The chain length of bacterial lipopolysaccharide O antigens is regulated to give a modal distribution that is critical for pathogenesis. This paper describes the process of chain length determination in the ATP-binding cassette (ABC) transporter-dependent pathway, a pathway that is widespread among Gram-negative bacteria. Escherichia coli O8 and O9/O9a polymannans are synthesized in the cytoplasm, and an ABC transporter exports the nascent polymer across the inner membrane prior to completion of the LPS molecule. The polymannan O antigens have nonreducing terminal methyl groups. The 3-O-methyl group in serotype O8 is transferred from S-adenosylmethionine by the WbdD_{O8} enzyme, and this modification terminates polymerization. Methyl groups are added to the 09a polymannan in a reaction dependent on preceding phosphorylation. The bifunctional WbdD_{O9a} catalyzes both reactions, but only the kinase activity controls chain length. Chain termination occurs in a mutant lacking the ABC transporter, indicating that it precedes export. An E. coli wbdD_{O9a} mutant accumulated O9a polymannan in the cytoplasm, indicating that WbdD activity coordinates polymannan chain termination with export across the inner membrane.

Lipopolysaccharide (LPS) is a unique and abundant glycolipid found in the outer membranes of Gram-negative bacteria. LPS has three structural domains (1). The hydrophobic lipid A forms the outer leaflet of the outer membrane and is responsible for the endotoxic properties of LPS. A short core oligosaccharide extends from lipid A. In many bacteria, the core is capped with a repeating unit glycan polymer known as the O polysaccharide (O-PS; O antigen). Lipid A is structurally conserved among Gram-negative bacteria, whereas limited variability of the core oligosaccharide is often observed within species. For example, five distinct core structures have been identified in different isolates of Escherichia coli (2). In contrast, O-PS structures vary extensively within a given species because of differences in the number and type of sugars in the repeat unit and the nature of glycosidic linkages within and between repeat units. Variation of the O-PS forms the basis of the O-antigen serotyping scheme. There are ~170 O serotypes in E. coli (3). LPS preparations from a given isolate contain a spectrum of molecular species with different sizes. The distribution can range from lipid A core molecules devoid of O-PS to LPS molecules with greater than 100 O-PS repeat units. However, most O-PS-substituted LPS molecules in a preparation fall within a limited size range (i.e. a modal distribution). Lipid A-core and O-PS are synthesized separately at the cytoplasmic face of the inner membrane. The two component parts are subsequently ligated at the periplasmic face of the inner membrane prior to export to the cell surface (reviewed in Ref. 1). Goldman and Hunt (4) first suggested that the modal distribution was established by competition between O-PS polymerization and termination (by ligation). However, subsequent work has implicated specific components of the O-PS biosynthesis systems in regulating the modal distribution of some O-PSs (see below).

O-PSs are assembled on a 55-carbon lipid acceptor, undecaprenol phosphate (und-P), and biosynthesis is initiated by transfer of a sugar-1-phosphate residue from its nucleotide diphosphosugar precursor to und-P. Subsequent extension and processing of the und-PP-linked intermediate proceeds through one of three distinct pathways. Although one of these pathways is currently confined to a single example (5), the other two are widespread among Gram-negative bacteria. The two major pathways are termed Wzy (polymerase)-dependent and ATP-binding cassette (ABC) transporter-dependent biosynthesis, respectively. They differ by the mechanisms involved in polymerization of the repeat units and in the process of translocation of the und-PP-linked polymer or intermediates across the inner membrane.

In the Wzy-dependent mechanism (reviewed in Ref. 1), single O-PS repeat units are assembled on an und-P carrier lipid by sequential glycosyltransferase reactions. A translocase (Wzx) then mobilizes und-PP-linked repeat units to the periplasmic face of the inner membrane, where the polymerase (Wzy) assembles those repeat units into und-PP-linked polysaccharide. Chain extension occurs by transfer of the growing glycan from the und-PP carrier to the nonreducing terminus of another und-PP-linked monomer, effectively extending the chain one repeat unit at a time. The extent of polymerization is controlled in a process that is not yet understood by the chain-length regulator protein, Wzz (formerly Rol or Cld) (6–8). Different Wzz proteins confer a characteristic modal distribution of O-PS chain length when expressed in a heterologous Wzy-dependent system (9, 10). The O-PS chains of wzz mutants are composed
predominantly of one or two repeat units, and the amount of each molecule is inversely proportional to its size, i.e. fully elongated LPS species are extremely rare (7, 11).

In the ABC transporter-dependent pathway, O-PS chains are elongated on the und-PP-linked intermediate by processive glycosyl transfer onto the nonreducing end of nascent polymer (reviewed in Ref. 1). Polymerization occurs within the cytoplasm, and an ABC transporter is required for export of the nascent polymer to the periplasmic face of the inner membrane, where it is ligated to lipid A-core (12). This system requires neither a Wzy polymerase enzyme nor a Wzx chain-length regulator. However, the resulting O-PSs still exhibit a modal chain-length distribution. Recent structural analysis has identified novel residues at the nonreducing end of some O-PSs synthesized by the ABC transporter-dependent pathway (Ref. 13 and the references therein). Here we describe for the first time the role played by these nonreducing terminal modifications in O-PS chain termination.

The polymann O-PSs of E. coli O8, O9, and O9a provide models for the ABC transporter-dependent pathway. These polymers share structural features and conserved biosynthesis steps, reflecting genes common to the O-PS biosynthesis genetic loci (Fig. 1). The polymann strains have nonreducing terminal O-methyl groups (13, 14). Identical O-PS structures and gene clusters are found in Klebsiella pneumoniae O3 (E. coli O9) and K. pneumoniae O5 (E. coli O8) as a result of lateral gene transfer (15). Structural and biosynthetic data for the E. coli and K. pneumoniae analogs are interchangeable. The mechanism of details of assembling the E. coli O8 and O9a O-PSs on the lipid carrier have not been fully elucidated, but a working biosynthetic model has been proposed based on the available structural and genetic evidence (13, 16). O-PS synthesis begins with the conserved formation of a “primer” by transfer of a GlcNac-1-phosphate residue to und-P. This step is catalyzed by the product of the wecA gene (17). WecA activity is not confined to O-PS biosynthesis, and the structural gene is located outside of the O-PS biosynthesis gene cluster (18). Using the primed lipid intermediate as an acceptor, an adaptor disaccharide is assembled by the mannosyltransferases WbdC and WbdB (16). Adaptor synthesis commits the lipid intermediate to the O-PS biosynthetic pathway. The remaining polymer containing the repeating unit is assembled by the processive activity of one or both of the WbdA and WbdB mannosyltransferase loci. The E. coli O9 and O9a structural variants arise from mutations affecting the WbdA mannosyltransferase, and a single amino acid change is sufficient to alter the structure of the O-PS repeat unit (Fig. 1) and convert serotype O9 to O9a (19). The primer, adaptor, and repeat-unit domains are clearly identified in structural studies of the LPSs (13). In this report we demonstrate that the WbdD proteins control the chain length of the E. coli O8 and O9a polymannans by modifying the nonreducing end of nascent und-PP-linked polymer. Additionally, we provide evidence that these terminal modifications couple polymerization/termination with the export of nascent O-PS across the inner membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains used in this study are described in Table I. Bacteria were grown at 37 °C in either LB medium (20) or M9 minimal medium (21). When required, media were supplemented with glucose (0.4% w/v), mannose (0.2 or 0.4% w/v), sucrose (5% w/v), histidine (22 µg/ml), tryptophan (200 µg/ml), or thiamine (1 µg/ml). The antibiotics ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), gentamicin (15 µg/ml), kanamycin (25 µg/ml), streptomycin (200 µg/ml), and tetracycline (15 µg/ml) were added when required. Strains harboring wbdD or wms-uzt mutations were maintained on media lacking mannose to avoid the high frequency acquisition of second-site mutations that relieved the growth defect by eliminating polymannan synthesis.

**Construction of manA Mutants—**Mutants defective in manA 6-phospho-α-mannose isomerase (manA) were constructed by transforming the manA allele from the Hfr donor E. coli CWH10 into CWG291 (O8) and CWG28 (O9a). Hfr-mediated chromosomal transfer was performed by plate mating and manA derivatives (CWG636, O8 and CWG634, O9a) were obtained by selecting for a Tn 10 deletion (Tc') linked to the manA allele (Table I). The ManA phenotype was confirmed by the lack of acid production on MacConkey-mannose agar and by showing that O-PS synthesis was dependent on the inclusion of mannose in the growth media.

**DNA Methods—**Custom-made oligonucleotides were obtained from Sigma-Genosys and used in PCR amplification under conditions optimal for each primer pair. Template DNA was obtained by resuspending bacterial cells from a single colony directly into water. DNA polymerase (Roche Applied Science) was used if amplification products were to be cloned, and Platinum Taq (Invitrogen) was used for screening of mutants and ligation products. PCR products and restriction fragments were purified either from agarose gels with the Ultraclean 15 DNA purification kit (MOBio Laboratories) or directly from the PCR with the Quick PCR purification kit (Qiagen). Plasmid DNA was purified with the GeneElute plasmid purification kit (Sigma). Restriction endonuclease digestions, DNA modification, and DNA ligation were all performed using standard methods as recommended by the enzyme manufacturers. DNA sequencing was performed by the Guelph Molecular Supercenter (University of Guelph, Guelph, Canada).

**Cloning and Overexpression of the wbdD Gene Products**—The genes encoding WbdD_o8 and WbdD_o9a were cloned on DNA fragments amplified by PCR from the chromosomies of E. coli 2775 and E69, respectively. Restriction sites designed in the primers were used to clone the amplified fragments in pBAD24 (22) to form pWQ53 (O8) and pWQ52 (O9a). The primers for wbdD_o8 were 5'-CCCAGAATTCCATATGCTGCT-CCCTTATTG3' (EcoRI site underlined) and 5'-CAGATTCTCTGACATTATTTCCGGTGAT-3' (SallI). Those for wbdD_o9a were 5'-GATCGAATTCCATAGCTTATCAAAAGCCGCT-CTG3' (EcoRI) and 5'-CTAGATTCTCTGATTTTCTGCAGTTACTTGTCGTCGTCCT-3' (BamHI). The wbdD_o8 gene was cloned into the one expression vector pUC-Gm (23) to form pWQ54. The wbdD_o9a insertion (Tcr) linked to the aacC1 gene from pUC-Gm (23) to form pWQ54. The aacC1 gene cassette is nonpolar when inserted in the same orientation as wbdD. A fragment containing the inactivated wbdD gene was isolated from pWQ52 and cloned into the temperature-sensitive suicide delivery vector pKO3 (24) to generate pWQ55. To transfer the wbdD_o8::aacC1 mutation to the E. coli O9a chromosome, CWG634(pWQ55) transformants were plated onto LB (glucose and chloramphenicol) agar and incubated at 45 °C. Colonies from the initial selection, potentially containing integrants, were pooled and subjected to further selection for plasmid excision on LB (glucose, sucrose, and gentamycin) agar at 30 °C.

For construction of a wms-uzt chromosomal mutation, a fragment containing both wms-uzt and flanking DNA was amplified by PCR from E. coli CWH26 chromosomal DNA using the primers, 5'-TTCCGTTAC-CATGACGAGGCGTCGTTTATCG-3' (KpnI site underlined) and 5'-CTCTAGATCTCGAGGAGGCT-3' (XbaI). The fragment blpIUScriptSK was modified by excising a HindIII-EcoRV fragment from the multiple cloning site. The unique restriction sites designed in the primers were used to insert the resulting FLAG-encoding fragment in-frame at the 3' end of the wbdD_o8 open reading frame.

**Construction of Chromosomal Insertion Mutations by Allelic Exchange—**The wbdD_o8 gene was inactivated by removing a 0.5-kb Smal fragment from the middle of the open reading frame cloned in pWQ52 and replacing it with a Smal fragment containing the aacC1 gene from pUC-Gm (23) to form pWQ54. The aacC1 gene cassette is nonpolar when inserted in the same orientation as wbdD. A fragment containing the inactivated wbdD gene was isolated from pWQ52 and cloned into the temperature-sensitive suicide delivery vector pKO3 (24) to generate pWQ55. To transfer the wbdD_o8::aacC1 mutation into the E. coli O9a chromosome, CWG634(pWQ55) transformants were plated onto LB (glucose and chloramphenicol) agar and incubated at 45 °C. Colonies from the initial selection, potentially containing integrants, were pooled and subjected to further selection for plasmid excision on LB (glucose, sucrose, and gentamycin) agar at 30 °C.
Fractions were separated from the cleared lysate by centrifugation at 100,000 g.

The reactions were incubated at 30 °C for 1 h, resuspended in 0.75 ml of assay buffer, and stored at -75 °C.

In Vitro Incorporation of Radiolabeled Substrates into Membrane Fractions—Time-dependent incorporation of [14C]mannose from GDP-[14C]mannose into polymannan was assayed in 1 ml of assay buffer (50 mM HEPES, pH 7.5). The cells were lysed by ultrasonication with intermittent cooling on ice. The cell lysate was collected onto MicronSep 0.45-

membranes were assayed in triplicate.

For experiments involving SDS-PAGE of radiolabeled O-PS intermediates, the reactions were performed in 0.1 ml of assay buffer containing membranes (0.1 mg of protein). Unless indicated otherwise, unlabeled substrates consisted of 1 μM GDP-mannose, 2.5 mM S-adenosylmethionine, and 10 μM ATP. In individual experiments, one of the unlabeled substrates was substituted with the respective radiolabeled substrate: GDP-[14C(U)]mannose (1 μM, 330 mCi/mmol; PerkinElmer Life Sciences); S-[methyl-3H]adenosylmethionine (1 μM, 55 Ci/mmol; PerkinElmer Life Sciences), and -[γ-32P]ATP (50 μM, 7000 Ci/mmol; ICN). The reactions were incubated at 30 °C for 1 h and stopped by the addition of 25 μl of SDS-PAGE sample buffer (312 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 0.0125% (w/v) bromphenol blue, 50% (v/v) glycerol, 1.8 M β-mercaptoethanol). The samples were heated in a boiling water bath for 5 min, and 20-μl aliquots were separated by SDS-PAGE. SDS-PAGE gels containing products labeled with [14C]mannose and [32P]were visualized using a PhosphorImager (Bio-Rad Molecular Imager FX). Products labeled with [3H]methyl residues were visualized by fluorography on Kodak BioMax MR 1 film and using Amplify (Amersham Biosciences) as the fluorographic reagent.

Preparation of Anti-O9a Antibody—New Zealand White rabbits were immunized with formalin-killed whole cells of E. coli CWG298. To prepare O9a-specific antibodies, immune serum was adsorbed with whole cells of both E. coli CWG291 and F470.

Immunofluorescence Microscopy—Immunofluorescence microscopy was used to visualize the O9a antigen in fixed cells with and without a permeabilizing step. Bacteria were grown in M9-glucose (0.2% w/v) broth at 37 °C to an A600 nm of 0.3. Mannose (0.4% w/v) was added to the cultures, and incubation was continued for 2 h to allow accumulation of O-PS. Cells (A450 nm) were collected by centrifugation at 4500 g, resuspended in 1 ml of formaldehyde solution (5% (v/v) in PBS), and incubated at 4 °C for 16 h. Fixed cells were collected by centrifugation, washed twice in 1 ml of PBS, and resuspended in 0.1 ml of PBS. The cell
suspension (0.01 ml) was applied to the well of a glass slide coated with poly-L-lysine and incubated at room temperature for 10 min. Bacterial cells were made permeable by a modification of a method described elsewhere (29). The fixed cell sample was incubated at room temperature for 15 min in 10 ml of lysozyme solution (0.5 mg/ml in 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and then at room temperature for 15 min in Triton X-100 (0.1% v/v in PBS). Antibody labeling of the O9a O-PS was performed by treating the slides at room temperature for 15 min with bovine serum albumin solution (1% w/v in PBS), washing extensively in PBS, and incubating with anti-O9a antiserum (1:100 in bovine serum albumin solution) at 37°C for 30 min. The slides were washed extensively in PBS and treated with rhodamine red-conjugated goat anti-rabbit antibody (1:50 in bovine serum albumin solution; Jackson Immunoresearch). The labeled slides were washed in PBS and mounted in Vectashield (Vector Laboratories). Bacteria were viewed on a Zeiss Axiovert 200 microscope using a 100x objective lens, and the images were processed using Openlab software (Improvision).

RESULTS

Conserved Motifs in the WbdD Proteins Implicate Them in the Addition of Nonreducing Terminal Residues to the O8 and O9a Polymannans—The predicted WbdD proteins from E. coli serotypes O8 and O9a differ in size at 48,630 Da (O8) and 81,731 Da (O9a), respectively. Although these proteins share only limited overall similarity, they do contain the same conserved motifs (Fig. 2). WbdD08 and WbdD09a share a region of sequence near their C termini that exhibits 34% identity and 53% similarity over 113 amino acids (Fig. 2). These domains are predicted to have a high probability of forming coiled-coil structures as determined by the COILS algorithm (30). Each exhibits the characteristic heptad repeat motif with hydrophobic residues at positions a and d and charged or polar residues at e and g. Residues a and d provide the interface between interacting α-helices, and different modes of interaction are possible. An additional putative coiled-coil domain (probability 95%) is found in WbdD O9a only (Fig. 2).

The N-terminal regions of the WbdD proteins share significant similarity with AdoMet-dependent methyltransferases. The WbdD proteins share the most similarity with a consensus motif from the UbiE family of methyltransferases that are involved in ubiquinone/menaquinone biosynthesis (32). Each exhibits the characteristic heptad repeat motif (31) with hydrophobic residues at positions a and d and charged or polar residues at e and g. Residues a and d provide the interface between interacting α-helices, and different modes of interaction are possible. An additional putative coiled-coil domain (probability >95%) is found in WbdD09a only (Fig. 2).

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To analyze the effect that overexpressing the WbdD proteins had on O-PS synthesis, strains containing either pWQ52 or pWQ53 were grown for 16 h in 5 ml of LB containing 0.4% (w/v) glucose to repress WbdD expression. The cultures were then diluted 1:100 into media containing 0.1% (w/v) L-arabinose (or no arabinox, respectively) and the cultures were grown to an A600 of 0.5. A, silver-stained SDS-PAGE of E. coli O8 and O9a LPS. The samples were prepared from transformants of E. coli CWG634 (O9a manA) and CWG636 (O8 manA) containing plasmids pWQ52 (wbdD<sub>08a</sub>) and pWQ53 (wbdD<sub>09a</sub>), respectively. Cultures were grown in the presence of 0.2% (w/v) l-arabinose, providing permissive conditions for polymer synthesis in the manA background. In the absence of arabinose (lanes 1 and 3), wbdD expression is noninduced, and the profile is identical to wild-type E. coli prototypes for the O8 and O9a antigens (data not shown). The position of high molecular weight O-PS-substituted LPS is indicated. Note the multiple clusters of LPS molecules with modal O-PS chain lengths (indicated by asterisks). The addition of 0.1% arabinose (and induction of wbdD expression; lanes 2 and 4) results in an altered nonmodal profile. B, effect of wbdD<sub>08a</sub> overexpression on the incorporation of GDP-[<sup>14</sup>C]mannose from GDP-[<sup>14</sup>C]mannose into polymannan with AdoMet and its control of polymannan chain length. The membranes from the cells overexpressing WbdD<sub>08a</sub> showed a significant (2.3-fold) reduction in [<sup>14</sup>C]mannose incorporation compared with membranes isolated from noninduced culture (Fig. 3B). The altered LPS SDS-PAGE profile resulting from WbdD<sub>08a</sub> overexpression could reflect either a direct effect on O-PS chain length or an alteration favoring preferential selection of short O-PS chains for ligation onto lipid A-core. To resolve these possibilities, E. coli CWG28 (O9a) membrane fractions were used to determine the effect of wbdD<sub>09a</sub> overexpression on the incorporation of [<sup>14</sup>C]mannose from GDP-[<sup>14</sup>C]mannose into polymannan in vitro. The membranes from the cells overexpressing WbdD<sub>09a</sub> showed a significant (2.3-fold) reduction in [<sup>14</sup>C]mannose incorporation compared with membranes isolated from noninduced culture (Fig. 3B).
intermediates synthesized in vitro by the ABC transporter-dependent mechanism are not ligated to lipid A-core, and recycling of und-PP does not occur, so the observed in vitro [14C]-mannose incorporation reflects a single round of extension of endogenous lipid-linked intermediates (34). Consequently, the low [14C]-mannose incorporation observed in membranes from cells overexpressing wbdD<sub>09a</sub> is consistent with control of O-PS chain length by WbdD at the level of chain elongation.

**Terminal Methylation Determines Chain Length of Nascent E. coli O8 Polymannan**—To determine whether methyl groups were transferred to und-PP-linked polymannan, further in vitro studies were performed. Membrane fractions from E. coli CWG636 were incubated with S-Ado-[3H]Met and unlabeled GDP-mannose, and the products were examined by SDS-PAGE. High molecular weight products were identified with a banding pattern typical of variable O-PS chain lengths (Fig. 4A). The radiolabeled bands corresponded in size and distribution to the products from a parallel reaction containing GDP-[14C]mannose and unlabeled AdoMet. The absence of S-Ado-[3H]Met-labeled products in reactions lacking GDP-mannose (data not shown) provided confirmation that the high molecular weight products were indeed polymannan O-PSs.

Titration of unlabeled AdoMet in an in vitro reaction with GDP-[14C]mannose showed a concentration-dependent reduction in O-PS chain length (Fig. 4B). In the absence of added AdoMet, the labeled products were aberrantly large. A substantial reduction in chain length was evident in the presence of 1 μM AdoMet, and a small additional reduction occurred when the amount of AdoMet was increased to 10 μM. Beyond that, no further reduction in polymannan chain length was detected. The concentration-dependent effect of AdoMet provided further support for the critical role of WbdD and 3-O-methylation in the regulation of chain length in serotype O8 polymannan.

**A Phosphorylation Process Determines Chain Length of Nascent E. coli O9a Polymannan**—The presence of putative kinase and methyltransferase domains in the WbdD<sub>09a</sub> protein suggested a role for both phosphorylation and methylation in the synthesis of the O9a O-PS. To establish whether und-PP-linked E. coli O9a O-PS was methylated, membranes from E. coli CWG634 were incubated with S-Ado-[3H]Met in various combinations with unlabeled ATP and GDP-mannose. Labeling of high molecular weight products occurred only in those reactions containing both GDP-mannose and ATP (Fig. 5A, lane 4) but not in reactions in which GDP-mannose and/or ATP was omitted (Fig. 5A, lanes 1–3), indicating that methylation was dependent on both polymer synthesis and phosphorylation. The products were also examined from corresponding reactions containing GDP-[14C]mannose as the source of label (Fig. 5B). Polymannan chain length was aberrantly large in the absence of AdoMet and ATP (Fig. 5B, lane 1). Chain length was reduced in reactions containing ATP plus AdoMet and ATP alone but...
Chain Length Determination and Export of O Antigen

not in those supplemented with only AdoMet (Fig. 5B, lanes 2–4). The chain length was reduced in an ATP concentration-dependent manner, with effects becoming apparent at a concentration of 0.02 mM (Fig. 5C). Further reductions were evident as the concentrations of ATP increased to 0.2 and 2 mM. These results explained the inability to establish direct connectivity between a specific mannose residue and the methyl group during NMR structural analyses of the K. oxytoca O5 polymannan (13).

The nature of the precursors for WbdD-mediated chain termination (AdoMet and ATP) and the evident effects in in vitro reactions suggested that these reactions occurred in the cytoplasm prior to export of nascent polymannan. To unequivocally verify that this was the case and provide further insight into the order of events, the effect of ATP on chain length was assessed in CWG638Δwzm-wzt:aphA-3. The absence of the ABC transporter ensured that the products examined were und-PP-linked glycans and that only those events preceding export would be examined. The ATP concentration-dependent reduction in chain length of in vitro reaction products synthesized by membranes from the export-deficient mutant was indistinguishable from that seen with membranes from the export-proficient parent (Fig. 5, compare C and D). These results indicated that chain-length determination preceded export and that chain length was not simply controlled by the rate of export of und-PP-O-PS through the ABC transporter.

To unequivocally confirm the involvement of WbD in chain-length determination, a chromosomal wbdD::aacC1 mutation (E. coli CWG635) was constructed by allelic exchange. As shown in Fig. 6A, membranes from E. coli CWG635 were still able to synthesize O9a polymannan in vitro, although the chain length was increased relative to that made by the parent. However, the polymeric product was not labeled by either S-Ado-[3H]Met or [32P]ATP (Fig. 6, B and C), nor was the chain length responsive to increasing ATP concentration in the reaction (Fig. 6D).

Terminal Modification of the O9a O-PS by WbdD Is Essential for Its Export from the Inner Membrane—Growth of the chromosomal wbdD::aacC1 mutant (E. coli CWG635) was impaired in the presence of mannose, i.e., permissive conditions for O9a polymannan synthesis in vivo (Fig. 7B). Complementation of the mutation with a plasmid carrying wbdD::aacC1 restored normal growth properties (data not shown), indicating that the impairment was due only to the wbdD::aacC1 defect and did not involve an unanticipated second-site mutation or any polar effects of the chromosomal insertion on downstream genes. To overcome the growth defect in E. coli CWG635 and examine O-PS synthesis, E. coli CWG634 and CWG635(wbdD::aacC1) were grown to early exponential phase in minimal-glucose media, and mannose was then added to activate polymannan synthesis. The LPS profiles were then assessed by SDS-PAGE analysis of whole cell lysates (Fig. 7A). The parent strain, E. coli CWG634, produced typical high molecular weight O9a LPS. In contrast, CWG635(wbdD::aacC1) did not exhibit lipid A-core-linked O9a O-PS under permissive conditions. When a functional wbdD::aacC1 gene was supplied in trans on plasmid pWQ52, expression of the high molecular weight O9a LPS was restored (Fig. 7A).

samples were taken, and whole cell lysates were prepared for silver-stained SDS-PAGE. B, growth impairment in E. coli CWG635 (wbdD::aacC1) under conditions permissive for O9a-polymannan synthesis. The growth of E. coli CWG635 in M9-glucose (0.2% w/v) was followed after the addition of either mannose (0.4% w/v) final concentration) or an equivalent volume of water. The growth curve was performed in triplicate, and the range of variation for individual time points was within ±10%. C, in vitro synthesis of O9a polymannan by membranes prepared from E. coli CWG635 (wbdD::aacC1) and its parent, CWG634.

Fig. 7. Phenotypic analysis of E. coli CWG635 (wbdD::aacC1). A, examination of the capability of E. coli CWG634, CWG635 (wbdD::aacC1), and CWG635 transformed with pWQ52 (expressing WbdD) to synthesize O9a antigen-substituted LPS. Cultures were grown to mid-exponential phase (OD600 = 0.4) in minimal medium containing 0.2% (w/v) glucose (i.e., conditions that were not permissive for O9a-polymannan synthesis, because of the manA mutations in each strain). At this point, each culture received mannose (final concentration, 0.4%) to allow O9a synthesis to proceed. After 2 h of further incubation,
The ability of membranes from *E. coli* CWG635-\(wbdD_{O9a}::aacC1\) to synthesize O9a polymannan was clearly established by PAGE analysis of the *in vitro* products reported in the preceding text. Direct comparative analysis of the mannosyltransferase activity in membranes, assayed in the absence of ATP, indicated that the \(wbdD_{O9a}\) membranes showed only slightly less activity than those of the parent (Fig. 7C). The reason for this slight reduction is unclear but could be related to minor polarity issues resulting from the insertion mutation or reduced amounts of mannosyltransferases because of altered stability of the mRNA in the mutant. Regardless, the absence of O-PS-substituted LPS in *E. coli* CWG635 (\(wbdD_{O9a}::aacC1\)) could only be explained by either an unexpected inability to form polymannan *in vivo* or an inability to export the polymer for ligation to lipid A-core at the periplasmic face of the membrane. To address these possibilities, the location of polymannan was determined by immunofluorescence microscopy (Fig. 8) using antibody specific for the O9a antigen. The parent, *E. coli* CWG634, showed strong immunofluorescence (Fig. 8A) at the cell periphery as expected for surface exposed LPS. In contrast, *E. coli* CWG635 (\(wbdD_{O9a}::aacC1\)) showed no surface immunofluorescence (Fig. 8B), a phenotype shared with *E. coli* CWG638 (\(wzm-wzt::aphA-3\)) (Fig. 8D). *E. coli* CWG638 also showed evidence of cell elongation and filamentation. To confirm that the \(wzm-wzt\) and \(wbdD_{O9a}\) mutants synthesized intracellular O9a polymannan, the cells were permeabilized, and diffuse internal immunofluorescence was detected throughout the cytoplasm (Fig. 8, C and E). Thus the inability of \(wbdD_{O9a}\) mutants to make O-PS-substituted LPS is due to a defect in export.

**DISCUSSION**

The regulation of O-PS chain length is critical for virulence of Gram-negative bacteria. Efficient chain-termination processes are required for the establishment of an appropriate modal chain length distribution and for ensuring that most lipid A-core is capped with O-PS. These characteristics dictate resistance to complement-mediated serum killing and virulence (35). The involvement of Wzz (formerly Rol or Cld) in regulating the chain length of O-PS synthesized by Wzy-dependent pathways is well accepted. Mutations in \(wzz\) make *Salmonella enterica* serovar Typhimurium (11), *E. coli* O75 (36), and *Shigella dysenteriae* serotype 2a (37) sensitive to complement and avirulent. *Yersinia enterocolitica* O:8 mutants with unregulated chain length are sensitive to killing by polymorphonuclear leukocytes and are attenuated for virulence (38). In *S. dysenteriae* 2a, Wzz creates precise modal O-PS chain length that is critical for the surface exposure of IcsA and therefore influences actin-based motility and intercell invasion (37, 39–42). Although the exact role of O-PS chain length may vary in different Gram-negative pathogens, it is anticipated that Wzz function is important in other bacterial pathogens whose O-PS is synthesized in a Wzy-dependent pathway. In the Wzy-dependent pathway, the polymerization of O-PS occurs at the periplasmic face of the inner membrane, and products of varying lengths are efficiently ligated to lipid A-core. Thus the influence of Wzz on O-PS chain length is readily apparent in SDS-PAGE profiles of LPS from a culture. The critical role for O-PS chain length in the biology of

![Fig. 8. O9a-polymannan export defect in *E. coli* CWG635 \(wbdD_{O9a}::aacC1\). Immunofluorescence microscopy displaying the presence of the O9a O-PS in whole and permeabilized cells of *E. coli*](#)
bacterial pathogens is not expected to be confined to those with Wzy-dependent O-PS biosynthesis, but the differences in the synthetic mechanism and location of polymerization dictate that a fundamentally different process must be involved for chain-length determination in the ABC transporter-dependent pathway. The data reported here give the first insight into the mechanism involved. Addition of a novel nonreducing terminal residue provides a simple process to terminate the action of processive glycosyltransferase activity. However, the process cannot be simply stochastic or random but must incorporate an ability to regulate (i.e. terminate when the chain length reaches the appropriate modal value). In in vitro experiments with the E. coli O8 and O9a polymannans, this can be achieved by titrating the amount of AdoMet or ATP precursors. This may be a factor in vivo. However, overexpression of WbdD in the parental background causes a reduction in chain length, and so cellular concentration (availability) of the terminating enzyme is also important in vivo. Chain termination is linked to ABC transporter-mediated export, and thus the chain-terminating enzyme is not the sole component involved in the process. Given the need for speed and efficiency in a rapidly growing bacterial culture, a coordinated multienzyme complex seems logical. The presence of a conserved C-terminal domain with the predicted propensity to form coiled-coils offers one possible avenue to establish protein-protein interactions in such a complex. Paired coiled-coils provide a prevalent mechanism (in 3–5% of cellular proteins) for intermolecular and intramolecular interactions. They are involved in important cellular events including cytoskeleton formation and motility and as receptors for molecular recognition in eukaryotes (reviewed in Ref. 43). In bacteria, coiled-coil domains are important for interactions within multienzyme complexes such as those involved in type III protein secretion systems (44–46). In the case of the O8/O9/O9a biosynthesis systems, WbdD proteins are the only dedicated O-PS biosynthesis components with predicted coiled-coil motifs. Therefore, the coiled-coil motifs are either involved in interactions between WbdD proteins to form a dimer or higher order structure or between WbdD and another presently unknown participant.

The data reported here cover two closely related O-serotypes in E. coli (and identical structures in K. pneumoniae). However, novel nonreducing terminal residues are found in other O-PS. For example, terminal 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo) residues with different linkages are present in the O4 and O12 serotypes of Klebsiella spp. (13). Bordetella bronchiseptica O antigen has a unique 2,3,4-triamino-2,3,4-trideoxy-a-galacturonamide derivative at the nonreducing terminus (47). Terminal modifications may be a common feature in the Bordetellae as Bordetella hinzii O antigen is terminated with a 4-O-methylated GalNAcNacAN residue (48). In the case of B. bronchiseptica, the O-antigen biosynthesis locus also contains homologs of wzm and wzt (49), but the importance of the terminal residues has not been examined. Rhizobium etli CE3 O antigen is terminated by 2,3-di- or 2,3,4-tri-O-methylfucose (50). The O antigen biosynthesis genes map to two loci with one adjacent to the wzm-wzt homologs (51–53), but the O antigen can apparently be made in the absence of the terminal methylated residue (53). 2-O-Methyl groups are found in Vibrio cholerae O1 (54). Although V. cholerae mutants lacking this residue are not impaired in the ability to synthesize O antigen, they do lose the epitope associated with seroconversion from type Ogawa to Inaba (55).

An obvious question is whether the phenomenon described applies to other cell surface polysaccharides formed by ABC transporter-dependent pathways. Many Gram-negative bacteria have capsules formed by this type of pathway, and the E. coli group 2 capsules provide prototypes (reviewed in Refs. 56 and 57). The capsules are distinguished from O antigens by the absence of terminal lipid A-core molecules. Furthermore, capsules have a pathway for translocation to the cell surface that is distinct from LPS (56, 58). Capsules from ABC transporter-dependent pathways are polymerized by processive glycosyltransferases, but the mechanisms involved in termination and potential chain-length regulation are currently unknown. Structural analyses have focused on the repeat unit structure and a conserved phosphatidyldiKdo substituent at the reducing terminus (59). There is currently no structural evidence for nonreducing terminal modifications in the capsules. Biosynthesis of B. subtilis cell wall teichoic acids also follows an ABC transporter-dependent pathway (60), but, as is the case with the capsules, structural studies specifically aimed at elucidating the details of the nonreducing terminal structures have not been performed. An ABC transporter-dependent system is also involved in the assembly of S-layer glycoproteins of the Gram-positive bacterium Geobacillus stearothermophilus (61). S-layers are regular crystalline proteinaceous arrays found on many bacterial surfaces (62), and some, like those in G. stearothermophilus, are modified with long glycan chains. The polymer in strain N2004/3a is a polyrhmanan of 15 trisaccharide repeat units and is terminated with 2-O-methylrhmanose (63). The structure also shows a reducing terminal domain, equivalent to the adaptor in the E. coli O8 and O9a antigens, that links the glycan to serine and threonine residues in the protein. The gene cluster for biosynthesis of this glycan encodes an ABC transporter and a predicted protein with a methyltransferase domain (61). The parallels between this system and ABC transporter dependent O-PS biosynthesis are striking and suggest that the chain regulation system that is described for the first time here may be more widespread in bacteria.

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