Klebsiella pneumoniae O1 and O2ac antigens provide prototypes for an unusual strategy for polysaccharide antigen diversification

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A limited range of different structures is observed in O-antigenic polysaccharides (OPSs) from Klebsiella pneumoniae lipopolysaccharides. Among these, several are based on modifications of a conserved core element of serotype O2a OPS, which has a disaccharide repeat structure \([-\rightarrow 3]\alpha\beta-D-Galp-(1\rightarrow 3)\beta\beta-D-Galf-(1\rightarrow)\]. Here, we describe the enzymatic pathways for a highly unusual modification strategy involving the attachment of a second glycan repeat-unit structure to the nonreducing terminus of O2a. This occurs by the addition of the O1 \([\rightarrow 3]\alpha\beta-D-Galp-(1\rightarrow 3)\beta\beta-D-Galp-(1\rightarrow)\) or O2c \([\rightarrow 3]\beta\beta-D-GlcpNac-(1\rightarrow 5)\beta\beta-D-Galp-(1\rightarrow)\] antigens. The organization of the enzyme activities performing these modifications differs, with the enzyme WbbY possessing two glycosyltransferase catalytic sites solely responsible for O1 antigen polymerization and forming a complex with the O2a glycosyltransferase WbbM. In contrast, O2c polymerization requires glycosyltransferases WbbM and WbbW, which interact with one another but apparently not with WbbM. Using defined synthetic acceptors and site-directed mutants to assign the activities of the WbbY catalytic sites, we found that the C-terminal WbbY domain is a UDP-Galp–dependent GT-A galactosyltransferase adding \(\beta\beta-(1\rightarrow3)\)-linked \(\beta\beta-D-Galp\), whereas the WbbY N terminus includes a GT-B enzyme adding \(\alpha\alpha-(1\rightarrow3)\)-linked \(\alpha\alpha-D-Galp\). These activities build the O1 antigen on a terminal \(\alpha\alpha-D-Galp\) in the O2a domain. Using similar approaches, we identified WbbV as the UDP-GlcNAc transferase and noted that WbbV represents a UDP-Galp–dependent enzyme and that both are GT-A members. WbbVW polymerizes the O2c antigen on a terminal Galp. Our results provide mechanistic and conceptual insights into an important strategy for polysaccharide antigen diversification in bacteria.

Antiobiotic-resistant bacterial pathogens are a leading concern in modern healthcare, leading the World Health Organization and Centers for Disease Control and Prevention to create lists of priority pathogens (1, 2). Klebsiella pneumoniae is a high-profile target due to the global dissemination of isolates producing carbapenemases and extended spectrum \(\beta\)-lactamases (3). This organism is a Gram-negative nosocomial pathogen, causing urinary tract infections, bacteremia, and sepsis (4). Immunosuppressed individuals are particularly at risk, and rapid-acting and highly-specific therapeutics are required for post-infection treatments.

K. pneumoniae isolates are typically enveloped in a layer of capsular polysaccharide, with varied structures defining more than 80 serotypes (5). The polysaccharide \((O)\) antigens attached to the lipid A–core oligosaccharide component of lipopolysaccharides are less diverse. Two studies proposed nine O-antigen serotypes in K. pneumoniae with slight differences in the included serotypes (6, 7), and recent structural (8, 9) and genomic data (10) suggest more diversity. However, in an investigation of 500 isolates from a diverse collection, 93% were assigned to six known serotypes (of which 83% were O1, O2, or O3) based on sequences of their O-antigen polysaccharide (OPS)5 genetic loci (10). Because of the relatively limited range of O-antigen structures in clinical isolates, they have been considered as feasible targets for immunotherapies for infection treatment and have recently been shown to be protective in animal models by passive immunization through administration of O-antigen–specific antibodies (11, 12).

In addition to their potential value in immunotherapeutic strategies, K. pneumoniae OPSs have provided important prototypes for elucidating the key concepts in bacterial polysaccharide biosynthesis and export by members of the ATP-binding cassette (ABC) transporter family (13, 14). These OPSs also offer insight into strategies used by bacteria in diversification of polysaccharide antigens. These processes create related struc-

5 The abbreviations used are: OPS, O-antigen polysaccharide; GT, glycosyltransferase; HMW, high-molecular weight; LPS, lipopolysaccharide; Und-PP, undecaprenyl diphosphate; ABC, ATP-binding cassette; EIC, extracted ion chromatogram; Ni-NTA, nickel-nitrilotriacetic acid; PDB, Protein Data Bank; BisTris, 2-[bis(2-hydroxyethyl)aminio]-2-[(hydroxymethyl)propane-1,3-diol; Galp, galactopyranose; Gal, galactofuranose; PS, polysaccharide; X-gal, 5-bromo-4-chloro indolyl galactopyranoside; Kdo, 3-deoxy-\(\alpha\)-manno-oct-2ulosonic acid; IPTG, isopropyl 1-thio-\(\beta\)-galactopyranoside.
Biosynthesis of Klebsiella O-antigens

tures that complicate serotype distinction among the clinically prevalent O1/O2 group (Fig. 1). They share a common repeat unit called O2a (sometimes referred to as D-Galactan I) (15) as part of their structures. The O2a repeat unit is composed of alternating α-(1→3)-linked galactopyranose (Galp) and β-(1→3)-linked galactofuranose (Galf) residues (16, 17). O2a antigen biosynthesis is directed by the rfb chromosomal locus (18), encoding six gene products that are necessary for the biosynthesis and transport of the O2a repeat unit (18). Wzm and Wzt make up the ABC transporter required for transport of the completed undecaprenyl diphosphate (Und-PP)-linked OPS (18). The remaining four proteins (Glf, WbbM, WbbN, and WbbO) are responsible for the biosynthesis of the O2a polysaccharide (Fig. 1) (18–21). Glf is a UDP-galactopyranose mutase that produces UDP-Galp from UDP-Galf (22), and WbbN, WbbO, and WbbM are glycosyltransferases (GTs) (19, 20, 23). After its biosynthesis, the Und-PP–linked OPS is exported to the periplasm by the ABC transporter, ligated to lipid A–core oligosaccharide, and translocated to the outer leaflet of the outer membrane (24, 25).

The O2 serotype is actually made up of a collection of four sub-serotypes that, along with O1, represent different modified versions of O2a. The O8 serotype has the same repeat-unit structure as O2a, but is nonstoichiometrically O-acetylated (26). The O2afg and O2aeh serotypes modify the O2a repeat unit by side-chain addition of (1→4)- or (1→2)-linked Galp residues, respectively (Fig. 1) (27). The side-chain modification is catalyzed by a system similar to bacteriophage-mediated OPS glucosylation in Salmonella and Shigella, and it is directed by a set of three genes, denoted gmlABC responsible for the (1→4)-linkage in O2afg and gmlABD responsible for the (1→2)-linkage in O2aeh (8, 28).

NMR spectroscopic investigations of the OPS structure revealed that the O1 antigen is covalently attached to the nonreducing terminus of O2a (16, 17) or O2afg (8) and is composed of a [→3]-α-D-Galp-(1→3)-β-D-Galp-(1→3)α-D-GlcNAc-(1→5)-β-D-Galf-(1→4) disaccharide repeat-unit structure (Fig. 1). A gene (wbbY) outside the rfb region has been reported as being responsible for production of the O1 antigen (28, 29), but the role of the WbbY gene product and the possible interplay between the O1 and O2a biosynthesis machinery is unknown. The O2c antigen is also extended from the O2a antigen and possesses a [→3]-β-D-GlcNAc-(1→5)-β-D-Galf-(1→3)α-D-Galp-(1→4) repeat unit (Fig. 1). Its production depends on an unlinked genetic locus denoted wbbmVWX (27, 28), but precise functions for these genes have not been resolved.

Although the minimum requirements for O1 and O2c production have been established, the biochemical function of WbbY and the individual roles of WbmVWX remain unclear. Here, we unequivocally establish the activities of the two GT catalytic modules of WbbY in O1 biosynthesis, and we assign the GT activities of WbmV and WbmW, which are sufficient for O2c biosynthesis. Completing the characterization of O2a modifications provides a fundamental understanding of the minimal genetic and biosynthetic requirements for clinically-important glycans. It also offers the first enzymatic insight into a novel strategy for diversification of polysaccharide antigens.

Figure 1. Repeat-unit structures of O1 and O2 serogroup antigens. O1 and O2c modifications occur on the nonreducing end of the O2a polymer. GTs responsible for biosynthesis of the O1, O2a, and O2c structure are identified above the relevant linkage; GTs for O2a biosynthesis have been described elsewhere (see text for details), and the O1 and O2c enzymes are reported in this study. Sugars contained in the repeat units are identified in the box.

Results

In vivo O1 antigen assembly requires WbbY and the full complement of O2a biosynthesis machinery

All available structural data are consistent with the O1 glycan being linked to the nonreducing end of the underlying OPS (e.g. O2a or O2afg) (Fig. 1) (8, 17), rather than being directly linked to lipid A–core oligosaccharide. Biosynthesis of the O1 antigen should therefore require the activities of each of the O2a assembly and export components in addition to WbbY. However, the shared linkages in the O2a and O1 antigens opened the possibility that WbbY makes one of the O2a GTs expendable. This was examined using a genetic approach in a recombinant Escherichia coli CWG286 host, which has its own OPS biosynthesis locus deleted, but it can assemble and export the O2a antigen from plasmid-encoded genes (22, 23, 28). The LPS products were examined in silver-stained SDS-PAGE and the corre-
sponding immunoblots using polyclonal anti-O2a and monoclonal anti-O1 antibodies (Fig. 2).

Plasmids containing individual mutations in each of the O2a biosynthesis genes were introduced together with a plasmid expressing wbbY into E. coli CWG286. When wbbY and a functional rfb2a gene cluster were introduced, E. coli GWG286 produced HMW OPS that was reactive only with a mAb specific for O1 PS (Fig. 2, A–C). In the absence of transport (∆wzm-wzt), Und-PP–OPS accumulates, and without a mechanism to transport it, it is readily modified to O1 in the presence of WbbY. Such intermediates are rarely detected by silver staining. rfb2a mutant plasmids are as follows: pWQ517 (∆wbbM); pWQ548 (wbbN*; frameshift mutation); pWQ516 (∆wbbO); pWQ289 (∆wzm wzt); and pWQ633 (∆glf).

**Figure 2.** Enzymes for O2a antigen biosynthesis are all required for production of the O1 antigen. E. coli CWG286 was transformed with plasmids containing the full O2a biosynthesis repertoire (rfb2a) or with individual deletion mutants of each gene in the locus, together with wbbY (pWQ991) for O1 biosynthesis. LPS profiles in whole-cell lysates were visualized using silver stain (A), and the O-antigens were detected by immunoblotting with anti-O2a (B) or anti-O1 (C) antibodies. In the absence of the ABC transporter (∆wzm-wzt), Und-PP–linked O-PS accumulates, and without a mechanism to transport it, it is readily modified to O1 in the presence of WbbY. Such intermediates are rarely detected by silver staining. rfb2a mutant plasmids are as follows: pWQ517 (∆wbbM); pWQ548 (wbbN*; frameshift mutation); pWQ516 (∆wbbO); pWQ289 (∆wzm wzt); and pWQ633 (∆glf).

WbbY uses α-Galp-(1→3)-β-Galf as an acceptor for in vitro polymerization of the O1 antigen. SDS-PAGE shows robust synthesis of high-molecular-weight O1 polysaccharide using membrane preparations containing His6–WbbY as a source of enzyme, UDP-Galp as substrate, and 2 as the acceptor. Acceptors and products were detected by fluorescence. NMR confirmed the product to be a O1 antigen. Only a trace amount of HMW material is evident when 1 is the acceptor. Control reactions were performed with membranes prepared from cells containing the pBAD-His/A expression vector. The acceptor structures (1 and 2) are shown.

**Figure 3.** WbbY uses α-Galp-(1→3)-β-Galf as an acceptor for in vitro polymerization of the O1 antigen. SDS-PAGE shows robust synthesis of high-molecular-weight O1 polysaccharide using membrane preparations containing His6–WbbY as a source of enzyme, UDP-Galp as substrate, and 2 as the acceptor. Acceptors and products were detected by fluorescence. NMR confirmed the product to be a O1 antigen. Only a trace amount of HMW material is evident when 1 is the acceptor. Control reactions were performed with membranes prepared from cells containing the pBAD-His/A expression vector. The acceptor structures (1 and 2) are shown.
Biosynthesis of *Klebsiella* O-antigens

| Sugar residue | H-1, C-1 | H-2, C-2 | H-3, C-3 | H-4, C-4 | H-5, C-5 | H-6 (6a,b), C-6 |
|---------------|---------|---------|---------|---------|---------|---------------|
| O1            |         |         |         |         |         |               |
| (3)-α-D-Galp-(1→3) | A 5.01, 96.7 | 4.07, 68.7 | 4.16, 80.4 | 4.29, 70.5 | 4.24, 71.9 | 3.75, 62.3 |
| (3)-β-D-Galp-(1→3) | B 4.71, 105.5 | 3.78, 71.0 | 3.80, 78.6 | 4.20, 66.2 | 3.70, 76.1 | 3.78, 82.6 |
| (3)-α-D-Gal-(1→3) | P 5.07, 100.9 | 4.00, 68.6 | 4.00, 80.5 | 4.25,   |         |               |
| (3)-β-D-Gal-(1→3) | F 4.97, 108.9 | 4.22, 80.5 | 4.04, 86.2 | 4.14, 83.4 | 3.86, 72.3 | 3.67, 64.2 |
| O2c           |         |         |         |         |         |               |
| (3)-β-D-Galp-(1→3) | A 5.00, 109.5 | 4.00, 82.3 | 4.26, 77.1 | 4.14, 82.5 | 3.98, 79.0 | 3.70, 62.5 |
| (3)-β-D-GlcNAc-(1→3) | B 4.72, 101.9 | 3.82, 56.4 | 3.63, 82.5 | 3.49, 69.8 | 3.48, 76.7 | 3.77, 93.2 |

5H,13C HSQC (O1, O2c) and HMBC (O1) spectra are shown in Figs. 4, 5, and S4. This arrangement implied that WbbY was bifunctional, consistent with its capacity to polymerize a glycan containing alternating α-(1→3)-Galp and β-(1→3)-Galp residues (Figs. 1 and 3). The Phyre2 server (32) and PDBeFold (http://www.ebi.ac.uk/msd-srv/ssm) were used to model the WbbY amino acid sequence on proteins with solved crystal structures (33). No single protein comprising homologs of both GT domains was identified by this method. However, structural homologs of the individual domains were found as follows: *Halothermothrix orenii* sucrose phosphate synthase (GT-B, PDB code 2R60) (34) and SpsA from *Bacillus subtilis* (GT-A, PDB code 1H7L) (35). These models were used to identify mutagenesis targets in WbbY to probe the activities of each domain.

The glycyltransferase domain of WbbY contains an EXE motif, which is conserved among many glycosyltransferases in this superfamily. The first glutamate in the motif (corresponding to Glu-318 in WbbY) contains a D metal-binding site (D502DD) and is structurally identical to authen- tic O1 PS from *K. pneumoniae* (16, 17). Furthermore, these results indicated that WbbY alone was sufficient for polymerization of the O1 antigen and that it is initiated on a terminal Galp residue in the O2a antigen.

### WbbY possesses dual GT domains

A query of the conserved domain database (30) revealed that WbbY contains two putative domains, each with homology to distinct GT superfamilies, GT-B and GT-A, respectively (Fig. 4A) (31). This arrangement implied that WbbY was bifunctional, consistent with its capacity to polymerize a glycan containing alternating α-(1→3)-Galp and β-(1→3)-Galp residues (Figs. 1 and 3). The Phyre2 server (32) and PDBeFold (http://www.ebi.ac.uk/msd-srv/ssm) were used to model the WbbY amino acid sequence on proteins with solved crystal structures (33). No single protein comprising homologs of both GT domains was identified by this method. However, structural homologs of the individual domains were found as follows: *Halothermothrix orenii* sucrose phosphate synthase (GT-B, PDB code 2R60) (34) and SpsA from *Bacillus subtilis* (GT-A, PDB code 1H7L) (35). These models were used to identify mutagenesis targets in WbbY to probe the activities of each domain.

The GT-B domain of WbbY contains an EXE motif, which is conserved among many glycosyltransferases in this superfamily. The first glutamate in the motif (corresponding to Glu-318 in WbbY) is involved in positioning the sugar moieties of the nucleotide-sugar donor within the active site (36, 37). GT-A members typically contain a DXD metal-binding site (D502DD in WbbY) required for stabilization of the nucleoside-diphosphate leaving group to promote sugar transfer (31). Glu-318 and Asp-504 were individually replaced with alanine residues to inactivate the GT catalytic sites in WbbY, allowing the identities of the GT modules to be established using *in vitro* reactions. Protein production was confirmed by detection of the His8-tag in the various derivatives, revealing a migration consistent with the predicted molecular mass of 88.5 kDa (Fig. 4C). After expression, *E. coli* K12 membranes containing WbbY5D04A were unable to add Galp residues to 2 (Fig. 4B). In contrast, the acceptor was modified by WbbY5E318A to yield a single major product of a size anticipated to result from the addition of a single galactose residue. This was confirmed by MS of the reaction products that showed a major [M + H]+ ion peak at *m/z* 1021.346 (Fig. 4D). WbbY5E318A produced a trace amount of material migrating slower than the major product observed in SDS-PAGE (Fig. 4B). The interband spacing between the major and minor products was consistent with an increase in two additional Galp residues. This was likely due to incomplete inactivation of the GT-B domain, which would produce a trace amount of α-Galp-β-Galp as acceptor for another Galp addition by the GT-A domain. Interestingly, this two-residue spacing is also preserved in the products of WT WbbY (Fig. 4B), suggesting the activity of the β-(1→2)-Galp GT module is higher than the α-(1→3)-Galp GT partner under these conditions. This leads to products that all have an odd number of sugar residues. Consistent with the SDS-PAGE analysis, the LC-MS spectra for WbbY5E318A showed minor peaks corresponding to 2 substituted with three ([M + H]+ at *m/z* 1345.452 and [M + 2H]+ at *m/z* 673.229) and five ([M + 2H]+ at *m/z* 835.280) hexose residues (Fig. S2). A trace amount of acceptor substituted with two hexoses was present, but no peaks corresponding to acceptor with an additional four hexoses were detected. A similar phenomenon has been observed with KpsC, a bacterial GT possessing two β-Kdo catalytic sites (38).

From these data, and the structure of the O1 repeat unit, the C-terminal GT-A domain of WbbY initiates polymerization of the O1 antigen by transfer of a β-(1→3)-linked d-Galp residue onto the terminal α-(1→3)-linked d-Galp of the O2a acceptor. The N-terminal GT-B domain subsequently adds the remaining α-(1→3)-linked d-Galp, and the coordinated activity of the two domains is sufficient for polymerization of the O1 PS.

### WbmVW is sufficient for in vivo O2c biosynthesis

Previously we reported that three genes in a genetic locus unlinked to the O2a rfb region were sufficient for O2c biosynthesis (28). To investigate the individual GT functions, constructs were made where pairs of the genes could be assessed for activity *in vivo*. Each pair was transformed, together with a plasmid expressing the O2a antigen, into *E. coli* CWG286, and the LPS products were examined by SDS-PAGE and immunoblotting (Fig. 5). Deletion of either *wbmV* or *wbmW* abolished O2c antigen production, and replacement of *wbmX* with the KanR cassette had no effect on O2c biosynthesis. This result was unexpected, as our previous work suggested all three genes were necessary. The difference is explained by a requirement for extended noncoding 5′ DNA missing from the earlier *wbmVW* construct.

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Assignment of GT activities to WbmV and WbmW

Protein localization studies (Fig. S3) revealed that WbmV and WbmW were present in the membrane fraction but also form large amounts of inclusion bodies when expressed separately. Protein purified from either the soluble or membrane fractions was inactive when tested with in vitro acceptors.
Biosynthesis of Klebsiella O-antigens

To assign individual activities to WbmV and WbmW, constructs were made to express the individual GTs. Membranes from cells expressing each GT expressed alone exhibited no activity in reactions with 1 (Fig. 6A). However, polymerization was restored using membranes prepared from cells co-transformed with the two constructs, indicating that the WbmV and WbmW constructs are both functional. This suggests that both proteins must be present to retain activity in these conditions.

To overcome this problem, a site-directed mutagenesis approach was used to eliminate the catalytic activity of either WbmV or WbmW, while retaining the proteins and preserving any required structural elements. Initial BLAST searches with WbmV and WbmW returned homologs of each protein that are predicted members of the GT-A family. As indicated above, GT-A members typically contain a critical DXD motif (31). Identification of putative DXD motifs in WbmV and WbmW was accomplished by multiple sequence alignment with known GT-A family members sharing sequence similarity with WbmV or WbmW, respectively (Fig. S7). A potential DXD motif was identified at position 100–102 in WbmV by comparison with Staphylococcus aureus TarS, wall teichoic acid GlcNAc transferase (PDB code 5TZ8); E. coli KfoC, chondroitin polymerase (PDB code 2Z86); and E. coli BcsA, cellulose synthase (Fig. S7A). In WbmW, a potential DXD motif was located at position 272–274 by comparison with E. coli WfgD, O152 antigen GT; Streptococcus parasanguinis GalT1 (PDB code 5HE1), putative glycosyltransferase; and Bacillus subtilis SpsA (PDB code 1H7L) (Fig. S7B). WbmV and WbmW both contained an additional aspartate residue after the proposed DXD motif, so all three aspartate residues in this motif were initially converted to alanine to ensure inactivation.

Membranes expressing WbmV<sup>D100A/D102A/D103A</sup>W generated no reaction products with 1 (Fig. 7A). To confirm that WbmW retained activity in this construct, 3 GlcNAc-Gal-Galp-FI was synthesized by E. coli K-12 membranes containing WbmVW, using 1 as the acceptor. Membranes containing WbmV<sup>D100A/D102A/D103A</sup>W generated a single product with 3; its migration in PAGE was consistent with addition of a single sugar. This was confirmed by LC-MS, which revealed an [M + H]<sup>+</sup> ion peak at m/z 1224.4, consistent with a Galp-GlcNAc-Galp-FI structure (Fig. 7B).

Membranes expressing WbmV<sup>D272A/D274A/D275A</sup>W were predicted to transfer a single GlcNAc residue to 1, but only low activity was observed. This was interpreted as a consequence of disruption of protein structure from the simultaneous mutation of three consecutive aspartate residues. To circumvent this problem, a WbmV<sup>D272A</sup> mutant derivative was made. Membranes expressing WbmV<sup>D272A</sup> retained robust activity and generated a product whose migration in PAGE was consistent with the transfer of a single GlcNAc residue to 1 (Fig. 7A). A [M + H]<sup>+</sup> ion peak at m/z 1062.4 observed in LC-MS analysis confirmed GlcNAc-Galp-Galp-FI (Fig. 7B).

**WbmV and WbmW form a membrane-associated heterocomplex**

The inability of WbmV or WbmW to retain function when expressed independently of its partner suggested the possibility of important protein–protein interactions. A bacterial two-hy-
biosynthesis of Klebsiella O-antigens

Figure 7. Site-directed mutants of WbmV and WbmW permit assignment of their GT specificities. GT activities of E. coli DH5α membranes containing different enzyme constructs were tested using 1 and 3 (shown), and the products were separated by SDS-PAGE and visualized by fluorescence (A). Both acceptors supported polymerization of O2c antigen by WbmVW. The structures of each of the new products synthesized in vitro were determined by LC-MS (B). Shown are overlapped EIC traces for 1 (red), 3 (blue), and Gal-GlcNAc-Gal–FLAG product (purple). Shown beside each EIC graph is the corresponding ESI mass spectrum for material at the indicated retention time. WbmVW272A (pWQ995) showed little to no activity with 1, but robust activity was observed with WbmVW272A (pWQ996), corresponding to the addition of one hexosamine residue. It was inactive with 3 as expected. WbmVD100A/D101A/D103AW (pWQ999) only possessed activity with 3, adding a single hexose. The enzymatically-produced material contained an orphan MS peak eluting at 5.9 min (mass spectrum shown major ion peaks at m/z 1062.488, 832.399, and 531.748). The origin of this peak is unknown, but it is not a substrate for O2c addition and does not compromise data interpretation. Control membranes were prepared from cells transformed with the plasmid vector pBR322.

Asbrid approach (39) was therefore used to assess any potential in vivo interactions between WbmV and WbmW. The T25-fragment of adenylate cyclase was fused to the N terminus of WbmV and WbmW, whereas the T18-fragment was fused to either the C or N terminus of the partners. Plating on LB–X-gal showed strong heterotypic interactions for some combinations, reflected in dark blue colonies comparable with the leucine zipper–positive control. Quantitative β-gal assays confirmed the interaction between WbmV and WbmW (Table 2). Robust β-gal activity was observed with T25–WbmW but interaction was weak when the T25 tag was fused to WbmW.

To confirm the interaction of WbmV and WbmW by a different method, a co-purification strategy was employed using His6–WbmV and WbmW–FLAG. The proteins were expressed under arabinose control from the same plasmid. On Ni-NTA chromatography, both His6–WbmV and WbmW–FLAG were retained, as evident by their detection in immunoblots with antibodies recognizing His6 or FLAG epitopes (Fig. 8A). The proteins eluted with imidazole were combined and purified with anti-FLAG beads. Again, both WbmV and WbmW were retained by the affinity resin and detected in the eluate by immunoblotting (Fig. 8B). The Coomassie Blue–stained eluates revealed approximately equal amounts of WbmV and WbmW, suggesting 1:1 stoichiometry. However, as this was not central to the main goal, it was not investigated further.

WbmVW and WbbY differ in demonstrable interactions with WbbM

Previously, we reported extensive interactions between WbbMNO in a heterocomplex for O2a OPS biosynthesis (23). The WbbM GT was a central hub in these interactions. To investigate possible interactions of the O2a and O1 or O2c sys-

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tems, WbbM was selected as bait in two-hybrid experiments (Table 2). Regardless of the position of the T18- and T25-fragments, no interactions were evident from LB–X-gal plates between WbbM and either WbmV or WbmW alone or in combination (data not shown). Quantitative \( \beta \)-galactosidase data were generated for constructs where the WbmVW complex was co-expressed to ensure that WbmV and WbmW were fully folded in an active conformation (Table 2). As anticipated from the white colonies on LB–X-gal, all combinations of WbmVW with WbbM showed no significant increase from the negative control indicating no detectable interaction between WbmVW and WbbM under these conditions.

In contrast, WbbY exhibited a strong \textit{in vivo} interaction with WbbM (Table 2). Three of four two-hybrid combinations produced \( \beta \)-gal activities equivalent to (or greater than) the leucine zipper–positive control. Collectively, these data demonstrated that polymerization of the O2a and O1 polysaccharides are closely coupled.

**Discussion**

All known \textit{K. pneumoniae} O-antigens are produced by biosynthetic pathways requiring an ABC transporter. Diversity in the O-PS repeat-unit structures, and resulting O serotypes, is achieved by several different mechanisms (Fig. 9). The simplest

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**Table 2**

Quantitative \( \beta \)-galactosidase values for bacterial two-hybrid analysis

|                                | N-terminal T25 | N- or C- terminal T-18 derivative | \( \beta \)-Galactosidase activity (Miller units) |
|--------------------------------|----------------|----------------------------------|-----------------------------------------------|
| Positive control               | Leucine zipper | Leucine zipper                   | 1419 ± 247                                    |
| Negative control               | Leucine zipper | Leucine zipper                   | 73 ± 22                                       |
| WbmV–W interactions            | WbmV           | WbmW                             | 910 ± 250                                     |
| Positive control               | WbmW           | WbmV                             | 148 ± 13                                      |
| WbmV–W interactions            | Leucine zipper | Leucine zipper                   | 910 ± 250                                     |
| Positive control               | WbmW           | Leucine zipper                   | 187 ± 11                                      |
| WbmV–WbbM interactions         | WbbM           | WbmV                             | 282 ± 32                                      |
| Positive control               | WbmVW          | WbbM                             | 271 ± 18                                      |
| Negative control               | Leucine zipper | Leucine zipper                   | 44 ± 5                                        |
| WbbY–WbbM interactions         | WbbM           | WbbY                             | 105 ± 1                                       |
| Positive control               | WbbM           | WbbY                             | 1212 ± 36                                     |

\( a \) Data were obtained from triplicate experiments.

\( b \) NA means not applicable.
involves the use of different GT enzymes to generate a novel structure based on monosaccharide components or linkage combinations. For example, in serotype O3, diversification occurs by mutations within the polymerase (WbdA) that change its activity to generate new structures and O-antigen subtypes (O3, 3a, and 3b) (9). Diversification of O2a can occur via side-chain modifications with Galp residues (Fig. 1) (8, 27, 28) or O-acetyl groups (26). These types of mechanisms of antigenic diversity are seen in other bacteria and are not confined to OPS. In contrast, the addition of a repeat-unit polysaccharide with a new structure onto an acceptor based on a different glycan is currently a rare strategy. The backbone of Brucella abortus OPS (also produced in an ABC transporter-dependent pathway) contains different structural motifs (40), but the mechanistic underpinning is unknown. The K. pneumoniae O1 and O2c antigens offer the first examples where the precise enzymatic activities are established.

Chain capping mechanisms occur in two different OPS strategies in K. pneumoniae. One involves the addition of a terminating residue (or residues) as part of a chain-length regulation strategy. Such is the case in serotypes O3 and O5, which add methyl phosphate and methyl groups, respectively, to the OPS backbone but was no longer resistant to serum killing (48). The most fundamental form of OPS variation is through variation of GTs, creating differing repeat units. GT mutations (O3, 3a, and 3b) can also result in the change in overall OPS structure by changing the number of sugar residues added. Side-chain additions to the main backbone have been demonstrated in O8 (O-acetyl) and in O2afg and O2aeh (Galp). The OPS backbone can also be modified at the nonreducing terminus assisting in termination of polymerization (O3, 3a, 3b, O5, O4, and O12). Finally, distinct glycans can also be added to the nonreducing end of the OPS; however, these are not produced for chain length regulation purposes.

Notably, the wbbY and wbmVW loci are both located between transposable elements, suggesting they originated from a lateral gene transfer event. The O1 and O2c modifications play no clear role in chain length regulation because O2a chain length is dictated by the stoichiometry of synthesis:export components (21).

Acquisition of the modifying genes from another source potentially provides an evolutionary advantage by masking the O2a antigen with a second, unrecognizable polymer. The efficacy of this can vary; O1 strains report only as O1 (and not O1,2a) in standard serological agglutination tests, whereas strains with the O2c antigen must expose both antigens to generate the O2ac serotype. This can influence recognition by pre-existing host immune response but also may offer resistance against O2a-specific bacteriophages, which are an important driver in glycan diversity (47). However, the glycan-extending modifications may offer important functional advantages. For example, a spontaneous O1-deficient mutant of an K. pneumoniae O1 isolate resulted in a strain that still produced O2a OPS but was no longer resistant to serum killing (48). Similarly, addition of α-(1→4)-linked Galp side-chain residues in serotype O2afg (found in the disseminated ST258 isolates) was correlated with substantially higher survival in normal human serum (49). Further serological complexity is possible through the co-expression of multiple O2a modifications. One example is the production of the O1 antigen in strains that also possess O2afg (8), and we have also reported K. pneumoniae clinical isolates from collections that possess both gmtPafg or wbbY together with wbmVW (10, 28). Although the OPS structures of these latter isolates are unknown, the apparent temperature regulation of WbmVW suggests the dominant modifications would be O2afg or O1 at 37°C.

The O1 and O2ac systems offer interesting contrasts in the underlying modification strategies. Both create glycans possessing a new disaccharide repeat-unit structure and extend off a specific residue in the O2a repeat unit: Galp for O1 and Gal for O2c. Both systems require the genetic capacity to synthesize full O2a antigen, as shown here for O1 and previously for O2c (28). However, the length of O2a structure present in these dual-domain polymers is uncertain. This question is difficult to resolve chemically, because cells producing O1- and O2c-

**Figure 9. Schematic diagram demonstrating the multiple points of O-antigen diversity in K. pneumoniae.** The most fundamental form of OPS variation is through variation of GTs, creating differing repeat units. GT mutations (O3, 3a, and 3b) can also result in the change in overall OPS structure by changing the number of sugar residues added. Side-chain additions to the main backbone have been demonstrated in O8 (O-acetyl) and in O2afg and O2aeh (Galp). The OPS backbone can also be modified at the nonreducing terminus assisting in termination of polymerization (O3, 3a, 3b, O5, O4, and O12). Finally, distinct glycans can also be added to the nonreducing end of the OPS; however, these are not produced for chain length regulation purposes.
Biosynthesis of Klebsiella O-antigens
capped glycans also produce and export a variable amount of LPS containing unmodified O2a antigen. Indeed, obtaining the original definitive data for the tandem arrangement of O2a and O1 glycans was challenging in the absence of genetic insight (16, 17). Generally, the longest chains of O1 and O2c show little reactivity with antibodies specific for O2a (see Figs. 2 and 5). This could reflect the small amount of O2a antigen in terms of the overall mass of these OPS chains, resulting from preferential extension of short O2a oligosaccharides. In both cases, the O2a-reactive glycan appears to have a shorter average chain-length distribution than that produced in the absence of O1 and O2c. The very long Und-PP–linked OPS produced in an ABC transporter-defective background shows no reactivity with anti-O2a antibodies (Fig. 2) (28). This observation is surprising as unmodified O2a antigen seen in the presence of the transporter should still be produced. Furthermore, in cells producing only O2a antigen, chain lengths exceeding the natural LPS-transporter should still be produced. These observations can be explained by a model in which WbbY takes over polymerization on acceptors containing one or few O2a repeat units. The appearance of O2a antigen-containing LPS species in the immunoblot profiles may reflect protection/sequestration of a subset of the nascent Und-PP–linked O2a glycan from WbbY-mediated modification by the ABC transporter. This protection would be lost in the absence of the transporter.

The O1 and O2c systems also identify different formats for the required GTs. O1 uses a dual-GT polymerase (WbbY), which is common for these systems. For example, multidomain polymerases are recognized for O8 and O9 (50) and in O12 (where the polymerase and terminating enzymes are combined in one protein) (43). In contrast, O2c requires two independent GTs, which seem to require interactions for proper function. Interaction would ensure the enzymes are properly coordinated at the site of polymerization. Whether the loss of function when the enzymes are expressed individually is due to incorrect localization, exposure to protease activity, or reflecting a need for both to be present for adopting proper conformations is currently unclear. This question was not central to this study and has not been pursued.

Previous studies in the O2a system described interactions in a membrane-associated multienzyme complex (23). WbbY shows strong interactions with WbbM, suggesting the possibility that these enzymes, which are encoded by unlinked loci, have coexisted (and possibly co-evolved) in the same background for a long period. Under the same conditions, no interactions were evident between WbmVW and WbbM, indicating a different strategy. A priority for this field is to understand the relative positioning of active sites in these multienzyme (and multidomain) complexes. The systems reported here are well-suited to begin to establish key structural concepts in organizing and coordinating multi-GT complexes. Moreover, the guiding principles may inform the development of efficient recombinant bacterial glycosylation systems for in vivo production of specific glycan structures for vaccine and other biomedical applications (51).

Experimental procedures

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table S1. Cultures were grown in lysogeny broth (LB) (52) supplemented with antibiotics as required. Final concentrations of antibiotics were 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. LB plates for bacterial two-hybrid experiments were supplementation with 0.5 mm isopropyl 1-thio-β-d-galactopyranoside (IPTG) and 40 μg/ml X-gal. Expression of Gal-containing OPS in E. coli CWG286-derived strains was conditional on the presence of galactose in the growth medium, due to the galE mutation (22). These strains were grown overnight in LB containing 0.4% (w/v) d-glucose and then subcultured into LB containing 0.1% (w/v) d-galactose. All strains for investigation of wbbY were grown at 37 °C, whereas expression from the native temperature-dependent promoter(s) of wbbM required growth at 30 °C.

Construction of recombinant plasmids

KOD Hot Start DNA polymerase (Novagen) was used for amplification of DNA fragments by PCR. Oligonucleotide primers (Table S2) were obtained from Sigma. PCR products were purified using the GeneJET PCR purification kit (Thermo Fisher Scientific), and plasmids were purified using the GeneJET plasmid mini-prep kit (Thermo Fisher Scientific). Recombinant plasmid construction was accomplished using restriction endonuclease digestions (enzymes from New England Biolabs) or Gibson assembly (New England Biolabs), as indicated in Table S1. Plasmids were confirmed by restriction digest and/or DNA sequencing performed by the Advanced Analysis Centre, University of Guelph. Lambda red recombination of multicopy plasmids was accomplished as described previously (53). Site-directed mutagenesis was accomplished using the Q5 site-directed mutagenesis kit with inverse PCR (New England Biolabs).

Bioinformatics analyses

BLAST analysis of protein sequences (54) was performed using the National Center for Biotechnology Information (NCBI) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). WbbY models were generated using the PHYRE2 protein fold recognition server (32) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Multiple sequence alignments were conducted using ClustalW (55) (https://www.genome.jp/tools-bin/clustalw) and displayed with ESPript 3.0 (56) (http://escript.ibcp.fr/ESPrRiPT/ESPrriPT/). The WbbY protein sequence used was from GenBankTM accession no. MG458672, and WbmVW was from GenBankTM accession no. MG602074.

SDS-PAGE and immunoblotting

LPS samples for SDS-PAGE were prepared as whole-cell lysates (57). One A460 nm unit of cells was resuspended in loading buffer, solubilized at 100 °C, and treated with proteinase K. Samples (10 μl) were separated on 12% gels using Tris-glycine buffer. LPS was either visualized using silver staining or by immunoblotting after transfer to nitrocellulose membranes.
(Protran, GE Healthcare). Electrophoretic transfer was accomplished with constant current at 200 mA for 45 min, using buffer containing 25 mM Tris, 150 mM glycine, and 20% (v/v) methanol. Membranes were blocked in 5% skim milk (BD Difco) or 5% BSA (GE Healthcare, fraction V) prepared in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.005% (v/v) Tween 20). The O1 antigen was detected with the mouse mAb O1-52.6 (16). Rabbit polyclonal antibodies specific for the O2a and O2c antigens were reported previously (28) and were diluted 1:1000 in 5% (w/v) skim milk or 5% (w/v) BSA prepared in TBST. Goat anti-rabbit or goat anti-mouse–conjugated alkaline phosphatase (Cedar Lane) was used as secondary antibodies, and development was achieved by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science).

Proteins were analyzed in solubilized whole-cell lysates without exposure to proteinase K and examined with minor modifications to the protocol above. Electrophoretic transfer of protein samples was achieved using a Pierce FastTransfer Power Blotter (Thermo Fisher Scientific) at 1.3 A for 7 min. The primary antibody was mouse anti-His$_6$ (1:1000, Qiagen) or mouse anti-FLAG (1:1000, Sigma). Goat anti-mouse alkaline phosphatase (Cedar Lane), or goat anti-mouse horseradish peroxidase were used as secondary antibodies. Detection was achieved by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) or Crescendo HRP substrate (Millipore).

**Identification of protein–protein interactions**

Complexes involving WbMV and WbmW were identified in co-purification experiments. *E. coli* cells DH5α harboring pWQ990 (His$_6$–WbMV–VW–FLAG) were subcultured 1:100 into 250-ml cultures and grown to $A_{600}$ of 0.6, and gene expression was induced by addition of 0.2% (w/v) of arabinose. The culture was then grown overnight at 18 °C. Cells were collected by centrifugation at 5000 × g, resuspended in buffer A (50 mM Tris, 50 mM NaCl, pH 7.4) containing 10 mM imidazole, and lysed by sonication. Cell-free lysates were then prepared by successive centrifugation at 4000 × g for 10 min followed by 12,000 × g. Membranes were removed by centrifugation at 100,000 × g for 1 h, and the supernatant was subjected to affinity chromatography with samples being prepared for SDS-PAGE at each step. Ni-NTA–agarose resin (2-ml bed volume; Qiagen) was washed twice with 10 volumes of buffer A containing 10 mM imidazole, and collected by centrifugation at 800 × g for 2 min. The resin was mixed with the enzyme-containing lysate and incubated at 4 °C with rocking. The resin was washed twice with 10 volumes of buffer A containing 25 mM imidazole, and the slurry was applied to a gravity column. Elution was performed with 10 volumes of buffer A containing 250 mM imidazole. Elution fractions 1 and 2 (1 ml each) were combined, buffer-exchanged, and concentrated into TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.4) using a Vivaspin 500 column with a 30,000 molecular weight cutoff (Sartorius). A 50-μl aliquot of anti-FLAG M2 magnetic beads (Sigma) was collected using a magnet and washed three times with 1 ml of TBS, and the Ni-NTA eluant (~500 μl) was added. FLAG-tagged protein was bound with rocking for 1 h at room temperature. The beads were washed three times with 1 ml of TBS, resuspended in 40 μl of SDS-PAGE loading buffer, and boiled for 5 min to release the protein fraction. The various fractions were then analyzed by SDS-PAGE, and immunoblots were probed with anti-His$_6$, and anti-FLAG antibodies.

**Bacterial two-hybrid analysis**

Overnight cultures of *E. coli* BTH101 co-expressing variations of two-hybrid constructs were subcultured 1:100 into LB containing kanamycin, ampicillin, 0.5 mM IPTG and grown at 30 °C to an $A_{600}$ nm of 0.5. The β-gal activity was measured using the microplate nonstopped protocol of the yeast β-galactosidase assay kit (Thermo Fisher Scientific), substituting the yeast protein extraction reagent (Y-PER) for bacterial protein extraction reagent (B-PER). Absorbance at 420 nm was measured using SynergyH1 microplate reader (BioTek).

**Preparation of membranes for in vitro GT reactions**

Membranes were prepared from 250-ml overnight cultures. Cells were resuspended in buffer B (50 mM HEPES, 150 mM NaCl, pH 7.4) and disrupted by sonication. Lysates were cleared by sequential centrifugation at 4000 × g for 10 min and 12,000 × g for 20 min. Membranes were collected by ultracentrifugation at 100,000 × g for 1 h, and resuspended in buffer B containing 2 mM dithiothreitol. Total protein content in the membrane preparations was estimated using the DC protein assay kit (Bio-Rad) with samples diluted in 1% (w/v) SDS. Bovine serum albumin was used as a protein concentration standard.

**Purification of Glf**

His$_6$–Glf was purified from 1 liter of *E. coli* Top10 containing pWQ1010. The culture was incubated at 37 °C with shaking at 200 rpm until the $A_{600}$ nm reached 0.6. l-Arabinose was added to 0.2% (w/v), and incubation was continued for 2.5 h. Bacteria were collected by centrifugation at 5000 × g for 10 min, resuspended in 50 ml of buffer C (50 mM BisTris, pH 7.0, 250 mM NaCl, pH 7.4) and disrupted by sonication. Membranes were prepared from 250-ml overnight cultures. Cells were resuspended in buffer B (50 mM HEPES, 150 mM NaCl, pH 7.4) and disrupted by sonication. Lysates were cleared by sequential centrifugation at 4000 × g for 10 min and 12,000 × g for 20 min. Membranes were collected by ultracentrifugation at 100,000 × g for 1 h, and resuspended in buffer B containing 2 mM dithiothreitol. Total protein content in the membrane preparations was estimated using the DC protein assay kit (Bio-Rad) with samples diluted in 1% (w/v) SDS. Bovine serum albumin was used as a protein concentration standard.

**In vitro glycan synthesis on synthetic acceptors**

The synthses of synthetic disaccharide acceptors 1 and 2 is described in Schemes S1–S3. Polymerization of the O1 polysaccharide on the synthetic glycan acceptors was performed in 20-μl reaction volumes containing 50 mM HEPES, pH 7.4, 20 mM MgCl$_2$, 2 mM DTT, 100 μM acceptor (1 or 2), and 5 mM...
Biosynthesis of Klebsiella O-antigens

UDP-Galp (Sigma), and membranes were isolated from E. coli Top10 (pWQ991) (1 mg/ml total protein). The reactions were incubated at 30 °C for 60 min. Large-scale reactions (500 μl) to prepare polysaccharide for NMR analysis contained 300 μM 2, 5 mM UDP-Galp, and membranes (455 μg/ml total protein). Analysis of WbY mutant derivatives for GT activity was performed using 9.75 μM 2, 5 mM UDP-Galp, and membranes were standardized to 65 μg/ml total protein. Reactions for in vitro synthesis of the O2c antigen contained 11 μM I, 5 mM UDP-Galp, 5 mM UDP-GlcNAc, 10 mM NADH, 37 μM Glf, and membranes (5 mg/ml total protein). O2c reaction were incubated at 30 °C for 16 h. A scaled-up 0.5-ml reaction containing 110 μM I was used to generate sufficient O2c polymer for analysis by NMR spectroscopy.

Reaction products (2 μl) were mixed 1:1 with 2× SDS-PAGE sample buffer (58), separated by SDS-PAGE, and visualized by UV transillumination.

In vitro synthesis of 3

In vitro enzymatic synthesis of 3 was accomplished using membranes from E. coli DH5α (pWQ987) cells expressing wbmVW. Scaled-up reactions contained 11 μM I, 5 mM UDP-GlcNAc, 2 mM DTT, 20 mM MgCl₂, 50 mM HEPES, pH 7.4, and WbmVW membranes (5 μg/ml total protein) in a reaction volume of 300 μl. The reaction mixtures were incubated for 12 h at 30 °C. The membranes were then removed as a pellet after centrifugation at 100,000 × g for 1 h, and the supernatant was treated with proteinase K treated (0.5 mg/ml) before the samples were frozen and lyophilized. The dried acceptor was dissolved in 15 μl of water to an approximate concentration (assuming full conversion and zero loss of product) of 2.2 mm. The presence of a single product (GlcNAc-Galp-Galp-Fl) was confirmed by LC-MS analysis.

Mass spectrometry

LC-MS analyses were performed on an Agilent 1260 HPLC interfaced with an Agilent UHD 6530 Q-TOF mass spectrometer (Advanced Analysis Centre, University of Guelph). In vitro reaction mixtures were filtered using a Microcon 10-kDa centrifugal filter or extracted with an equal amount of chloroform followed by centrifugation. Samples were separated on a C18 column (Agilent Poroshell 120, EC-C18 50 mm × 0.7 cm) in water. Elution was monitored by a refractometer and by TLC using a plate developed with ethyl acetate/water/1-butanol/acetic acid (5:4:4:2.5). Fluorescent reaction products were detected with a hand-held UV lamp. Fractions containing high-molecular-weight polymer (remaining at the origin of TLC plate) were pooled. Fractionation of O2c was deemed unnecessary because SDS-PAGE revealed the majority of I was converted to high-molecular-weight polymer. Both samples were deuterium-exchanged by lyophilizing twice with 99.9% D₂O. NMR spectra were obtained at 50 °C (O1 polymer) or 30 °C (O2c polymer) in 99.9% D₂O using a Bruker Avance III 600 MHz spectrometer, equipped with a cryoprobe (Advanced Analysis Centre, University of Guelph). Chemical shifts were referenced to 3-trimethylsilylpropanoate-2,2,3,3-d₄ (δH 0 ppm, δC −1.6 ppm) as an internal standard. The O1 polysaccharide was analyzed using 1H, COSY, TOCSY, ROESY, 1H, 13C HSQC, and HMBC experiments. The identity of O2c polysaccharide was confirmed using 1H and HSQC experiments.

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