Coordination of Polymerization, Chain Termination, and Export in Assembly of the Escherichia coli Lipopolysaccharide O9a Antigen in an ATP-binding Cassette Transporter-dependent Pathway*

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Received for publication, August 5, 2009, and in revised September 4, 2009. Published, JBC Papers in Press, September 4, 2009, DOI 10.1074/jbc.M109.052878

The Escherichia coli O9a O-polysaccharide (O-PS) is a prototype for O-PS synthesis and export by the ATP-binding cassette transporter-dependent pathway. Comparable systems are widespread in Gram-negative bacteria. The polymannose O9a O-PS is assembled on a polyisoprenoid lipid intermediate by mannosyltransferases located at the cytoplasmic membrane, and the final polysaccharide chain length is determined by the chain terminating dual kinase/methyltransferase, WbdD. The WbdD protein is tethered to the membrane via a C-terminal region containing amphipathic helices located between residues 601 and 669. Here, we establish that the C-terminal domain of WbdD plays an additional pivotal role in assembly of the O-PS by forming a complex with the chain-extending mannosyltransferase, WbdA. Membrane preparations from a ΔwbdD mutant had severely diminished mannosyltransferase activity in vitro, and no significant amounts of the WbdA protein are targeted to the membrane fraction. Expression of a polypeptide comprising the WbdD C-terminal region was sufficient to restore both proper localization of WbdA and mannosyltransferase activity. In contrast to WbdA, the other required mannosyltransferases (WbdBC) are targeted to the membrane independent of WbdD. A bacterial two-hybrid system confirmed the interaction of WbdD and WbdA and identified two regions in the C terminus of WbdD that contributed to the interaction. Therefore, in the O9a assembly export system, the WbdD protein orchestrates the critical localization and coordination of activities involved in O-PS chain extension and termination at the cytoplasmic membrane.

Lipopolysaccharide (LPS) is a glycolipid unique to the outer membranes of Gram-negative bacteria. LPS has three structural domains in most bacteria (1). Hydrophobic lipid A is a major component of the outer leaflet of the outer membrane. A short core oligosaccharide (OS) serves as a linker between lipid A and a repeat unit polymer termed the O-polysaccharide (O-PS; O-antigen). The structure of lipid A is conserved among Gram-negative bacteria, whereas limited variability is observed among the core OSs of a given species. For example, five closely related core oligosaccharides have been described for Escherichia coli (2). In contrast, the O-PS structures vary extensively within species. O-PS structural variations include differences in the number and type of sugars in the repeat unit and the nature of the glycosidic linkages within and between repeat units. O-PS variations provide the basis for the O-antigen serotyping system, and there are over 180 O-antigen serogroups proposed for E. coli (3, 4).

Lipid A-core OS and O-PS are synthesized independently at the cytoplasmic membrane and are subsequently linked together in the periplasm (reviewed in Ref. 1). O-PS assembly is initiated by transfer of a sugar-1-phosphate from a nucleotide sugar precursor to the 55-carbon lipid acceptor, undecaprenol phosphate. In the majority of E. coli serotypes, the initiating reaction is performed by the GlcNAc:Und-P GlcNAc-1-P transferase, WecA (5, 6). WecA is an integral membrane protein and is also essential for initiating synthesis of the enterobacterial common antigen (7). In E. coli, elongation and export of the undecaprenol-PP-linked intermediate proceeds through one of two fundamentally different O-PS assembly pathways. These pathways have been termed Wzy (polymerase)-dependent and ATP-binding cassette (ABC) transporter-dependent biosynthesis, respectively (reviewed in Ref. 1). In Wzy-dependent O-PS biosynthesis, single repeat units are assembled on the undecaprenol-PP-linked intermediate at the cytoplasmic face of the inner membrane. The lipid-linked repeat units are subsequently reoriented to the periplasm where they are assembled into polysaccharide by a process involving Wzy and a chain length regulator, Wzz. In contrast, in the ABC transporter-dependent pathway, the O-PS is elongated on the undecaprenol-PP-linked intermediate in the cytoplasm by sequential glycosyl transfer. Depending on the system, chain extension is terminated by the addition of a nonreducing terminal residue or by interaction with the ABC transporter (8). Full-length O-PS chains are then translocated across the inner membrane by the ABC transporter. The two O-PS assembly pathways converge at a ligation reaction, which transfers the O-PS from undecaprenol-PP to lipid A-core OS at the periplasmic face of the membrane.

* This work was supported in part by the Natural Sciences and Engineering Research Council (to C.W.).

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3 The abbreviations used are: LPS, lipopolysaccharide; O-PS, O-polysaccharide; OS, oligosaccharide; PS, polysaccharide; ABC, ATP-binding cassette; LB, Luria-Bertani; S-LPS, smooth lipopolysaccharide; TIR, transferase-interacting region; IPTG, isopropyl β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ORF, open reading frame.
inner membrane. Once assembled, LPS molecules are shuttled to the outer membrane through a process involving the Lpt-ABCDE complex (reviewed in Ref. 9).

The polymannose O-PS of *E. coli* O9a provides a model system for ABC transporter-dependent O-PS biosynthesis. The *E. coli* O9a PS biosynthesis gene cluster (see Fig. 1A) encodes three GDP-mannose-dependent mannosyltransferases (WbdA, WbdB, and WbdC) that assemble the O-PS on undecaprenol-PP-GlcNAc (10). Structural studies identify terminal capping residues in a number of O-PSs synthesized by the ABC transporter-dependent pathway (11). It has been proposed that the addition of a capping residue to the nonreducing end of the undecaprenol-PP-linked PS serves to regulate O-PS chain length by terminating elongation. In the case of the O9a PS, termination involves methylation and phosphorylation. The chain length of the O9a PS is strictly controlled by the activity of WbdD, and O-PS-substituted LPS molecules expressed on the cell surface exhibit a narrow size distribution. The *E. coli* O9a WbdD protein contains putative kinase and methyltransferase domains, and these activities have been confirmed in biochemical studies (12). In addition to the role in O-PS chain regulation, methyl and/or phosphoryl modification is required for binding of the O9a PS to the nucleotide-binding component (Wzt) of the ABC transporter (13, 14), a crucial initial step in O-PS export. Unmodified polysaccharide in the cytoplasm.

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in Luria-Bertani (LB) medium (17) or M9 minimal medium (18). Minimal medium was supplemented with glycerol (0.4% w/v), thiamine HCl (0.5 μg/ml), nicinamide (0.5 μg/ml), adenine (20 μg/ml), uracil (20 μg/ml), l-histidine (20 μg/ml), and l-tryptophan (20 μg/ml). D-glucose (0.4% w/v), D-mannose (0.1% w/v), D-ribonose (0.001–0.2% w/v), maltose (1% w/v), X-gal (40 μg/ml), IPTG (0.5 mm), or neutral red (30 μg/ml) was added to media as required. The antibiotic, ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), gentamycin (15 μg/ml), or kanamycin (50 μg/ml) was added where appropriate.

**TABLE 1**

| Strain/plasmid | Description or genotype | Ref. or source |
|----------------|-------------------------|---------------|
| BTH101        | F− cya-854, recA1, endA1, gyrA96, thi1, hsdR17, spoT1, rfbD1, glmV44(AS); Na1 | Ref. 33 |
| E69           | *E. coli* O9aK30        | F. Oreskov    |
| CWG28         | E69 derivative; O9aK−; trp his lac rpsL cps3500 Sm− | Ref. 46 |
| CWG634        | CWG28 manA; Sm+, Tc−    | Ref. 12 |
| CWG635        | CWG634 wbdD::aacC1; Sm+, Tc+, Gm+ | Ref. 12 |
| CWG900        | CWG634 ΔwbdD; Sm+, Tc+ | This study |
| CWG901        | CWG634 wbdA::aacC1; Sm+, Tc+, Gm+ | This study |
| CWG910        | CWG634 ΔwbdD; Sm+, Tc+ | This study |
| CWG917        | CWG634 ΔwbdD; Sm+, Tc+ | This study |
| pBAD24        | t-arabinose-inducible plasmid; Ap′ | Ref. 47 |
| pET30a        | Cloning/expression vector; Kc− | Novagen |
| pUC-Gm        | Source of the aacC1 gentamycin− cassette; Ap′ | Ref. 21 |
| pKT25         | Bacterial two-hybrid vector that encodes the T25 fragment of *B. pertussis* anissuty cyclase S to a multiple cloning site; Kc− | Ref. 33 |
| pUT18C        | Bacterial two-hybrid vector that encodes the T18 fragment of *B. pertussis* anissuty cyclase S to a multiple cloning site; Ap′ | Ref. 33 |
| pKT25-Zip     | pKT25 derivative encoding the CyaA T25 fragment fused to the leucine zipper from the yeast GCN4 protein; Kc− | Ref. 33 |
| pUT18C-Zip    | pUT18C derivative encoding the CyaA T18 fragment fused to the leucine zipper from the yeast GCN4 protein; Ap′ | Ref. 33 |
| pWQ173        | Counterselectable, temperature-sensitive suicide vector; Cm′ | Ref. 22 |
| pWQ470        | pBAD24 derivative encoding His6-WbdD from *E. coli* O9a; Ap′ | This study |
| pWQ471        | pBAD24 derivative encoding His6-WbdD1–686; Ap′ | This study |
| pWQ472        | pBAD24 derivative encoding His6-WbdD475–708; Ap′ | This study |
| pWQ473        | pBAD24 derivative encoding WbdC-FLAG; Ap′ | This study |
| pWQ474        | pBAD24 derivative encoding WbdB-FLAG; Ap′ | This study |
| pWQ486        | pKT25 derivative encoding T25-WbdD; Kc− | This study |
| pWQ487        | pUT18C derivative encoding T18-WbdA; Ap′ | This study |
| pWQ488        | pKT25 derivative encoding T25-WbdD; Kc− | This study |
| pWQ489        | pKT25 derivative encoding T25-WbdD1–686; Kc− | This study |
| pWQ490        | pKT25 derivative encoding T25-WbdD1–600; Kc− | This study |
| pWQ491        | pKT25 derivative encoding T25-WbdD519–708; Kc− | This study |
| pWQ492        | pBAD24 derivative encoding WbdA-His10 | This study |
| pWQ493        | pET30a derivative containing the wbdA gene on a Saci/Ndel PCR fragment; Km− | This study |
| pWQ494        | pWQ493 derivative with a BamHI fragment containing the aacC1 gene of *pUC-Gm* inserted into wbdA; Kc− | This study |
| pWQ495        | pWQ491 derivative containing a NotI/Notl fragment including the wbdA::aacC1 insertion from pWQ494; Cm′ | This study |
| pWQ496        | pBAD24 derivative encoding His6-WbdD1–600; Ap′ | This study |
| pWQ497        | pBAD24 derivative encoding His6-WbdD1–686; Ap′ | This study |
| pWQ498        | pKT25 derivative encoding T25-WbdD1–686; Kc− | This study |

**Cloning and Mutagenesis Methods**—DNA fragments used to make recombinant plasmids were PCR-amplified from
genomic DNA using Pwo DNA polymerase (Roche Applied Sciences) and custom oligonucleotide primers (Sigma). Restriction endonuclease sites and epitope tag (poly-His or FLAG) coding sequences were included in the primer sequences. Genomic DNA was prepared using the Instagene Matrix (Bio-Rad). Plasmid DNA was purified using the PureLink Plasmid Mini-prep Kit (Invitrogen), and DNA from PCRs and restriction digestions was purified using the PureLink PCR purification kit (Invitrogen). Restriction endonucleases (Invitrogen) and T4 DNA ligase (New England Biolabs) were used according to the manufacturer’s directions. All of the constructs were confirmed by restriction endonuclease digestion or DNA sequencing (Guelph Molecular Supercenter).

The λ-red recombine system (19, 20) was used to create mutants in the CWG634 background. To make CWG900 (ΔwbdD), primers, 5′-ATGACATAAAAGACCTTAAACACGCT-GGTACGAGATTTATCAGTGAGGCT-GGGAGCTGCC-3′ and 5′-TCCGACGTTAAGCCTCGCTTTCCGGCACGTTCATATG-AGCTGCTTCG-3′ were used to amplify the kanamycin resistance cassette of pKD4 and T4 DNA ligase (New England Biolabs) were used according to the manufacturer’s directions. All of the constructs were confirmed by restriction endonuclease digestion or DNA sequencing (Guelph Molecular Supercenter).

Gene-specific PCR fragments on wbdD were used to replace the entire ORF with primers, 5′-ATGACATAAAAGACCTTAAACACGCT-GGTACGAGATTTATCAGTGAGGCT-GGGAGCTGCC-3′ and 5′-TCCGACGTTAAGCCTCGCTTTCCGGCACGTTCATATGAGCTGCTTCG-3′. The fragments were inserted into the suicide delivery vector, pCP20, was used to remove the antibiotic marker cassette from pUC-Gm (21). Primers, 5′-AGCCGGATAAGCCTCGCTTTCCGGCACGTTCATATGAGCTGCTTCG-3′ and 5′-ATGACATAAAAGACCTTAAACACGCT-GGTACGAGATTTATCAGTGAGGCT-GGGAGCTGCC-3′, were used to amplify a PCR fragment containing the chloramphenicol resistance cassette of pKD4 with primers, 5′-GATGTTGGCGGAAATACAGCTATGACGGTGACAAATACGGCAGTACGTCGGACTGTCCTCG-3′ and 5′-GTTTCCGATAAGATTTGTGCGAGCCGATTACGCTTTGTTGACGCTGTCCTCG-3′. Primers were used to PCR fragment was used to replace the entire wbdD ORF on the CWG634 chromosome. Similarly, CWG910 (ΔcyA) was made by amplifying the kanamycin resistance cassette of pKD4 with primers, 5′-GATGTTGGCGGAAATACAGCTATGACGGTGACAAATACGGCAGTACGTCGGACTGTCCTCG-3′ and 5′-GTTTCCGATAAGATTTGTGCGAGCCGATTACGCTTTGTTGACGCTGTCCTCG-3′. The ORF on wbdD was amplified as Miller units (23).

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The reactions were initiated by the addition of membranes (600 μg of membrane protein), and 100-μl samples were taken at various time points over a 30-min incubation period. The reaction was stopped by mixing with 1 ml of ice-cold 12% (v/v) acetic acid, and the membranes were collected onto MicronSep 0.45-μm cellulose filters (Osmonics). The filters were washed with 2 ml of 12% (v/v) acetic acid, dried, and then submerged in 5 ml of EcoLite scintillant (ICN Biomedicals). The radioactivity on the filters was determined by liquid scintillation counting.

β-Galactosidase Assays for Bacterial Two-hybrid Analysis—Bacterial cultures (5 ml in LB) were inoculated 1:50 from overnight growth and incubated at 30 °C for 4.5 h (A600 nm = 0.4–0.8). Samples of the cultures (70 μl) were assayed for β-galactosidase activity with the Pierce β-galactosidase assay kit and using the stopped microplate assay protocol as described by the manufacturer. Enzyme activity was calculated as Miller units (23).

Purification of Anti-WbdA Antibody—Antiserum was raised by immunizing New Zealand White rabbits with purified WbdA-His10 protein. For purification of WbdA-His10, 4 liters of CWG917[pWQ492] culture (A600 nm = 0.6) was induced with 0.2% (w/v) l-arabinose for 16 h at 20 °C. The cells were collected by centrifugation at 5,000 × g for 10 min. The cells were resuspended in 200 ml of buffer A (50 mM sodium phosphate, 500 mM NaCl, pH 7.0) containing 50 mM imidazole and disrupted by ultrasonication. The lysate was cleared by successive centrifugation steps at 12,000 × g for 30 min and 75,000 × g for 2 h. WbdA-His10 was purified from the supernatant using nickel-nitrilotriacetic acid resin (Qiagen). The column was washed with buffer A containing 125 mM imidazole, and WbdA-His10 was eluted with buffer A containing 250 mM imidazole. Elution fractions containing purified WbdA-His10 were pooled, and a PD-10 column (GE Healthcare) was used to exchange the protein into 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, for storage. The polyclonal antiserum recognized...
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WbdA-His\textsubscript{10} but did not react with any other His-tagged proteins. However, the antiserum did exhibit significant nonspecific reactivity with other proteins present in *E. coli* O9a whole cell lysates. Therefore, WbdA-specific antibodies were purified from the antiserum by affinity chromatography using immobilized WbdA-His\textsubscript{10}. Purified WbdA-His\textsubscript{10} (4 mg) was exchanged into 1 ml of 100 mM NaHCO\textsubscript{3}, 500 mM NaCl, pH 8.3, using a Vivaspin 15R filtration unit (30,000 molecular weight cutoff; Sartorius Biolab Products). The WbdA-His\textsubscript{10} protein was conjugated to CNBr-activated Sepharose 4B (0.75 g; GE Healthcare) according to the instructions from the manufacturer. For purification of anti-WbdA antibody, antiserum was diluted 1:5 care) according to the instructions from the manufacturer. For purification of anti-WbdA antibody, antiserum was diluted 1:5 in phosphate-buffered saline (13.7 mM NaCl, 0.27 mM KCl, 1 m

**RESULTS**

The C Terminus of WbdD Is Essential for Assembly of the O9a Polysaccharide—A *wbdD* mutant, CWG635, was previously constructed from *E. coli* O9a (CWG634) by replacing a 500-bp Smal restriction fragment within the *wbdD* ORF with the *aacC1* gentamycin resistance cassette (Fig. 1B) (12). This mutation was lethal under conditions permissive for O-PS biosynthesis. All of the mutations were made in a *manA* background, making synthesis of GDP-mannose (and hence O-PS) dependent on the addition of mannose to the growth medium (32). In contrast to the *wbdD::aacC1* mutation, a strain with a complete deletion of *wbdD* (CWG900) showed no lethal effects. However, CWG900 possessed the same O-antigen-deficient LPS phenotype as CWG635 when pulsed with mannose. In both cases, the SDS-PAGE profiles of cell lysates lacked the high molecular weight ladder pattern characteristic of S-LPS (*i.e.*, lipid A-core OS substituted with O-PS chains of varying length) (Fig. 2).

CWG635 (*wbdD::aacC1*) lacks the ability to chain polymerization (32). However, CWG635 does accumulate intracellular O-PS (12). As expected, isolated membrane fractions from CWG635 incorporated [\textsuperscript{14}C]mannose from GDP-[\textsuperscript{14}C]mannose in vitro, indicating that polymerization of undecaprenol-PP-linked mannan is not affected by the *wbdD::aacC1* mutation. Surprisingly, membranes from CWG900 (*\Delta wbdD*) showed no significant polymer synthesis, in contrast to the robust synthesis evident in membranes from the CWG634 parent (Fig. 3).

To determine whether the distinct phenotypes and biochemical properties of CWG635 (*wbdD::aacC1*) and CWG900 (*\Delta wbdD*) were due only to differences in the nature of the corresponding *wbdD* defects, both mutants were transformed by plasmid pWQ470 containing the full-length *wbdD* gene. In both cases, the resulting SDS-PAGE profiles showed complete restoration of S-LPS, ruling out the possibility of unexpected second site mutations contributing to the different characteristics of the mutants (Fig. 2). The consequences of the insertion in the *wbdD::aacC1* mutation were therefore examined by sequence analysis. In CWG635, the genomic DNA sequence located 3’ to the *aacC1* cassette contains a putative ribosome-binding site (GGAG) located 6 bp upstream of an ATG codon (Fig. 1B). Potentially, this ATG codon could initiate translation of a 208-amino acid polypeptide comprising the C terminus of WbdD (Fig. 1B). The potential contribution of this domain to O-PS biosynthesis was therefore assessed by complementation experiments using plasmids expressing truncated derivatives of WbdD.

Two derivatives were constructed (Fig. 1C). pWQ471 expresses the N-terminal catalytic domain (*WbdD\textsubscript{1–600}*) which contains the methyltransferase and kinase elements whose activities are essential for chain termination and subsequent export (12). In contrast, pWQ472 expresses only the C-terminal domain (*WbdD\textsubscript{675–709}*) in a region including the proposed residual WbdD peptide remaining in CWG635 (*wbdD::aacC1*). Production of S-LPS was fully restored by expressing *WbdD\textsubscript{1–600}* in CWG635 (Fig. 2), confirming that the O-PS biosynthesis defect is directly attributable to the absence of chain termination and export. In contrast, neither of the truncated derivatives restored S-LPS formation in CWG900 (*\Delta wbdD*) (Fig. 2 and data not shown). However, determination of mannosyltransferase activity in membranes.
from CWG900 expressing the WbdD truncated derivatives yielded an intriguing result (Fig. 3). In the presence of WbdD_{1–600} (pWQ471), no mannosyltransferase activity was detected, similar to the CWG900 \((\text{wbdD}^{-}/H9004\text{wbdD}^{-})\) membrane control. In contrast, the expression of WbdD_{475–708} (pWQ472) resulted in robust mannosyltransferase activity. Together, these data indicated that WbdD contains domains that participate at different points in O9a biosynthesis. The N-terminal part of the protein contains the methyltransferase and kinase essential for chain termination and polymer export, as reported previously (12), whereas the C-terminal 108 residues are essential for functional mannosyltransferase activity at the inner (cytoplasmic) membrane.

The C Terminus of WbdD Contains a Predicted α-Helical Membrane-anchoring Domain—Examination of the cellular localization of His6-WbdD showed that it was almost exclusively confined to the membrane fraction when it was overexpressed in CWG900 \((\Delta\text{wbdD})\) (Fig. 4). The same location was identified for the C-terminal domain (His-WbdD_{475–708}), whereas the N-terminal domain (His-WbdD_{1–600}) containing the methyltransferase and kinase activities was found entirely in the soluble fraction.

Given the data showing that the C-terminal region of WbdD was responsible for membrane binding, the primary sequence of this segment was subjected to computer analysis to identify structural features that could potentially target the protein to the cytoplasmic membrane. Secondary structure prediction was performed using the programs PSIPred, PredictProtein, Jpred, and Prof (27–30). The data from each analysis indicated that the C-terminal region of WbdD was predominantly α-helical (Fig. 5). However, none of the α-helices were predicted to be transmembrane structures. Ten α-helices (H1–H10) were

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**FIGURE 1. Structure and biosynthesis of the E. coli O9a PS and schematic showing WbdD and mutant derivatives.** A, the structure of the O9a PS shows the adaptor region, repeat unit, and terminating residues. The nonreducing end of the O-PS is capped by methylation and phosphorylation, but the nature of the linkage between capping residues and the repeat unit is unknown (11, 12). The O9a-PS biosynthesis and export genes are shown together with the functions of the encoded proteins. B, a linear representation of the wild-type WbdD protein from CWG634 is shown in context with the genomic wbdD mutations in CWG635 and CWG900. The methyltransferase (MTase) and kinase domains are shown within WbdD and have been described previously (12). In CWG635, the chromosomal wbdD ORF was disrupted by replacing a 500-bp SmaI restriction fragment with the \(\text{aacC1}\) cassette. A potential ribosomal-binding site, initiation codon, and stop codon are shown and together define an ORF encoding amino acids 501–708 of WbdD. In CWG900, the entire wbdD ORF has been removed from the chromosome. C, a schematic of the truncated WbdD polypeptide derivatives encoded by plasmids used in this study. The numbers shown above the polypeptides refer to amino acid positions in the native WbdD protein. Each polypeptide contained either an N-terminal His6 tag or the T25 fragment of \(B. pertussis\) adenylate cyclase (see plasmids in Table 1).
predicted within the last 234 amino acids of WbdD, and as described above, amino acids 601–708 comprise a region sufficient to direct the association of WbdD with the cytoplasmic membrane (Fig. 5). Four \( \alpha \)-helices (H5–H8) within the C-terminal 108 amino acids had asymmetrical charge distributions, and some could potentially be amphipathic and involved in membrane binding. Helical wheel representations of H5–H8 show that one side of each \( \alpha \)-helix is composed of hydrophobic and uncharged polar amino acids, whereas the opposite side predominantly contains positively charged amino acids (Fig. 5). In contrast, by the same criteria, helices H9 and H10 were not predicted to have amphipathic characters.

The C Terminus of WbdD Is Responsible for the Membrane Association of the Mannosyltransferase, WbdA

Incorporation of radioactivity from GDP-[\(^{14}\)C]mannose into the membrane fraction of wild-type \( E. coli \) 09a implied that the mannosyltransferases essential for polymer formation (WbdA, WbdB, and WbdC) are physically associated with the cytoplasmic membrane. One possible interpretation of the different properties of CWG635 (\( \text{wbdD}::\text{aacC1} \)) and CWG900 (\( \text{wbdD}^{\text{H9004}} \)) is that CWG900 fails to target mannosyltransferase enzymes to the membrane. Secondary structure analysis of the WbdABC proteins did not reveal any potential transmembrane \( \alpha \)-helices (data not shown). However, the mannosyltransferases could be peripheral membrane proteins.

To test their locations, WbdB and WbdC were independently expressed as fusion proteins containing a FLAG epitope tag. Western immunoblotting using anti-FLAG monoclonal antibody showed that both WbdB-FLAG and WbdC-FLAG were associated with the membrane fraction when these proteins were overexpressed in CWG634 and CWG900 (Fig. 6). Low amounts of WbdB-FLAG and WbdC-FLAG were also found in the soluble fractions from both strains (Fig. 6).

This may have been an artifact of protein overexpression, or alternatively, WbdB and WbdC may normally be present in both subcellular fractions. The distribution of WbdB-FLAG and WbdC-FLAG among the membrane and soluble fractions was similar in both strain backgrounds (Fig. 6). Therefore, expression of WbdD was not a prerequisite for the physical association of the WbdC and WbdB mannosyltransferases with the cytoplasmic membrane and the decreased level of in vitro...
[14C]mannose incorporation into CWG900 membranes (Fig. 3) was likely not due to the absence of WbdC or WbdB. Similar experiments with plasmid-encoded WbdA-FLAG suggested a massive shift from the membrane to the soluble fractions in the absence of WbdD. However, traces of WbdA-FLAG still remained in the membrane fraction of CWG900 (H9004wbdD) (data not shown). To test the possibility that this resulted from overexpression, polyclonal antiserum was raised against WbdA to detect chromosomally encoded WbdA in the soluble and membrane fractions of the parent strain, CWG634. In a Western blot, a protein corresponding with the theoretical molecular mass of WbdA (94.2 kDa) was detected in the cleared lysate from CWG634 (Fig. 7A). The 94-kDa protein band was absent from a lysate of the negative-control (H9004wbdA) strain, CWG901. WbdA was found predominantly in the membrane fraction of CWG634, although some WbdA was also present in the soluble fraction. The WbdA protein in the membrane fraction of CWG634 consistently migrated more slowly in SDS-PAGE compared with the soluble form of WbdA. Both fractions were derived from the same CWG634 cleared lysate, in which only one WbdA species was observed. This aberrant WbdA migration was observed in all strains in which membrane-bound WbdA was detected, and the underlying cause is unknown. WbdA was detected in the cleared lysate from the ΔwbdD mutant, CWG900, albeit at a lower level compared with the CWG634 lysate. In contrast to CWG634, WbdA was found exclusively in the soluble fraction of CWG900 (Fig. 7A). These results correspond with the detected mannosyltransferase activity (Fig. 3).

To establish the region of WbdD responsible for membrane association of WbdA, the truncated WbdD derivatives were used (Fig. 7B). WbdA targeted to the membrane of CWG900 when the C terminus of WbdD (Wbd475–708; pWQ472) was present, but not when the N terminus (WbdD 1–600) was expressed. These results therefore mirrored the requirements for the proper localization of WbdD itself. Collectively, the data indicate that expression of the C-terminal region of WbdD is essential (and sufficient) for targeting both WbdD and WbdA to the membrane.

To further define the WbdD C-terminal region involved in membrane targeting of WbdA, plasmids pWQ496 and pWQ497, encoding C-terminal truncation derivatives His6-WbdD1–686 and His6-WbdD1–669, respectively, were introduced into CWG900. His6-WbdD1–686 and His6-WbdD1–669 were both found in the membrane fractions of CWG900, yet WbdA was confined to the soluble fractions of these strains.

**FIGURE 5. Secondary structure prediction of the WbdD C-terminal region.** The amino acid sequence comprises residues 475–708 at the WbdD C terminus. The gray rectangles shown below the sequence represent α-helices (H1–H10) predicted using the PSIPred application. Helical wheel diagrams are shown for the putative α-helices H5–H8 within the membrane-binding region (MB). Note the distribution of positively charged (basic) amino acids to one side of each helix. H6, H7, and H8 also contain hydrophobic faces and are potentially amphipathic helices. Gray circles in the helical wheel diagrams represent empty positions and are results of α-helical sequences that were shorter than the window size within the helical wheel drawing application. Regions predicted to form coiled-coil structures are represented by open rectangles. TIR1 and TIR2 indicate transferase interactive regions. Deletion of either TIR1 or TIR2 abrogates WbdD-WbdA interaction.
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**FIGURE 6.** Membrane localization of the WbdB and WbdC mannosyltransferases is not dependent on the presence of WbdD. CWG634 (wild-type O9a PS) and CWG900 (ΔwbdD) overexpressing WbdB-FLAG (pWQ474) (A) or WbdC-FLAG (pWQ473) (B) were grown in LB containing 0.02% (w/v) L-arabinose. The cell lysates were separated into soluble and membrane-containing fractions, and the fusion proteins were detected in Western blots using an anti-FLAG monoclonal antibody as a probe. The sizes of the detected polypeptides were consistent with the predicted values for WbdB-FLAG (44.6 kDa) and WbdC-FLAG (43.5 kDa), respectively. L, cleared cell lysate; S, soluble fraction; M, membrane fraction.

**FIGURE 7.** WbdD is essential for targeting the mannosyltransferase, WbdA, to the cytoplasmic membrane. A, Western blot of subcellular fractions from the E. coli O9a parental strain, CWG634, and mutant derivatives. The blot was probed with anti-WbdD antiserum to detect chromosomally expressed WbdA. B, Western immunoblot showing the subcellular localization of WbdA in CWG900 (ΔwbdD) overexpressing His6-WbdD and its truncated derivatives. Plasmid-encoded His6-WbdD polypeptides were induced with 0.001% (w/v) L-arabinose, and chromosomally expressed WbdA was detected using anti-WbdA antiserum. In C, His6-WbdD was detected using anti-His6 monoclonal antibody. The sizes of the detected polypeptides were consistent with the predicted values for WbdD (94.2 kDa), His6-WbdD1–686 (79.8 kDa), and His6-WbdD1–669 (78.0 kDa), respectively. L, cleared cell lysate; S, soluble fraction; M, membrane fraction.

The C-terminal Region of WbdD Interacts with WbdA—The requirement for WbdD in the membrane localization of the WbdB mannosyltransferase could have been a consequence of a physical interaction between these two proteins. To investigate potential interactions in vivo, a bacterial two-hybrid assay was employed. In this system, interacting partners that bring together the two functional domains (T25 and T18) of Bordetella pertussis adenylate cyclase reconstitute its activity. The reporter for this activity is β-galactosidase (33). WbdD and WbdA were expressed as fusion proteins C-terminal to the T25 and T18 fragments, respectively, of the B. pertussis adenylate cyclase. BTH101[pKT25, pUT18C] served as a negative control, the T25 and T18 fragments, respectively, of the B. pertussis adenylate cyclase. BTH101[pKT25, pUT18C] was employed. In this system, interacting partners that bring together the two functional domains (T25 and T18) of Bordetella pertussis adenylate cyclase reconstitute its activity.

Using the same system, potential interactions between WbdA and truncated WbdD derivatives were investigated. The β-galactosidase activity from BTH101[pWQ488, pWQ487] (T25–WbdD1–669-T18–WbdA) was indistinguishable from blue colonies (Fig. 8A) and 328 ± 21 units of β-galactosidase activity (Fig. 8C), providing strong evidence for a physical interaction between WbdD and WbdA. This interaction was not facilitated by indirect interactions with other dedicated O9a PS biosynthesis proteins because they were absent from the reporter strain, BTH101. However, indirect interaction involving “housekeeping” proteins common to BTH101 and E. coli O9a cannot be ruled out. To confirm that WbdD and WbdA interact in the native E. coli O9a background, a cyA mutant (CGW910) was constructed from CWG634 and cotransformed with plasmids pWQ486 and pWQ487. The CWG634 lineage contained a defect in the lac operon, and thus, cAMP-dependent maltose utilization was used as an alternative reporter to detect adenylate cyclase activity. CGW910[pWQ486, pWQ487] grew as red colonies on LB maltose indicator agar (Fig. 8B), verifying that the WbdD-WbdA interaction was not an artifact of expression in E. coli BTH101.
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In the biosynthesis of the O9a O-PS, WbdD plays a pivotal role in adding nonreducing terminal modifications that terminate chain extension and create a polymer that is competent for export (12, 13). Here we identify a new role for WbdD in controlling polymerization by coordinating the correct membrane association required for activity of one of the critical mannosyltransferases, WbdA.

The interaction between WbdA and WbdD likely occurs through multiple contact sites. A polypeptide comprising the C-terminal 190 amino acids of WbdD (WbdD_{519–708}) was sufficient to form a productive interaction with WbdA in the two-hybrid assay. Within this region we have defined two segments (TIR1 and TIR2; Fig. 5) that must both be present to maintain the WbdD-WbdA interaction. Output data from the COILS algorithm (31) indicated that the WbdD sequence between residues 487 and 588 (including TIR1) had a high probability of forming multiple coiled-coils (Fig. 5). Although coiled-coil structures are well known as mediators of protein-protein interactions (34), the WbdD-WbdA interaction cannot be attributed to direct coiled-coil interactions because the predicted WbdA secondary structure does not contain complementary coiled-coils. However, intra-protein coiled-coil interactions may be important to maintain the WbdD tertiary structure and contribute indirectly to the interaction with WbdA. Under such circumstances, the role of TIR1 may be indirect; deletion of the putative coiled-coil domain within TIR1 may influence the folding of the truncated WbdD derivative and prevent interaction with WbdA through TIR2. Despite this uncertainty the data unequivocally implicate the C-terminal domain in mediating the interaction with WbdA.

We have also identified a membrane-binding region (amino acids 601–669) near the C terminus of WbdD. One striking feature of the primary sequence of this region is the high number of positively charged amino acids. The outputs from secondary structure prediction programs identify four α-helices (H5–H8) within this region, and helical wheel projections demonstrated that basic amino acids are clustered on one side of each predicted helix (Fig. 5). Helices H6, H7, and H8 could be considered amphipathic, and collectively, they could possibly form a membrane-interactive structure. The putative α-helices could potentially be partially embedded into the membrane with the basic amino acids stabilizing the structure by interacting with negatively charged phospholipid head groups. Alternatively, α-helices H5–H8 could adopt a fold in which the nonpolar residues are embedded in the interior of the structure.
with the basic residues exposed on the surface where they could form ionic interactions with anionic phospholipid head groups.

In the generally accepted view of bacterial cell surface polysaccharide biosynthesis, glycosyltransferase reactions occur at the inner face of the cytoplasmic membrane, and this has been consistently supported in the literature. In most systems, the nascent sugar chains are assembled on the C55 carrier lipid, undecaprenol-P (reviewed in Ref. 1). Presumably, tethering the growing polymers to the membrane places them in proximity to the export apparatus and thus promotes efficient transport out of the cytoplasm. It is still uncertain how this might be achieved, but in one proposal, undecaprenol itself may play a role in organizing a complex (35). Polysaccharides synthesized by ABC transporter-dependent pathways are completely elongated prior to export. Maintaining the glycosyltransferases and modifying enzymes within a multiprotein complex is reasonably assumed to be advantageous for the cell, because tight coordination of the assembly reactions would maintain the efficiency and fidelity of the polymerization process. We have demonstrated that the 

E. coli O9a O-PS chain terminator enzyme WbdD and the mannansyltransferase WbdA form a complex at the cytoplasmic membrane. The O9a-PS chain length is strictly regulated by the kinase/methyltransferase activity of WbdD (12). Intimate interaction between WbdD and WbdA could possibly facilitate synchronization of chain termination with the polysaccharide elongation reaction. Notably, the other mannansyltransferases, WbdC and WbdB, target to the membrane without WbdD, and they do not show any interactions with WbdD in two-hybrid analyses. In the current understanding of O9a biosynthesis, WbdD is reported to transfer the first mannosyl residue to the und-PP-GlcNAc acceptor, followed by a combination of WbdB and WbdA (10). In preliminary experiments, WbdB appears to add a second mannosyl residue to complete the adaptor region (Fig. 1A). This is followed by chain extension mediated by WbdA alone. This is consistent with the proposal that WbdA contains two mannansyltransferase domains (36) and with detailed structural information for the resulting O-PS (11). An early step in the export of the O9a PS across the inner membrane involves binding of the terminated undecaprenol-PP-linked PS with the nucleotide-binding component (Wzt) of the ABC transporter complex (13). The presence of the WbdD-WbdA termination/elongation complex on the membrane could facilitate delivery of newly terminated O-PS to the transporter complex. Investigations into potential interactions between the ABC transporter enzymes and the WbdD-WbdA complex will be required to test this hypothesis.

Polysaccharide assembly complexes have been described for other bacterial systems. The 

E. coli 2 capsular polysaccharides are synthesized by an ABC transporter pathway with assembly and export mechanisms similar to O9a-PS biosynthesis (37). The capsule biosynthesis proteins KpsC and KpsS are membrane-associated and mutations in either kpsC or kpsS abrogated export of both the 

E. coli K5 PS (38) and the closely related K1 PS (39). Both KpsC and KpsS were shown to be essential for membrane localization of the K5 glycosyltransferase enzymes KfiA and KfiC (40). Furthermore, cross-linking experiments provided evidence for a biosynthetic complex involving the export proteins in the inner and outer membranes, the glycosyltransferases, and the ABC transporter proteins (15). In 

E. coli O9a O-PS biosynthesis WbdD may have a role analogous to KpsC. However, there are some significant differences between the K1 and K5 systems, despite conservation in many of the export/assembly proteins. For example, in K1, the KpsC and KpsS homologs were not essential for membrane localization of the NeuS glycosyltransferase, (41), although KpsC was shown to interact with NeuS and with the export protein, KpsE (16). Also, biosynthesis of the 

E. coli K1 polysaccharide capsule occurs within a putative complex that protects newly synthesized polymer from degradation by a specific glycanase (16). The same is not the case in K5 (42). It is apparent that group 2 capsule biosynthesis involves a complex network of protein-protein interactions that may vary depending on the context (i.e. the precise polysaccharide structure). To understand the general principles involved in complex formation, it will therefore be necessary to have a complete view of the interactions within several model systems. This work represents an important first step for the O9a O-PS, the prototype for the widely distributed ABC transporter-dependent O-antigen biosynthesis pathway.

How common might this type of system be? A subset of bacterial glycans, synthesized by ABC transporter-dependent pathways, have modifications at the nonreducing termini. These glycans include O-PS (11, 43, 44) and S-layer glycoprotein examples (45). Such terminal residues are often overlooked in structural analyses that typically focus on the residues in the repeat unit domain of the polymers. Presumably, synthesis of these polymers involves the activities of chain terminating enzymes analogous to WbdD. The 

E. coli O8 polysaccharide biosynthesis gene cluster encodes a WbdD paralog, which adds a terminal methyl group to terminate polymerization (12). Biosynthesis of the 

Geobacillus stearothermophilus S-layer (glycoprotein) glycan involves essentially the same pathway. One enzyme, WsaE, contains domains for both glycosyltransferase and chain termination activity (45), providing an ideal mechanism to coordinate elongation and termination. In another example, assembly of the 

E. coli O52 O-PS may involve a bifunctional glycosyltransferase/chain termination enzyme (43). It is possible that the O-PS elongation/termination complex described here for 

E. coli O9a O-PS assembly represents a common biosynthetic mechanism that is shared by numerous ABC transporter-dependent systems.

Acknowledgment—We thank Lisa Bertolo for technical assistance.

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