**In Vitro Reconstruction of the Chain Termination Reaction in Biosynthesis of the Escherichia coli O9a O-Polysaccharide**

**The Chain-Length Regulator, WbdD, Catalyzes the Addition of Methyl Phosphate to the Non-Reducing Terminus of the Growing Glycan**

Received for publication, August 26, 2011, and in revised form, October 4, 2011. Published, JBC Papers in Press, October 11, 2011, DOI 10.1074/jbc.M111.295857

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**Background:** WbdD is a chain-length regulator that modifies the non-reducing terminus of the *Escherichia coli* O9a glycan.

**Results:** WbdD phosphorylates and methylates a synthetic O9a repeating unit acceptor.

**Conclusion:** The acceptor is modified with a terminal methyl phosphate.

**Significance:** Determining the terminal structure is crucial to understanding the quality control processes of chain-length regulation and export of this prototypical glycan.

The *Escherichia coli* O9a O-polysaccharide (O-PS) represents a model system for glycan biosynthesis and export by the ATP-binding cassette (ABC) transporter-dependent pathway. The polymannose O9a O-PS is synthesized using a decaprenol-diphosphate-linked acceptor by mannosyltransferases located at the cytoplasmic membrane. An ABC-transporter subsequently exports the polymer to the periplasm where it is assembled onto lipopolysaccharide prior to translocation to the cell surface. The chain length of the O9a O-PS is regulated by the dual kinase/methyltransferase activity of the WbdD enzyme and modification of the polymer is crucial for binding and export by the ABC-transporter. Previous biochemical data provided evidence for phosphorylation/methylation at the non-reducing end of the O9a O-PS but the structure of the terminus has not been determined. Here, we describe the exploitation of a synthetic O9a O-PS repeating unit carrying a fluorescent tag as an acceptor for *in vitro* phosphorylation and methylation by a purified soluble form of WbdD. Phosphorylation of the acceptor was evident by both a mobility shift in thin layer chromatography and radiolabeling of the acceptor using [γ-33P]ATP. Methylation of the acceptor was dependent on phosphorylation and was demonstrated by radiolabeling using S-[methyl-3H]adenosylmethionine as a substrate, in the presence of ATP. NMR spectroscopic and mass spectrometric methods were used to determine the precise structure of the terminal modification, leading to the conclusion that WbdD catalyzes the addition of a novel methyl phosphate group to the 3-position of the non-reducing terminal mannos of the O9a O-PS repeating unit.

Lipopolysaccharide (LPS) is a glycolipid that forms a major component of the outer membranes of most Gram-negative bacteria (1). In the Enterobacteriaceae (and many other bacteria), this molecule comprises three structural domains: the hydrophobic lipid A, which forms the outer leaflet of the membrane, a short core oligosaccharide, and a repeating-unit glycan known as the O-polysaccharide (O-PS) or O-antigen, which extends from the cell surface. The O-PS in LPS from a single species can show extensive diversity and these have been used to distinguish isolates by serotyping methods. More than 180 O-antigen serotypes have been identified in *Escherichia coli* (2) and the structures of many of these glycans have been determined (3). LPS molecules isolated from culture reveal a heterogeneous population, ranging from smaller species representing lipid A substituted with core oligosaccharide to longer forms containing 1 to >100 O-PS repeating units. However, the O-PS-substituted species typically fall within particular size ranges reflected in a tight cluster (or clusters) of bands when LPS preparations are examined by SDS-PAGE (4). In pathogens, the O-PS often plays a role in protection against complement-mediating killing and other host defenses (reviewed in

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*The abbreviations used are: LPS, lipopolysaccharide; O-PS, O-polysaccharide; ABC, adenosine triphosphate-binding cassette; FITC, fluorescein isothiocyanate; HR ESI, high resolution electron spray ionization; 5AM, S-adenosyl-methionine; MALDI, matrix-assisted laser desorption/ionization; gCOSY, gradient-enhanced correlation spectroscopy; gTOCSY gradient-enhanced total correlation spectroscopy; tROESY, transverse rotating-frame Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum correlation; MBR, membrane binding region; CBM, carbohydrate binding module.*
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Ref. 5). In this context, the length of the O-PS is critical for function (6–9).

O-PS chain length is determined by specific components of the biosynthetic machinery according to the particular assembly pathway involved. Most O-PSs are synthesized by one of two widely distributed pathways; the Wzy-dependent pathway and the ABC transporter-dependent pathway (reviewed in Ref. 1). The polymannose O-PSs of E. coli O8, O9 and O9α provide well-studied prototypes for the ABC transporter-dependent pathway (10–14). The O8/O9/O9α glycans differ in the linkage sequence within the repeating unit (3) and polysaccharides identical to the O8 and O9 antigens are also found in Klebsiella pneumoniae (15). In each case, the glycan is assembled on a 55-carbon lipid acceptor (undecaprenol phosphate) in the inner (cytoplasmic) membrane (Fig. 1). Chain extension is mediated by three mannosyltransferases, designated WbdCBA, and occurs by the addition of mannoside residues to the non-reducing terminus of the glycan (13, 16). The chain length of the O9α O-PS is controlled by the activity of the WbdD protein, which modifies the non-reducing terminus of the glycan (10). In serotype O8 (and K. pneumoniae O5), WbdD is a methyltransferase and the 3-O-methyl group has been identified at the polymer terminus (15, 17). The E. coli O9 and O9α WbdD proteins are dual kinase-methyltransferases. Methyl and/or phosphorylation modification is required for binding of the nascent undecaprenol pyrophosphate-linked O9α O-PS to the nucleotide-binding component (Wzt) of the ABC-transporter and initiation of polymer export (11, 12). Once at the periplasmic face of the inner membrane, the O-PS is linked to lipid A-core and translocated to the cell surface (1, 18). Related systems participate in the biosynthesis of glycans in diverse bacteria, including O-PSs, capsular/extracellular polysaccharides, glycans for bacterial protein glycosylation and other glycoconjugates of unknown structure or function (19).

The addition of O-PS non-reducing terminal modifications therefore has a dual purpose in O-PS assembly. In addition to establishing the appropriate chain length in the O-PS, this processing provides an essential quality control step, ensuring the appropriate chain length in the O-PS, this processing provides an essential quality control step, initiating the assembly and extending the polysaccharide chain until the appropriate length is established (11, 12). Once at the periplasmic face of the inner membrane, the O-PS is linked to lipid A-core and translocated to the cell surface (1, 18). Related systems participate in the biosynthesis of glycans in diverse bacteria, including O-PSs, capsular/extracellular polysaccharides, glycans for bacterial protein glycosylation and other glycoconjugates of unknown structure or function (19).

The addition of O-PS non-reducing terminal modifications therefore has a dual purpose in O-PS assembly. In addition to establishing the appropriate chain length in the O-PS, this processing provides an essential quality control step, ensuring that only chains of the proper length are exported for completion of the LPS molecule. The E. coli O9α O-PS has been an influential model for these processes; however, the precise details of the chain termination reaction remain unknown. An in vitro study using membranes supplied with radiolabeled substrates has established that phosphorylation of the non-reducing terminus of the glycan is a prerequisite for methylation (10) but the structure of the non-reducing terminus was not resolved in analyses of the native glycan (15, 20, 21). Here we have used a synthetic tetrasaccharide acceptor to reconstitute the chain termination reaction with a soluble form of WbdD to establish the structure of the chain terminus.

**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 either Luria Bertani medium (22) or in Neidhardt’s MOPS minimal medium (23) supplemented with thiamine-HCl (1 μg/ml), uracil (20 μg/ml), adenine (20 μg/ml), histidine (20 μg/ml), tryptophan (20 μg/ml), and glycerol (0.4% w/v). Ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) was added to media when appropriate.

**DNA 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). Genomic DNA was prepared using the Instagene Matrix (Bio-Rad). Plasmid DNA was purified using the PureLink Plasmid Mini-prep Kit (Invitrogen) and DNA fragments from PCRs and restriction digests were 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 constructs were verified using DNA sequencing.

To construct the manA mutation in CWG1005, the manA gene was amplified from E. coli Sφ874 genomic DNA using primers, 5′-GAGGGGAGGTCCATGCAAAAAACTCATTAACTGAGTCG-3′ and 5′-TTCCGATGCCACTCAGCTTTGTTGTAACACCGGT-3′, and the PCR fragment was cloned into pBR322 using the BamH1 sites introduced in the primer sequences (underlined), to form pWQ716. The manA gene was inactivated by removing an internal 147 bp MluI-BssHII fragment (ManA amino acids 148–198) to form pWQ717. The mutated manA gene was excised from pWQ717 with BamH1 and cloned into the BamH1 site of the suicide delivery vector, pKO3 (24) to generate pWQ718. Gene replacement was performed by plating Sφ874[pWQ718] transformants onto LB (chloramphenicol) agar and incubating at 45 °C to select for
integrants. Colonies from the initial selection were then plated onto LB containing 5% (w/v) sucrose at 30 °C to select for plasmid excision. Surrogate-resistant isolates containing the manA defect were screened by selecting white colonies on MacConkey-mannose agar. To mutate the wecA gene, a wecA::Tn10-48 allele was transferred by P1 bacteriophage transduction from E. coli 21548 into CWG1005 to generate CWG1101.

Plasmid pWQ719 was constructed by amplification of a 6649 bp fragment encoding WbdD475-708 and WbdABC from CWG28 genomic DNA with the primers, 5'-GATCAGATTCCACATGGAGCTTACCGGAGGAAGT-3' (EcoRI) and 5'-GATCAAGCTTTACAGATTTTGCTTCACGTAATGATGTG-3' (HindIII) and cloning the PCR fragment into EcoRI-HindIII-digested pBAD24.

**Purification of His6-WbdD1–600**—His6-WbdD1–600 was overexpressed in E. coli BL21[pWQ471] by inducing 500 ml of culture (A600 nm = 0.6) with 1-arabinose (0.1% w/v final concentration) for 1.5 h. The bacterial cells were collected by centrifugation at 5,000 × g and resuspended in 25 ml of 20 mM Bis-Tris, pH 7.0, 250 mM NaCl, 5% (w/v) glycerol (Buffer A), containing 50 mM imidazole. After disrupting the cells by ultrasonication, a cleared lysate was obtained by successive centrifugation steps at 12,000 × g and 100,000 × g. The cleared lysate was applied to a 5 ml HiTrap chelating (Ni2+) column (GE healthcare), washed with Buffer A containing 75 mM imidazole, and His6-WbdD1–600 was eluted with Buffer A containing 250 mM imidazole. A PD-10 column (GE Healthcare) was used to exchange the purified protein into 20 mM Bis-Tris, pH 7.0, 150 mM NaCl (Buffer B). The volume of the protein solution (His6-WbdD1–600) was reduced to ~1 ml with a Vivaspin 15R (30,000 MWCO) filtration unit (SartoriusStedim Biotech), and 50-μl aliquots were frozen in dry ice/ethanol and then stored at −80 °C. The protein concentration (19 mg/ml) was determined from the A280 nm, and the theoretical extinction coefficient of His6-WbdD1–600 (105,880 m−1 cm−1) was determined using the program, ProtParam (25). SDS-PAGE of hexahistidine-tagged proteins was performed in Tris-glycine buffer (26), and gels were stained with Simply Blue (Invitrogen). For Western immunoblotting, hexahistidine-tagged protein was transferred to nitrocellulose membrane as described previously (27). Membranes were probed with anti-Penta-His antibody (Qiagen). Alkaline phosphatase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) was used as a secondary antibody, and the chromogenic substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Roche Applied Science) were used for detection.

**Synthesis of the Synthetic E. coli O9a Repeating Unit Acceptor**—The synthesis of the tetrasaccharide repeating unit of the O9a LPS as an azido-ocyt 3-glycoside has been described elsewhere (28). The azido group in this oligosaccharide was reduced by treatment with hydrogen and palladium hydroxide providing the corresponding amine, which was then reacted with fluorescein isothiocyanate (FITC) to afford the fluorescein-tagged tetrasaccharide, 1 (Scheme 1, supplemental Fig. S1). HR ESI mass spectrometry (negative ion) of 1 identified the expected m/z (1181.3865) calculated for C53H69N2O26S [M-H]−.

**In Vitro Modification of the Synthetic E. coli O9a Repeating Unit Acceptor**—Labeling reactions (20 μl) were performed in Buffer C (50 mM HEPES, pH 7.0, 50 mM NaCl, 20 mM MgCl2, 2 mM dithiothreitol) and contained 100 μM 1 and 10 μM purified His6-WbdD1–600. For phosphorylation reactions, 1 mM unlabeled ATP and 5 × 10−5 mM [γ-33P]ATP (111 TBq/mmol; PerkinElmer Life Sciences) were added to the reaction. For methylation, ATP (5 mM) and S-[methyl-3H]adenosyl-methionine ([3H]SAM, 3.5 × 10−4 μM, 2.904 TBq/mmol; PerkinElmer Life Sciences) were both added. The reactions were performed at 30 °C for 2 h and then stopped by the addition of 20 μl of 50% (v/v) acetonitrile, 1% (v/v) sodium dodecylsulfate, 10 mM EDTA. Samples (1.5 μl) were spotted onto aluminum-backed Silica G thin layer chromatography (TLC) plates (Whatman) and developed in ethyl acetate-water-butanol-acetic acid (5:4:2.5). Fluorescent reaction products were detected with a hand-held UV lamp. A Bio-Rad FX phosphorimager and Kodak
BioMax MR film were used to detect $^{33}$P- and $^3$H-labeled reaction products, respectively.

Time-dependent incorporation of $[^{33}P]$phosphate into the synthetic acceptor was assayed at 30 °C in 35 μl of Buffer C containing 100 μM I, 0.1 μM His$_6$-WbdD$_{1-600}$, 2.5 mM unlabeled ATP, and $5 \times 10^{-4}$ mM $[^{33}P]$ATP (111 TBq/mmol; PerkinElmer Life Sciences). Samples (5 μl) were removed at various times during the reaction and added to 5 μl of 50% (v/v) acetonitrile, 1% (w/v) sodium dodecylsulfate, 10 mM EDTA. Samples (1.5 μl) were subjected to TLC as described above. Known amounts of $[^{33}P]$ATP were also loaded on the same TLC plate as standards for quantitation. The $^{33}$P-labeled products were detected by phosphorimaging and densitometry was performed using ImageJ software (29).

Phosphorylation and Methylation of the Synthetic O9a Repeat Unit Acceptor for Structural Analysis—Phosphorylated acceptor was prepared in two 100 μl reactions, each containing Buffer C, 140 μg I, 3.5 mM ATP, and 20 μM His$_6$-WbdD$_{1-600}$. The reactions mixtures were incubated for 2 h at 30 °C and stopped by adding EDTA (10 mM final concentration). Modification of the acceptor was confirmed by TLC as described above. The stopped reactions were pooled, diluted to 10 ml with water, and applied to a Sep-Pak Plus C18 column (Waters) that was previously washed with 10 ml of acetonitrile, followed by 20 ml of water. The sample was washed on the column with 20 ml of water and eluted with 50% (v/v) acetonitrile. Elution fractions that exhibited an absorbance at 494 nm (fluorescein) were pooled and dried.

Dually phosphorylated/methylated acceptor was prepared as described for the phosphorylated acceptor with the exception that the reactions consisted of Buffer C, 95 μg I, 2 mM ATP, 0.16 mM S-adenosyl-methionine (New England BioLabs), and 11 μM His$_6$-WbdD$_{1-600}$.

NMR Spectroscopy and Mass Spectrometry of the Modified Acceptors—All NMR spectra for 2 and 3 were acquired in D$_2$O at 27 °C and referenced to an external standard of acetone (2.22 ppm). The 1D, gCOSY, gTOCSY, and tROESY $^1$H spectra were run on a 700 MHz spectrometer equipped with a cryoprobe. For both compounds, the spectral window for the 1D $^1$H spectrum was 8446 Hz (from 10.8 to −1.3 ppm), and the intensity of the residual HOD peak was decreased using a presaturation pulse sequence, irradiating at 4.76 ppm.

For 2, the spectral windows for the gCOSY and gTOCSY experiments were 6313 Hz (from 9 to 0 ppm) in both dimensions; 37 increments in the F1 dimension and 8 transients in the F2 dimension were obtained. A mixing time of 0.4 s was used for the tROESY. For all of the 2D spectra, sine-bell functions were applied interactively to improve signal-to-noise; no line broadening was used.
The 1D $^{31}$P($^1$H) spectrum for 2 was on a 400 MHz spectrometer (162 MHz for $^{31}$P). The spectral window was 32.4 kHz (from 100 to −100 ppm), and line broadening of 2 Hz was applied to improve the signal-to-noise.

For 3, the spectral windows for the gCOSY and gTOCSY experiments were also 6313 Hz (from 9.5 to 0.5 ppm) in both dimensions. 337 increments in the F1 dimension and 32 transients in the F2 dimension were obtained for the gCOSY, and 674 increments in the F1 dimension and 32 transients in the F2 dimension were obtained for the gTOCSY. A mixing time of 0.1 s was used for the gTOCSY. The spectral window for the tROESY was 7022 Hz (9.5 to −0.5 ppm) in both dimensions; 375 increments in the F1 dimension and 32 transients in the F2 dimension were obtained. A mixing time of 0.4 s was used for the tROESY. For all of the 2D spectra, sine-bell functions were applied interactively to improve signal-to-noise; no line broadening was used. Both the 1D $^{31}$P($^1$H) and 1D $^1$H–$^{31}$P HSQC spectra for 3 were acquired on a 500 MHz spectrometer (202 MHz for $^{31}$P). The spectral window for the 1D $^{31}$P($^1$H) spectrum was 60.6 kHz (from 150 to −150 ppm), and line broadening of 2 Hz was applied to improve the signal-to-noise. The spectral window for the 1D $^1$H–$^{31}$P HSQC spectrum was 5971 Hz (from 11 to −1 ppm), and the proton signals were decoupled during acquisition. The $^3$J$_{H,P}$ value of the O-methyl group (11.0 Hz, measured from the 1D $^1$H spectrum) was used to set up the appropriate delays in the $^1$H–$^{31}$P HSQC experiment.

The MALDI-TOF mass spectrum for 2 was obtained on a Bruker Daltonics Apex-Qe FTICR MS in negative ion mode. The electrospray mass spectrum for 3 was obtained on an Agilent Technologies 6220 oaTOF in negative ion mode.

**In Vitro Mannan Polymerization on the Synthetic Acceptor Using E. coli Cell Envelope Preparations**—To prepare the acceptor as a substrate for elongation, 1 (2 nmol) was phosphorylated in 20 μl of Buffer C containing 5 mM ATP and 10 μM His$_6$-WbdD$_{1-600}$ for 30° C. The reaction was stopped by addition of 20 μl of 50% (v/v) acetonitrile, 1% (w/v) sodium dodecylsulfate, 10 mM EDTA. The entire reaction mixture was applied to a TLC plate and developed as described above. The major band corresponding to phosphorylated acceptor was scraped from the TLC plate, eluted with 500 μl of 50% (v/v) acetonitrile and dried. As a non-phosphorylated control, 1 was purified from a reaction lacking ATP.

To prepare cell envelope fractions containing mannosyltransferase activity, CWG1101[pWQ719] was grown in 1 liter of supplemented Neidhardt’s MOPS minimal medium to exponential phase (OD$_{600}$ nm = 0.4) and induced for 2.5 h with L-arabinose (0.2% w/v, final concentration). Bacteria were collected by centrifugation at 5,000 g, resuspended in 20 ml of Buffer D (50 mM HEPES, pH 7.0, 20 mM MgCl$_2$, 2 mM dithiothreitol), and 50 μl of Buffer D at 25° C. The phosphorylation reaction was stopped at various time points and the reaction products were separated by TLC and visualized by phosphorimaging. The $^{32}$P-labeled reaction products were quantified by densitometry.

**FIGURE 3.** His$_6$-WbdD$_{1-600}$ phosphorylates a synthetic derivative of the E. coli O9a O-polysaccharide repeating unit in vitro. The acceptor (1) contains a fluorescent substituent allowing detection by exposure to ultraviolet light. Completed reaction mixtures were analyzed by TLC. A, fluorescent detection showing 1 and modified products after incubation with His$_6$-WbdD$_{1-600}$ and ATP[$^32$P]ATP (20000:1). B, autoradiogram of A showing $^{32}$P-labeling of the modified acceptor. The apparent "transparent" regions near the origin in B were due to saturation of the phosphorimaging screen with unincorporated [$^32$P]ATP. C, time course of [$^32$P]phosphate incorporation into the synthetic acceptor. The phosphorylation reaction was stopped at various time points and the reaction products were separated by TLC and visualized by phosphorimaging. The $^{32}$P-labeled reaction products were quantified by densitometry.
disrupted by ultrasonication. The lysate was cleared by centrifugation at 12,000 × g for 30 min. The membrane fraction was collected by ultracentrifugation at 100,000 × g for 1 h and resuspended in 500 μl of Buffer D. The protein concentration of the cell envelope fraction was determined with the Bio-Rad DC protein assay kit using bovine serum albumin as a standard.

Mannan polymerization on both unmodified and phosphorylated synthetic acceptor was performed by adding 20 μl of Buffer D, containing 4 mM GDP-mannose and cell envelope fraction (1 mg protein/ml), directly to the dried purified acceptor. The reactions were incubated at 30 °C for 30 min and stopped reaction mixture was subjected to TLC as described above. A control reaction was performed with unmodified acceptor together with membranes from CWG1101 containing no plasmid. An additional control contained CWG1101[pWQ719] membranes incubated with unmodified acceptor but in the absence of GDP-mannose. The fluorescent reaction products were detected using a hand-held UV lamp.

RESULTS

His<sub>6</sub>-WbdD<sub>1–600</sub> Can Phosphorylate a Synthetic Derivative of the E. coli O9a O-PS Repeating Unit—Our previous studies demonstrated that the E. coli O9a chain-length regulator, WbdD, can phosphorylate and methylate endogenous undeca-prenol-linked O9a mannan synthesized de novo in isolated membrane fractions (10). However, the nature of the linkage between the phosphate/methyl residues and the O9a O-PS was not resolved (15). Structural studies ruled out methylation throughout the chain and the K. pneumoniae O5 (E. coli O8) polymannose O-PS carries a methyl group at the non-reducing terminus (15, 17). Therefore, it was hypothesized that WbdD-dependent modification blocked chain extension by modification of the non-reducing end of nascent O9a O-PS. We therefore developed an in vitro method for WbdD-dependent modification of a small synthetic acceptor comprising an 8-aminoctyl glycoside derivative of the O9a repeating unit conjugated to fluorescein (1). The use of this acceptor provided the advantage of straightforward purification and structural analysis to unequivocally determine the nature of the O9a O-PS modification.

WbdD is tightly associated with the cytoplasmic membrane via a helix-rich region (MBR) spanning amino acids 601 to 669 near the C terminus (14) (Fig. 2A). Additionally, the C-terminal portion of WbdD is necessary for recruiting the WbdA mannosyltransferase to the membrane and enabling synthesis of the O9a O-PS (14). Plasmid pWQ471 encodes a soluble His<sub>6</sub>-tagged truncated derivative comprising amino acids 1 to 600 of WbdD (His<sub>6</sub>-WbdD<sub>1–600</sub>). His<sub>6</sub>-WbdD<sub>1–600</sub> contains functional N-terminal methyltransferase and kinase elements and over-expression of the protein can restore O9a O-PS chain termination and export to an E. coli wbdD::aacC1 mutant in which the WbdD C-terminal membrane-binding region is independently expressed from the genome (14) (Fig. 2B). We have previously shown that the O9a O-PS chain length is decreased when full-length WbdD is overexpressed in E. coli O9a (10). Short O-PS chains were also observed in the wbdD::aacC1 mutant (CWG635) when His<sub>6</sub>-WbdD<sub>1–600</sub> was expressed at high copy from the pBAD promoter; i.e. 0.1% (w/v) l-arabinose (Fig. 2B). However, CWG635 expressing His<sub>6</sub>-WbdD<sub>1–600</sub> at a low level, produces O-PS chains comparable to the wild type O9a strain (14). His<sub>6</sub>-WbdD<sub>1–600</sub> was overexpressed in E. coli BL21 and purified (Fig. 2C). Purified His<sub>6</sub>-WbdD<sub>1–600</sub> migrated in SDS-PAGE with an apparent molecular mass of 65.5 kDa.
compared with the theoretical molecular mass of 69.5 kDa. The reason for this aberrant migration is unclear. Western blotting using anti-His5 antibody confirmed the identity of the purified His6-tagged protein (data not shown).

Purified His6-WbdD1–600 was assayed for the ability to modify 1 in a reaction containing a mixture of ATP and [γ-33P]ATP (20000:1). The reaction products were analyzed by TLC. In the absence of His6-WbdD1–600, 1 resolved as two major species in TLC with $R_f$ values of 0.50 and 0.48, respectively (Fig. 3A, lane 3). Prior to the conjugation to fluorescein, the tetrasaccharide derivative was shown to be a single component by $^1$H NMR spectroscopy (data not shown) and we therefore attributed the presence of two TLC bands to heterogeneity arising from the fluorescein component of the acceptor. Purified His6-WbdD1–600 converted both acceptor species into more polar reaction products (Fig. 3A, lane 2). This modification was dependent on the presence of ATP in the reaction (Fig. 3A; compare lanes 1 and 2). The reaction products were both labeled with $^{31}$P ($^1$H) NMR spectrum of 2 showed a single peak at 4.20 ppm (data not shown), indicating the presence of a single phosphorus residue. This was confirmed by MALDI mass spectrometry, which produced the expected peak for a sodium adduct of monophosphorylated acceptor [C$_5$H$_{68}$N$_2$NaO$_{29}$PS]$^+$ with an $m/z$ of 1283.3 (supplemental Fig. S2). Using a combination of one-dimensional (Fig. 4), gCOSY, and gTOCSY (supplemental Fig. S2) $^1$H NMR experiments, the phosphate group was determined to be on the 3-position of one of the sugar ring structures. This conclusion was based on the 1D $^1$H NMR spectrum (Fig. 4), which contained a resonance at 4.28 ppm (doublet of doublet of doublets, $J_{2,3} = 3.1$ Hz, $J_{3,4} = 8.9$ Hz, $J_{1,4} = 8.9$ Hz). Rings A and B of 2 are circled.

His$_6$-WbdD1–600 Adds a Phosphate to the 3-Position of the Non-reducing Terminal Mannose of the O9a Repeating Unit—The phosphorylated acceptor (2) was purified by reverse-phase C18 chromatography from a scaled up reaction containing His$_6$-WbdD1–600, ATP, and 1. The established structure of the 8-azidooctyl precursor to 1 (28) facilitated the structural determination of 2. A $^{31}$P($^1$H) NMR spectrum of 2 showed a single peak at 4.20 ppm (data not shown), indicating the presence of a single phosphorus residue. This was confirmed by MALDI mass spectrometry, which produced the expected peak for a sodium adduct of monophosphorylated acceptor [C$_5$H$_{68}$N$_2$NaO$_{29}$PS]$^+$ with an $m/z$ of 1283.3 (supplemental Fig. S2). Using a combination of one-dimensional (Fig. 4), gCOSY, and gTOCSY (supplemental Fig. S2) $^1$H NMR experiments, the phosphate group was determined to be on the 3-position of one of the sugar ring structures. This conclusion was based on the 1D $^1$H NMR spectrum (Fig. 4), which contained a resonance at 4.28 ppm (doublet of doublet of doublets, $J_{2,3} = 3.1$ Hz, $J_{3,4} = 8.9$ Hz, $J_{1,4} = 8.9$ Hz). Rings A and B of 2 are circled.

FIGURE 5. tROESY $^1$H NMR spectrum of the phosphorylated synthetic O9a repeating unit acceptor (2). The spectrum was recorded at 27 °C on a 700 MHz spectrometer with D$_2$O as solvent and a mixing time of 0.4 s. When the data were transformed, a sine-bell window function was applied, but no line broadening was used. The NOE cross peaks due to the $\alpha$-(1→2) linkage between rings D and C and the $\alpha$-(1→3) linkage between rings C and B of 2 are circled.
Methyl Phosphate Modification by WbdD

|   | 1                | 2                | 3                | 4                |
|---|------------------|------------------|------------------|------------------|
| [H]SAM | −                | −                | +                | +                |
| ATP     | +                | −                | +                | −                |

**FIGURE 6.** His$_6$-WbdD$_{1−600}$-dependent methylation of the synthetic O9a repeating unit acceptor requires prior phosphorylation. Acceptor (1) was incubated in reactions with His$_6$-WbdD$_{1−600}$ and varying combinations of ATP and [H]SAM. Samples from completed reactions were separated by TLC. A, fluorescent detection showing the relative mobilities of the bands in the reaction products. B, autoradiogram of A showing showing labeling with [H]methyl residues when 1 was incubated with both ATP and [H]SAM. In lane 3, the two radiolabeled bands in B comigrate with the two major fluorescent bands in A (R$_f$ = 0.45/0.41).

A free C3 hydroxyl thus confining the phosphate moiety to either of rings C or D. A tROESY $^1$H NMR spectrum was used to identify interactions between individual anomeric protons and protons of adjacent ring systems (Fig. 5). A strong cross peak was observed between the anomeric proton of one ring (5.05 ppm) and H2 on a different ring (4.10 ppm). This interaction was attributed to the only α-(1→2)-linked residues in 2 (i.e. rings D and C). Another strong cross peak was observed between a different anomeric proton (5.44 ppm) and H3 on a separate ring (4.00 ppm). From gCOSY and gTOCSY spectra (supplemental Fig. S2) the anomeric signal at 5.44 ppm was determined to be on ring C. Therefore, the tROESY correlation between the 5.44 ppm and 4.00 ppm resonances were attributed to the α-(1→3) linkage between rings C and B. Given these two key cross peaks, the anomeric protons of all four ring systems were identified and it was concluded that the phosphate group was attached at the 3-position of ring D, the non-reducing end mannose.

His$_6$-WbdD$_{1−600}$ Catalyzes the Addition of a Methyl Phosphate to the O9a Repeating Unit—[H]SAM was used as a methyl donor to determine whether 1 could be methylated by purified His$_6$-WbdD$_{1−600}$. A reaction containing both ATP and SAM yielded products with $R_f$s indistinguishable to those seen with ATP alone (Fig. 6A, compare lanes 3 and 1). However, the corresponding autoradiogram revealed radiolabeled products that comigrated ($R_f$ 0.45 and 0.41) with the phosphorylated acceptor compounds on TLC (Fig. 6B, lane 3). These data demonstrated that the acceptor was methylated by His$_6$-WbdD$_{1−600}$. Methylation of the acceptor was not evident in a reaction containing [H]SAM but lacking ATP (Fig. 6B, lane 4), an observation consistent with previous findings showing that phosphorylation of the O9a O-PS was a prerequisite for methylation (10).

To determine the structure of the dually phosphorylated/methylated acceptor, 1 was modified in a scaled-up reaction containing His$_6$-WbdD$_{1−600}$, ATP, and SAM. ESI MS data from the purified reaction product revealed signals with $m/z$ values expected for dually phosphorylated/methylated acceptor (Table 2, supplemental Fig. S3). The 1D $^1$H NMR spectrum (Fig. 7) of the modified acceptor (3) was similar to that obtained for 2 (Fig. 4). The resonance due to the phosphate (Fig. 7, H-3D, $\delta$ = 4.31 ppm) previously assigned in 2 was present as a doublet of doublet of doublets and had similar coupling constants ($J_{3,4} = 8.2$ Hz, $J_{3,5} = 9.1$ Hz). The key tROESY $^1$H NMR correlations observed for 2 (Fig. 5) were also observed for 3 (H-1D (5.06 ppm)/H-2C (4.10 ppm) and H-3B (5.64 ppm)/H-3B (4.00 ppm)). These data, together with the 2D gCOSY and gTOCSY $^1$H NMR spectra (supplemental Fig. S3) confirmed that 3 had the same backbone structure as 2. However, the 1D $^1$H NMR spectrum of 3 contained a doublet at 3.63 ppm (Fig. 7, $J = 11.0$ Hz) that was not present in the spectrum of 2 (Fig. 4).

To determine whether the resonance at 3.63 ppm was due to a methyl group attached to phosphate, a 1D $^1$H $^{31}$P HSQC spectrum was acquired (Fig. 8). Two resonances appeared in this spectrum, one at 4.31 ppm (H-3D) and another at 3.63 ppm, thus indicating that there were only two proton resonances with three-bond coupling to phosphorus. In addition, a $^{31}$P[1H] NMR spectrum of 3 showed only one signal indicating the presence of only a single phosphate group (1.06 ppm, data not shown). Therefore, it was concluded that the methyl group had been added to the phosphate attached to the 3-position of ring D at the non-reducing end of the synthetic O9a repeat unit tetrasaccharide.

**TABLE 2**

| $m/z$      | Observed $m/z$ | Abundance |
|------------|----------------|-----------|
| [C$_{54}$H$_{70}$N$_2$O$_{29}$PS]                 | 1275.3685         | 2.19%     |
| [C$_{54}$H$_{72}$N$_2$O$_{29}$PS]                 | 1275.3641         | 2.19%     |
| [C$_{54}$H$_{74}$N$_2$O$_{29}$PS]                 | 424.4513          | 15.71%    |

Elongation of the O9a O-PS on the Synthetic Acceptor Is Blocked by Non-reducing Terminal Phosphorylation—we have previously shown that the O9a O-PS could be synthesized in vitro from GDP-mannose and E. coli O9a cell membrane fractions containing undecaprenol-linked acceptor, WbdD, and mannosyltransferase activity (10). In these experiments, the length of the O9a O-PS chains synthesized de novo was dependent on the concentration of ATP present in the reaction. These data were interpreted as evidence for a chain length control mechanism, whereby phosphorylation of the non-reducing terminus by WbdD blocked further mannoside incorporation into the nascent O-PS. Thus, the rate of phosphorylation determined the chain length of the O-PS. To determine the effect of...
O-PS phosphorylation on polymer elongation, both 1 and 2 were analyzed for the ability to serve as acceptors for elongation of the O9a O-PS, using GDP-mannose as a sugar donor. Membrane fractions from CWG1101[pWQ719] overexpressing WbdABC and the WbdD C-terminal region (required for membrane recruitment of WbdA; see above) were used as a source of mannosyltransferase activity. Upon incubation with CWG1101[pWQ719] membranes, 1 was converted into a product that did not migrate from the origin in TLC (Fig. 9, lanes 3 and 4). The acceptor (1) was not modified in a reaction containing CWG1101[pWQ719] membranes but lacking GDP-mannose (Fig. 9, lane 2) nor was it modified by CWG1101 membranes lacking mannosyltransferase activity (Fig. 9, lane 1). Therefore, the lack of mobility was attributed to WbdABC-
discontinuous enzyme activities. In the O9a and O8 O-PS systems, a C-terminal extension of Wzt contains a carbohydrate-binding module (CBM) that establishes the specificity for the cognate O-PSs to engage the ABC-transporter (12). For example, the polymannose E. coli O8 O-PS contains a 3-O-methyl group at the non-reducing terminus (15) and this polymer can bind to the CBM domain from the serotype O8 but not to the corresponding CBM from the O9a Wzt protein. The converse is true for the O9a O-PS. The O9a CBM domain only binds O9a O-PS that has been modified by WbdD (12). One interpretation is, the terminal modification serves to both stop polymer elongation and identify the nascent polymer as being competent for export by the ABC-transporter.

Considering that phosphorylation is the limiting process in O9a O-PS chain-length regulation, it is not apparent what role is served by subsequent methylation of the terminal phosphate. It is possible that phosphorylation and methylation have individual roles whereby phosphorylation blocks O-PS chain elongation and the methyl modification is required for glycan export. Phenotypical analysis of site-directed wbdD mutants in which the kinase and/or methyltransferase activities have been inactivated would aid in elucidating the individual roles of phosphorylation and methylation in O9a O-PS export. How-
ever, there is inadequate amino acid sequence conservation between the putative methyltransferase domain of WbdD (10) and other known methyltransferases to rationally design methyltransferase active site mutations. A detailed three-dimensional structure of WbdD will be critical for designing and interpreting such future experiments.

The presence of the C-terminal extensions on Wzt proteins in glycan biosynthesis systems from other bacteria is also correlated with the presence of non-reducing terminal modifications on the cognate polysaccharides, suggesting that the general strategy for glycan export and quality control described for the O9a O-PS is conserved in other systems (19). For example, the O-PS of E. coli O8 and the S-layer (glycoprotein) glycan from Geobacillus tepidamans both have non-reducing terminal methyl residues (15, 17, 36) and very similar biosynthesis components. The Klebsiella O12 antigen contains a terminal 3-deoxy-D-manno-octulosonic acid residue, a sugar predominantly present in the LPS core (15). An S-layer glycan from Geobacillus tepidamans is terminated with both N-acetylglucosamine and N-acetylmuramic acid (37). N-Acetylmuramic acid was generally thought to be unique to peptidoglycan in the bacterial cell wall. A mechanistic understanding of the components of the E. coli O9a O-PS prototype now provides an opportunity to investigate the interplay between mannosyltransferase activity and chain termination to understand how these processes are balanced in vivo to generate glycans with specific sizes to fulfill their functional roles.

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