

Biosynthesis of the Polymannose Lipopolysaccharide O-antigens from *Escherichia coli* Serotypes O8 and O9a Requires a Unique Combination of Single- and Multiple-active Site Mannosyltransferases^{*[5]}

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Background: The *Escherichia coli* O8 and O9a antigens are influential models for bacterial glycan assembly.

Results: O8 and O9a glycan biosynthesis requires three mannosyltransferases, whose activities were defined.

Conclusion: Two conserved mannosyltransferases (WbdCB) produce an adaptor trisaccharide on which each WbdA polymerizes a serotype-specific repeat unit polysaccharide.

Significance: These systems require multiple mannosyltransferase modules in a flexible arrangement.

The *Escherichia coli* O9a and O8 O-antigen serotypes represent model systems for the ABC transporter-dependent synthesis of bacterial polysaccharides. The O9a and O8 antigens are linear mannose homopolymers containing conserved reducing termini (the primer-adaptor), a serotype-specific repeat unit domain, and a terminator. Synthesis of these glycans occurs on the polyisoprenoid lipid-linked primer, undecaprenol pyrophosphoryl-Glc₆NAc, by two conserved mannosyltransferases, WbdC and WbdB, and a serotype-specific mannosyltransferase, WbdA. The glycan structure and pattern of conservation in the O9a and O8 mannosyltransferases are not consistent with the existing model of O9a biosynthesis. Here we establish a revised pathway using a combination of *in vivo* (mutant complementation) experiments and *in vitro* strategies with purified enzymes and synthetic acceptors. WbdC and WbdB synthesize the adaptor region, where they transfer one and two α -(1 \rightarrow 3)-linked mannose residues, respectively. The WbdA enzymes are solely responsible for forming the repeat unit domains of these O-antigens. WbdA^{O9a} has two predicted active sites and polymerizes a tetrasaccharide repeat unit containing two α -(1 \rightarrow 3)- and two α -(1 \rightarrow 2)-linked mannopyranose residues. In contrast, WbdA^{O8} polymerizes trisaccharide repeat units containing single α -(1 \rightarrow 3)-, α -(1 \rightarrow 2)-, and β -(1 \rightarrow 2)-mannopyranoses. These studies illustrate assembly systems exploiting several mannosyl-

transferases with flexible active sites, arranged in single- and multiple-domain formats.

Lipopolysaccharide (LPS) is an essential constituent of the outer membrane of most Gram-negative bacteria. The prototypical LPS molecule consists of three structural regions: the lipid A, core oligosaccharide, and O-antigenic polysaccharide (O-PS)⁵ (1). The structure of lipid A is well conserved, as are some features of the core oligosaccharide. In contrast, O-PSs are hypervariable glycans of variable chain length that consist of repeat units containing one or more sugars and non-carbohydrate components. Unique O-PS structures define more than 180 different O-antigen serotypes in *Escherichia coli* (2, 3). The lipid A-core and O-PS components of LPS are assembled in separate pathways that converge in a ligation reaction at the periplasmic face of the inner membrane (1), and the completed molecule is then transported to the cell surface (4). Despite the structural diversity, all O-PS glycans are thought to be assembled by one of only three pathways, all of which involve biosynthetic intermediates built on the 55-carbon polyisoprenoid lipid acceptor, undecaprenol phosphate (reviewed in Ref. 1).

The O-PSs of *E. coli* O9, O9a, and O8 are a family of related structures comprising linear homopolymers of mannopyranose (Man_p) (3) (Fig. 1A). Investigation of their biosynthesis began in 1971 (5), and they represent influential prototypes for O-PSs synthesized via the well distributed ATP-binding cassette (ABC) transporter-dependent pathway (6). The *E. coli*

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[5] This article contains supplemental Tables S1 and S2 and Figs. S1–S4.

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⁵ The abbreviations used are: O-PS, O-polysaccharide; und-PP, undecaprenol pyrophosphate; ABC, adenosine triphosphate-binding cassette; Man_p, mannose; Glc₆NAc, N-acetylglucosamine; Rha_p, rhamnose; Glc₆, glucose; Glc₆A, glucuronic acid; MalE, maltose-binding protein; CE, capillary electrophoresis; gCOSY, gradient-enhanced correlation spectroscopy; tROESY, transverse rotating-frame Overhauser enhancement spectroscopy; gHSQC, gradient-enhanced heteronuclear single quantum correlation; Glc₆NAc-PP-C₁₃, β -Glc₆NAc-pyrophosphoryl-C₁₃; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

TABLE 1
Bacterial strains and plasmids

Strain/plasmid	Description or genotype	Reference or source
Strains		
Top10	<i>E. coli</i> F ⁻ , <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>), φ80, <i>lacZ</i> ΔM15, Δ <i>lacX74</i> , <i>deoR</i> , <i>nupG</i> , <i>recA1</i> , <i>araD139</i> , Δ(<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>repSL</i> (Str ^r), <i>endA1</i>	Invitrogen
BL21	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>gal</i> [malB ⁺] _{K12} (λ ^S)	Novagen
CWG634	<i>E. coli</i> O9a:K ⁻ ; <i>trp his lac rpsL cps</i> _{K30} ; <i>manA</i> ; Sm ^r ; Tc ^r	Ref. 15
CWG636	<i>E. coli</i> O8:K ⁻ ; <i>ugd::aacC1 manA</i> ; Gm ^r ; Tc ^r	Ref. 15
CWG1005	K-12 <i>lacZ trp</i> Δ(<i>sbcB-rfb</i>) <i>upp rel rpsL manA</i>	Ref. 12
CWG1006	CWG1005 derivative; <i>waaL::cat</i> ; Cm ^r	This study
CWG1007	CWG636 derivative; Δ <i>wbdB</i> ; Gm ^r ; Tc ^r	This study
CWG1008	CWG636 derivative; Δ <i>wbdC</i> ; Gm ^r ; Tc ^r	This study
CWG1009	CWG634 derivative; Δ <i>wbdB</i> ; Sm ^r ; Tc ^r	This study
CWG1010	CWG634 derivative; Δ <i>wbdC</i> ; Sm ^r ; Tc ^r	This study
CWG1104	CWG636 derivative; Δ <i>wbdA</i> ; Gm ^r ; Tc ^r	This study
CWG1105	CWG634 derivative; Δ <i>wbdA</i> ; Sm ^r ; Tc ^r	This study
Plasmids		
pMAL-c2X	IPTG-inducible plasmid for expressing cytoplasmic maltose-binding protein fusions; Ap ^r	New England Biolabs
pBADHisA	L-Arabinose-inducible plasmid to express N-terminal hexahistidine-tagged constructs; Ap ^r	Invitrogen
pBAD24	L-Arabinose-inducible plasmid; Ap ^r	Ref. 23
pKD3	Source of Cm ^r resistance cassette	Ref. 22
pKD46	Helper plasmid encoding the Red recombinase genes, γ, β, and <i>exo</i>	Ref. 22
pCP20	Helper plasmid encoding the FLP recombinase	Ref. 22
pWQ492	pBAD24 derivative containing an EcoRI/HindIII fragment encoding WbdA ^{O9a} -His ₁₀ ; Ap ^r	Ref. 58
pWQ575	pMAL-c2X derivative containing an XmnI/HindIII fragment encoding WbdC ^{O9a} ; Ap ^r	This study
pWQ576	pMALc-2X derivative containing an EcoRI/HindIII fragment encoding WbdB ^{O9a} ; Ap ^r	This study
pWQ577	pMALc-2X derivative containing an EcoRI/HindIII fragment encoding WbdC ^{O8} ; Ap ^r	This study
pWQ578	pMALc-2X derivative containing an EcoRI/HindIII fragment encoding WbdB ^{O8} ; Ap ^r	This study
pWQ579	pBAD24 derivative containing an EcoRI/HindIII fragment encoding WbdC ^{O9a} ; Ap ^r	This study
pWQ580	pBAD24 derivative containing an EcoRI/HindIII fragment encoding WbdC ^{O9a} -WbdB ^{O9a} ; Ap ^r	This study
pWQ581	pBAD24 derivative containing an EcoRI/HindIII fragment encoding WbdC ^{O8} ; Ap ^r	This study
pWQ582	pBAD24 derivative containing an EcoRI/HindIII fragment encoding WbdC ^{O8} -WbdB ^{O8} ; Ap ^r	This study
pWQ587	pBADHisA derivative containing an XhoI and PvuII fragment encoding WbdA ^{O8} ; Ap ^r	This study
pWQ588	pBAD24 derivative containing an NcoI/XbaI fragment encoding His ₁₀ -WbdA ^{O8} ; Ap ^r	This study

O9a and O8 O-PSs are identical to the *Klebsiella pneumoniae* O3 and O5 O-PSs, respectively, and the genetic loci encoding the corresponding O-PS biosynthesis enzymes are highly conserved (7). The close evolutionary relationships in these antigens are believed to reflect lateral gene transfer between the two species (8, 9). The *E. coli* O9a O-PS is a variant of the *E. coli* O9 structure. Seroconversion of O9a to O9 requires a single amino acid substitution in WbdA, one of three mannosyltransferases required for their synthesis (10). Detailed structural studies have identified four domains in the *K. pneumoniae* O3 and O5 O-PS molecules: a conserved β-Glc₁NAc residue (the primer) at the reducing terminus, an adapter region containing two or more α-linked Man₉ residues, the variable serotype-specific repeat unit domain, and the terminator comprising an O-methyl group (O8) or a methyl phosphate modification (O9 and O9a) (Fig. 1A) (11–13).

The gene clusters responsible for expression of these O-PSs encode proteins involved in synthesis of the nucleotide precursor, GDP-Man (ManBC), the transmembrane and nucleotide-binding domains of the ABC transporter (Wzm and Wzt, respectively), a chain terminator (WbdD), and three mannosyltransferases (WbdC, WbdB, and WbdA; formerly MtfCBA) (14) (Fig. 1B). WbdC and WbdB are highly conserved in these serotypes. The WbdA^{O8} and WbdA^{O9/O9a} proteins differ in size and sequence, and experimental evidence supports the conclusion that WbdA is the serotype-defining mannosyltransferase (10). WbdD^{O9/O9a} is a bifunctional kinase-methyl transferase that adds a phosphomethyl group to the O9/O9a non-reducing terminus, whereas WbdD^{O8} adds only a methyl group in O8 (12, 15). The terminated glycan is then recognized by a serotype-specific carbohydrate-binding module located at the

C terminus of Wzt, prior to its export via the ABC transporter (16). Chain termination is essential for recognition and export.

The proposed biosynthesis pathway of these O-PS glycans is based primarily on data from *E. coli* O9a; this was initially reported as O9 in earlier studies and corrected later (14, 17). In the published model, WbdC is thought to add the first α-Man₉ residue to the β-Glc₁NAc-PP-und acceptor that is synthesized by WecA (14). WecA is part of the machinery for biosynthesis of the enterobacterial common antigen glycan, and the *wecA* gene is not located in the O-PS locus (18, 19). The activity of WbdC is proposed to be followed by the addition of two α-(1→3)-linked Man₉ residues by WbdB. Further chain extension then occurs through the combined alternating activities of WbdB and WbdA (14). However, this pathway is difficult to rationalize with the O-PS structures and the pattern of conserved (WbdC/B) and variable (WbdA) mannosyltransferases.

Here, we establish a revised biosynthetic pathway using purified proteins and defined acceptors. We conclude that biosynthesis of these important model O-PSs involves a unique combination of two enzymes containing a single mannosyltransferase domain, one of which is capable of adding two Man₉ residues in succession, and multidomain-polymerizing mannosyltransferases.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) medium (20) or M9 minimal medium (21). M9 minimal medium was supplemented, where appropriate, with glycerol (0.4%, w/v), thiamine HCl (0.5 μg/ml), niacinamide (0.5 μg/ml),

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L-histidine (20 $\mu\text{g/ml}$), L-tryptophan (20 $\mu\text{g/ml}$), D-glucose (0.4%, w/v), D-mannose (0.1%, w/v), L-arabinose (0.2%, w/v), isopropyl β -D-1-thiogalactopyranoside (0.5 mM), ampicillin (100 $\mu\text{g/ml}$), or chloramphenicol (34 $\mu\text{g/ml}$).

DNA Methods—InstaGene Matrix (Bio-Rad) or DNAzol reagent (Invitrogen) was used to purify chromosomal DNA. DNA fragments were PCR-amplified using *Pwo* DNA polymerase (Roche Applied Science) or *Pfu* Ultra high fidelity DNA polymerase (Stratagene), using custom oligonucleotide primers (Sigma) containing restriction sites to facilitate cloning. The sequences and features of the oligonucleotide primers are described in supplemental Table S1. The PureLink PCR purification kit (Invitrogen) was used to purify DNA fragments from PCRs or restriction enzyme digests, and DNA fragments from agarose gels were purified using the PureLink quick gel extraction kit (Invitrogen). Plasmid DNA was purified using either the PureLink quick plasmid miniprep kit (Invitrogen) or the Qia-gen HiSpeed Midi kit (Qiagen). Restriction endonucleases (Invitrogen and New England Biolabs) and T4 DNA ligase (New England Biolabs) were used according to the manufacturer's instructions. DNA sequencing was performed by the AAC Genomics Facility (University of Guelph, Guelph, Canada).

Construction of Chromosomal Mutants—The λ red-recombinase system (22) was used to generate CWG1006 (*waaL::cat*), CWG1105 ($\Delta wbdA^{O9a}$), CWG1104 ($\Delta wbdA^{O8}$), CWG1009 ($\Delta wbdB^{O9a}$), CWG1007 ($\Delta wbdB^{O8}$), CWG1010 ($\Delta wbdC^{O9a}$), and CWG1008 ($\Delta wbdC^{O8}$). The chloramphenicol resistance cassette was amplified by PCR from pKD3 using oligonucleotide primers containing 50 nucleotide homology extensions upstream and downstream of the target gene. The oligonucleotide primers were designed to generate deletions of the entire open reading frame. After recombination to replace the targeted gene with an antibiotic resistance marker, the FLP-recombinase plasmid (pCP20) was used to remove the marker from the chromosomes of mutants. The exception was CWG1006 (*waaL::cat*), where removal of the marker was unnecessary because any potential polar effects would not influence experiments conducted in this study.

Cloning of *wbdC*, *wbdB*, and *wbdA* Mannosyltransferase Genes from *E. coli* O9a and O8—The *wbdC* and *wbdB* genes were amplified from *E. coli* CWG634 (O9a) and CWG636 (O8) chromosomal DNA and cloned into the isopropyl β -D-1-thiogalactopyranoside-inducible pMAL-c2X vector (New England Biolabs) to generate N-terminal maltose-binding protein (MalE) fusions. The *wbdC* and *wbdB* genes from both serotypes were also cloned into the arabinose-inducible pBAD24 vector (23). The *wbdA* genes from serotypes O8 and O9a were cloned into the pBAD24 or pBADHisA (Invitrogen) vector to produce proteins with polyhistidine tags. All constructs were confirmed by restriction endonuclease digestion or by sequence and by their ability to complement their corresponding chromosomal mutation.

Purification of MalE-WbdB and MalE-WbdC Fusion Proteins—A 250-ml culture of *E. coli* Top10 transformed with the appropriate pMAL-c2X-based plasmid was grown in LB medium at 37 °C until an A_{600} of ~ 0.3 was reached. Cultures were then transferred to an incubator set at 20 °C and grown until midexponential phase ($A_{600} = 0.6$). 0.5 mM isopropyl β -D-

1-thiogalactopyranoside was then added to induce expression of the MalE fusion protein, and incubation was continued overnight at 20 °C. Cells were then collected by centrifugation and resuspended in 25 ml of buffer A (20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5), prior to lysis by sonication with intermittent cooling on ice. The cell lysate was cleared by sequential centrifugation steps at 3000 $\times g$ for 10 min and 27,000 $\times g$ for 30 min. The resulting supernatant was loaded onto a column containing amylose resin (New England Biolabs) and washed extensively with buffer A. The bound MalE fusion protein was eluted using buffer A containing 10 mM maltose. The protein concentrations were determined using the A_{280} , based on theoretical extinction coefficients of MalE-WbdC^{O8/O9a} (105,910 $\text{M}^{-1} \text{cm}^{-1}$) and MalE-WbdB^{O8/O9a} (140,985 $\text{M}^{-1} \text{cm}^{-1}$), predicted by the ProtParam program (24). The elution fraction containing the most protein typically contained 2–4 mg/ml protein and was stored in buffer A containing 10 mM maltose at -80 °C.

Purification of *WbdA*^{O9a}-His₁₀ and His₁₀-*WbdA*^{O8}—A 500-ml culture of *E. coli* BL21 transformed with the appropriate pBAD24-based plasmid was grown in LB medium at 37 °C until an A_{600} of ~ 0.3 was reached. Gene expression was induced as described above, with the exception that 0.2% (w/v) L-arabinose was used as inducer. Cells were then collected by centrifugation and resuspended in 25 ml of buffer B (20 mM BisTris, 250 mM NaCl, 5% (w/v) glycerol, pH 7.0) prior to lysis by sonication with intermittent cooling on ice. The cell lysate was cleared by sequential centrifugation steps at 5000 $\times g$ for 10 min and 12,000 $\times g$ for 20 min. The resulting supernatant was centrifuged at 100,000 $\times g$ for 60 min, and the soluble fraction was loaded onto a 5-ml HiTrap chelating (Ni^{2+}) column (GE Healthcare). For His₁₀-WbdA^{O9a}, the column was washed sequentially with buffer B containing 50 and 75 mM imidazole, and the bound protein was eluted in buffer B containing 125 mM imidazole. The column loaded with His₁₀-WbdA^{O8} was washed with buffer B and then eluted in the same buffer containing 50 mM imidazole. A PD-10 column (GE Healthcare) was used to exchange the purified proteins into buffer C (20 mM BisTris, 50 mM NaCl, pH 7.0), and aliquots were frozen at -80 °C. The protein concentrations were determined using the A_{280} , based on theoretical extinction coefficients of His₁₀-WbdA^{O9a} (123,230 $\text{M}^{-1} \text{cm}^{-1}$) and His₁₀-WbdA^{O8} (160,420 $\text{M}^{-1} \text{cm}^{-1}$) predicted by the ProtParam program (24). The protein concentration in frozen aliquots of His₁₀-WbdA^{O9a} and His₁₀-WbdA^{O8} was typically 2 mg/ml.

Preparation of Membrane Fractions—A 200-ml culture of CWG1006 containing the desired pBAD24-derived plasmid was grown in M9 minimal medium at 37 °C until an A_{600} of ~ 0.4 was achieved. Recombinant protein expression was induced by the addition of 0.2% (w/v) L-arabinose, and the culture was grown for a further 3.5 h. Cells were collected by centrifugation and resuspended in 20 ml of buffer D (20 mM HEPES, pH 7.5) prior to lysis by sonication. The lysate was cleared by sequential centrifugation steps at 4000 $\times g$ for 10 min and 12,000 $\times g$ for 20 min. The resulting supernatant was centrifuged at 100,000 $\times g$ for 60 min, and the membrane pellet was resuspended in 0.2 ml of buffer E (50 mM HEPES, 20 mM MgCl_2 , 1 mM dithiothreitol, pH 7.5). The total membrane pro-

tein concentration was typically 5–10 mg/ml, based on the DC Protein Assay (Bio-Rad), and membrane preparations were stored in buffer E at -80°C .

In Vitro WbdC and WbdB Mannosyltransferase Reactions Using a Synthetic Acceptor—A synthetic acceptor lipid analog β -Glc₁NAc-pyrophosphoryl-C₁₃ (Glc₁NAc-PP-C₁₃) (25) was generously provided by Dr. E. D. Brown (McMaster University, Hamilton, Canada) and used as a substrate for MalE-WbdC and/or MalE-WbdB. Radiolabeled mannosyltransferase assays were carried out for 1 h at 25°C in 10- μl reaction volumes of buffer E containing 0.5 mM Glc₁NAc-PP-C₁₃, 0.31 μM GDP-[¹⁴C(U)]Man (262 mCi/mmol; PerkinElmer Life Sciences) and 10 μg of enzyme. Reactions were terminated by the addition of an equal volume of stop solution (50% (v/v) aqueous acetonitrile, 1% (w/v) SDS, 10 mM EDTA). Radiolabeled reaction products were analyzed by thin layer chromatography (TLC). Aliquots (2–4 μl) of stopped reaction mixtures were spotted on AL SIL G TLC plates (Whatman) and developed in solvent A (ethyl acetate/methanol/water/acetic acid, 4:2:1:0.1). Dried TLC plates were exposed to film (Kodak Biomax MR film, Amersham Biosciences) for 1 week.

To prepare non-radiolabeled products for mass spectrometry (MS), reactions were carried out for 3 h at 25°C in 50- μl reaction volumes consisting of buffer E containing 1 mM Glc₁NAc-PP-C₁₃, 1 mM GDP-Man, and 50 μg of enzyme. After termination in stop solution, reaction products were diluted in 1 ml of H₂O and loaded onto a C₁₈ Sep-Pak cartridge (Waters). After washing extensively with water, the products were eluted in 60% acetonitrile and concentrated using a SpeedVac concentrator. Capillary electrophoresis-mass spectrometry (CE-MS) of these products was conducted on a Prince CE system (Prince Technologies, Netherlands) coupled to a 4000Qtrap mass spectrometer (AB/Sciex, Concord, Canada), via a micro-ion spray interface. Survey scan spectra of the products were obtained in negative ion mode. Sheath solution (isopropyl alcohol/methanol, 2:1) was delivered at a flow rate of 2.0 $\mu\text{l}/\text{min}$. Separations were obtained on a ~ 90 -cm length bare fused silica capillary, using 10 mM ammonium acetate in chloroform/methanol (2:1). Separations were performed by applying a voltage of 30 kV together with 500 millibars of pressure. MS/MS experiments were performed in positive ion mode. The instrument was operated with low (0.7 atomic mass unit peak width at half height) resolution on quadrupole 1, and typical collision energy of 50 eV was applied to induce fragmentation of ions selected for collision-induced dissociation. Nitrogen was used as the collision gas with peak width at half-height.

In Vitro WbdC and WbdB Mannosyltransferase Reactions Using Endogenous Acceptors in Membranes—The use of membranes from CWG1006 (*manA waaL::cat*) in these experiments ensured that the reaction products in these experiments remained as und-PP-linked intermediates rather than being transferred to lipid A core by the WaaL ligase enzyme (1). The *manA* mutation prevented synthesis of GDP-Man in the absence of mannose in the growth medium (15), allowing *de novo* synthesis in membranes following the addition of the radiolabeled sugar donor. Mannosyltransferase assays were carried out for 1.5 h at 25°C in 100- μl reaction volumes of buffer E containing 0.31 μM GDP-[¹⁴C(U)]Man and 100 μg of

membrane protein. The und-PP-linked oligosaccharides were extracted from membranes in an equal volume of 1-butanol (26). The organic (upper phase) was kept, and the extractions were repeated. The combined organic phases were washed once with an equal volume of water and then dried to completeness. 100 μl of 1-propanol, 2 M trifluoroacetic acid (1:1) was added to each of the dried lipid extracts, and the samples were heated to 50°C for 15 min to liberate the oligosaccharide phosphates from the und-PP-linked products. The samples were dried and then treated with 10 units of calf intestinal alkaline phosphatase (2000 units/mg; Roche Applied Science) in dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) for ~ 2 h at 37°C .

The dephosphorylation reaction mixtures were diluted to 1 ml with water and then loaded onto a column (75 \times 2.5 cm) containing Toyopearl HW-40(S) resin (Tosoh Bioscience LLC). 100 μl of radiolabeled WbdC (~ 5500 cpm) or WbdCB ($\sim 10,000$ cpm) products were mixed with 25 μl each of Man₉, maltose, maltotriose, and maltotetraose (10 mg/ml) standards. The mixture was separated, and eluates were collected in 1-ml fractions using 0.1 M acetic acid as the eluant. The radiolabeled oligosaccharides were detected by scintillation counting of 0.4 ml of each fraction in Ecolite scintillation fluid (ICN Biomedicals). The non-radiolabeled standards were detected using the colorimetric Dubois assay for total sugars (27).

In Vitro WbdA Mannosyltransferase Reactions Using Synthetic Substrates—Two synthetic acceptors were used. Acceptor A (α -Man_p-(1 \rightarrow 2)- α -Man_p-(1 \rightarrow 2)- α -Man_p-(1 \rightarrow 3)- α -Man_p) represents the repeat unit of the O9a antigen, whereas Acceptor B (α -Man_p-(1 \rightarrow 3)- α -Man_p-(1 \rightarrow 3)- β -Glc₁NAc) is the conserved reducing terminal trisaccharide of the O8 and O9a antigens (Fig. 1A). Their synthesis as 8-azido-octyl glycoside derivatives has been described elsewhere (28, 29), and both were used as fluorescein-tagged derivatives. Preparation of a fluorescein-tagged derivative of Acceptor A and its use in characterizing the WbdD^{O9a}-mediated chain-terminating activity has been described previously (12). The synthesis of the fluorescein-tagged derivative of Acceptor B followed the same protocol.

Initial assay conditions were established using WbdA^{O9a}. The enzyme's activity was determined to be optimal at room temperature ($\sim 25^{\circ}\text{C}$) and declined rapidly at temperatures above 30°C (data not shown). Approximate K_m values for the acceptor and GDP-Man were determined using a CE-based assay. The percentage conversion of Acceptor A into products was determined by integration of the product peak areas on electropherograms. The percentage conversion was used to establish reaction conditions at which the rate of product formation was linear with time (*i.e.* the initial velocity). Keeping within the linear range, apparent K_m values for Acceptor A and GDP-Man were determined in two separate trials. The percentage conversion was used to calculate the enzyme activity for each substrate concentration, and the data were fit to the Michaelis-Menten equation in GraphPad Prism version 4.0. The $K_{m(\text{app})}$ values for Acceptor A in the independent experiments were 1.5 ± 0.2 and 1.2 ± 0.3 mM. The $K_{m(\text{app})}$ values for GDP-Man were 0.5 ± 0.05 and 0.6 ± 0.08 mM (data not shown). Insufficient acceptor was available for a complete kinetic anal-

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ysis, but these experiments defined conditions of excess substrate used for standard reactions. These reactions were carried out for 30 min at 25 °C in 10- μ l volumes of buffer E containing 0.5 mM acceptor, 5 mM GDP-mannose, and 2 μ M purified His₁₀-WbdA^{O9a} or 10 μ M purified His₁₀-WbdA^{O8}. Reactions were terminated by the addition of an equal volume of stop solution, diluted 1:4 with 50% (v/v) aqueous acetonitrile, and 2- μ l aliquots were spotted on AL SIL G TLC plates (Whatman). The plates were developed in solvent B (ethyl acetate/water/1-butanol/acetic acid, 5:4:4:2.5) and fluorescent reaction products were detected with a hand-held UV lamp.

CE analysis of the WbdA^{O9a} products synthesized using Acceptor A was conducted according to published methods (30). The stopped reactions were centrifuged at 13,000 rpm for 5 min and then diluted 1:250 in water (to 1 μ M acceptor concentration). The samples were pressure-injected for 10 s into a bare silica capillary (50 μ m \times 50 cm) and separated in CE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, 20 mM SDS, pH 8.27) over 15 min. The samples were resolved at 30 kV using a Beckman-Coulter P/ACE MDQ CE system equipped with an argon ion laser-induced fluorescence detector (λ_{Ex} = 488 nm, with emission filter at 520 nm).

Structural Analyses of the Products of WbdA Proteins Using Synthetic Acceptors—To generate sufficient products for NMR analyses, 250- μ l reaction volumes were used. After 30 min, the reactions were diluted in 1 ml of H₂O and loaded onto a C₁₈ Sep-Pak cartridge (Waters), and the cartridge was washed extensively with water. The products were eluted in 2 ml of 60% (v/v) aqueous acetonitrile and concentrated using a SpeedVac concentrator.

MALDI-TOF mass spectra of the reaction products were obtained on a Bruker Ultraflextreme MALDI TOF/TOF. The MALDI spectra of the reaction products of WbdA^{O9a} with Acceptor B were obtained in positive ion mode, whereas the reaction products of WbdA^{O8} or WbdA^{O9a} with Acceptor A were obtained in negative ion mode.

NMR spectra for the reaction products were acquired in D₂O at 27 °C on a 700-MHz spectrometer equipped with a cryoprobe. The spectra were referenced to an external standard of acetone (2.22 ppm for ¹H, 31.07 for ¹³C). One-dimensional, gCOSY, ¹H-tROESY, and ¹H-¹³C gHSQC spectra were obtained. For all of the ¹H spectra, the intensity of the residual HOD peak was decreased using a presaturation pulse sequence, irradiating at 4.76 ppm. The spectral window for the one-dimensional ¹H spectra was 8446 Hz (from 10.8 to -1.3 ppm), and a line broadening function of 0.5 Hz was applied to improve the signal/noise ratio. For all of the two-dimensional spectra, sine-bell functions were applied interactively to improve signal/noise, but no line-broadening was used. For the ¹H-¹³C gHSQC spectra, proton signals were decoupled during acquisition, and the ¹J_{C,H} value was set to 140 Hz to determine the appropriate delays. Additional gradient-enhanced total correlation spectroscopy spectra were also acquired to facilitate interpretation.

For the O9a WbdA products, the spectral window for the gCOSY was 8446 Hz (from 10.8 to -1.3 ppm) in both dimensions, with 512 increments in F1 and 64 transients in F2. The tROESY was acquired with a spectral window of 5605 Hz (8.0 to 0.0 ppm) in both dimensions, 299 increments in the F1 dimen-

sion, 64 transients in the F2 dimension, and a mixing time of 0.4 s. The spectral window for the ¹H-¹³C gHSQC was 4223 Hz (from 6.0 to 0.0 ppm) in F2 (¹H dimension, 64 transients) and 24.6 kHz (from 130 to -10 ppm) in F1 (¹³C dimension, 128 increments).

To investigate the structure of the O8 WbdA products, the spectral windows for the gCOSY and tROESY were 5605 Hz (from 9.0 to 1.0 ppm) in both dimensions. The gCOSY was acquired with 600 increments in F1 and 16 transients in F2, and the tROESY was acquired with 550 increments in F1, 16 transients in F2, and a mixing time of 0.4 s. The spectral window for the ¹H-¹³C gHSQC was 5605 Hz (from 9.0 to 1.0 ppm) in F2 (¹H dimension, 16 transients) and 28.2 kHz (from 160 to 0 ppm) in F1 (¹³C dimension, 432 increments).

Protein and LPS PAGE—Protein and LPS samples were analyzed by SDS-PAGE in Tris/glycine buffer (31). Protein was visualized using Simply Blue stain (Invitrogen). LPS samples were prepared by proteinase K digestion of whole-cell lysates according to the method of Hitchcock and Brown (32). LPS was visualized by silver staining (33). Western immunoblots of LPS were prepared by transferring samples to PROTRAN nitrocellulose membranes (PerkinElmer Life Science). Samples were probed at a 1:500 dilution with O9a-specific or O8-specific antiserum (15). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Cedar Lane Laboratories) was used at a dilution of 1:3000, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) were used as substrates for detection.

RESULTS

WbdC^{O8/O9a} Is a Monofunctional Mannosyltransferase—Previous experimental data from *E. coli* O9a support the conclusion that WbdC^{O9a} performs the first committed step in biosynthesis of this polymannose O-PS, by transferring a single Manp residue to the multifunctional acceptor, β -Glc pNac-PP-und (14). This is consistent with both of the O-PS structures (Fig. 1A), which contain an α -Manp-(1 \rightarrow 3)- β -Glc pNac disaccharide at their reducing termini (11). The predicted WbdC^{O8} and WbdC^{O9a} proteins share 96% identity, and, as expected, each gene could rescue O-PS synthesis in the Δ wbdC mutants constructed in the O8 and O9a serotypes (Fig. 2, A–D), suggesting an identical function *in vivo*. O-PS was even synthesized in the absence of inducer, due to the “leaky” promoter (34). Induced cultures generated reduced amounts of O-PS, which may be a consequence of high levels of protein expression. This may reflect increased formation of inactive enzyme aggregates or altered stoichiometry of components in an enzyme complex. However, this does not influence interpretation of the current data and was not pursued further.

To confirm the function of WbdC, *in vitro* synthesis was performed in reactions containing membranes from cells expressing WbdC^{O8/O9a}, GDP-[¹⁴C]Man, and endogenous acceptor. TLC and autoradiography revealed a single major co-migrating oligosaccharide product from each enzyme (data not shown). The products from WbdC^{O9a} were subjected to gel filtration chromatography, revealing a product co-migrating with a disaccharide standard, consistent with an α -Manp-(1 \rightarrow 3)- β -Glc pNac disaccharide (Fig. 3A).

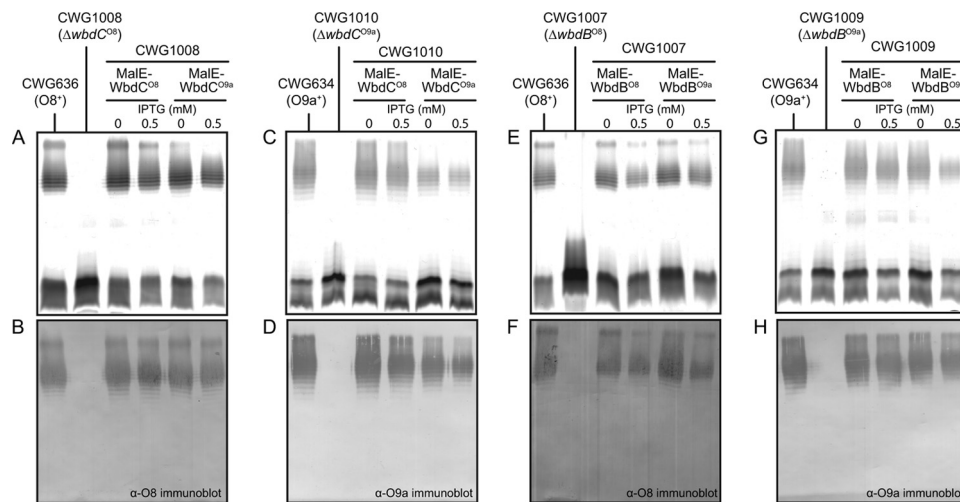


FIGURE 2. The O8 and O9a *wbdC* and *wbdB* genes are functionally exchangeable. The results show gene complementation experiments with CWG1008 ($\Delta wbdC^{O8}$) (A and B), CWG1010 ($\Delta wbdC^{O9a}$) (C and D), CWG1007 ($wbdB^{O8}$) (E and F), and CWG1009 ($\Delta wbdB^{O9a}$) (G and H). The top panels in each pair show the silver-stained SDS-polyacrylamide gel of LPS samples from whole-cell lysates, and the bottom panels show the corresponding confirmatory Western immunoblots using O8- or O9a-specific antisera. In each case, native O-PS biosynthesis was restored by the introduction of plasmids carrying the relevant gene from either serotype O8 or O9a. The plasmids used were as follows: pWQ577 (*malE-wbdC^{O8}*), pWQ575 (*malE-wbdC^{O9a}*), pWQ578 (*malE-wbdB^{O8}*), and pWQ576 (*malE-wbdB^{O9a}*).

range of sizes on TLC (Fig. 4A). At high GDP-Man/acceptor ratios, the product was confined to the origin of a TLC plate, whereas 3–4 faster migrating products were observed when the molar concentrations of donor and acceptor were 1:1. A CE trace of samples taken at various time points from a reaction containing Acceptor A demonstrated the accumulation of larger products over time (data not shown). These observations are consistent with a reaction mechanism that is distributive rather than processive.

The reaction products obtained with Acceptor A and WbdA^{O9a} were examined by MALDI MS, and a range of products was detected. These consist of Acceptor A alone (m/z 1182) and acceptor plus increasing numbers of Man p residues (data not shown). The largest product (m/z 4911) corresponds to the mass of the acceptor plus 23 Man p residues. Each peak was separated by the mass of one Man p residue (162.1). MALDI MS of the products made by WbdA^{O9a} with Acceptor B also revealed products of increasing size. The largest (m/z 4487.5) corresponds to the acceptor plus 21 Man p residues (Fig. 4B). The smallest product (m/z 1246.5) is Acceptor B plus one added Man p ; no unmodified acceptor was detected, indicating that all of the starting material was converted into product by the enzyme.

WbdA^{O9a} is capable of polymerizing sufficient Man p residues to encompass multiple repeat units of the O-PS, but MS offers no insight into the linkage sequence in the product. To resolve this question, reaction products generated using Acceptor B were examined by ¹H NMR spectroscopy. The one-dimensional ¹H spectrum revealed that at least two compounds were produced in the reaction (Fig. 4C). Integration of the anomeric signals in the ¹H spectrum showed that these compounds were present in an approximate ratio of 3:1. Four signals were obtained in the anomeric region for the major product, indicating that the H1 protons are present in four distinct chemical environments. This is consistent with the O9a repeat unit that contains blocks of two α -(1 \rightarrow 2)- and two

α -(1 \rightarrow 3)-linked mannose residues. The chemical shifts for the anomeric protons and carbons of the major species closely match those published for the O9a antigen (Table 2) (40).

To confirm the linkages of the major product, gCOSY and tROESY spectra were acquired (supplemental Fig. S2). The gCOSY spectrum was used to establish through-bond correlations between the protons of each ring system. The H2 and H3 resonances of each mannose residue were correlated to the anomeric signals in the spectrum. This information was used to interpret the tROESY spectrum, which established the through space correlations between protons of adjacent ring systems. tROESY showed a correlation between the anomeric signal at 5.37 ppm and a signal at 3.99 ppm, which was assigned as the H3 proton in the same Man p residue as the anomeric signal at 5.12 ppm. Thus, the Man p residues corresponding to the H1 at 5.37 and 5.12 ppm are linked (1 \rightarrow 3). Similarly, there is a correlation between the anomeric signal at 5.12 ppm and the peak at 3.93 ppm, which was assigned as the H3 in the same residue as the anomeric signal at 5.04 ppm. Therefore, the Man p residues corresponding to the H1 at 5.12 ppm and 5.04 ppm are also linked (1 \rightarrow 3). The anomeric signal at 5.04 ppm was correlated to 4.11 ppm, which was assigned as H2 in the residue corresponding to the remaining anomeric peak at 5.30 ppm. This indicated that the Man p residues of the H1 at 5.04 and 5.30 ppm are linked (1 \rightarrow 2). The final important correlation was between the anomeric signal at 5.30 ppm and a resonance at 4.09 ppm, which was assigned as the H2 in the mannose with an anomeric peak at 5.37 ppm. Thus, the last linkage between the residues of the H1 at 5.30 and 5.37 ppm is also (1 \rightarrow 2). Key correlations from the tROESY spectrum are labeled in supplemental Fig. S2B. From these data, the structure of the major product produced by WbdA_{O9a} with the Acceptor B was identified as a repeating chain of the following tetrasaccharide: \rightarrow 3)- α -D-Man p -(1 \rightarrow 2)- α -D-Man p -(1 \rightarrow 2)- α -D-Man p -(1 \rightarrow 3)- α -D-Man p -(1). This structure is identical to the repeat unit of the O9a antigen (40).

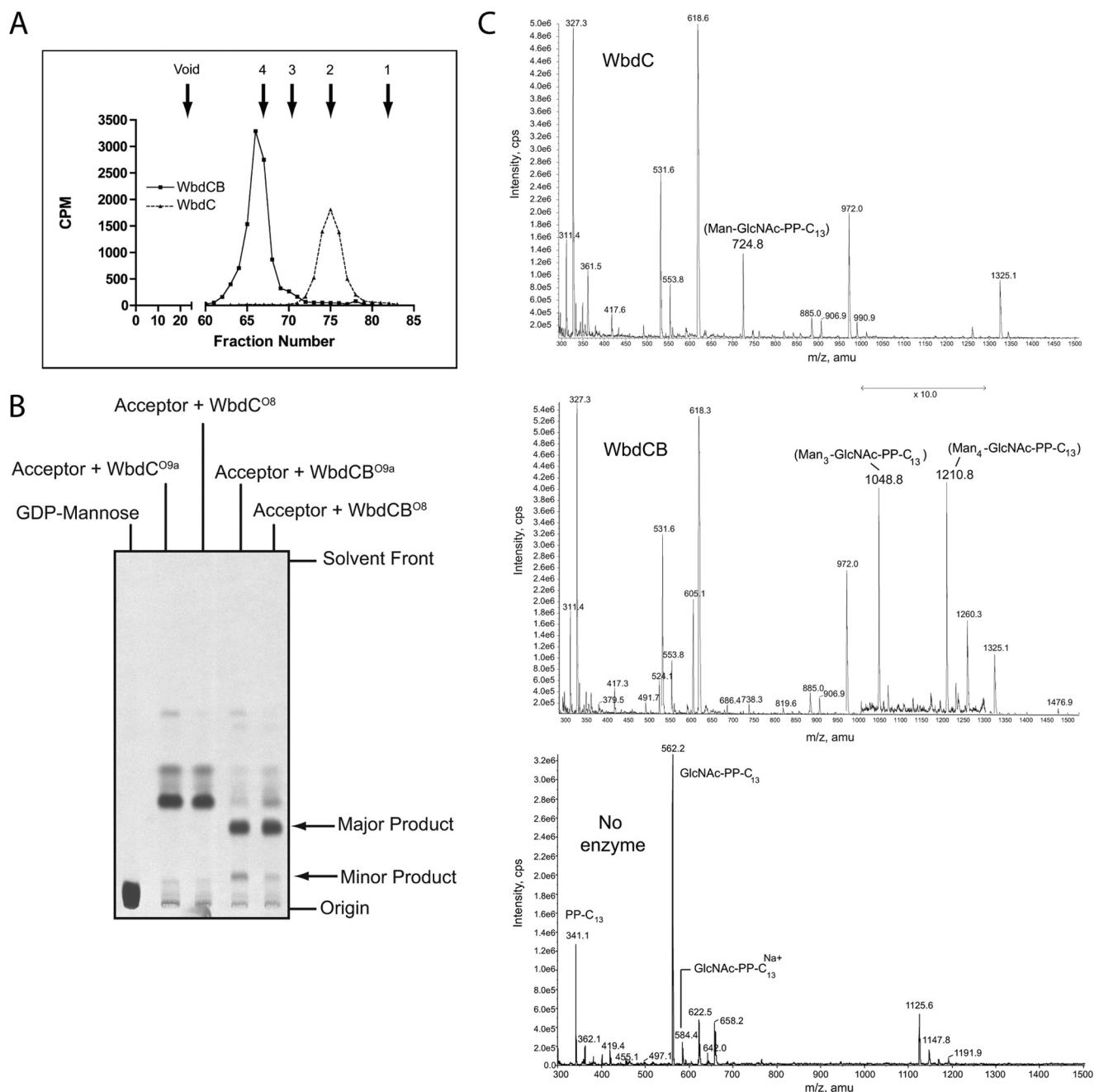


FIGURE 3. Analysis of the *in vitro* products from WbdC and WbdCB mannosyltransferase activities using an endogenous acceptor (membrane fraction) and a synthetic acceptor. A, gel filtration chromatography of the WbdC^{O9a} and WbdCB^{O9a} oligosaccharide products generated using the endogenous (β -GlcNAc-PP-und) acceptor in membranes. The standards were mannose (1), maltose (2), maltotriose (3), and maltotetraose (4). B, autoradiograms of TLC-separated reactions synthesized by WbdC^{O8}, WbdC^{O9a}, WbdCB^{O8}, and WbdCB^{O9a} using the synthetic GlcNAc-PP-C₁₃ acceptor. The CE-MS spectra of the reaction products generated by WbdC^{O9a} and the WbdCB^{O9a} pair using the synthetic GlcNAc-PP-C₁₃ acceptor and the unmodified acceptor are shown in C.

The structure of the minor reaction product was more difficult to determine. Resonances for five anomeric protons were visible in the one-dimensional ¹H spectrum (Fig. 4C), but many of the correlations in the two-dimensional spectra were similar in intensity to the noise. Therefore, to aid in the interpretation of the data, the computer program CASPER (41, 42) was used. This software predicts ¹H and ¹³C chemical shifts from a database built from mono-, di-, and trisaccharides. Sequences were entered into the program for several penta- and hexasaccharides of various linkages with the Acceptor B trisaccharide (α -D-Manp-(1 \rightarrow 3)- α -D-Manp-(1 \rightarrow 3)- β -D-GlcpNAc) at the

reducing end. The best match between the anomeric shifts of the minor reaction product and the chemical shifts predicted by CASPER occurred for the following hexasaccharide: α -D-Manp-(1 \rightarrow 3)- α -D-Manp-(1 \rightarrow 2)- α -D-Manp-(1 \rightarrow 2)- α -D-Manp-(1 \rightarrow 3)- α -D-Manp-(1 \rightarrow 3)- β -D-GlcpNAc (Table 3). For comparison, the sequence of a dodecasaccharide containing three repeating units of the major structure was entered into CASPER. The experimental and predicted chemical shifts agreed remarkably well (Table 3). Therefore, we propose that the minor reaction product is a truncated version of the major product with the structure of the hexasaccharide above. Its syn-

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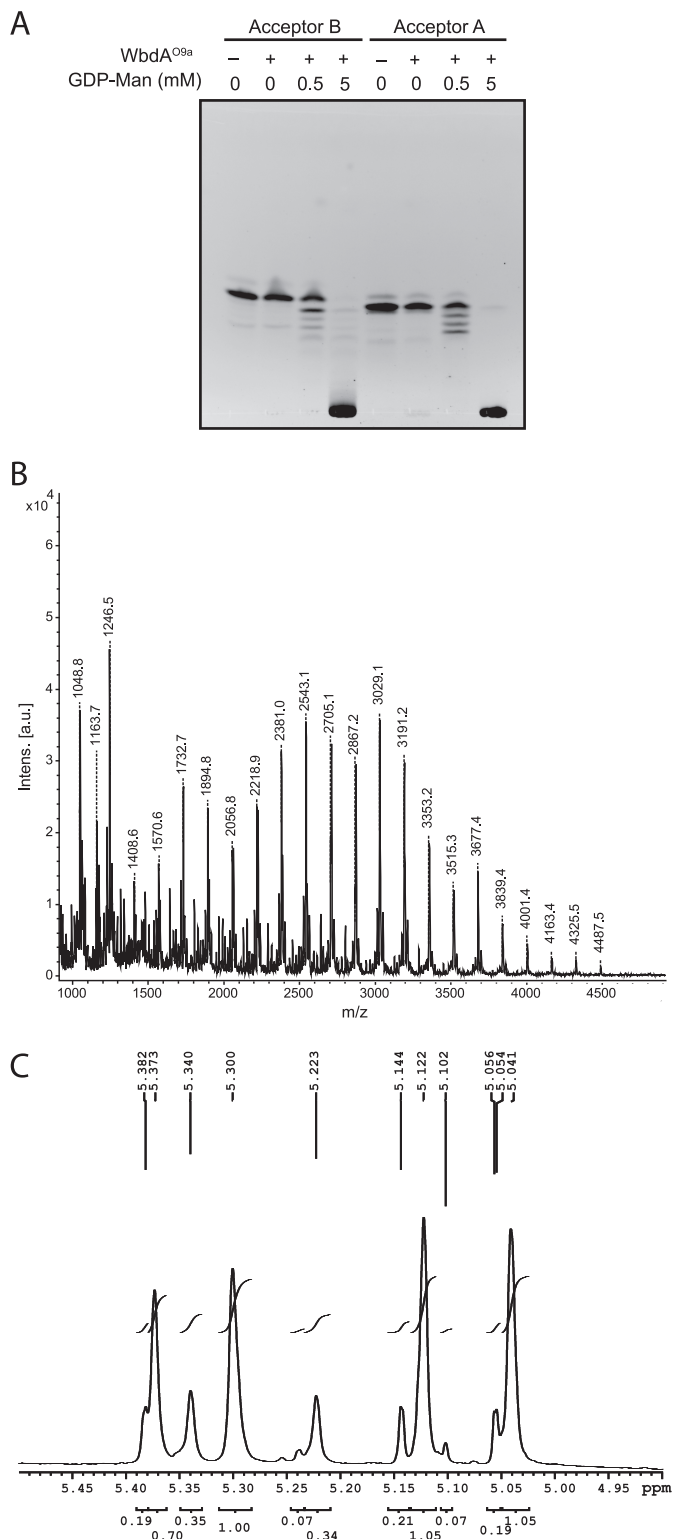


FIGURE 4. Analysis of the *in vitro* products from WbdA^{O9a} mannosyltransferase activity using synthetic acceptors. The reaction products were separated by TLC (A), using the fluorescein tag on the acceptors for detection. The products generated with Acceptor B were examined by MALDI MS (B), revealing a series of incrementally sized products that differ by the addition of one Manp residue. C, anomeric region from the ¹H NMR spectrum of the reaction products generated by WbdA^{O9a} using Acceptor B.

thesis may result from relaxed fidelity in the *in vitro* reaction conditions because there is no evidence for such a product in the native O-PS.

TABLE 2

Comparison of the chemical shifts for the anomeric protons and carbons of the major WbdA^{O9a} product generated using Acceptor B with those previously published for the O9a O-PS

	A	B	C	D
	→3)-α-Manp-(1→2)-α-Manp-(1→2)-α-Manp-(1→3)-α-Manp-(1			
Experimental δ _H (ppm)	5.04	5.30	5.37	5.12
Experimental δ _C (ppm) ^a	102.7	101.1	101.1	102.7
Literature δ _H (ppm) ^b	5.07	5.28	5.35	5.14
Literature δ _C (ppm) ^b	102.8	101.4	101.4	102.8

^a The chemical shift values were determined from the ¹H-¹³C gHSQC spectrum (supplemental Fig. S1).

^b Values from Ref. 40.

TABLE 3

Comparison of experimental ¹H chemical shifts (ppm) with those predicted by CASPER for the anomeric protons of the major WbdA^{O9a} product (1) and predicted minor WbdA^{O9a} product (2) generated using Acceptor B

(1) O9a repeat-unit:	1A	1B	1C	1D		
	→3)-α-Manp-(1→2)-α-Manp-(1→2)-α-Manp-(1→3)-α-Manp-(1					
(2) Hexasaccharide (truncated O9a product):	2A	2B	2C	2D	2E	2F
	α-Manp-(1→3)-α-Manp-(1→2)-α-Manp-(1→2)-α-Manp-(1→3)-α-Manp-(1→3)-β-GlcpNAc					
	A	B	C	D	E	F
1, Experimental	5.04	5.30	5.37	5.12	–	–
1, CASPER	5.02	5.29	5.33	5.09	–	–
1, Difference	0.02	0.01	0.04	0.03	–	–
2, Experimental	5.14	5.04	5.34	5.38	5.22	– ^a
2, CASPER	5.11	5.02	5.29	5.33	5.22	4.74
2, Difference	0.03	0.02	0.05	0.05	0.00	–

^a The HOD peak at 4.76 ppm is too large to determine if the anomeric signal for the β-G1cNAc residue is present.

Similar experiments were performed using WbdA^{O8}. This enzyme could not extend Acceptor B under the conditions tested but, perhaps surprisingly, yielded a range of different sized products with Acceptor A (Fig. 5, A and B). The reaction products obtained with Acceptor A and WbdA^{O8} were examined by MALDI MS, and a range of products were detected consisting of Acceptor A alone (*m/z* 1183) and acceptor plus increasing numbers of Manp residues (Fig. 5B). The largest product (*m/z* 4427) corresponds to the mass of the acceptor plus 20 Manp residues. There is a non-statistical distribution of intensities in the MALDI spectrum, indicating varied substrate activity for the different length oligosaccharides. For example, the intensity corresponding to the addition of seven Manp residues (*m/z* 2318) is much lower than that of nine additional residues (*m/z* 2643).

The anomeric signals of the one-dimensional ¹H spectrum showed that at least two compounds were present in the reaction mixture generated by WbdA^{O8} using Acceptor A (Fig. 5C). The signals for the three anomeric protons of the major product integrate to one proton each, and the chemical shifts for the corresponding anomeric protons and carbons closely match those published for the O8 antigen (Table 4) (43). The signals for the minor compound are consistent with unmodified acceptor.

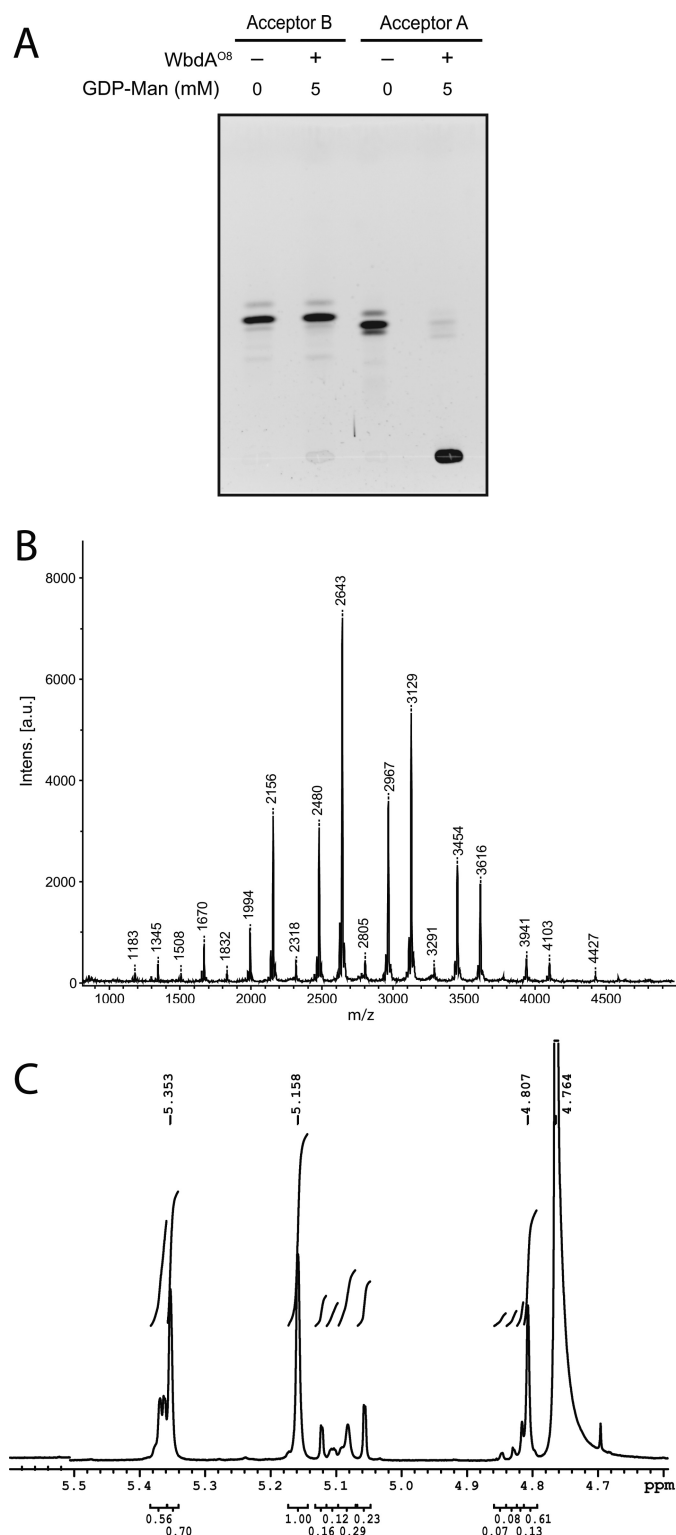


FIGURE 5. Analysis of the *in vitro* products from WbdA^{O8} mannosyltransferase activity using synthetic acceptors. The reaction products were separated by TLC (A), using the fluorescein tag on the acceptors for detection. The reaction products generated with Acceptor A were examined by MALDI MS (B), revealing a series of incrementally sized products that differ by the addition of one Manp residue. Some of the product masses are 1 or 2 mass units higher than expected because the MS analysis was performed after their NMR spectra were acquired, and there were some residual deuterons remaining in the sample. C, the anomeric region from the ¹H NMR spectrum of the reaction products generated by WbdA^{O8} using Acceptor A.

TABLE 4

Comparison of the chemical shifts for the anomeric protons and carbons of the WbdA^{O8} product generated using Acceptor A with those previously published for the O8 O-PS

	→3)-β-Manp-(1→2)-α-Manp-(1→2)-α-Manp-(1		
	A	B	C
Experimental δ _H (ppm)	4.81	5.16	5.35
Experimental δ _C (ppm) ^a	98.3	99.9	100.7
Literature δ _H (ppm) ^b	4.75	5.12	5.30
Literature δ _C (ppm) ^b	99.1	100.8	101.5

^a The chemical shift values were determined from the ¹H-¹³C gHSQC spectrum (supplemental Fig. S3).

^b Values from Ref. 43.

Further analysis of the gCOSY and tROESY spectra confirmed the linkages between each of the Manp residues. As with the major WbdA^{O9a} product, the resonances for H2 and H3 for each of the residues in O8 product could be correlated to each anomeric signal from the gCOSY spectrum (supplemental Fig. S4A). This information was used to interpret the tROESY spectrum and assign the linkages. Key correlations from the tROESY are labeled supplemental Fig. S4B. The anomeric signal at 5.35 ppm correlated to a signal at 3.75 ppm, which was assigned as the H3 proton in the same residue as the signal at 4.81 ppm. Thus, the resonance at 5.35 ppm is indicative of a (1→3)-linked moiety. The anomeric signal at 5.16 ppm had correlations to protons on different residues, one to the anomeric proton at 4.81 ppm and a second to the signal at 4.13 ppm. This signal was assigned to H2 on the same residue as the anomeric proton at 5.35 ppm, and the correlation is consistent with a (1→2)-linked residue. The anomeric signal at 4.81 correlated to a signal at 4.30 ppm, which is proton H2 on the same ring as the signal at 5.16 ppm. This resonance indicates another (1→2) linkage. From these data, we conclude that the major compound in the NMR spectrum is the O8 antigen.

DISCUSSION

The O8, O9, and O9a antigens were the first representatives of O-PS synthesized via an ABC transporter-dependent system to be studied biochemically. These systems have been influential in the development of an understanding of the shared features of this biosynthetic strategy in O-PS and in other cell surface glycans from a wide range of bacteria with different lifestyles (44). Biosynthesis of the *E. coli* O9a and O8 polymannose O-PSs occurs through the sequential transfer of Manp residues to β-GlcpNAc-PP-und, and this is catalyzed by three mannosyltransferases, WbdC, WbdB, and WbdA (14). WbdC and WbdB are highly conserved in serotypes O8 and O9a, and all experimental evidence indicates that they perform the same reaction in each serotype. Although the published model correctly assigned the catalytic activity of the WbdC mannosyltransferase, the context of the activity of WbdB was misinterpreted as being involved in repeat unit synthesis, because the adaptor region at the reducing terminus of the O-PS was unknown at the time. Furthermore, definitive assignments can

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only be made when purified enzymes are used with defined acceptors. Our data confirm that, operating together, WbdC and WbdB are responsible for completing the und-PP-linked tetrasaccharide (α -Manp-(1 \rightarrow 3)- α -Manp-(1 \rightarrow 3)- α -Manp-(1 \rightarrow 3)- β -GlcNac), to form a conserved reducing terminal structure (the adaptor-primer) in the serotype O8 and O9a O-PSs. In our earlier report of the O-PS structure (11), we suggested that the conserved region may be confined to a trisaccharide (α -Manp-(1 \rightarrow 3)- α -Manp-(1 \rightarrow 3)- β -GlcNac). Both possibilities are accommodated by the reported structure, but they result in a different position of the first Manp residue in the repeat unit domain, essentially shifting the register of the repeat unit. However, only the conserved reducing terminal tetrasaccharide reconciles all of the structural, genetic, and biochemical data.

WbdC and WbdB are single active site glycosyltransferases belonging to the GT4 family (39). Several monospecific GT4 representatives from cell surface glycoconjugate assembly systems have been characterized, and solved structures are available for some. Selected examples include WaaG (*E. coli* LPS biosynthesis (45)), WasF (*Geobacillus stearothermophilus* NRS 2004/3A S-layer glycan (46)), and PimA (mycobacterial phosphoinositol mannoside (47)). In contrast, WbdB transfers two Manp residues to the native acceptor *in vivo*. Although less common, this is not without precedent. One well characterized example of this phenomenon is seen in PglH (another GT4 family member), which is involved in the synthesis of an und-PP-linked glycan that is used for *N*-glycosylation of proteins in *Campylobacter jejuni* (48). In a situation that is reminiscent of the activity of WbdB, PglH transfers three GalpNac residues to a terminal GalpNac in the growing glycan (49). Other examples of this type of activity are provided by GlfT1, an enzyme that transfers two galactofuranosyl residues to a decaprenol-P-linked acceptor in the biosynthesis of cell wall arabinogalactan in *Mycobacterium tuberculosis* (50, 51) and KdtA in *E. coli*, which adds two 2-deoxy-D-manno-octulosonic acid residues to lipid A in LPS biosynthesis (52). Examples are not confined to prokaryotes, because Alg11 of *Saccharomyces cerevisiae* adds two Manp residues to the dolichol-PP-linked intermediate used for *N*-glycosylation (53, 54). These enzymes are distinguished from single-site processive polymerases by the unique ability to limit the number of sequential glycosyl transfers to 2–3 residues. PglH offers insight into how this may occur. It has been proposed that the transfer of a discrete number of residues is controlled by the relative binding affinities for the growing acceptor (49). These increase with size, resulting in a lower k_{cat} . A counting mechanism for PglH is effectively provided by inhibition of enzymatic activity after the acceptor has been elongated by three GalpNac residues. Whether this applies to other examples remains to be established.

WbdB adds only two Manp residues to the endogenous acceptor *in vivo*. In contrast, using a purified enzyme and soluble acceptor WbdB also generates a minor product with three Manp residues transferred. This relaxed activity presumably results from the artificial (soluble) reaction conditions and/or the absence of other enzymes in the pathway. A similar phe-

nomenon has been observed with the bifunctional α -(2 \rightarrow 3), α -(2 \rightarrow 8) sialyltransferase, Cst-II. This enzyme normally adds one or two sialic acid residues to lipooligosaccharide of *C. jejuni* (55), but the purified enzyme can add up to four sialyl residues to a synthetic acceptor (56).

The studies described here indicate that the earlier proposal that WbdB participates in repeat unit synthesis (14) is incorrect. Instead, our data unequivocally demonstrate that WbdA^{O9a} is a bifunctional α -(1 \rightarrow 2), α -(1 \rightarrow 3)-mannosyltransferase and that WbdA^{O8} is a trifunctional enzyme with α -(1 \rightarrow 2), α -(1 \rightarrow 3), and β -(1 \rightarrow 2) mannosyltransferase activities. These enzymes are sufficient for formation of the repeat unit domain of the cognate O-PS. WbdA^{O9a} can polymerize the authentic O9a mannan using either Acceptor A or Acceptor B, indicating that it can elongate acceptors containing either α -(1 \rightarrow 3)- or α -(1 \rightarrow 2)-linked terminal Manp residues. This might be expected from the repeat unit structure. The accumulation of larger products over time and the altered size distribution with varying acceptor/donor ratios are consistent with an enzyme that is non-processive, or distributive. In this mechanism, the enzyme dissociates from the growing polymer after each Manp transfer. Surprisingly, WbdA^{O8} could also use Acceptor A (the O9a repeat unit), although this enzyme does see an α -(1 \rightarrow 2)-linked terminal Manp in the biosynthesis of the native glycan. In principle, the enzyme should also be able to add to an α -(1 \rightarrow 3)-linked terminal Manp, but no activity was seen with Acceptor B. Because neither of the available synthetic oligosaccharides represented a true physiological acceptor for WbdA^{O8}, the difference in the capacity to use these acceptors cannot be readily interpreted. Nevertheless, the extension of Acceptor A to generate an O8 glycan does provide clear evidence that WbdA^{O8}, like its counterpart from serotype O9a, is sufficient for assembly of the corresponding repeat unit domain.

Unlike WbdC and WbdB, the WbdA mannosyltransferases are not functionally exchangeable *in vivo* (data not shown). This result is expected, given that the ability of the ABC transporter to recognize the nascent O-PS (and form completed, O-PS-substituted LPS molecules) requires that the O-PS be first capped by the action of the cognate WbdD enzyme (15, 57). These enzymes differ in specificity and create different terminal structures (11, 13, 15, 43) (Fig. 1A). Furthermore, in O9a, a C-terminal region of WbdD is essential for recruiting the cognate WbdA to the membrane (58), and specificity in these interactions might be anticipated based on sequence differences between the two serotypes.

In silico predictions using the NCBI Conserved Domain Database (59) identify two putative glycosyltransferase domains in WbdA^{O9a} and three in WbdA^{O8}. These predictions correlate with sizes of the WbdA^{O9a} (95.5 kDa) and WbdA^{O8} (137 kDa) proteins. WbdA^{O9a} is approximately twice the size of a typical monofunctional mannosyltransferase, and WbdA^{O8} is roughly 3 times as large. For reference, WbdC and WbdB have predicted sizes of 42.5 and 43.9 kDa, respectively. Previous studies have shown that the domains in WbdA^{O9a} can be separated; however, both are required for O9a biosynthesis (35), and it is currently unknown whether a specific mannosyltransferase activity is associated with each domain.

In light of the observations reported here, other glycan biosynthesis systems following the same general theme can be proposed. One example is the *E. coli* O99 antigen, which has the following repeat unit backbone: $\rightarrow 3$ - α -D-Rhap-(1 \rightarrow 2)- α -D-Rhap-(1 \rightarrow 2)- α -D-Rhap-(1 \rightarrow 3)- α -D-Rhap-(1 (60). The structure carries additional α -(1 \rightarrow 2)-linked Glcp substituents on the first and fourth Rhap residues in the repeat unit. Genetic analyses suggest that like other glycans containing D-Rhap, the backbone is synthesized using GDP-D-Rhap as the donor and is therefore very similar to the O9a antigen. The fine structure of the reducing terminus of the O99 glycan has not been reported, but its biosynthesis involves three glycosyltransferases, WejKJI, which are similar to WbdCBA, respectively (60). Catalytic assignments have been proposed based on the earlier model of O9a biosynthesis (60), but WejI contains two putative glycosyltransferase domains like WbdA and may be solely responsible for synthesis of the repeat unit domain. Another multidomain glycosyltransferase is predicted in synthesis of the *E. coli* O52 antigen (61), but further investigation is needed to confirm the activities of the enzymes involved.

Some Gram-positive S-layer glycoproteins provide non-O-PS systems that share striking similarities with the *E. coli* O9a and O8 antigens in both structure and biosynthesis. For example, the O-linked S-layer glycan of *G. steaerothermophilus* NRS 2004/3a contains an adaptor and a repeat unit domain. The glycan repeat unit is composed of the trisaccharide $\rightarrow 2$ - α -L-Rhap-(1 \rightarrow 3)- β -L-Rhap-(1 \rightarrow 2)- α -L-Rhap-(1 and is terminated with a methyl group (62). Biosynthesis requires a multidomain glycosyltransferase that also contains the chain-terminating methyltransferase (63).

Despite these examples, it is not possible to make a reliable prediction of the synthesis strategy and the organization of glycosyltransferase modules based on the glycan structure alone. For example, the A-band O-PS of *Pseudomonas aeruginosa* possesses a trisaccharide, $\rightarrow 2$ - α -D-Rhap-(1 \rightarrow 3)- α -D-Rhap-(1 \rightarrow 3)- α -D-Rhap-(1 (64), reminiscent of both the O99 antigen and the *G. steaerothermophilus* NRS 2004/3a glycan. However, the three glycosyltransferases involved, WbpZYX (65), all appear to be single-domain enzymes based on predictions using their primary sequence. In the biosynthesis of the *E. coli* K4 capsular polysaccharide, a two-domain enzyme polymerizes a chondroitin backbone composed of a disaccharide repeat unit, $\rightarrow 4$ -D-GlcpA- β -(1 \rightarrow 3)- β -D-GalpNac-(1 (66, 67). In contrast, in *E. coli* K5, the modified heparosan backbone, $\rightarrow 4$ - α -D-GlcpA-(1 \rightarrow 4)- β -D-GlcpNac-(1, requires the concerted activity of two glycosyltransferases, KfiC and KfiA (68–70). KfiC and KfiA are both single-domain glycosyltransferases, and they must associate with one another to polymerize the K5 capsule (70).

In summary, the *E. coli* O9a and O8 polymannose O-PSs represent important model systems for the ABC transporter-dependent assembly of O-PS. They provide examples where a combination of two enzymes containing a single mannosyltransferase domain, one of which is capable of adding two Manp residues in succession, and multidomain-polymerizing mannosyltransferases is exploited to build a single glycan. Although these features have been seen individually in other glycan biosynthesis systems, we are unaware of any others where all appear in the same dedicated assembly pathway.

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