Substrate recognition by a carbohydrate-binding module in the prototypical ABC transporter for lipopolysaccharide O-antigen from *Escherichia coli* O9a

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*Escherichia coli* serotype O9a provides a model for export of lipopolysaccharide (LPS) O-antigen polysaccharide (O-PS) via ABC transporters. In O9a biosynthesis, a chain-terminator enzyme, WbdD, caps the nonreducing end of the glycan with a methylphosphate moiety and thereby establishes chain-length distribution. A carbohydrate-binding module (CBM) in the ABC transporter recognizes terminated glycans, ensuring that only mature O-PS is exported and incorporated into LPS. Here, we addressed two questions arising from this model. Are both residues in the binary terminator necessary for termination and export? And is a terminal methylphosphate moiety sufficient for export of heterologous glycans? To answer the first question, we uncoupled WbdD kinase and methyltransferase activities. WbdD mutants revealed that although the kinase activity is solely responsible for chain-length regulation, both activities are essential for CBM recognition and export. Consistent with this observation, a saturation transfer difference NMR experiment revealed a direct interaction between the CBM and the terminal methyl group. To determine whether methylphosphate is the sole determinant of substrate recognition by the CBM, we exploited *Klebsiella pneumoniae* O7, whose O-PS repeat-unit structure differs from O9a, but, as shown here, offers the second confirmed example of a terminal methylphosphate serving in substrate recognition. *In vitro* and *in vivo* experiments indicated that each CBM can bind the O-PS only with the native repeat unit, revealing that methylphosphate is essential but not sufficient for substrate recognition and export. Our findings provide important new insight into the structural determinants in a prototypical quality control system for glycan assembly and export.

Lipopolysaccharide (LPS) is the major glycolipid of the Gram-negative bacterial outer membrane (1). It is almost always essential for cellular viability and is important for pathogenicity. The tripartite structure of LPS consists of the outer membrane–embedded component, lipid A (also known as endotoxin), a core oligosaccharide, and a structurally variable and immunogenic sugar polymer termed O-antigen (O-PS) (2). In human pathogens, O-PS is one of the first molecules encountered by the host, and it provides protection to the bacterium from immune processes such as the complement system (3). For resistance to serum killing, the length of the O-PS is a critical factor in promoting a successful defense, with longer O-PS providing a more effective barrier (4, 5). Loss of O-PS chain-length regulation has resulted in loss of serum resistance and susceptibility to leukocytes in some Gram-negative bacteria (5). There are many different O-PS structures with the diversity driven by selective environmental factors, such as host immune response and bacteriophages that exploit O-PSs as receptors (6).

Many O-PSs are assembled by a mechanism that requires an ATP-binding cassette (ABC) transporter to export lipid-linked O-PS intermediates from the site of synthesis in the cytosol to the periplasm (7, 8), where the LPS molecule is completed before being translocated to the cell surface by the Lpt (LPS transport) machinery (9, 10). The ABC transporter provides an important role in determining the chain-length distribution of the O-PS products, with two main mechanisms being described. In the simpler process exhibited by *Klebsiella pneumoniae* serotype O2a (11), the O-PS is polymerized to completion within the cytosol by a biosynthetic enzyme complex. The chain-length distribution is controlled by the relative activities of a complex of glycosyltransferase (GT) enzymes and the ABC transporter. The O2a ABC transporter does not possess strict O-PS specificity, and it can export polymers with diverse...
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During O-PS biosynthesis in both systems, the 55-carbon polyisoprene carrier lipid, undecaprenol phosphate (Und-P) serves as the acceptor for the shared initiating phosphoglyco-

repeat-unit structures, but polymerization and export are obligatorily coupled (11, 12). The more intricate Escherichia coli serotype O9a O-PS assembly system incorporates an additional mechanism that imposes a stricter level of control over chain length. This requires the installation of a chain-terminating residue that creates an export signal recognized by a carbo-

prokaryotic exporters, and it shares similarity with

larity ruler, the coiled-coil structure facilitates multimerization of exporter, and it shares similarity with

The structural require-

sments for glycan recognition by the CBM provide the focus of this study.

During O-PS biosynthesis in both systems, the 55-carbon polyisoprene carrier lipid, undecaprenol phosphate (Und-P) serves as the acceptor for the shared initiating phosphoglyco-

sulky transferase enzyme, named WecA (2). In serotype O9a, the resulting Und-PP-GlcNAc product is then extended by sero-

type-specific mannosyltransferases to create the O9a O-PS backbone (Fig. 1). Central to polymerization is WbdA, a dual-

domain GT polymerase that catalyzes formation of alternating pairs of α1→2- and α1→3-linkages (13, 14). WbdA operates in a distributive (rather than processive) manner, where the growing chain is released from the enzyme at each step (14, 15). The length of the O9a O-PS is dictated by WbdD. The C terminus of WbdD contains a membrane-anchoring helix and a region that recruits WbdA into an active membrane-bound heterocomplex (Fig. 1C) (14, 16). The N terminus of WbdD possesses kinase and methyltransferase catalytic sites that add a phosphomethyl moiety to the nonreducing terminus of the O-PS, blocking further extension by WbdA (17–19). The physical separa-
tion of the chain-terminating domain from the membrane by an extended coiled-coil ensures that only O-PS of a minimum length can be capped (20).

In addition to operating as a molecular ruler, the coiled-coil structure facilitates multimerization of functional WbdD trimers. The relative abundance of WbdA and WbdD also affects the distribution of O-PS lengths, and this is explained by the variable geometry model that describes the outcome of changes in the stoichiometries of the WbdA-WbdD heterocomplexes (21).

Essential to this chain-length regulation strategy is a quality control mechanism on the ABC transporter protein complex, which ensures that only terminated (and chain-regulated) O-PS is exported for assembly on the cell surface. O-PS ABC transporters are composed of homodimers of the transmem-
brane domain protein (Wzm) and the nucleotide-binding domain protein (Wzt). In Escherichia coli O9a and related assembly sys-

Figure 1. Structure and biosynthesis of the E. coli O9a O-PS. A, organization of the O-PS synthesis (wb* operon). B, structure of the Und-PP-linked O9a biosynthetic intermediate. The square brackets delineate the tetrasaccharide O-PS repeat unit. Man, D-mannose; GlcNAc, D-GlcNAc.

C, domain organization of the dual kinase methyltransferase WbdD. The coiled-coil domain acts as a molecular ruler separating WbdA, which is recruited to its interaction site at the C terminus of WbdD (indicated), from the chain-terminating catalytic sites of WbdD.
Substrate recognition by a glycan ABC transporter

Figure 2. The kinase domain of WbdD is sufficient for chain termination in vivo. Levels of WbdD-His\(_5\) (expressed from pWQ470) (A) and its mutants with inactivated methyltransferase (H132\(^{→}\)Ala; pWQ829) (B) or kinase (D351\(^{→}\)Ala; pWQ830) (C) catalytic sites were titrated in *E. coli* CWG638 manA Δwzm-wzt:aphA-3. The addition of 0.4% D-glucose was used to repress the pBAD promoter, whereas varying concentrations of L-arabinose induced expression of the WbdD variants. To overcome second site mutations occurring during prolonged growth in the absence of functional O-PS export (17), a manA mutant was used, making O9a O-PS biosynthesis conditional on the addition of mannose to the growth medium at the beginning of the experiment. Und-PP–linked O-PS intermediates (accumulating in the absence of transport) were detected in proteinase K–digested whole-cell lysates by SDS-PAGE and immunoblotting with O9a-specific antibodies (top panels). The control lane is authentic O9a LPS from the manA strain (CWG634). The bottom panels show the detection of WbdD-His\(_5\) variants by immunoblotting with anti-His\(_5\) antibodies.

Similar chain termination/export strategies exist in other species and/or serotypes. For example, O-PS chain regulation is achieved by the addition of a methyl group in *E. coli* O8 or a sugar (3-deoxy-D-manno-oct-2-ulosonic acid; Kdo) in *K. pneumoniae* O12 (25). The involvement of both phosphate and methyl residues in chain-length regulation in *E. coli* O9a appears unnecessarily complicated; in principle, a single terminating residue should suffice. Given the pivotal role the *E. coli* O9a O-PS system plays as an influential prototype for bacterial glycan export, it is essential to understand the details and limitations of substrate recognition and export. This provides the goal of the current study.

Results

**WbdD kinase activity is solely responsible for arresting O9a O-PS polymerization**

The modal distribution of O9a-PS chain lengths is established by the coiled-coil domain, which physically separates the WbdD termination enzymes from membrane-anchored WbdA (20). Previous work established that WbdD halts WbdA-mediated O-PS extension by installation of a phosphomethyl moiety to the nonreducing terminus, where new backbone residues are added (17, 20, 21). Logically, the addition of a single terminating residue to the site of O-PS extension should be sufficient for regulation of chain-length distribution. To confirm this hypothesis and rule out any unanticipated requirements for a functional methyltransferase, we exploited the observation that overexpression of a functional WbdD terminator decreases the average chain length of O-PS (21). Mathematical modeling supports the hypothesis that O-PS chain length responds to altered stoichiometry within the WbdA::WbdD heterocomplex, with the resulting changes in geometry of the complex altering access of the glycan to termination catalytic sites (21). The O-PS phenotype is readily assessed by SDS-PAGE profiles of Und-PP–linked O9a intermediates in whole-cell lysates. These intermediates are not visible in silver-stained SDS-PAGE but are readily visualized in immunoblots using O-PS–specific antibodies (11, 12, 26).

These experiments were performed in a manA Δwzm-wzt:aphA-3 background, where O-PS production is conditionally dependent on the addition of D-mannose to the growth medium (17). This is necessary to avoid any unexpected effects on viability of the transformants, known to occur when O9a export is eliminated (22). After SDS-PAGE of whole-cell lysates, O9a intermediates were detected using O9a-specific antibodies (Fig. 2). Prior *in vitro* experiments verified that WbdD\(^{H132A}\) and WbdD\(^{D351A}\) lack methyltransferase and kinase activities, respectively (19). Each variant was expressed from a pBAD promoter by titrating the L-arabinose inducer, and the expected increase in protein production was confirmed by exploiting the His\(_5\) tag for Western immunoblotting. As shown previously (21), increasing levels of WT WbdD resulted in an inducer-dependent reduction in the average O-PS intermediate chain-length (Fig. 2A). The profiles of O9a intermediates in cells overexpressing the methyltransferase mutant (WbdD\(^{H132A}\)) were qualitatively similar to those with WT WbdD (Fig. 2B). In contrast, the kinase mutant (WbdD\(^{D351A}\)) had no effect on the chain-length distribution of O-PS intermediates (Fig. 2C). These results show that the addition of the activity of the kinase is sufficient to determine O-PS chain length *in vivo* and that the kinase and methyltransferase activities can be uncoupled.

**The terminating methyl group is essential for export by Wzm-Wzt**

To evaluate whether O9a O-PS lacking normal nonreducing terminal modification could be transported across the inner
membrane, an *E. coli* O9a *manA ΔwbdD* mutant was transformed with plasmids expressing the WbdD mutant variants. Export activity was monitored by silver-stained LPS profiles in whole-cell lysates separated by SDS-PAGE (Fig. 3A). Whereas plasmids expressing WT WbdD restored the production of O-PS–containing LPS visible in silver-stained gels, only O-PS–deficient LPS was evident in cells expressing either WbdD<sup>H132A</sup> or WbdD<sup>D351A</sup>. However, as expected, small amounts of Und-P–linked O-PS were still detected by Western immunoblotting with polyclonal primary antibodies directed against O9a (note that immunoblotting detects both Und-PP–linked and lipid A–linked O-PS). Due to the high signal from complete LPS compared with Und-PP–linked intermediates in equivalent amounts of cells, a longer exposure of the lanes containing the Und-PP–linked intermediates is shown on the right. The middle panel is an immunoblot using polyclonal primary antibodies directed against O9a (note that immunoblotting detects both Und-PP–linked and lipid A–linked O-PS). To determine whether the requirement for export either the nonmethylated O-PS or the nonterminated (nonphosphorylated and nonmethylated) O-PS.

The carbohydrate-binding module on Wzt is unable to recognize nonmethylated O9a O-PS

Previous work demonstrated that the C-terminal domain of Wzt functions as a CBM (23). In that study, an *in vitro* pulldown approach revealed the CBM binds its cognate O-PS, but not O-PS lacking the terminal methylphosphate or O-PS from *E. coli* O8 possessing a polymannose structure (with a repeat-unit structure different from that of O9a) capped with a terminal methyl group. To determine whether the requirement for O-PS methylation for export is dictated by the CBM-binding step, purified LPSs lacking either terminal phosphomethyl or methyl modifications were tested using the same pulldown approach. LPS molecules with chemically distinct nonreducing termini were generated using a strategy exploited previously to generate nonterminated *K. pneumoniae* O12 LPS (12). This
Substrate recognition by a glycan ABC transporter

Figure 4. The Wzt CBM does not recognize nonmethylated LPS. Pulldown experiments were performed using purified E. coli O9a CBM (Wzt-C-His6) and one of three LPS species: WT O9a (A), nonmethylated O9a (B), or nonterminated O9a (C). Reaction mixtures consisting of 200 μg of CBM and 200 μg of LPS were mixed with nickel magnetic beads and collected using a magnet. The samples are unbound material in the supernatant (S) and material released by sequential washes of the beads in the absence (W1–W3) or presence of imidazole (E1–E3). Aliquots of the collected samples were subjected to SDS-PAGE. LPS was detected by silver staining (top panels) and protein was detected by simply blue staining (bottom panels).

method takes advantage of the ability of the K. pneumoniae O2a ABC transporter to export heterologous O-PS intermediates. This transporter lacks a CBM and has no specificity for either the repeat-unit structure or terminal modifications of O-PS substrates (11, 27). It can therefore export LPS molecules with varying terminal modifications. LPS species were purified from E. coli O9a ΔwbdD transformed with plasmids to express the K. pneumoniae O2a transporter and appropriate WbdD variants.

The pulldown experiments follow retention (or not) of LPS by His6-tagged CBM using nickel magnetic beads (Fig. 4). In the control experiments with WT O9a LPS, a considerable amount of LPS eluted in fractions containing CBM as reported previously (23). However, when CBM was incubated with either nonmethylated or nonmethylated/nonphosphorylated LPS, no binding was evident, indicating that the methyl group is essential for recognition by the CBM, and the phosphate group alone is insufficient. The inability of the CBM to bind improperly terminated O-PS is entirely consistent with the transport defects described above. These data support the notion that the impaired transport reflects altered substrate recognition but cannot exclude additional defects in later transport stages resulting from a terminally exposed phosphate.

Direct interaction between the nonreducing terminal methylphosphate and the CBM

During the characterization of the O9a CBM, residues in a putative binding pocket were identified that abrogated O-PS binding in vitro or had adverse effects on export in vivo (23). A saturation transfer difference (STD) NMR experiment (28) was used to provide more insight into the interaction and to formally verify the direct role of the methyl group in WztO9a-C; O9a binding. Similar experiments have been performed previously to precisely define the interaction sites of antibodies with glycans by “epitope mapping” (e.g. see Refs. 29–31). The method relies on the application of precise radiofrequency pulses to selectively saturate the NMR signals of the protein; this saturation is then transferred to ligands that transiently bind the protein, attenuating the NMR signals of ligand protons at and near the binding site. To better observe small (>5%) attenuations, STD spectra are collected as the difference of an “on-resonance” spectrum (i.e. saturation pulses applied on the protein signals) and an “off-resonance” reference spectrum (i.e. saturation pulses applied far away from any NMR signals). STD spectra therefore display only signals corresponding to ligand protons that are at and near the protein-binding site. To address complications arising from saturation in the presence of larger ligands (30), a saturation transfer double difference spectrum (STDD) (32) is obtained by subtracting an STD spectrum obtained from a sample without protein from the protein-ligand STD spectra.

Serotype O9a O-PS was purified, and its structure was confirmed by comparison against the reported NMR data (13, 33) (data not shown). For STD NMR experiments, two samples were prepared. The first sample contained an approximately 100-fold excess of O9a polysaccharide mixed with WztO9a-C, whereas the second sample contained O9a polysaccharide without protein. Saturation times were tested at 1, 2, 3, and 6 s (Fig. 5A). It was determined that 3 s was sufficient for magnetization transfer, and data were collected. The most intense signal from the obtained STDD spectrum (aside from a differentiating artifact arising from the Tris buffer) was a doublet at δH 3.63 ppm with coupling constant J = 11 Hz (Fig. 5B), which precisely corresponds to the chemical shift of the methyl protons on the nonreducing terminal methylphosphate group from O9a O-PS (33). These findings offer clear and direct data for the interaction of the nonreducing terminal methyl group with WztO9a-C and are entirely consistent with its requirement for WztO9a-C-binding in LPS pulldown experiments as well as its requirement for display of the O9a polysaccharide on the cell surface.

Identification of a terminal methylphosphate moiety in the K. pneumoniae O7 O-PS

The data above reveal the importance of the methylphosphate group for substrate recognition and export but do not reveal whether the terminal modification is sufficient by itself for these processes. To address this question, we examined the ability of the O9a CBM to bind an O-PS possessing a terminal methylphosphate group but a different repeat-unit structure. This was provided by K. pneumoniae O7, whose detailed structure has not been reported previously. Examination of the K. pneumoniae O7 wb locus (Fig. S1) revealed features consis-
investigated. We used $^1$H, $^{13}$C, and $^{31}$P NMR spectroscopy, including two-dimensional $^1$H,$^1$H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), NOE spectroscopy (NOESY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and HMQC-TOCSY experiments to unequivocally determine the structure of the repeat unit and the nonreducing terminus of the O-PS. NMR spectra of O-PS contained the signals for four major spin systems, which were assigned to a Ribf residue (A) and three Rha residues (B, C, and D) (Table 1, Fig. 7A, and Figs. S2–S4). The $\beta$-anomeric configuration of Rib was confirmed by the position of the Rib C-1 signal at $\delta$ 108.2 (compare published data (36) of $\delta$ 103.1 and 108.0 for methyl glycosides of $\alpha$- and $\beta$-Ribf, respectively). The $\alpha$-configuration of all three Rha residues were inferred from the H-5 and C-5 chemical shifts at $\delta_{HH}$ 70.4–70.7 (compare published data (37) of $\delta_{HH}$ 72.8 for $\beta$-Rhap). These data, together with the sequence of the sugar residues determined by HMBC and NOESY (Fig. S2) experiments, confirmed the structure of O7 O-PS reported earlier (Fig. 7A).

In addition to the signals for sugar residues, the NMR spectra contained a characteristic sharp signal at $\delta_{HP}$/C 3.63/54.3 with $J_{HP}$ = 11 Hz, which was previously assigned to a methylphosphate group at the nonreducing terminus of E. coli O9a O-PS (33). The presence of a methylphosphate group was confirmed by correlation between methyl protons and phosphate at $\delta_{HP}$/C 3.64/1.75 observed in the $^1$H,$^{31}$P HMBC spectrum (Fig. 7D). Furthermore, a $^1$H,$^{31}$P HMQC-TOCSY experiment showed correlation between phosphate and H-1 to H-5 of a minor $\alpha$-Rhap spin system denoted as B'. The selective one-dimen-
sional TOCSY (Fig. 7B) and NOESY experiments (not shown) confirmed the assignment of B' H-1 to H-6 signals. A relatively low-field position of the signal for B' C-3, together with strong HMBC correlation between phosphate and B' H-3, demonstrate that the methylphosphate group is attached at position O3 of B'. The linkage between B' and the next sugar residue could not be determined unambiguously, although the HMBC correlation at $\delta_{HP}$/C 5.09/79.3 indicates that B' is linked to another Rha residue. Thus, the NMR spectroscopic analysis confirmed the structure of the O7 repeat unit reported earlier (35) and established the second known example of a methylphosphate O-PS terminal modification.

### The methylphosphate group is necessary but not sufficient for CBM binding and transport

To examine whether the nonreducing terminal modification is the sole contributor to WztO9a-C O-PS recognition, we performed a pulldown experiment using the E. coli O9a CBM with K. pneumoniae O7 LPS (Fig. 8, A and B). No O7 LPS was detected in elution fractions, indicating that the CBM did not bind O7 LPS. To validate these results, the reciprocal experiments were done using purified WztO7-C. Evident from the silver stain, the O7 CBM was only able to bind its cognate O7 LPS (Fig. 8, C and D). Finally, in vivo mutant complementation experiments were performed in E. coli O9a mutants lacking a functional O-PS ABC transporter (manA $\Delta$wzm-wzt::aphA-3) (Fig. 9). Expression of the K. pneumoniae O7 ABC
transporter in this mutant could not restore translocation of the O-PS across the inner membrane, as evidenced by the absence of banding on silver-stained SDS-polyacrylamide gels, consistent with results from in vitro pulldown experiments. Because the O7 and O9a LPS structures contain the same nonreducing terminal modification, the lack of O-PS binding by WztO9a-C (23). A, cartoon representation overlay of WztO7-C model (orange) with WztO9a-C crystal structure (blue). The structural overlay was generated using PyMOL (Schrodinger, LLC).

### Table 1

| Sugar residue | H-1 C-1 | H-2 C-2 | H-3 C-3 | H-4 C-4 | H-5 C-5 | H-6 C-6 |
|---------------|--------|--------|--------|--------|--------|--------|
| **Repeat unit** |        |        |        |        |        |        |
| a-D-ribose (1→A) | 3.57 | 4.22 | 4.44 | 4.07 | 3.87 | 3.71 |
| a-D-rhamnose (1→B) | 5.06 | 4.24 | 3.91 | 3.53 | 3.90 | 1.31 |
| a-D-rhamnose (1→C) | 4.98 | 4.18 | 3.85 | 3.57 | 3.80 | 1.29 |
| a-D-rhamnose (1→D) | 5.15 | 4.10 | 3.93 | 3.53 | 3.76 | 1.32 |
| **Non-reducing end** |        |        |        |        |        |        |
| a-D-rhamnose (1→B') | 5.09 | 4.27 | 4.29 | 3.60 | 3.90 | 1.31 |

* The signals for the methylphosphate group are at δH 3.63, δC 54.3, and δC 1.75.

Comparable systems have been identified in *E. coli* O8 and *K. pneumoniae* O12 and in S-layer glycoprotein assembly in *Geobacillus stearothermophilus* NRS2004/3a, *Geobacillus tepidamans* GSS5-97T, and *Anapirinibacillus thermoanophilus* L420–911T (27). In most examples, a single terminal residue (and presumed termination-export signal) is used. Examples include an O-methyl group on *E. coli* O8 O-PS and on the S-layer glycans from *G. stearothermophilus* NRS2004/3a (38) and *A. thermaeus* L420–911T (39), as well as a Kdo on *K. pneumoniae* O12 (25). An exception is the addition of GlcNAc and N-acetylmuramic acid residues onto separate positions of the final rhamnose in *G. tepidamans* GSS5-97T (40), but only one of these residues is positioned to directly impede chain extension. The overall strategy for the biosynthesis of the terminally methylated o-rhamnose polymer in *G. stearothermophilus* NRS2004/3a is remarkably similar to that used for the methylated o-mannose *E. coli* O8 antigen (41). In contrast, *E. coli* O9a utilizes a binary terminator, consisting of a phosphate and an O-methyl group (18, 25). An identical structure terminates the related mannans produced by *E. coli* O9, *K. pneumoniae* O3a/O3b/O3c (42), and *Hafnia alvei* PCM1223 (33). This reflects lateral gene transfer of the locus, followed by diversification of the repeat-unit structure by mutations in *wbdA*. *K. pneumoniae* O7 uses the same methylphosphate terminating moiety to cap a different repeat-unit structure.

### Discussion

The *E. coli* O9a O-PS biosynthesis and export system represents an elegant model for regulating polymer chain length distribution. By combining a molecular ruler with polymerization-termination and exporter-recognition mechanisms, the cell ensures an appropriate O-PS length distribution to optimize survival within the host. This system has become an important and influential prototype for similar strategies involved in the assembly of a variety of O-PS and other cell-surface glycans from Gram-negative and Gram-positive bacteria. The experimental platform for investigating substrate engagement by the *E. coli* O9a transporter therefore has broad relevance in microbial glycobiology.
study and is not required for data interpretation, it was not pursued here.

We demonstrated that the WbdD kinase domain was sufficient for chain length determination. However, the quality control mechanism conferred by the CBM shows an additional dependence on methyltransferase activity. STDD NMR experiments demonstrated that WztO9a-C interacts directly with the methyl substituent at the nonreducing terminus, and this likely provides the most prominent hydrophobic interaction involved in O9a polysaccharide recognition by Wzt. The O9a CBM crystal structure has been solved, and the binding pocket was characterized using site-directed mutagenesis in conjugation with transport and CBM binding experiments (23). Several residues were necessary for substrate binding. Aromatic residues (Trp-286 and Tyr-404) presumably contribute via \( \pi \)-stacking with the monosaccharide rings, whereas polar residues (Asn-348, Arg-402, and Asp-405) may interact with sugar hydroxyl moieties or the terminal unit. Because these are conserved between WztO9a and WztO7, additional side chains distinct to each homologue must provide the noncovalent interactions critical for serotype specificity. However, these are not sufficient for robust productive binding in the absence of any terminal modification, as seen here and in previous experiments using in vitro-synthesized O9a glycan with or without complete terminal modification (23). STDD NMR experiments implicated the terminal methyl group as an interaction group with the CBM. Unfortunately, these experiments could not identify additional hydrophobic interactions arising from the main polymer, due to inefficient magnetization transfer. Furthermore, STD approaches are not suitable for identification of electrostatic or polar interactions. Interactions contributed by the main-chain polymer may be predominantly polar, arising from hydroxyl groups, and these are not detected because of the fast exchange of hydroxyl protons with solvent. It is likely that the phosphate moiety on the O9a glycan provides contact points within the binding pocket (e.g. through electrostatic interaction with the critical Arg-402 residue) (23) and therefore still contributes to recognition of the fully modified glycan. The extensive binding pocket likely accommodates main-chain sugars that contribute to the binding interface.

The O9a and O7 O-PS structures contain identical termination groups on the C-3 position of the nonreducing terminal rhamnose residue. A, the determined repeat unit and nonreducing terminal structure of the O7 O-PS. Rha, L-rhamnose; Rib, D-ribose. B, part of the one-dimensional selective TOCSY spectrum obtained after irradiation of \( \delta \) H-1 at 5.09. \( ^1H,^{31}P \) HMQC-TOCSY (C) and \( ^1H,^{31}P \) HMBC (D) spectra demonstrate correlations between phosphate and methyl protons as well as protons within the \( \delta \) spin system. MeP, methylphosphate. The corresponding part of the \( ^1H \) NMR spectrum is shown along the horizontal axis. 

**Figure 7.** *K. pneumoniae* O7 O-PS is terminated with a methylphosphate group on C3 of the nonreducing terminal rhamnose residue. A, the determined repeat unit and nonreducing terminal structure of the O7 O-PS. Rha, L-rhamnose; Rib, D-ribose. B, part of the one-dimensional selective TOCSY spectrum obtained after irradiation of \( \delta \) H-1 at 5.09. \( ^1H,^{31}P \) HMQC-TOCSY (C) and \( ^1H,^{31}P \) HMBC (D) spectra demonstrate correlations between phosphate and methyl protons as well as protons within the \( \delta \) spin system. MeP, methylphosphate. The corresponding part of the \( ^1H \) NMR spectrum is shown along the horizontal axis.
resolved. The ultimate goal of this and related studies is to recapitulate the export process with purified proteins and substrates in proteoliposomes, and, in this context, resolution of the chemical determinants for export is a critical step toward this objective. However, why \textit{E. coli} O9a has evolved and maintained a more complex strategy, compared with the simple methylation process in the related \textit{E. coli} O8, remains unanswered.

**Experimental procedures**

**Strains, plasmids, and growth conditions**

The bacterial strains and plasmids used in this study are described in Table 2. Cultures were grown in lysogeny broth (LB) at 37 °C with aeration and were supplemented with 100 µg ml⁻¹ of ampicillin, D-glucose (0.4% w/v), D-mannose (0.4% w/v), or L-arabinose (0.0002–0.2% (w/v)) where appropriate, unless otherwise specified.

**DNA methods**

KOD Hot Start DNA polymerase (Novagen) was used for cloning DNA fragments. Oligonucleotide primers were obtained from Sigma and are listed in Table S1. Amplified products were purified using a GeneJET purification kit (Thermo Fisher Scientific), and restriction endonuclease digests were performed using enzymes from New England Biolabs. Restriction sites used are indicated in Table 1. Plasmids were confirmed by restriction digests and by DNA sequencing performed by the Advanced Analysis Centre, University of Guelph.

**Construction of \textit{E. coli} CWG1412**

\textit{E. coli} CWG1412 (\textit{manA wbdD::aacC1 Δwzm-wzt::aphA-3}) was constructed using the same primers and protocol as described previously for construction of CWG638 (17). However, CWG635 was used as the parent strain instead of CWG634.

**Genomic DNA sequencing**

Whole-genome shotgun sequence data for \textit{K. pneumoniae} 264(1) was generated using the Solexa paired-end sequencing platform (Illumina) and genomic DNA prepared using the Illumina sample preparation kit. \textit{De novo} assembly of sequencing reads (~100× coverage) was performed with Velvet (45). The
immunoblotting, bacterial cells (10 μg) were fractionated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with primary antibodies and subsequently reacted with a horseradish peroxidase-conjugated secondary antibody (Cedar Lane Laboratories). Bands were detected using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific). Controls were performed using preimmune sera. O7 LPS was purified from overnight cultures of E. coli CWG28 (O9a O-PS control) and E. coli CWG1412 transformed with pWQ391, which lacks innate terminating O-PS modification. L-Asparagine was added to the culture medium to facilitate WbdD synthesis, and growth was continued for an additional 1 h before cells were harvested. Microscopy was performed on formaldehyde-fixed whole cells as described previously (17), using rabbit-anti-O9a primary antibody and FITC-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific).

SDS-PAGE and immunoblotting

To prepare whole-cell lysates for SDS-PAGE and Western immunoblotting, bacterial cells (1 A600 nm unit equivalent) were collected by centrifugation, solubilized in SDS-PAGE loading buffer, and heated to 100 °C for 10 min prior to electrophoresis. For LPS analysis, the whole-cell lysates were treated with proteinase K (Invitrogen) for 1 h at 55 °C (46). SDS-PAGE was performed using 12% acrylamide resolving gels in Tris-glycine buffer (47). LPS was visualized by staining with a silver nitrate solution (48), and protein was detected using SimplyBlue SafeStain (Invitrogen).

For immunoblot analyses, material resolved by SDS-PAGE was transferred to nitrocellulose membranes (Protran; PerkinElmer Life Sciences). O9a antigen was detected using polyclonal antibodies raised in rabbits (17). Alkaline phosphatase–conjugated goat anti-rabbit was used as a secondary antibody (Cedar Lane Laboratories). Hexahistidine-tagged proteins were detected using mouse-anti-His5 mAb (Qiagen) and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Cedar Lane Laboratories). Immunoblots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (Roche Applied Science).

Immunofluorescence microscopy

Overnight cultures grown in LB, 0.4% d-glucose were subcultured 1:100 into fresh LB, glucose. After 4 h, cells were resuspended in fresh medium containing 0.4% d-mannose and 0.02% L-arabinose to facilitate WbdD synthesis, and growth was continued for an additional 1 h before cells were harvested. Microscopy was performed on formaldehyde-fixed whole cells as described previously (17), using rabbit-anti-O9a primary antibody and FITC-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific).

Wzt-C purification

WztO9a-C was purified as described previously (23). Briefly, E. coli Top10 harboring pWQ424 was grown at 37 °C with aeration to mid-exponential growth phase and induced with 0.3% L-arabinose for an additional 2 h. Cells were collected by centrifugation, resuspended in buffer A (25 mM Tris, pH 7.5, containing 100 mM NaCl), and lysed by sonication. Cleared lysate was applied to 1-ml columns of nickel affinity resin. Resin was washed with buffer A containing 20 mM imidazole, and protein was eluted using buffer A containing 200 mM imidazole. WztO7-C was purified from E. coli Top10 harboring pWQ1013, using the same growth and induction conditions. WztO7-C was also purified by nickel-affinity chromatography, but buffer B (25 mM Bis-Tris, pH 7.0, 250 mM NaCl) was used.

LPS purification

WT E. coli O9a LPS was purified from overnight cultures of E. coli CWG28 and E69 for pulldown experiments and STDD NMR experiments, respectively. WT K. pneumoniae O7 LPS was purified from K. pneumoniae 264(1). LPS containing non-terminated O-PS was prepared from E. coli CWG1412 (manA wbdD::aacC1 Δwzm-wzt::aphA-3), which lacks innate termination and transport activities, transformed with pWQ391, which provides the ABC transporter from K. pneumoniae O2a. This transporter operates independently of O-PS repeat-unit structure and does not require a terminating moiety (11, 12). LPS lacking a nonreducing terminal O-PS modification was purified from E. coli CWG1412 transformed with pWQ391 and either pWQ830 (WbdDY16F) or pWQ831 (wbdDY16F). The WbdD(Y16F) variant lacks methyltransferase activity (19). L-Arabinose (0.1%) was added to induce the recombinant K. pneumoniae O2a ABC transporter. After 30 min of growth, D-mannose was added as an inducer (11, 12). LPS was purified from overnight cultures of E. coli CWG28 and E69 for pulldown experiments. WbdD(Y16F) is a result of nonexported O-PS, evident from its lack of silver staining.

Substrate recognition by a glycan ABC transporter

sequence of the gene cluster for O7 O-PS biosynthesis has been deposited in NCBI (GenBank accession no. MN173773).

E. coli O9a manA

E. coli O9a manA, Δwzm-wzt::aphA-3

silver stain

anti-O9a

anti-His5

Figure 9. The K. pneumoniae O7 ABC transporter is unable to export the E. coli O9a O-PS. Overnight cultures of E. coli manA (CWG634; O9a O-PS control) and E. coli manA Δwzm-wzt::aph3-A (CWG638) transformed with plasmids encoding either the O9a (pWQ332) or O7 (pWQ1014) transporter (Wzm-Wzt-His6) were subcultured for 3 h in medium supplemented with 0.1% D-mannose and 0.1% L-arabinose to induce O-PS biosynthesis and transporter expression, respectively. LPS profiles were examined in proteinase K–treated whole-cell lysates using silver-stained SDS-polyacrylamide gels (top). α-His5 Western blots of whole cell lysates were used to confirm transporter expression (bottom). O-PS present in lanes 2 and 4 of the Western blot is a result of nonexported O-PS, evident from its lack of silver staining.
ride mixture was applied to a DEAE Fast Flow (GE Healthcare) column, LPS was sedimented by ultracentrifugation for 18 h at 100,000 × g and 20 mM NaOAc, pH 7.0.

O-PS for NMR analysis was isolated by acid hydrolysis of LPS at 100 °C in 2% (v/v) acetic acid, followed by centrifugation at 13,000 × g to remove lipid precipitate. The carbohydrate-containing supernatant was fractionated on a Sephadex G-50 superfine column (2.5 × 75 cm) in 50 mM pyridinium acetate buffer (pH 4.5) at a flow rate of 0.6 ml min⁻¹. Elution was monitored with a Smartline 2300 refractive index detector (Knauer). The O9a O-PS–containing fraction was acidic (potentially due to contamination of capsule; data not shown), so the polysaccharide mixture was applied to a DEAE Fast Flow (GE Healthcare) column and eluted with a 5–500 mM sodium phosphate gradient at pH 6.3 monitored using absorbance spectroscopy at a wavelength of 208 nm (50). The O9a polysaccharide–containing fractions were pooled and buffer-exchanged by chromatography on a Sephadex G-50 superfine column equilibrated with water.

**Binding assays**

In vitro binding assays were performed as described previously (12, 23). 1-ml reaction volumes of buffer B (25 mM Bis-Tris, pH 7.0, 250 mM NaCl) containing 200 μg of LPS and 200 μg of Wzt-C were incubated on a rotary shaker for 30 min at room temperature. This mixture was added to 50 μl of PureProteome nickel magnetic beads (Millipore), which were pre-equilibrated with buffer B, and incubated for 30 min at room temperature on a rotary shaker. The beads were collected with a magnet and washed three times with 500 μl of buffer B. Protein was eluted stepwise from the beads using three washes with 100 μl of buffer B containing 500 mM imidazole.

## Table 2

| Strain/plasmid name | Description | Source |
|---------------------|-------------|--------|
| **Strain** | | |
| E. coli E69 | Serotype O9a K30 | F. Orskov |
| K. pneumoniae 2641(CW28) | Serotype O7 K67 | F. Orskov |
| CWG634 | E. coli E69 derivative; trp his lac rpsL cph242 Smr' | Ref. 52 |
| CWG635 | CWG634 derivative; wbdD::aacC1; Smr', Tc', Gm' | Ref. 17 |
| CWG638 | CWG634 derivative; Δwzm-wzt::ApA-3; Smr' Tc' Km' | Ref. 16 |
| CWG900 | CWG634 derivative; ΔwbdD; Smr', Tc' | Ref. 17 |
| CWG1412 | CWG635 derivative; Δwzm-wzt::ApA-3; Smr' Tc' Km', Gm' | This study |
| **Plasmid** | | |
| pBad24 | Plasmid vector with L-arabinose-inducible promoter; Ap' | Ref. 53 |
| pKD3 | Source of chloramphenicol resistance cassette; Cm' | Ref. 54 |
| pWQ470 | pBAD2 derivative containing His6-wbdD | Ref. 16 |
| pWQ829 | pWQ470 derivative containing His6-wbdD(D112A) | Ref. 19 |
| pWQ830 | pWQ470 derivative containing His6-wbdD(D192A) | Ref. 19 |
| pWQ831 | pWQ470 derivative containing His6-wbdD(Y348A) | Ref. 19 |
| pWQ391 | pBAD2 derivative containing a fragment including wzm-wzt from K. pneumoniae | Ref. 11 |
| pWQ1013 | O2a and the Cm' cassette from pKD3 cloned into the Scal site; Cm' | This study |
| pWQ1014 | pBAD2 derivative containing the open-reading frame encoding His6-Wzt-C (amino acids 288–464) from K. pneumoniae O7 cloned between the NheI and Ncol sites | This study |
| pWQ332 | pBAD2 derivative containing Wzt-O9a | Ref. 22 |
| pWQ424 | pBAD2 derivative containing E. coli O9a Wzm-Wzt-His6 | Ref. 23 |

**STDD NMR**

NMR studies were performed at the University of Guelph Advanced Analysis Centre. The chemical shifts are referenced to 3-trimethylsilylpropanoate-2,2,3,3-d₄ (δ₄s 0, δC = -1.6) added as an internal standard. The Bruker TopSpin 3.2 program was used to acquire and process the NMR data.

STD NMR samples contained 22.2 mg/ml (estimated 2.2 mM) O9a polysaccharide and 0.022 mM WztO9a-C (omitted for the “double difference” sample) in 25 mM Tris, pH 7.4, 100 mM NaCl. Deuterium oxide was added to 10% (v/v). NMR spectra were collected on a Bruker AVANCE III 600-MHz spectrometer equipped with a 5-mm TCI cryoprobe. The sample temperature was regulated at 298.2 ± 1 K. STD spectra (28) were acquired using the Bruker pulse sequence stddiffseq, which suppresses the water resonance via an excitation sculpting scheme (51). WztO9a-C was saturated with a train of Gaussian-shaped pulses of 25 ms each (calibrated to the correct power level for a 90° Gaussian pulse), applied at 0.67 ppm for the “on-resonance” spectrum and at −11.66 ppm for the “off-resonance” spectrum. Four different irradiation times of 1, 2, 3, and 6 s were employed, and the total prescan delay (i.e. including the irradiation time) was kept constant at 7 s. The acquisition time was 1.7 s. STD data were obtained by subtracting the on-resonance FID from the off-resonance FID using the stdsplit macro provided by the Bruker software program TopSpin.

The frequency-selective pulse employed in the excitation sculpting scheme is not infinitely narrow and can cause undesired attenuation of nearby ligand resonances (e.g. anomeric protons). In the default implementation, the frequency-selective pulse duration is 2 ms, which resulted in the undesired suppression of the anomeric proton resonance at 5.04 ppm to 6% of its nominal intensity. For the experiments presented in this paper, the frequency-selective pulse duration was increased to 5 ms; the reduction in the corresponding pulse bandwidth restored the intensity of the 5.04-ppm anomeric proton to 80% of the nominal value.
When performed on larger ligands, the STD experiment can result in partial saturation of the ligand. To correct for this, a set of "ligand-only" STD experiments were performed on a second sample not containing WztO9a-C (but otherwise identical to the sample described above), and the resulting “ligand-only” STD data were subtracted from the STD data of O9a in the presence of WztO9a-C to produce the STDD spectra reported (32).

**Elucidation of the *K. pneumoniae* O7 O-PS structure**

O-PS samples were lyophilized twice in 99.9% D2O for deuterium exchange and then analyzed in 99.9% D2O at 30 °C. Data were collected using a Bruker Avance III 600-MHz spectrometer equipped with a 5-mm TCI cryoprobe, with the exception of 31P-based NMR experiments, performed on a Bruker 400-MHz Avance III spectrometer equipped with a 5-mm broadband Prodigy cryoprobe. Mixing times of 100 and 200 ms were used in TOCSY and NOESY experiments, respectively. The HMBC experiment was optimized for the J_H,C coupling constant of 8 Hz and a J_H,LP coupling constant of 11 Hz. Mixing times of 100 and 80 ms were used for selective TOCSY and 1H,31P HMQC-TOCSY, respectively.

**Author contributions**—E. M., S. D. K., and C. W. conceived the study. E. M., S. D. K., and S. A. performed the experiments. B. L. provided genome sequence data. E. M., S. D. K., S. A., B. R. C., O. G. O., and C. W. analyzed and interpreted the data. E. M., S. D. K., S. A., O. G. O., and C. W. prepared the initial manuscript draft, and all authors provided input for the final version.

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