

Bacteriophage-mediated Glucosylation Can Modify Lipopolysaccharide O-Antigens Synthesized by an ATP-binding Cassette (ABC) Transporter-dependent Assembly Mechanism^{*[5]}

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Background: Bacteriophage-mediated seroconversion by glucosylation is currently unknown for O-antigens synthesized by ABC transporter-dependent pathways.

Results: *Raoultella terrigena* O-antigen is modified with a glucose side chain when expressed in *E. coli* K-12.

Conclusion: The ABC transporter-dependent pathway poses no intrinsic mechanistic barrier to phage-mediated glucosylation.

Significance: O-antigen glucosylation has implications for evolution of antigenic diversity and vaccine development.

Lysogenic bacteriophages may encode enzymes that modify the structures of lipopolysaccharide O-antigen glycans, altering the structure of the bacteriophage receptor and resulting in serotype conversion. This can enhance virulence and has implications for antigenic diversity and vaccine development. Side chain glucosylation is a common modification strategy found in a number of bacterial species. To date, glucosylation has only been observed in O-antigens synthesized by Wzy-dependent pathways, one of the two most prevalent O-antigen synthesis systems. Here we exploited a heterologous system to study the glucosylation potential of a model O-antigen produced in an ATP-binding cassette (ABC) transporter-dependent system. Although O-antigen production is cryptic in *Escherichia coli* K-12, because of a mutation in the synthesis genes, it possesses a prophage glucosylation cluster, which modifies the GlcNAc residue in an α -L-Rha-(1→3)-D-GlcNAc motif found in the original O16 antigen. *Raoultella terrigena* ATCC 33257 produces an O-antigen possessing the same disaccharide motif, but its assembly uses an ABC transporter-dependent system. *E. coli* harboring the *R. terrigena* O-antigen biosynthesis genes produced an O-antigen displaying reduced reactivity toward antisera raised against the native *R. terrigena* repeat structure, indicative of an altered chemical structure. Structural determination using NMR revealed the addition of glucose side chains to the repeat units. O-antigen modification was dependent on a functional ABC transporter, consistent with modification in the periplasm, and was eliminated by deletion of the glucosylation genes from the *E. coli* chromosome, restoring native level antisera sensitivity and structure. There are therefore no intrinsic

mechanistic barriers for bacteriophage-mediated O-antigen glucosylation in ABC transporter-dependent pathways.

Bacterial surfaces possess a range of different macromolecules that contain complex carbohydrates. These glycoconjugates mediate contact with the external environment and represent a remarkably diverse spectrum of carbohydrate structures. Significant progress has been made in establishing their precise structures and modes of assembly. As an example, there are more than 180 chemically and serotypically distinct glycans, giving rise to LPS O-antigens in *Escherichia coli*, and the structures, genes, and biosynthetic enzymes have been catalogued (1–4). Diversity in glycoconjugate structures results from recombination events within genetic loci responsible for the synthesis of polysaccharides to generate a new structure and by acquisition of unlinked genes whose products modify existing structures. One mechanism of diversification, illustrated by certain O-antigens in the genera *Escherichia*, *Salmonella*, and *Shigella*, involves the addition of a side branch glucose to the main chain of the glycan (see Fig. 1). The genes responsible for glucosylation are encoded by the genomes of lysogenic bacteriophages, so phage infection leads to serotype conversion. Glucosylation has a particularly profound effect on serotype in *Shigella*. Of 15 recognized serotypes, all but two contain the same glycan backbone (serotype Y). Diversity arises mainly from glucosylation and/or O-acetylation of this backbone (reviewed in Ref. 5), but other modifications are also possible (6, 7). Depending on the serotype, any of the sugars in the tetrasaccharide repeat unit may be modified. *Salmonella* isolates can contain up to four glucosylation systems, modifying different parts of the O-antigen structures (8). Clearly, these modification processes have a significant impact on diversity.

O-antigens currently known to be subject to glucosylation are united by a common assembly mechanism, known as the Wzy-dependent pathway (reviewed in Ref. 9). In this process, repeat units of the O-antigen are built at the cytoplasmic face of

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the cell membrane on an undecaprenol diphosphate (Und-PP)⁴ lipid carrier. A transporter (Wzx) then exports these lipid-linked intermediates to the external face of the membrane, where they provide substrates for the pathway-defining polymerase (Wzy). The polymerization reaction involves transfer of a growing glycan chain from its Und-PP carrier to the nonreducing end of the incoming Und-PP-linked repeat unit. After polymerization, the O-antigen is transferred to a lipid A core acceptor, and the completed molecule is translocated to the outer membrane (10). The glucosylation modification reaction was first reported in the seroconversion of *Salmonella* O serotypes. Although the activated initial donor of the glucose residue is UDP-glucose, the direct donor is undecaprenol phosphate-glucose (11–14). Early studies established an association between glucosylation modification and lysogenic bacteriophages (13, 15), and the same is true in *Shigella* (5). It is now known that the modification reaction requires acquisition of only three genes. The GtrB enzyme synthesizes undecaprenol phosphate-Glc, and GtrA is the putative exporter for the lipid-linked donor; the corresponding genes are conserved across glucosylation systems. A third gene (designated *gtrC* or *gtr**) is variable and encodes a serotype-specific glucosyltransferase (16, 17).

To date, glucosylation machinery of this type is only known to modify O-antigens assembled by the well distributed Wzy-dependent processes. However, many O-antigens follow a different synthetic pathway, defined by the involvement of an ATP-binding cassette (ABC) transporter, composed of dimers of both the nucleotide-binding domain (NBD) polypeptide, which is designated Wzt, and of the transmembrane domain (TMD), known as Wzm (18). In this pathway, the O-antigens are still assembled on an Und-PP acceptor, but polymerization is completed in the cytoplasm by the sequential action of glycosyltransferase enzymes. Once complete, the Und-PP-linked glycan is exported to the periplasm by the ABC transporter. The nascent O-antigen is then transferred to the lipid A core molecule and translocated to the outer membrane by processes that operate independently of the mode of O-antigen synthesis. The association of glucosylation modification with the Wzy-dependent pathway may reflect the limits to which systems have been characterized at a bioinformatics and biochemical level or may result from the glycosylation system being critically dependent on elements of the Wzy-dependent machinery. Here, we examine the possibility that the ABC transporter-dependent pathway provides an intrinsic mechanistic barrier for the glucosylation process by examining the potential for the native *E. coli* K-12 glucosylation machinery encoded by prophage Kp1E1 (19) to modify target residues supplied by a heterologous ABC transporter-dependent O-antigen from *Raoultella terrigena* ATCC 33257 (see Fig. 2). This O-antigen is built up of repeat units containing an α -L-Rha-(1 \rightarrow 3)-D-GlcNAc disaccharide, and this represents a motif that is modifiable by glucosylation systems in both *E. coli* K-12 and *Shigella flexneri* (see Fig. 1).

Experimental Procedures

Bacterial Strains and Growth Conditions—Bacterial cultures were grown with aeration at 37 or 30 °C in LB base (Invitrogen), supplemented with appropriate antibiotics (100 μ g ml⁻¹ ampicillin, 34 μ g ml⁻¹ chloramphenicol, 100 μ g ml⁻¹ kanamycin). *R. terrigena* ATCC 33257 was a gift from U. Mamat (Research Centre Borstel, Leibnitz Centre for Medicine and Biosciences, Borstel, Germany). The *E. coli* strains used in this study are derivatives of strain TOP10 (F⁻, *mcrA*, Δ (*mrr-hsdRMS-mcrBC*), ϕ 80, *lacZ* Δ M15, Δ *lacX74*, *deoR*, *nupG*, *recA1*, *araD139*, Δ (*ara-leu*)7697, *galU*, *galK*, *rpsL*(Str^r), *endA1*) from Invitrogen. Deletion mutagenesis was performed using the λ -red recombination system (20). To remove part of the K-12 *wb** gene cluster in *E. coli* Top10 to create *E. coli* CWG1217 (Δ *wzx-wbbK*), pKD4 (21) was used as a template to amplify the kanamycin resistance cassette using oligonucleotide primers containing 50 nucleotides of homology with *wzx* and *wbbK* genes (identified by lowercase); the primer sequences were 5'-acgaataaattatctttaagaagaacgttatatctggtgctgcttcaGTGTAG-GCTGGAGCTGCTTC-3' and 5'-atgttcttcagtaataaaactagttcatcaaacccaactaatacatCATATGAATATCCTCCTTAG-3'. Electrocompetent cells of *E. coli* Top10 containing pSIM6 (22) were transformed with the linear PCR product, and after recovery overnight with aeration at 30 °C, mutants were selected on LB agar containing 15 μ g ml⁻¹ kanamycin grown for 2 days at 30 °C. The correct deletion was confirmed by a series of diagnostic PCR amplification reactions using primers with sequences located outside and within the deleted regions. Sensitivity to ampicillin indicated loss of pSIM6.

To construct *E. coli* CWG1218 (Δ *wzx-wbbK* Δ *gtrA*), the chloramphenicol acetyltransferase gene was amplified from pKD3 (21) using oligonucleotide primers OL1019 (5'-aagacttggatgatagacttcattccttggattattagctgatagaagaaGTGTAGGCTG-GAGCTGCTTC-3') and OL1020 (5'-aattggtatcgcttcttcttcattgaagacaggaactacaagagatatctCATATGAATATCCTCCTTAG-3'), containing ~50-nucleotide extensions identical to the sequences flanking the target open reading frame, which is designated *yfdG* in *E. coli* DH10b (GenBankTM accession number NC_010473.1). The process for mutagenesis, confirmation, and curing of pSIM6 was the same as that described for the parent, CWG1217, except mutants were selected on LB agar containing 10 μ g ml⁻¹ chloramphenicol at 30 °C. Potential downstream (polarity) effects of the cassettes replacing *gtrA* or *wzx-wbbK* had no influence on the experimental strategy, so they were left in place in strains used to isolate polysaccharides for NMR experiments. However, they were subsequently excised using the pCP20 helper plasmid (21) to construct *E. coli* CWG1219 (Δ *wzx-wbbK* Δ *gtrA*), to avoid conflicts in selectable markers for the Wzt titration and *gtr* complementation experiments.

DNA Methods—Custom oligonucleotide primers were obtained from Sigma. PCR amplification was performed using Pwo DNA polymerase (Roche Applied Science). The PureLink PCR purification kit (Invitrogen) was used to clean the PCR product. Plasmid DNA was purified from overnight cultures using the PureLink quick plasmid miniprep kit (Invitrogen). Chromosomal DNA was obtained using the PureLink genomic

⁴ The abbreviations used are: Und-PP, undecaprenol diphosphate; ABC, ATP-binding cassette; NBD, nucleotide-binding domain.

DNA mini kit (Invitrogen). Restriction digest and ligation reactions were performed according to the manufacturer's instructions. DNA sequencing was performed in the Genomics Facility of the Advanced Analysis Center at the University of Guelph.

Plasmid Constructs—pKM114 is a pMBL19 derivative containing the 10.3-kb *R. terrigena* *wb** gene cluster, encoding production of the O-antigen, and was a gift from U. Mamat (Research Centre Borstel, Leibnitz Centre for Medicine and Biosciences, Borstel, Germany).

The *wzt* gene was PCR-amplified from pKM114 using primers designed to include an N-terminal FLAG tag in the gene product. The primer sequences (forward primer: 5'-gatcgaattcATGgattacaaggatgacgacgataagTCCTCTAATGAAATTGC-TATCCAGGTCAC-3'; reverse primer: 5'-gatcaagcttTACTCTCCATTCGAAATAATTTTCAACGGAG-3') incorporated EcoRI and HindIII restriction sites (underlined) for cloning (chromosomal sequences are indicated by uppercase letters, and FLAG tag sequences are italicized). The PCR product was digested with EcoRI and HindIII and ligated into pWQ573 to generate pWQ114 (23). The vector contains an L-arabinose-inducible pBAD promoter (24) and a chloramphenicol resistance cassette.

The pWQ811 vector is a pMBL19 (25) derivative containing a tetracycline-inducible promoter and an ampicillin resistance cassette. It was generated by cloning the tetracycline-inducible operator *tetRA* into pMBL19. The *tetRA* genes were PCR-amplified from *E. coli* BL21 (DE3) (forward primer, 5'-gtcgaagaattc-atATGACTTTTCTCTATCACTGATAGGGAGTGG-3'; reverse primer, 5'-agaacagctagCATTTAGGTGGGTACGTTG-GAGC-3') and digested with EcoRI and NheI using the sites incorporated into the primers. The kanamycin resistance cassette from pBSL15 (26) was amplified (forward primer, 5'-aaaaaataataaagcttgcagctgcagctgactgCGTTGCTGTCGCT-AGCTTCAC-3'; reverse primer, 5'-aaaaaagaattccatggtaccgg-gatcctctagactgGTTGCTGTCGCGAACCCAG-3'), digested with EcoRI and AseI (end cohesive with NdeI digest) and ligated into NdeI/NheI-digested pMBL19 concurrently with the *tetRA* digest. This construct was digested with SpeI and self-ligated to remove the kanamycin selection marker.

pWQ677 contains *wbbL-wzm-rbs-wbbB* cloned into pWQ811. The added *rbs* (ribosome binding site) is required for expression of *WbbB* in this construct. *wbbL-wzm-rbs* (forward primer, 5'-aaaaaagaattcctATGACTTATGAAGCAATGAAG-CC-3'; reverse primer, 5'-aaaaaaaactagtgtaccacacctctagagga-CATCAGCAAATCCC-3'; a ribosome binding site is indicated in italics) and *wbbB* (forward primer, 5'-aaaaagctagcaggaggt-tggtaccATGCTGGCTGTATTTTACCTCCC-3'; reverse primer, 5'-aaaaaaagcttccatggaCTAGCGGTTGCGCTTAAA-CTCC-3') were PCR-amplified from pKM114. The PCR fragments were ligated individually into pWQ811, using the EcoRI and KpnI sites for *wbbL-wzm-rbs* and the KpnI and HindIII sites for *wbbB*.

The *gtr* gene cluster from *E. coli* $\Delta wzx-wbbK$ was PCR-amplified from genomic DNA (forward primer, 5'-taatggtaccAC-AGCAAGTATCGAT-3'; reverse primer, 5'-ttagatccCGCA-ATTCTATCAGGAG-3') following a *gtr*-cloning strategy described elsewhere (27)). PCR fragments were cleaved with KpnI and ligated into pWQ573 cleaved with KpnI and SmaI to

produce pWQ115. This plasmid contains a fragment carrying the *gtrA*, *gtrB* and *gtr** genes for complementation because the chromosomal deletion of *gtrA* also eliminated expression of the overlapping *gtrB* gene.

SDS-PAGE and Immunoblotting—To examine LPS in whole cell lysates, equivalent amounts of cells (determined by optical density at 600 nm), were solubilized in SDS-PAGE loading buffer and heated to 100 °C for 10 min. Samples were then treated with proteinase K prior to SDS-PAGE with 12% polyacrylamide resolving gels in Tris-glycine buffer (28, 29). LPS was visualized with silver staining (30). For O-antigen immunoblot analysis, LPS was transferred to nitrocellulose membranes (Protran; PerkinElmer Life Sciences) and probed with rabbit antiserum generated against *Klebsiella pneumoniae* O12 formalin-fixed whole cells. The *K. pneumoniae* O12 antigen is structurally and serologically identical to the O-antigen in *R. terrigena* ATCC 33257 (31). Goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Cedarlane Laboratories) was used, and the immunoblot was developed with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Roche Applied Science). For Western blot analysis, samples were solubilized in SDS-PAGE loading buffer, heated to 100 °C for 10 min, and subjected to SDS-PAGE. Protein was transferred to nitrocellulose membrane and probed with mouse polyclonal anti-FLAG antibodies (Sigma-Aldrich). Goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Cedarlane Laboratories) was used as previously described.

Titration of Wzt—Glucose-supplemented (0.4%) overnight cultures of *E. coli* CWG1217 ($\Delta wzx-wbbK$) and CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) transformed with both pWQ114 and pWQ677 were diluted 1/100 and grown to an A_{600} of ~ 0.8 following induction of *wbbL-wzm-wbbB* expression by adding 2.5 ng ml⁻¹ anhydrotetracycline. At the same time, expression of *wzt* from pWQ114 was varied by repression with 0.4% glucose or induction with a range of (0.002–0.2%) L-arabinose concentrations. LPS and Wzt levels were analyzed by SDS-PAGE, using a 12% resolving gel, and immunoblotting.

Complementation of the *gtr* Mutation—CWG1219 was transformed with pKM114 and pWQ115. The cultures were grown overnight, and LPS was examined by SDS-PAGE and immunoblotting.

Isolation of LPS and O-polysaccharide—Cultures of *E. coli* CWG1217 ($\Delta wzx-wbbK$) and CWG1218 ($\Delta wzx-wbbK \Delta gtrA$) transformed with pKM114 were grown at 37 °C overnight. Cells were harvested by centrifugation, washed with distilled water, and lyophilized. LPS was isolated by hot phenol-water extraction (32), followed by dialysis of the extract without separation of the phenol and water phases, and freed from insoluble material by centrifugation. The resulting crude LPS solution was concentrated and purified by precipitation of proteins and nucleic acids with cold aqueous 50% CCl₃CO₂H (33). After centrifugation, the supernatant was dialyzed against distilled water and lyophilized. The O-polysaccharide was released by heating an LPS sample (150 mg) with 2% acetic acid at 100 °C for 2 h. The lipid precipitate was removed by centrifugation, and the carbohydrate-containing supernatant was fractionated on a Sephadex G-50 superfine column (2.5 cm × 75 cm) in 50 mM pyridinium acetate buffer (pH 4.5) at a flow rate of 0.6 ml

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min^{-1} . Elution was monitored with a differential refractometer (Knauer).

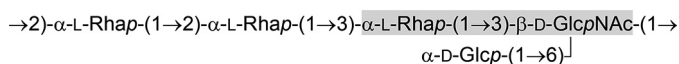
Nuclear Magnetic Resonance Spectroscopy—NMR studies were performed in the Advanced Analysis Centre at the University of Guelph. Polysaccharide samples were deuterium-exchanged by lyophilizing twice from 99.9% D_2O and then examined as solutions in 99.96% D_2O . NMR spectra were recorded at 35 °C on a Bruker Avance II 600 MHz spectrometer equipped with a cryoprobe, using internal sodium 3-trimethylsilylpropanoate-2,2,3,3- d_4 (δ_{H} 0, δ_{C} -1.6) as a reference. Two-dimensional experiments were performed using standard Bruker soft-

ware, and the Bruker TopSpin 2.1 program was used to acquire and process the NMR data. Mixing times of 100 and 200 ms were used in total correlation spectroscopy and NOESY experiments, respectively. The heteronuclear multiple-bond correlation spectroscopy experiment was optimized for the $J_{\text{H,C}}$ coupling constant 8 Hz.

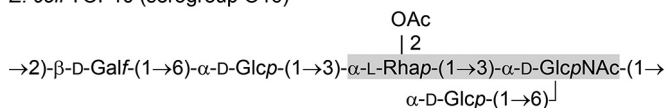
Results

Generation of Recombinant Strains Expressing the O-antigen from *R. terrigena* ATCC 33257—The O-antigen of *R. terrigena* ATCC 33257 was selected to probe the ability of a phage-mediated glucosylation system to interact with an ABC transporter-dependent O-antigen biosynthesis pathway. The repeating structure of this O-antigen possesses an α -L-Rha-(1→3)-D-GlcNAc disaccharide motif that is subject to glucosylation in *E. coli* O16 and *S. flexnerii* 4a (Fig. 1). The *R. terrigena* O-antigen gene cluster cloned in pKM114 (31) contains eight genes involved in O-antigen formation (Fig. 2). The same O-antigen biosynthesis genes are present in *K. pneumoniae* O12 (34). The *rmlBADC* genes encode the enzymes for production of the dTDP-L-rhamnose precursor (35) and *wbbL* encodes a rhamnosyltransferase (34). Homologues of these genes are found in the O-antigen biosynthesis gene locus in *E. coli* K-12 (including Top10). Located downstream of *wbbL* are the *wzm* and *wzt* genes encoding the TMD and NBDs of the ABC transporter, respectively. The predicted *wbbB* gene product possesses domains with homology to glycosyltransferases sharing homology with GT1 and GT25 families, as well as a putative β -Kdo transferase resembling KpsS and KpsC from *E. coli* capsule systems (36). The *R. terrigena* O-antigen shares the same repeating unit (and serological cross-reactivity) with the *K. pneumoniae* O12 antigen, where the same repeat unit polysaccharide is terminated with a single β -Kdo residue at the nonreducing terminus (37). Signals consistent with this terminator were reported in the NMR spectrum of *R. terrigena* O-antigen (31). The sequence data are consistent with WbbB partici-

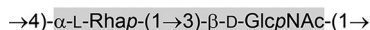
S. flexnerii 4a



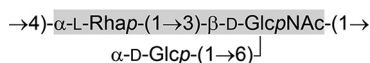
E. coli TOP10 (serogroup O16)



R. terrigena ATCC 33257 and *K. pneumoniae* O12



E. coli CWG1217 ($\Delta wzx\text{-wbbK}$); pKM114



E. coli CWG1218 ($\Delta wzx\text{-wbbK } \Delta gtrA$); pKM114

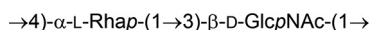


FIGURE 1. Structures of the O-polysaccharide repeat units. The component sugars are glucose (Glc), 2-acetyl-amino-2-deoxyglucose (N-acetylglucosamine; GlcNAc), and 6-deoxymannose (rhamnose; Rha).

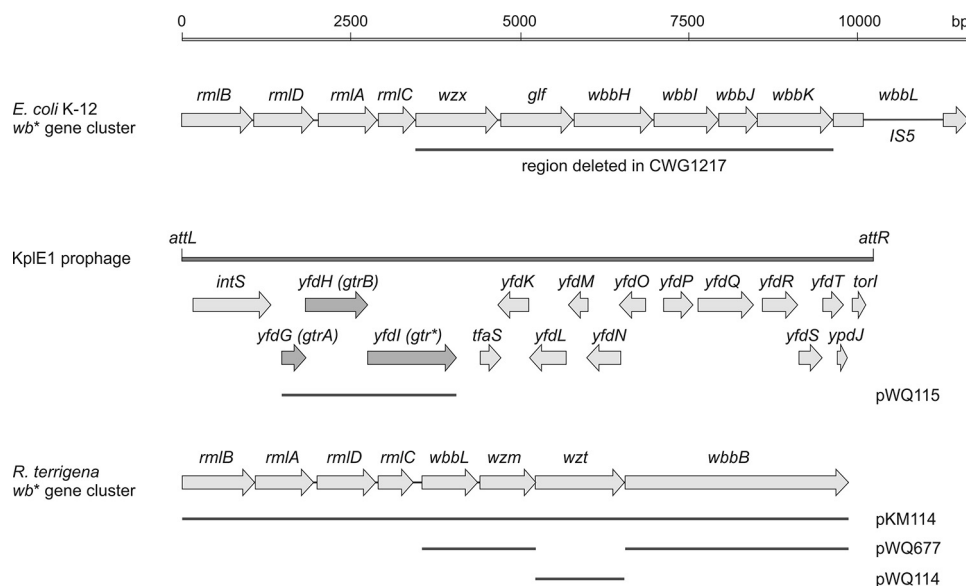


FIGURE 2. Organization of the O-antigen biosynthesis genes in *E. coli*. Shown are the *wb** O-antigen biosynthesis gene clusters from *E. coli* K-12 (GenBank™ accession number NC_010473) and *R. terrigena* ATCC 33257 (GenBank™ accession number AY376146), as well as the KpIE1 prophage (also named CPS-53) in the *E. coli* K-12 genome, encoding the three Gtr proteins required for glucosylation of O-antigens.

pating in both chain extension and chain termination, but this remains to be confirmed biochemically, and the precise activities giving rise to the O-antigen backbone are not central to the current study.

Although *E. coli* K-12 strains lack inherent O-antigen synthesis because of mutation(s) in the biosynthesis gene cluster (38), the ancestral O-antigen was serotype O16 (39) (Fig. 1). To exclude any interference between *R. terrigena* and *E. coli* glycosyltransferases and ensure that a homogeneous glycan backbone was synthesized, pKM114 was introduced into *E. coli* CWG1217 ($\Delta wzx-wbbK$), which lacks all of the glycosyltransferase activities, transport (Wzx) and polymerization machinery (Wzy) encoded by the chromosomal O-antigen biosynthesis locus.

Differential Reactivities of Native *R. terrigena* LPS and LPS from the Recombinant Strains with O-specific Antibodies—Whole cell lysate LPS samples were probed in immunoblots with antibodies raised against the *K. pneumoniae* O12 antigen, which is identical to the O-antigen from *R. terrigena* ATCC 33257. The immunoreactivity of LPS in CWG1217 ($\Delta wzx-wbbK$) containing pKM114 was dramatically reduced in comparison to *R. terrigena* ATCC 33257 whole cell lysate, despite the evident signal in the corresponding silver-stained LPS gel, suggesting an alteration in O-antigen structure (Fig. 3). This anomaly was noted previously in a different *E. coli* background, but the molecular basis was not examined (31).

Differential Antibody Reactivity Depends on Bacteriophage-derived Glucosylation Genes—Addition of the glucosyl side chains in the O16 antigen of *E. coli* K-12 has been attributed to prophage-mediated modification (5). Examination of the deposited genome of *E. coli* DH10b (the parent of Top10) (GenBankTM accession number NC_010473.1) revealed a three-ORF cluster with high sequence identity to *S. flexneri* bacteriophage (*gtr*) glucosylation clusters, belonging to the prophage KplE1 (19). The λ -red recombination system was used to remove the first gene in this cluster, a *gtrA* homologue annotated *yfdG*, which overlaps with the first four nucleotides of the *gtrB* homologue, *yfdH*. GtrA performs an essential step in glucosylation (17). Whole cell lysates from *E. coli* CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) and its *gtrA*⁺ parent, CWG1217, transformed with pKM114 showed the production of equivalent amounts of O-antigen-substituted LPS, as judged by silver stain. However, LPS from the CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) background reacted significantly more strongly with O12 antisera (Fig. 3). Reintroduction of the *gtr* cluster (on pWQ115) into CWG1219 resulted in decreased immunoreactivity, similar to the LPS from the *gtrA*⁺ CWG1217 background. Notably, the banding of O-antigen-substituted LPS molecules is out of register in *gtrA*⁺ and *gtrA*⁻ backgrounds, as expected from the increased size of modified repeat units. We therefore concluded that the decreased immunoreactivity toward the heterologous O-antigen may be due to glucosylation of the polysaccharide chain by a lysogenic bacteriophage-derived *gtr* cluster within the *E. coli* K-12 chromosome.

Elucidation of the Structures of *R. terrigena* O-antigens Expressed in *E. coli* Mutants—LPS was isolated from *E. coli* CWG1217 ($\Delta wzx-wbbK$) and CWG1218 ($\Delta wzx-wbbK \Delta gtrA$) transformants, both harboring pKM114. Purified LPS samples

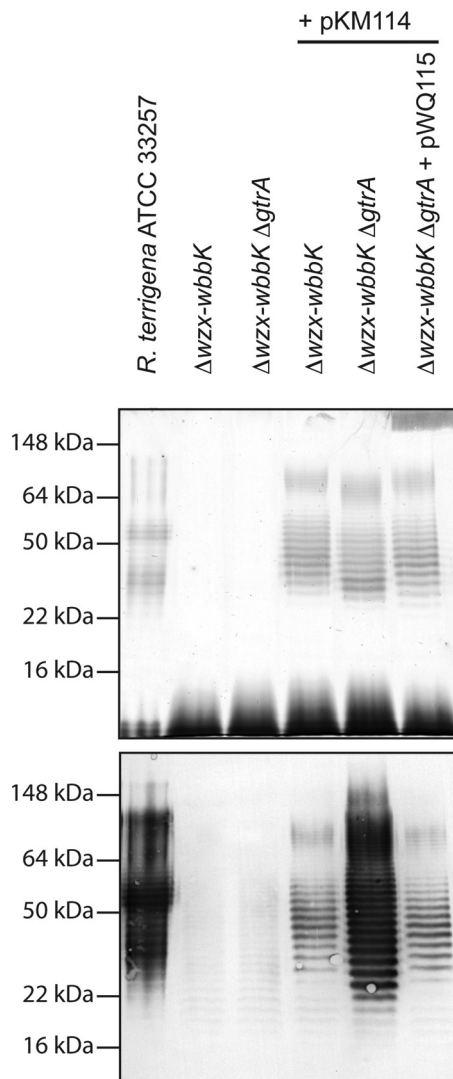


FIGURE 3. Reaction of LPS from *R. terrigena* ATCC 33257 and *E. coli* recombinant strains with antibodies raised against the *K. pneumoniae* O12 antigen. The figure shows the silver-stained SDS-PAGE LPS profile (upper panel) and the corresponding immunoblot (lower panel). The samples were proteinase K-treated whole cell lysates. The markers indicated on the left are protein standards to allow comparison of LPS migrations in different backgrounds.

were degraded with 2% AcOH, and the released polysaccharides were isolated by gel chromatography on Sephadex G-50 for NMR spectroscopy.

Analysis of ¹H and ¹³C NMR spectra (Fig. 4, bottom panel, and Table 1), as well as the ¹H,¹³C heteronuclear single-quantum coherence spectrum of the O-antigen expressed in CWG1218 ($\Delta wzx-wbbK \Delta gtrA$) and comparison with data reported previously (31), confirmed that it possesses a disaccharide repeat unit structurally identical to that of the *R. terrigena* ATCC 33257 and *K. pneumoniae* O12 O-antigen (Fig. 1). This finding is in agreement with the observed wild-type level reactivity of the recombinant LPS with anti-*K. pneumoniae* O12 antibodies (Fig. 3).

In contrast, the ¹³C NMR spectrum of the O-antigen expressed in *E. coli* CWG1217 ($\Delta wzx-wbbK$) demonstrated a regular structure with a trisaccharide repeat unit (Fig. 4, top panel). The spectrum contained signals for three sugar resi-

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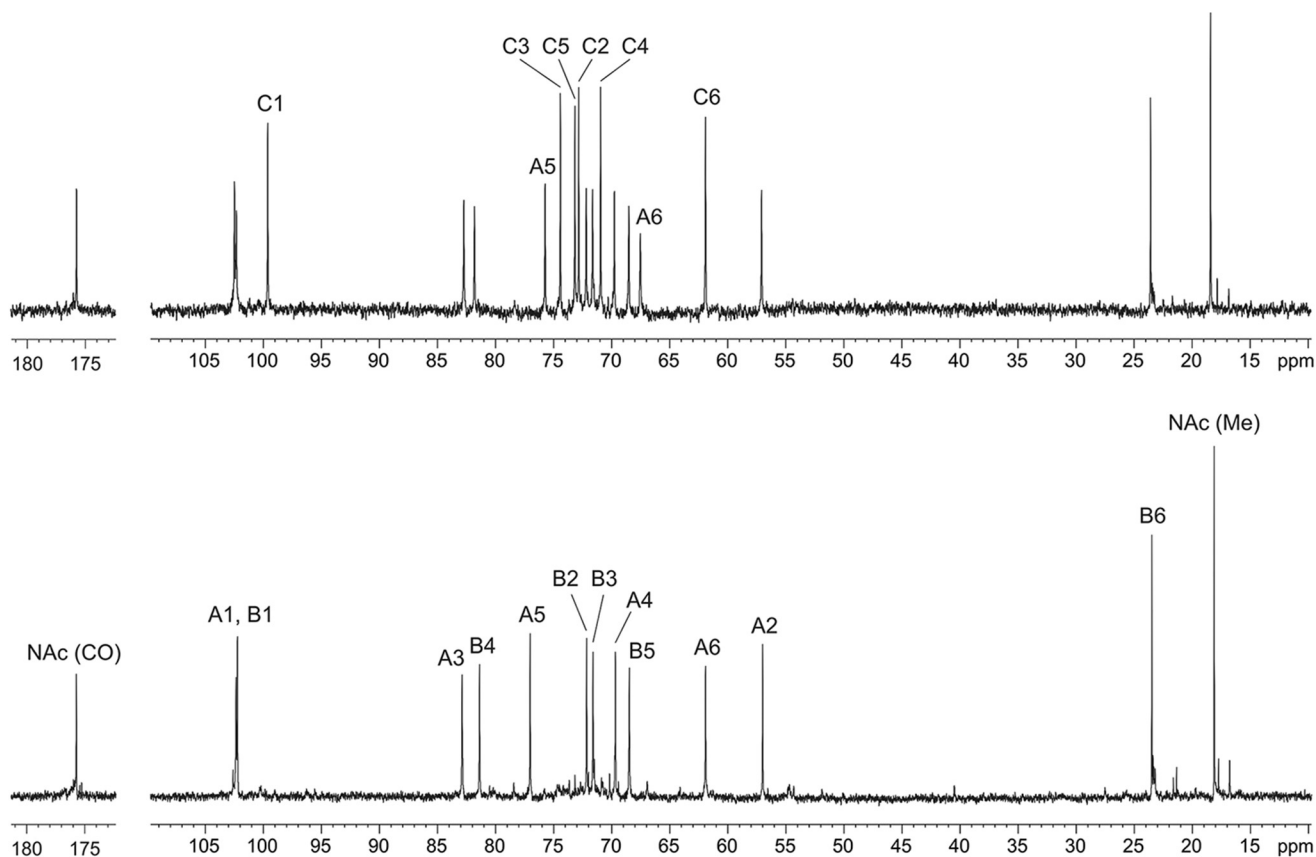


FIGURE 4. ^{13}C NMR spectra of the O-polysaccharides. The spectra represent samples from *E. coli* CWG1217 ($\Delta wzx-wbbK$) (top panel) and CWG1218 ($\Delta wzx-wbbK \Delta gtrA$) (bottom panel) harboring pKM114. The numbers refer to carbons in sugar residues designated as shown in Table 1. The displacement of the A6 and A5 signals (C-6 and C-5 of GlcNAc) is due to positive α -effect on the linkage carbon and negative β -effect on the neighboring carbon and indicates the position of glucosylation.

TABLE 1
 ^1H and ^{13}C NMR chemical shifts (δ , ppm)

Sugar residue	C-1, H-1	C-2, H-2	C-3, H-3	C-4, H-4	C-5, H-5	C-6, H-6 (6a, 6b)
<i>R. terrigena</i> O-polysaccharide produced in <i>E. coli</i> CWG1217 ($\Delta wzx-wbbK$)						
$\rightarrow 3$ - β -D-GlcpNAc-(1 \rightarrow ^a)	A 102.5, 4.82	57.0, 3.80	82.7, 3.61	69.7, 3.60	75.7, 3.62	67.5, 3.78, 3.94
$\rightarrow 4$ - α -L-Rhap-(1 \rightarrow)	B 102.3, 4.85	72.2, 3.74	71.6, 3.81	81.8, 3.59	68.4, 3.99	18.4, 1.26
α -D-Glcp-(1 \rightarrow)	C 99.6, 4.96	72.8, 3.55	74.4, 3.74	70.9, 3.41	73.1, 3.69	61.8, 3.75; 3.84
<i>R. terrigena</i> O-polysaccharide produced in <i>E. coli</i> CWG1218 ($\Delta wzx-wbbK \Delta gtrA$)						
$\rightarrow 3$ - β -D-GlcpNAc-(1 \rightarrow ^a)	A 102.2, 4.80	57.0, 3.78	82.9, 3.60	69.6, 3.50	77.0, 3.42	61.9, 3.75, 3.91
$\rightarrow 4$ - α -L-Rhap-(1 \rightarrow)	B 102.3, 4.85	72.1, 3.74	71.6, 3.81	81.4, 3.60	68.4, 3.99	18.1, 1.27

^a Signals for NAc groups are at δ_{H} 2.05, δ_{C} 23.5 (CH_3), and 175.7 (CO).

dues, including those for anomeric carbons at δ 99.6, 102.3, and 102.5; one nitrogen-bearing carbon at δ 57.0, one $\text{H}_3\text{C}-\text{CH}$ group at δ 18.4; two HOCH_2-C groups at δ 61.8 and 67.5; and one *N*-acetyl group at δ 23.5 (CH_3) and 175.7 (CO). The ^1H NMR spectrum showed signals for three anomeric protons at δ 4.82, 4.85, and 4.96; one $\text{C}-\text{CH}_3$ group at δ 1.26; one *N*-acetyl group at δ 2.05; and other protons in the region δ 3.41–3.94 (Table 1). All signals in the NMR spectra were assigned by using two-dimensional $^1\text{H}, ^1\text{H}$ correlation spectroscopy, total correlation spectroscopy, NOESY (supplemental Figs. S1–S3), $^1\text{H}, ^{13}\text{C}$ heteronuclear single-quantum coherence (Fig. 5), and heteronuclear multiple-bond correlation spectroscopy experiments (supplemental Fig. S4) (Table 1). Analysis of ^1H and ^{13}C NMR chemical shifts and comparison with data on free monosaccharides reported previously (40), intraresidue H,H and H,C correlations, and the coupling constants revealed spin systems for β -GlcN

(residue A), α -Rha (residue B), and α -Glc (residue C), all in the pyranose form. The presence of a GlcN H-2/*N*Ac CO correlation at δ 3.80/175.7 observed in the heteronuclear multiple-bond correlation spectroscopy experiment indicated that GlcN residues are *N*-acetylated (supplemental Fig. S4).

The glycosylation pattern in the repeat unit was inferred from significant downfield displacements of the signals for the linkage carbons, C-3 and C-6 of β -GlcNAc and C-4 of α -Rha (at δ_{C} 82.7, 67.5, and δ_{C} 81.8, respectively), compared with their positions at δ_{C} 74.8, 61.8, and 73.2, in the corresponding non-substituted monosaccharides (40). The chemical shifts for C-2 to C-6 of α -Glc revealed no significant differences, indicating that glucose occupies the terminal position in a side chain. Finally, the monosaccharide sequence in the repeat unit was determined by the following inter-residue cross-peaks between anomeric protons and linkage carbons in the heteronuclear

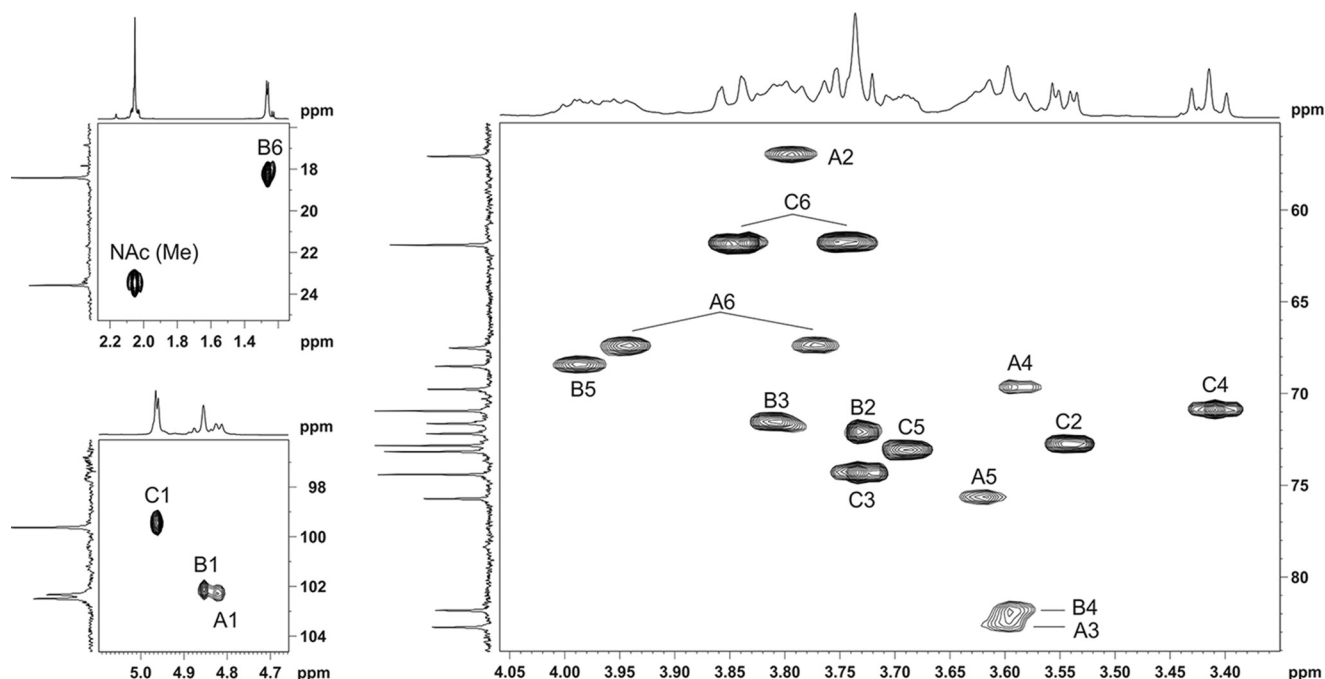


FIGURE 5. Partial ^1H , ^{13}C heteronuclear single-quantum coherence spectrum of the O-polysaccharide of *E. coli* CWG1217 $\Delta wzx-wbbK$ harboring the *R. terrigena* O-antigen gene cluster. The corresponding parts of the ^1H and ^{13}C NMR spectra are shown along the axes. The numbers refer to H/C pairs in sugar residues, designated as shown in Table 1.

multiple-bond correlation spectroscopy spectrum: GlcNAc H-1/Rha C-4, Rha H1/GlcNAc C-3, and Glc H-1/GlcNAc C-6 (supplemental Fig. S4).

Thus, the O-antigen expressed in *E. coli* CWG1217 $\Delta wzx-wbbK$ has the structure shown in Fig. 1. It differs from *R. terrigena* O-antigen by the presence of α -Glc residue attached to C-6 of GlcNAc, which is the same position observed in the *E. coli* O16 antigen. No minor signals that would correspond to nonglycosylated polysaccharide were observed in the NMR spectra.

O-antigen Modification Is Dependent on a Functional ABC Transporter—A key feature of the phage-mediated glucosylation reaction is its periplasmic location. To confirm the same location in the recombinant bacteria, we varied the amount of expression of the *wzt* gene (encoding the NBD of the ABC transporter) to control the flow of completed O-antigen across the inner membrane to the expected site of glucosylation and examined the effect on immunoreactivity. In *E. coli* K-12 hosts, the transport of an O-antigen produced by this type of pathway is dependent on the cognate ABC transporter (18). *E. coli* CWG1217 ($\Delta wzx-wbbK$) and CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) were transformed with pWQ677 carrying *wbbL-wzm-wbbB* under the control of the Tet promoter and pWQ114 where expression of a N-terminally FLAG-tagged version of Wzt was regulated by the arabinose-inducible pBAD promoter. Expression of the biosynthesis genes was induced with a constant amount of anhydrotetracycline, whereas *wzt* was induced with varying L-arabinose. In the presence of 0.4% glucose, *wzt* (NBD) expression was repressed, and the silver-stained gel revealed no detectable high molecular weight laddering indicative of O-antigen-substituted lipid A core in either CWG1217 or CWG1219 (Fig. 6, top panel). In the absence of transport, cytosolic Und-PP-linked glycan is expected to accumulate, but these mole-

cules are not seen in silver-stained profiles. The Und-PP-linked intermediates seen under these conditions show strong reactivity with the antibody in the immunoblot, consistent with the absence of glucosylation (Fig. 6, middle panel). Induction of *wzt* expression led to detectable amounts of FLAG-Wzt in the cell lysates (Fig. 6, bottom panel) and the formation of O-antigen-substituted lipid A core in both strain backgrounds. Comparison of the silver-stained profiles shows the individual bands of O-antigen-containing LPS molecules in CWG1217 and CWG1219 are not in register, as expected for molecules that differ in the presence or absence of glucose residues. Following activation of transport in *E. coli* CWG1217 ($\Delta wzx-wbbK$), the immunoreactive signal, consisting of both Und-PP and lipid A core-linked glycan, is reduced. This is the expected outcome if modification of reactive nonglycosylated intermediates occurs in the periplasm prior to their transition into the poorly reactive mature LPS containing glucosylated O-antigen. In contrast, the high level of immunoreactivity of the glycans in CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) remained unchanged when transport and completion of the LPS molecule were activated. These observations are therefore entirely consistent with the currently understood mechanism and location of *gtr* glucosylation.

Discussion

Bacteriophage-encoded activities play an important role in serological diversity in Gram-negative bacteria. However, attributing O-antigen glucosylation to lysogenic bacteriophages requires both a solved glycan structure and identification of the context and content of the O-antigen biosynthesis loci. Although these types of comprehensive data are available for some species (e.g. *E. coli*, *Salmonella*, *Shigella*), this is not the case for many other bacteria. In all of the known examples, the target O-antigen for glucosylation is synthesized in a Wzy-

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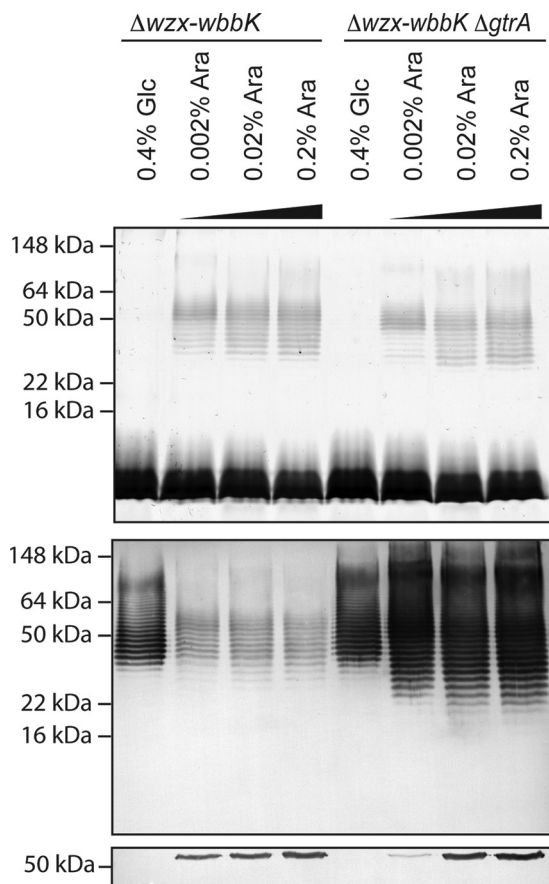


FIGURE 6. O-antigen modification is dependent on a functional ABC transporter. The samples were proteinase K-treated whole cell lysates of *E. coli* CWG1217 ($\Delta wzx-wbbK$) and CWG1219 ($\Delta wzx-wbbK \Delta gtrA$) transformed with pWQ677 (carrying *wbbL-wzm-wbbB*) and pWQ114 (encoding FLAG-Wzt). Gene expression was induced with 2.5 ng ml^{-1} anhydrotetracycline to give a constant level of the biosynthesis enzymes. The amount of FLAG-Wzt (NBD) was varied by repression with glucose or induction with different concentrations of L-arabinose. The presence of the O12 antigen in LPS and Und-PP-linked glycans was assessed using silver stain (top panel) and immunoblotting (middle panel) of proteinase K-treated whole cell lysates. The silver stain profile detects only lipid-A linked O-antigen, whereas the immunoblot detects O-antigen linked to both lipid-A and UndPP. The Western immunoblot of FLAG-tagged Wzt confirmed the presence of the NBD (bottom panel). The markers indicated on the left are protein standards to allow comparison of LPS migrations in different backgrounds.

dependent pathway. This study exploited a heterologous system, to provide the proof of principle that an ABC transporter-dependent mechanism poses no intrinsic mechanistic barrier to phage-mediated glucosylation and could potentially occur in natural isolates or in vaccine strains producing recombinant O-antigens. Glucosylation was dependent on genes encoded by a prophage in *E. coli* K-12, and the O-antigen was found to be α -glucosylated at the position C-6 of the GlcNAc residues in the O-antigen repeat unit, the same site seen in the *E. coli* O16 antigen. That is the same position modified in the O-antigen of *S. flexneri* isolates infected with lysogenic phage SfIV (5) (Fig. 1).

Given its effect on serotype, glucosylation is an important consideration in vaccine strategies (41, 42). Molecular modeling studies have established that glucosylation alters the conformation of *Shigella* O-antigens. In general, it affects the conformation of the nearest backbone linkage, but the influence

varies, depending on the precise site modified (41). In some serotypes, glucosylation can lead to a substantial (up to 50%) physical shortening of the O-antigen chain (43). In many Gram-negative bacteria, the O-antigen is a critical determinant in resistance to serum killing (44). Although glucosylation does not appear to change this role in *Salmonella* and *Shigella* (43, 45, 46), other cellular properties are certainly impacted in a species-specific manner. In *Shigella*, glucosylation leads to greater invasion, which is correlated to enhanced function and exposure of the type 3 secretion system (43), and elevated acid tolerance (47). In *Salmonella*, glucosylation is associated with virulent isolates (48) but is not a stable property leading to antigenic (form) variation in the O serotype (45, 46, 49). Glucosylation is induced by exposure to macrophages, and although not required for invasion and systemic spread, it appears to enhance long term colonization (46).

The results presented here raise interesting questions about the glucosylation process itself. Biosynthetic experiments suggested that the acceptor for the modification reaction is the Und-PP-linked O-antigen polymerization product rather than the individual Und-PP-linked repeat units (12). Consistent with this proposal, the LPS products of *wzy* mutants, which contain a single O-repeat unit, are not modified in *Salmonella* (50, 51). Likewise, the first repeat unit of *S. flexneri* O-antigen is not subject to glucosylation (7, 53, 54). However, in bacteria producing full-length LPS forms, both glucosylated and nonglycosylated glycans can be produced by the same culture, and when they were separated, the modification was confined to LPS molecules with O-antigens exceeding ~ 6 repeat units in length (55). In Wzy-dependent systems, growth of the glycan chain occurs one repeat unit at a time at the reducing terminus, building on new Und-PP-linked intermediates delivered by Wzx. The distribution of glucosyl modifications has been interpreted as reflecting a process where the catalytic site of the GtrC/Gtr* enzyme (mediating the final transfer) has no access to shorter polymerization products or lacks sufficient affinity for those products to allow modification. Only when the glycan chain reaches a particular size would it become a substrate for modification. The structure of the recombinant glucosylated O-antigen described here is not consistent with a large region of unmodified material, but NMR analysis lacks the sensitivity to reveal one or two unmodified repeat units at the reducing terminus of long chains. Each O-antigen chain retains part of the core oligosaccharide at its reducing terminus, which is released from lipid A with acetic acid. Signals for the core region are not evident because the NMR spectra are dominated by signals from the repeat units of long chains, and the same would apply to a core oligosaccharide linked to one or two unmodified core-proximal repeat units. In *Salmonella*, the extent of the unmodified region was determined by comparing mobilities of LPS molecules bearing very short O-antigen chains by SDS-PAGE in the presence or absence of glucosylation. Unfortunately, the *R. terrigena* assembly pathway precludes a similar approach because it involves a chain termination mechanism designed to generate only longer glycan chains, where the limits of SDS-PAGE resolution preclude such comparisons (Fig. 6). The underlying strategy for this type of chain length regulation has recently been resolved for *E. coli* O9a antigen, a prototype for

the ABC transporter-dependent pathway (56, 57). What is clear from NMR spectra is that the recombinant system in *E. coli* lacks the phase variation that results in a combination of modified and unmodified chains in some *Salmonella* isolates (8, 55).

The WaaL enzyme that ligates nascent O-antigens to lipid A is specific for Und-PP-linked glycan donors, and its mechanism and architecture are conserved across species (58). This enzyme is unaffected by the pathway of O-antigen biosynthesis. For example, in addition to native Wzy-dependent O-antigens, *E. coli* K-12 hosts effectively ligate the products of ABC transporter pathways. This property is shown here with the *R. terrigena* glycan and in previous reports with different O-antigens of *Klebsiella* (34, 52). Although the preceding steps may vary, immediately prior to ligation the Und-PP-linked glycans from either of the assembly strategies are similarly confined to the interface of the cytoplasmic membrane and the periplasm. This presumably offers comparable access of the glucosylation machinery to its glycan substrate but rules out any essential targeted interaction of Gtr proteins with specific components of an O-antigen assembly pathway.

In conclusion, the ABC transporter-dependent pathway provides no intrinsic mechanistic barrier for bacteriophage-derived modification systems. This possibility must now be considered in studies correlating O-antigen structures with genetic determinants and in the use of recombinant strains in vaccine strategies.

Author Contributions—E. M., O. G. O., J. D. K., and C. W. conceived the study. E. M., O. G. O., and J. D. K. performed the experiments. E. M., O. G. O., J. D. K., and C. W. analyzed and interpreted the data. E. M., O. G. O., and C. W. wrote the manuscript, and J. D. K. provided input for the final draft.

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