—Chromosomal insertion mutants of \textit{E. coli} CWG713 (wzm\textsubscript{O8}-wzt\textsubscript{O8}), CWG710 (wzt\textsubscript{O8}), CWG711 (wzm\textsubscript{O8}), CWG709 (wzm\textsubscript{O8}-wzt\textsubscript{O8}) were created through allelic replacement using the suicide delivery vector pRE112 (43). Insertion mutations were selected on M9–0.2% glycerol agar at room temperature and confirmed by mini-Tn5 insertion. Insertion mutants were selected on M9 (glucose, sucrose, threitol, 606 nM GDP-[14C]mannose (330 mCi/mmol; PerkinElmer Life Sciences), and the membrane equivalent of 0.5 mg of total protein (determined using the Bio-Rad DC protein assay kit). At each time interval, 0.1 ml was removed and added to 0.3 ml of cold 10% acetic acid to stop the reaction. Samples were filtered onto MicroSep 0.45-µm cellulose filters (Osmonics) and washed twice with 10% acetic acid to remove residual radioactive GDP-[14C]mannose. Filters were dried and placed in 5 ml of Ecolite scintillation fluid (ICN Biomedicals), and radioactivity incorporated into polymer was measured using a scintillation counter.

Analytical Methods—To examine LPS, proteinase K-digested whole cells lysates were prepared for SDS-PAGE by the method of Hitchcock and Brown (45) and visualized by silver staining (46). For antibody detection, LPS was transfected by immunoblotting to PROTRAN nitrocellulose membranes (PerkinElmer Life Sciences) and detected using O9a-specific polyclonal antiserum (36) and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Sigma).

Immunofluorescence Microscopy—Cells were subcultured 1:25 from a 5-ml overnight culture grown in LB 0.4% glucose into 5 ml of LB supplemented with ampicillin and 0.4% arabinose where required. Cultures were grown for ~3 h. Immunofluorescence labeling of intact and permeabilized cells was performed as described previously using O9a-specific polyclonal antiserum as the primary antibody and rhodamine red-conjugated goat anti-rabbit IgG (H + L) (Jackson Immunoresearch) as the secondary antibody (36).

RESULTS

\textbf{Wzt\textsubscript{O8} and Wzt\textsubscript{O9a} Contain a C-terminal Region Not Present in either KpsTK1 or KpsTK5.}—The \textit{wzm} genes from \textit{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 \textit{E. coli} O8 and O9a Wzm proteins with \textit{KpsM} from \textit{E. coli} K1 (Fig. 3) shows that the O-PS exporter homologues share only very limited primary sequence similarity with the capsule exporter. However, hydrophathy plots for the three ABC transporter TDMS are very similar, with all three proteins predicted to contain six membrane-spanning segments at similar positions (data not shown).

The \textit{wzt} homologues 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 KpsTK1. Comparison of the amino acid sequences of Wzt\textsubscript{O8}, Wzt\textsubscript{O9a}, and KpsTK1 reveals that the variable C-terminal domain present in the O8 and O9a Wzt homologues is absent from KpsTK1.
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>O8</sub>, wzm<sub>O9a</sub>, and wzt<sub>O8</sub> 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.

![Fig. 4. Alignment of the NBD homologues from E. coli O8, O9a, and K1. Identical residues are highlighted in black, and similar residues are highlighted in gray. Conserved sequence motifs common to all ABC transporter NBD proteins are indicated.](http://www.jbc.org/)

**FIG. 4.** Alignment of the NBD homologues from E. coli O8, O9a, and K1. Identical residues are highlighted in black, and similar residues are highlighted in gray. Conserved sequence motifs common to all ABC transporter NBD proteins are indicated.
radioactive incorporation seen in the wild type and ABC transport mutants is therefore due only to incorporation of radioactive label 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 E. coli O8 and O9a transporters were functionally interchangeable, Wzm and Wzt were expressed from plasmid-encoded genes in O8 and O9a transporters being O-antigen-specific. The corresponding genes encoding transporter proteins from E. coli K5, O8, or O9a in trans. A sample of each culture was cross-streaked on the LB-agar plate and spotted with 5 μl of the K1-specific bacteriophage (indicated by the arrow). Clearing due to cell lysis indicated complementation of K1 surface expression.

To test whether substrate specificity was a function of the TMD component of the transporter (Wzt), or both, plasmid-encoded copies of the transporter (Wzm), the NBD component of the transporter (Wzt), or both, plasmid-encoded copies of the genes were expressed in the O8 and O9a single mutant with the O8 and O9a transporters being O-antigen-specific. The corresponding genes encoding transporter proteins from E. coli K5, O8, or O9a in trans. A sample of each culture was cross-streaked on the LB-agar plate and spotted with 5 μl of the K1-specific bacteriophage (indicated by the arrow). Clearing due to cell lysis indicated complementation of K1 surface expression.

To determine whether the Wzm mutants affecting either or both of the O-PS transporter components in E. coli O8 and O9a resulted in growth inhibition when cells were grown under conditions permissive to O-PS biosynthesis. A, viability of E. coli CWG638 (wzm-wzt) was followed after the addition (indicated by the arrow) of 0.2% mannosamine or the same volume of water to cells growing in M9–0.2% glycerol. CWG638 cells were prepared for microscopy at 510 min after the addition of mannosamine, and differential interference contrast images are shown in B, C. The mannosyltransferase activity of mutants with ABC transporter defects, measured by the incorporation of radioactivity from GDP-[14C]mannose into polymeric product.

Substrate Specificity of O-antigen Export

FIG. 5. Complementation of E. coli K1 transporter mutations. Sensitivity to K1 specific bacteriophage was tested on LB-0.2% arabinose agar at 37 °C for RS2804 (kpsMΔO8) and RS2436 (kpsTΔO9a) expressing the corresponding genes encoding transporter proteins from E. coli K5, O8, or O9a in trans. A sample of each culture was cross-streaked on the LB-agar plate and spotted with 5 μl of the K1-specific bacteriophage (indicated by the arrow). Clearing due to cell lysis indicated complementation of K1 surface expression.

FIG. 6. Growth phenotype and mannosyltransferase activity of E. coli CWG638 (wzm-wzt). A chromosomal mutation affecting either or both of the O-PS transporter components in E. coli O8 and O9a resulted in growth inhibition when cells were grown under conditions permissive to O-PS biosynthesis. A, viability of E. coli CWG638 (wzm-wzt) was followed after the addition (indicated by the arrow) of 0.2% mannosamine or the same volume of water to cells growing in M9–0.2% glycerol. CWG638 cells were prepared for microscopy at 510 min after the addition of mannosamine, and differential interference contrast images are shown in B, C. The mannosyltransferase activity of mutants with ABC transporter defects, measured by the incorporation of radioactivity from GDP-[14C]mannose into polymeric product.

Chimeric Proteins Implicate the C-terminal Domain of Wzt in O-PS Recognition in E. coli O8 and O9a—To further localize cellular 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 (Wzm), the NBD 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, only was able to function in the export of its cognate polymer (Figs. 7B and 8, G–K). A wzmO9a mutant (CWG709) showed the same phenotype as the wzm-wztO9a 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 wzmO9a (pWQ336) was supplied in trans in CWG709 (wzmO9a) (Fig. 8O). In contrast, expression of wzmO9a (pWQ335) in trans restored surface expression of O-PS in CWG709 (wzmO9a), 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.
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 WzmO9a (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 WzmO9a. 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 WztO8 were exchanged with the corresponding amino acids from WztO9a (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 capsular 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 phosphatidic acid 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. 9. Complementation of CWG708 (wztO9a) and CWG638 (wzm-wztO9a) /H11545 pWQ335 (wzmO8) 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.

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.
specific gene products responsible for this modification, however, remain undetermined (29–31).

The fundamental differences in the capsule and O-PS systems are exemplified by the inability of Wzm-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 undecaprenol pyrophosphate-linked polymer from ABC transporter-dependent systems onto the lipid A core (and therefore recycling of undecaprenol 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>KpO12</sub> and wbbA<sub>SmO4</sub> proteins are predicted to encode glycosyltransferases (65). The O-PS of K. pneumoniae O12 contains a terminal β-3-deoxy-α-manno-oct-2-ulosonic acid residue (66), whereas the possibility of a terminating residue on the S. marcescens O4 antigen has not been examined. One interpretation of these results is that the large, probably multifunctional WbbB<sub>KO12</sub> and WbbA<sub>SmO4</sub> proteins are responsible for the addition of terminal modifications to their cognate O-PS. Therefore, the possibility of a similar nonreducing terminal modification export-signaling pathway may extend beyond the O-PSs of E. coli and K. pneumoniae.

A key issue is whether the situation observed with the polymannan O-PS extends to other glycan biosynthetic systems. The S-layer glycan of the Gram-positive Geobacillus stearothermophilus NRS 2004/3a is exported across the inner membrane by an ABC transporter (67), also containing an extended C-terminal domain. ORFG106 found in the S-layer glycan biosynthesis cluster encodes a protein of 1127 amino acids. ORFG106 is probably trunctional, containing two putative glycosyltransferase domains with a third region showing homology to methyltransferases (67). Like the O-PS of E. coli O8 and O9a, the G. stearothermophilus S-layer glycan terminates in a methyl moiety (68). The methyltransferase domain of ORFG106 is probably responsible for this terminal modification and may be involved in polysaccharide chain-length regulation analogous to WbdD<sub>O8</sub> and WbdD<sub>O9a</sub> (36). Recently, the S-layer glycan of Geobacillus tepidamans GSS-9T was found to terminate in an N-acetylmuramic acid moiety (69). Thus, the significance of the results reported for the polymannan O8 and O9a export system may extend beyond O-PS.

Examination of ABC transporter classification by the Transporter Commission (available on the World Wide Web at www.tcdb.org) shows that many of the NBD proteins from both bacterial importers and exporters also contain a C-terminal domain extending beyond the conserved region recognized for all NBDs. Some or all of the NBD proteins in the CUT1, PepT, SulT, MolT, FeT, POPT, QAT, NitT, TauT, BIT, and MUT importer families contain an extended C-terminal domain. The function of these domains has been investigated in only a few cases. The MalK protein of E. coli belongs to the CUT1 family of transporters and is involved in maltose import. The 135-amino acid C-terminal domain of MalK is involved in interactions with enzyme IIA<sup>Glc</sup> and the transcriptional regulator MalT (70–73). One of the NBDs from the nitrate/nitrite uptake system of Synechococcus sp. strain PCC 7942, NrtC, which belongs to the NitT family of importers, contains a C-terminal domain of 380 amino acids. This domain is not required for import but is required for the inhibition of transport in the presence of ammonium (74). The P<sub>n</sub> protein (glnB) is also required for this inhibition (75). The structures of three NBD proteins containing an extra C-terminal domain, namely MalK (76), GlcV (77), and CysA (5), have been solved. Despite differences in primary sequence, the C-terminal domain of each NBD protein takes on a similar fold, and it has been speculated that this domain may be involved in signal transduction in each system (5). The specific roles of the C-terminal domain of GlcV (CUT1 family) and CysA (SulT family) have not been examined. Given that all of the above mentioned NBD proteins are components of importer systems, it is difficult to make conclusive comparisons between them and the E. coli O8 and O9a Wzt homologues, which are involved in substrate export. However, these examples provide an alternate possibility for the mechanism imparting specificity in the ABC transporter: that Wzt may not be involved in direct interactions with the O-PS but could instead have essential interactions with the enzyme that adds the novel nonreducing terminal residues (i.e. WbdD (36)). Signaling between Wzt and WbdD may regulate WbdD activity and therefore O-PS chain length. This study provides the first evidence of an extended C-terminal domain of an ABC transporter NBD protein in substrate specificity.
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 WtOSS and WtOSS. One example is WtO of P. pneumoniae O1. This raises the fascinating possibility that more than one mechanism may be used for export in the ABC transporter-dependent O-PSs, and this will only be resolved by detailed functional analysis of additional systems.

Acknowledgments—We thank Dr. E. R. Vinr and R. P. Silver for generously providing bacterial strains and bacteriophage and Dr. J. S. Lam for providing access to the Zeiss Axiovert 200 microscope. The assistance of Catriona Bouwman in the creation of CWG672 is gratefully acknowledged.

REFERENCES

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

Leslie Cuthbertson, Jacqueline Powers and Chris Whitfield

doi: 10.1074/jbc.M504371200 originally published online June 24, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504371200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 76 references, 46 of which can be accessed free at
http://www.jbc.org/content/280/34/30310.full.html#ref-list-1