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Roles of Predicted Glycosyltransferases in the Biosynthesis of the *Rhizobium etli* CE3 O Antigen

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The *Rhizobium etli* CE3 O antigen is a fixed-length heteropolymer. The genetic regions required for its synthesis have been identified, and the nucleotide sequences are known. The structure of the O antigen has been determined, but the roles of specific genes in synthesizing this structure are relatively unclear. Within the known O-antigen genetic clusters of this strain, nine open reading frames (ORFs) were found to contain a conserved glycosyltransferase domain. Each ORF was mutated, and the resulting mutant lipopolysaccharide (LPS) was analyzed. Tricine SDS-PAGE revealed stepwise truncations of the O antigen that were consistent with differences in mutant LPS sugar compositions and reactivity with O-antigen-specific monoclonal antibodies. Based on these results and current theories of O-antigen synthesis, specific roles were deduced for each of the nine glycosyltransferases, and a model for biosynthesis of the *R. etli* CE3 O antigen was proposed. In this model, O-antigen biosynthesis is initiated with the addition of *N*-acetyl-quinovosamine-phosphate (QuiNAc-P) to bactoprenol-phosphate by glycosyltransferase WreU. Glycosyltransferases WreG, WreE, WreS, and WreT would each act once to attach mannose, fucose, a second fucose, and 3-*O*-methyl-6-deoxytalose (3OMe6dTal), respectively. WreH would then catalyze the addition of methyl glucuronate (MeGlcA) to complete the first instance of the O-antigen repeat unit. Four subsequent repeats of this unit composed of fucose, 3OMe6dTal, and MeGlcA would be assembled by a cycle of reactions catalyzed by two additional glycosyltransferases, WreM and WreL, along with WreH. Finally, the O antigen would be capped by attachment of di- or tri-*O*-methylated fucose as catalyzed by glycosyltransferase WreB.

Lipopolysaccharide (LPS) is the major surface component of Gram-negative bacteria and consists of lipid A, a core oligosaccharide, and an O-specific polysaccharide or O antigen (1). While the composition, structure, and synthesis of lipid A and core regions are often similar, especially between closely related species, the O-antigen portions can vary considerably even between strains of the same species (1, 2). O antigens can consist of a polymer of a single sugar (homopolymer) or many different sugars (heteropolymer). In both types of polymers, the number of repeating units can be either fixed or varied, giving rise to O antigens of uniform and modal lengths, respectively (1, 2).

Further variation occurs due to the fact that a given O antigen is synthesized by one of two main types of pathways, one dependent on Wzy polymerase and the other involving an ATP-binding cassette (ABC) transporter (1). In each pathway, the initial reaction attaches a sugar-phosphate to bactoprenol phosphate (BpP), creating the first in a series of pyrophosphoryl-bactoprenol-linked (BpPP) oligosaccharide intermediates (3–5). O-antigen biosynthesis by the Wzy pathway assembles on the cytoplasmic face of the inner membrane a single repeating unit on BpPP, which is then "flipped" across the inner membrane by protein Wzx. In the periplasm, a growing O-antigen chain on another BpPP is transferred, through the action of an O-antigen polymerase encoded by *wzy*, to the nonreducing end of the newly "flipped" repeating unit. Thus, elongation of the O antigen occurs by extension at the reducing end in this type of O-antigen synthesis, like peptidoglycan synthesis. In contrast, O-antigen biosynthesis by the ABC transporter pathway assembles the *complete* O antigen on BpPP at the cytoplasmic face of the inner membrane. The subsequent glycosyltransferase reactions elongate the O antigen by the addition of sugars at the nonreducing end (6). An ABC transporter, typically composed of subunits Wzm and Wzt (7, 8), translocates the completed O-antigen-BpPP intermediate to the periplasmic face of the inner membrane. Both pathways converge again at the point when the O antigen is ligated to the lipid A-core regions (9, 10), and the mature LPS is transported to the outer membrane (11, 12).

The O-antigen portion of the *Rhizobium etli* CE3 LPS is a heteropolymer of uniform length, most molecules having five repeats of a three-sugar unit (Fig. 1) (13). Having homologs of *wzm* and *wzt* in the chromosomal O-antigen genetic cluster (Fig. 2), *R. etli* CE3 is postulated to synthesize its O antigen by the ABC transporter pathway (2, 14, 15). For this type of synthesis, the terms primer, adaptor, repeat-unit domain, and chain terminator have been proposed for different portions of the O-chain (1, 4), as indicated in Fig. 1. Based on considerations discussed below, it is likely that the primer sugar of the CE3 O antigen is 2-*N*-acetamido-2,6-dideoxyglucose (*N*-acetyl-quinovosamine [QuiNAc]). Attached to the primer are the four sugars of the adaptor region, which are defined here as sugars added through the action of glycosyltransferases that act once per O antigen. In strain CE3, mannose and the first fucose (Fuc^{I}) are obvious components of the adaptor region, but perhaps less obvious are two sugars in the first instance of the repeat unit. One of these latter two sugars is a fucose (Fuc^{II}), which is attached to Fuc^{I}, whereas the remaining repeat-unit fucoses (Fuc^{III-VI}) are attached to methyl glucuronate (MeGlcA). Therefore, the enzyme that catalyzes the addition of Fuc^{II} should act only once. The last sugar of the adaptor, either a

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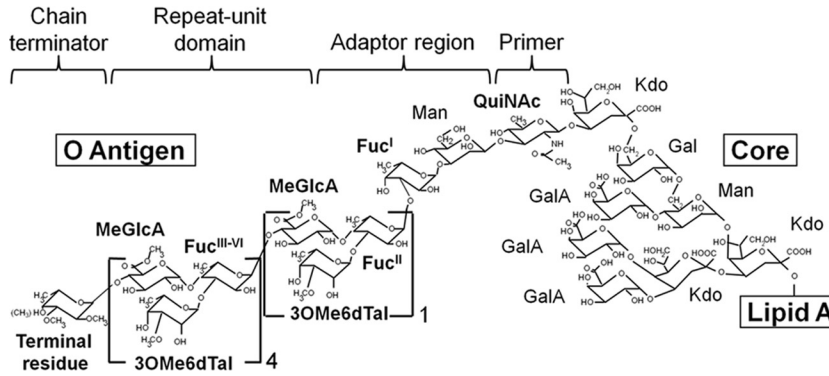

FIG 1 *R. etli* CE3 LPS I structure (13, 45). The O antigen is hypothesized to begin with *N*-acetyl-quinovosamine (QuiNAc) at the reducing end (see the text) followed by mannose (Man) and fucose (Fuc) residues. After that are five repeats (in most molecules) of a unit consisting of Fuc, 3-*O*-methyl-6-deoxytalose residue (3OMe6dTal), and methyl glucuronate (MeGlcA). One repeating unit is shown separately in order to emphasize that two of its residues are added by glycosyltransferases that likely act only once and therefore belong to the biosynthetic adaptor region (see the text). Nonstoichiometric *O*-methylation occurs at position 2 of the fucose (2OMeFuc) of the repeating units (not shown) (13, 35). The O-antigen backbone is capped with a 2,3-di-*O*-methylfucose terminal residue that is variably *O*-methylated at position 4, as indicated by parentheses. O-antigen-specific sugars are shown in boldface type, and residues within the primer, adaptor region, repeat-unit domain, and chain terminator are indicated. The structure of the core region, consisting of sugars 3-deoxy-D-manno-2-octulonic acid (Kdo), galacturonic acid (GalA), mannose (Man), and galactose (Gal), is also shown.

MeGlcA or 3-*O*-methyl-6-deoxytalose (3OMe6dTal), is also added to an L-fucose, but only to Fuc^{II} and not to Fuc^{I} (13). One way to prevent its addition to Fuc^{I} uses the fact that the responsible enzyme acts on the acceptor substrate only if it ends in two linked fucoses (Fuc^{II}-Fuc^{I}-), which is true only at this point in the synthesis. An enzyme with such a specificity should act only once in the formation of an O-antigen chain. After completion of the four-sugar adaptor, synthesis would then shift to three additional enzymes, which would recognize portions of the acceptor substrate that repeatedly appear in a cycle of three reactions resulting in the assembly of the complete repeat-unit domain. A final enzyme would catalyze the addition of the terminal residue, 2,3-di-*O*-methylfucose, which is variably *O*-methylated at position 4. This model of CE3 O-antigen synthesis predicts nine different glycosyltransferases.

Nearly all the genes required to synthesize the known *R. etli* CE3 O-antigen structure are located in three separate loci in the genome (2, 14, 16). Except for well-known orthologous genes, such as *fcl* and *gmd*, genes devoted to *R. etli* CE3 O-antigen synthesis have been given the designation *wre* (Fig. 2) (17) in order to conform with the conventions of naming O-antigen-related genes (18). (For example, genes in Fig. 2 designated *fcl*, *gmd*, *wreE*, *wreU*, and *wreV* in various previous contexts have been referred to as *nolK*, *noeL*, *exoU*, *lps*β*1*, and *lps*β*2*, respectively [14, 19].) Besides genes for the ABC O-antigen transporter (15), the *wre* loci include genes predicted to encode proteins for the synthesis of nucleotides of O-antigen-specific sugars (2, 19–21). Also within two of these loci are *wre* genes predicted to encode the glycosyltransferases needed to connect together the sugars from these sugar nucleotides. The chromosomal genetic cluster contains eight open reading frames (ORFs) whose sequences are predicted to encode glycosyltransferases, and one such ORF is located on plasmid pCFN42b (Fig. 2). One of these ORFs (*wreB*) has been shown to be required for the addition of the terminal residue of the O antigen (17). In this study, the LPSs from mutants defective in each of the other predicted glycosyltransferase ORFs were analyzed. Each ORF was shown to be required for O-antigen synthesis. From the

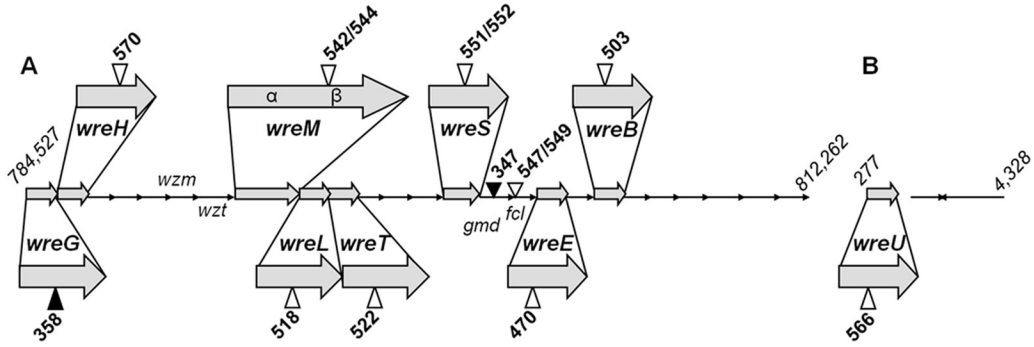

FIG 2 *R. etli* CE3 O-antigen genetic clusters encoding putative glycosyltransferases. (A) The *R. etli* CE3 chromosomal O-antigen genetic cluster spans nucleotides 784,527 to 812,262 of the genome sequence (14) and consists of 25 putative ORFs. (B) The O-antigen genetic cluster on plasmid pCFN42b spans nucleotides 277 to 4,328 (14) and consists of 3 putative ORFs. The predicted glycosyltransferases are enlarged, and other related ORFs are indicated. ORFs RHE_PB00001, RHE_CH00748, RHE_CH00749, RHE_CH00755, RHE_CH00756, RHE_CH00757, RHE_CH00761, RHE_CH00764, and RHE_CH00766 were named *wreU*, *wreG*, *wreH*, *wreM*, *wreL*, *wreT*, *wreS*, *wreE*, and *wreB*, respectively. The relative locations of mutations are indicated by white triangles for mutations created by insertion of antibiotic cassettes and black triangles for mutations created by Tn*5* mutagenesis. The strain numbers carrying these mutations are indicated above or below the triangles.

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description and/or genotype or phenotype[a]	Reference or source
Bacterial strains		
R. etli strains		
CE3	Wild-type strain; *str-1*	22
CE113	CE3 derivative; *str-1* Δ*wreU*	22
CE347	CE3 derivative; *str-1 gmd*::Tn*5*	23
CE358	CE3 derivative; *str-1 wreG*::Tn*5*	23
CE470	CE3 derivative; *str-1 wreE*::Km	24
CE503	CE3 derivative; *str-1 wreB*::Gm	17
CE518	CE3 derivative; *str-1 wreL*::Km	This work
CE522	CE3 derivative; *str-1 wreT*::Km	This work
CE532	CE3 derivative; *str-1 lpcA*::Km	This work
CE542	CE3 derivative; *str-1 wreM*β::Km	17
CE544	CE3 derivative; *str-1 wreM*β::Gm	17
CE547	CE3 derivative; *str-1 fcl*::Km	This work
CE549	CE3 derivative; *str-1 fcl*::Gm	This work
CE551	CE3 derivative; *str-1 wreS*::Gm	This work
CE552	CE3 derivative; *str-1 wreS*::Km	This work
CE566	CE3 derivative; *str-1 wreU*::Km	This work
CE570	CE3 derivative; *str-1 wreH*::Km	This work
E. coli strains		
INVαF′	F′ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169* λ^-	Invitrogen
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z*ΔM15]	Promega
MT616	*pro thi endA hsdR supE44 recA*-J6 pRK2013Km::Tn*9*	25
Plasmids		
pEX18Tc	Suicide plasmid; Tc^r *oriT*$^+$ *sacB*$^+$	26
pUCGm	*aaC1* gene cassette; Gm^r	27
pBSL15	*nptII* gene cassette; Km^r	28
pBSL86	*nptII* gene cassette; Km^r	28
pKT72	*fcl* with Gm cassette inserted at SmaI site in pEX18Tc	This work
pKT73	*fcl* with Km cassette inserted at SmaI site in pEX18Tc	This work
pKT59	*lpcA* with Km cassette inserted at SalI site in pEX18Tc	This work
pLS15	*wreH* with Km cassette inserted at BamHI site in pEX18Tc	This work
pJB45	*wreL* with Gm cassette inserted at SmaI site in pEX18Tc	This work
pKT81	*wreS* with Km cassette inserted in EcoRV deletion in pEX18Tc	This work
pKT82	*wreS* with Gm cassette inserted in EcoRV deletion in pEX18Tc	This work
pJB47	*wreT* with Gm cassette inserted at XhoI site in pEX18Tc	This work
pKT98	*wreU* with Km cassette inserted in SalI deletion in pEX18Tc	This work
pFAJ1708	Expression vector; Tc^r	29

[a] The *str-1* gene confers resistance to streptomycin. Km, insertion of kanamycin resistance cassette from pBSL86 or pBSL15; Gm, insertion of gentamicin resistance cassette from pUCGm.

LPS phenotypes, the encoded glycosyltransferases were assigned particular roles in synthesis of the primer, adaptor, or repeat-unit domains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *Rhizobium etli* strains (Table 1) were derived from strain CE3, which is itself a streptomycin-resistant derivative (22) of wild-type strain CFN42, whose genome nucleotide sequence has been determined (14). *R. etli* strains were grown to stationary phase at 30°C on a rotating shaker in TY liquid medium (0.5% tryptone [Difco Laboratories], 0.3% yeast extract [Difco], and 10 mM $CaCl_2$). *Escherichia coli* strains were grown in Luria-Bertani (LB) liquid medium (1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl) to stationary phase at 37°C on a rotating shaker. Agar medium contained 1.5% Bacto agar (Difco).

Computer analysis of predicted protein sequences. Amino acid sequences of open reading frames (ORFs) deduced from *R. etli* CE3 genomic DNA sequence (14) were compared to nonredundant (nr) protein sequences by the BLAST search tool at the National Center for Biotechnology Information (NCBI) (30) and the Carbohydrate Active Enzyme (CAZy) database (http://www.cazy.org/). Alignment of amino acid sequences was performed using ClustalW2, version 2.0.10.

Materials and techniques for DNA isolation. Genomic DNA was isolated from *R. etli* strains using the AquaPure genomic DNA isolation kit (Bio-Rad) for use in cloning. *E. coli* JM109 (Promega) or INVαF′ (Invitrogen) competent cells were transformed (31), and plasmids were isolated from *E. coli* using QIAprep spin miniprep (Qiagen). DNA was recovered from agarose gels using Geneclean II kit (MP Biomedicals, LLC.) and modified with restriction enzymes purchased from New England BioLabs (NEB) (Beverly, MA). Custom primers were synthesized by Eurofins MWG Operon (Huntsville, AL).

Cloning and site-directed mutagenesis. Using PCR, *fcl*, *lpcA*, *wreH*, *wreL*, *wreS*, *wreT*, and *wreU* were amplified separately from *R. etli* CE3 genomic DNA (for primer sequences, see Fig. S1 in the supplemental material). PCR products were inserted into plasmid pEX18Tc (26). Either the gentamicin resistance cassette (Gm) from plasmid pUCGm (27) or the

kanamycin resistance cassette (Km) from plasmid pBSL15 (28) or plasmid pBSL86 (28) was inserted (Table 1).

Separately, plasmids carrying the mutated ORFs were transferred into *R. etli* CE3 by triparental mating (32) with plasmid-mobilizer strain MT616 (25) on TY agar plates. CE3 transconjugants containing these constructs were selected and purified as previously described (17, 26). Double-crossover recombinants were screened on TY agar plates supplemented with 1 μg of tetracycline/ml. Of the recombinants that were sensitive to 1 μg of tetracycline/ml and resistant to 8% sucrose on TY agar, colonies were saved after verifying by PCR the absence of the wild-type allele and the presence of a mutant allele. The resulting *R. etli* strains were CE549 and CE547 (*fcl*), CE532 (*lpcA*), CE570 (*wreH*), CE518 (*wreL*), CE551 and CE552 (*wreS*), CE522 (*wreT*), and CE566 (*wreU*) (Table 1).

Complementation of *wre* mutants with wild-type DNA. Wild-type *wre* gene sequences were cloned separately into plasmid pFAJ1708 (29). A recombinant plasmid carrying a particular wild-type gene was transferred by triparental mating, as described above, into a strain mutated in that particular gene. Transconjugants were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, 5 μg of tetracycline/ml, and 30 μg of kanamycin/ml or 30 μg of gentamicin/ml. The presence of the respective wild-type gene restored wild-type LPS I, as determined by SDS-PAGE (see Fig. S3 in the supplemental material).

SDS-PAGE analysis. Bacterial cells from 0.5-ml portions of fully grown cultures were pelleted and extracted in 0.1 ml of 1× SDS-PAGE sample buffer (16) and treated with proteinase K (0.25 mg/ml) at 37°C for 1 h. After centrifugation to remove insoluble material, the SDS extracts were subjected to electrophoresis by discontinuous Laemmli SDS-PAGE (33) with 18% (wt/vol) acrylamide resolving minigels or by discontinuous Tricine SDS-PAGE as described previously (34). LPS in the gels was stained by a previously described periodate-silver method (35).

Immunoblot analysis with MAb. After SDS-PAGE, some minigels were electrotransferred to nitrocellulose before periodate-silver staining of the residual gel contents. The dried nitrocellulose blots were immunostained using monoclonal antibodies (MAb) JIM28 and JIM26 (generous gifts from N. J. Brewin, Norwich, United Kingdom) (17, 36) and anti-rat immunoglobulin M conjugated with alkaline phosphatase as previously described (23, 36).

Purification of LPS. LPS was extracted from washed bacterial cell pellets (from 6.0 liters of culture) by the hot phenol-water method (PWE) (37). LPS was then subjected to dialysis and treated with 0.05 mg/ml RNase A, 15 units/ml DNase I, and 0.05 mg/ml proteinase K. After lyophilization, the LPS from the PWE aqueous phase was purified by gel filtration chromatography using a Sepharose 4B column with TEA-EDTA buffer (0.03 M triethylamine [TEA], 0.01 M EDTA [pH 7.0]), a Sepharose 4B column with ammonium formate buffer (5.0 mM ammonium formate [pH 5.0]), a Sephadex G-150 column with deoxycholic acid (DOC) buffer (0.2 M NaCl, 1.0 mM EDTA, 10 mM Tris, 0.25% DOC) (16, 38), or a Bio-Rad P-100 column with DOC buffer. The LPS content in fractions eluted from the columns was assessed by SDS-PAGE of selected fractions. The LPS-containing fractions were pooled, dialyzed against water, and lyophilized. In the case of DOC buffer, the pooled fractions were dialyzed first against DOC removal buffer (0.004 M Tris, 0.25% NaCl, 10% ethanol [pH 9.3]) and second against water. The LPS from strains CE503 (17) and CE566 was not subjected to purification by gel filtration, rather the PWE aqueous phase was used for sugar composition analysis directly.

LPS compositions. For determination of neutral and amino sugars, LPS preparations were treated with 2 M trifluoroacetic acid for 2 h at 121°C. After reduction with $NaBD_4$ and acetylation, alditol acetate derivatives of the LPS sugars were analyzed by gas chromatography (GC) on a fused silica capillary SP2300 column (60 m long; 0.53-mm inner diameter; 0.20-μm film thickness) (Supelco) (13). The oven temperature was raised 1°C min^{-1} from 150 to 185°C, 10°C min^{-1} from 185 to 245°C, and then maintained at 245°C for 35 min (35). For determination of glucuronic acid and other acidic sugars, purified LPS was subjected to mild acid hydrolysis to effect cleavage at 3-deoxy-D-manno-2-octulonic acid (Kdo) residues. Up to 4 mg of purified LPS was dissolved in 1.0 ml of glass-distilled water and incubated at 100°C for 10 min. Glacial acetic acid was added to a concentration of 1%, and heating at 100°C was continued for an additional 6 h. Lipid A was removed by extracting three times with 500 μl of chloroform. The resulting aqueous phase, containing the O-antigen and core regions, was dried. After methanolysis in methanolic 1 M HCl, reaction with trimethylsilane, and extraction into hexane, the resulting trimethylsilyl-methylglycosides were analyzed by gas chromatography on an SPB-1 column (Supelco) (13). The oven temperature was raised 2°C min^{-1} from 150 to 180°C, 1°C min^{-1} from 180 to 200°C, 5°C min^{-1} from 200 to 300°C, and then maintained at 300°C for 25 min. Identification of GC peaks as particular sugars was confirmed by gas chromatography coupled to mass spectrometry (GC-MS) (data not shown).

Permeabilized cells and GDP-[^{3}H]fucose labeling. Cells were grown in TY medium to stationary phase, inoculated 1:20 into 50 ml of fresh TY medium, and grown at 30°C for about 24 h. Cell growth was measured (optical density at 595 nm [OD_{595}] of ~0.5 to 0.6), and cells were collected at 4,300 × *g* for 20 min. The cell pellet was washed once with 50 ml of 70 mM Tris (pH 8.2) and resuspended in a volume of 70 mM Tris containing 10 mM EDTA (pH 8.2) that gives a final cell concentration corresponding to 100 times that of a suspension giving an OD_{595} of 1.0 (typically 250 to 280 μl) (39, 40). The cell suspension was transferred to a 1.5-ml centrifuge tube, and cells were frozen in a dry ice-ethanol bath and thawed in a 37°C water bath a total of three times. The permeabilized cells were stored at −80°C until use.

The standard reaction mixture (100 μl) contained 70 μl of permeabilized cells, 10 μCi of GDP-[^{3}H]fucose (PerkinElmer; 0.1 mCi/ml, 15 to 35 Ci/mmol), 500 μM UDP-*N*-acetylglucosamine, 500 μM GDP-mannose, and 100 μM NADPH (39, 40). Reaction mixtures were incubated at 12°C for 30 min and stopped by the addition of 0.5 ml of ice-cold 70 mM Tris containing 10 mM EDTA (pH 8.2). After centrifugation, cell pellets were washed twice with 0.3 ml of 70 mM Tris (pH 8.2) and extracted twice with 100 μl of chloroform-methanol-water (1:2:0.3). The combined extracts were dried in a vacuum centrifuge and resuspended in 10 μl of the chloroform-methanol-water mixture. The entire volume was spotted on an aluminum-backed precoated Silica-60 thin-layer chromatography (TLC) plate and eluted in isobutyric acid–1.0 M ammonium hydroxide (5:3) for 8 h (41). The TLC plates were exposed to film at −80°C.

RESULTS

The predicted glycosyltransferase ORFs and mutant constructions. The protein sequences encoded by ORFs *wreG*, *wreH*, *wreL*, *wreT*, *wreS*, *wreE*, *wreB*, and the β domain (17) of *wreM* (*wreM*β) within the O-antigen chromosomal genetic cluster and the *wreU* ORF on endogenous plasmid pCFN42b of *R. etli* CE3 were each predicted to contain a glycosyltransferase domain (see Fig. S2 in the supplemental material). WreU best matched enzymes that specifically transfer sugar phosphates, while the other eight proteins were similar to families of enzymes that transfer sugars from sugar-nucleotide substrates.

Strains were constructed with mutations in each of the nine predicted glycosyltransferase ORFs (Table 1 and Fig. 2; see Fig. S1 in the supplemental material). The approach was to insert antibiotic resistance cassettes that in past work have been shown to not cause polar effects on genes downstream in an operon (17). Lack of polarity was confirmed by complementation of the mutants to the wild-type LPS phenotype when a plasmid carrying only the wild-type allele corresponding to the mutated allele was transferred into a given mutant (Fig. S3). LPSs from these mutant strains were analyzed by SDS-PAGE, immunoblotting, and examining the sugar composition.

SDS-PAGE. SDS-PAGE resolves the LPS of *R. etli* CE3 into two distinct mobility classes (37), LPS I (containing the O antigen as

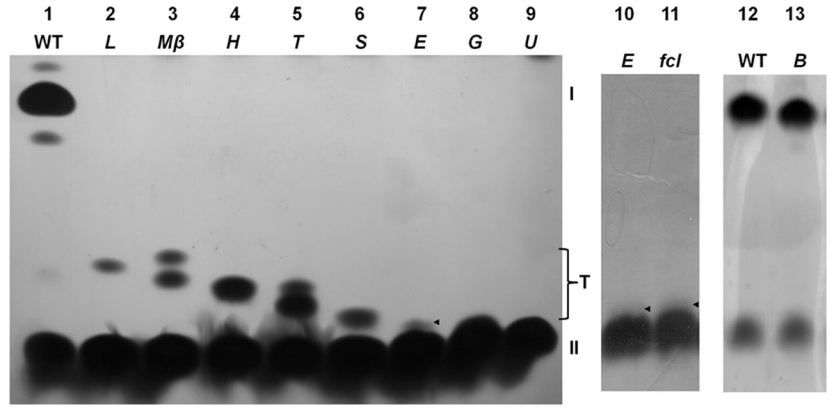

FIG 3 Tricine SDS-PAGE of LPSs from mutants with lesions in the genes encoding the nine predicted glycosyltransferases. The O antigen on average consists of five repeat-unit domains, thereby conferring the major LPS I band in the wild type (WT) (lane 1). The less-abundant bands above and below this major band may be due to O antigens having six and four repeat units, respectively. None of the glycosyltransferase mutants produced LPS I, and some synthesized truncated LPSs of various sizes and abundances. The truncated LPSs of *wreE* and *fcl* mutants are visible above LPS II (small black arrowheads), while *wreG* and *wreU* mutants did not produce a visible truncated LPS. The positions of LPS bands are indicated to the right of the gel as follows: I, LPS I; II, LPS II; T, truncated LPS. Lane 1, WT (CE3); lane 2, *wreL* mutant (CE518); lane 3, *wreM*β mutant (CE542); lane 4, *wreH* mutant (CE570); lane 5, *wreT* mutant (CE522); lane 6, *wreS* mutant (CE551); lane 7, *wreE* mutant (CE470); lane 8, *wreG* mutant (CE358); lane 9, *wreU* mutant (CE566); lane 10, *wreE* mutant (CE470); lane 11, *fcl* mutant (CE549); lane 12, WT (CE3); lane 13, *wreB* mutant (CE503). Lanes 1 to 9, lanes 10 and 11, and lanes 12 and 13 are from gels run separately.

well as the lipid A and core regions) and LPS II (containing only lipid A and core regions). All of the glycosyltransferase mutants lacked the wild-type LPS I band, and several mutants synthesized truncated LPS, which was usually less abundant than LPS I of the wild type as indicated by silver staining (Fig. 3). *wreU* and *wreG* mutants produced only LPS II and no visible, slower-moving bands. On the other hand, *wreE*, *wreS*, *wreT*, and *wreH* mutants gave rise to progressively longer versions of the O antigen, as inferred from the presence of progressively slower-migrating bands, and *wreM*β and *wreL* mutants produced the slowest-moving truncated LPSs. LPS I of the *wreB* mutant was produced in the same abundance as the wild-type LPS I but exhibited a slightly higher mobility (17).

The truncated LPS of the *wreE* mutant was compared to LPS produced by mutants defective in the *fcl* and *gmd* genes. Because Fcl and Gmd are required to convert GDP-mannose to GDP-fucose (2, 42), mutants lacking either enzyme are expected to stop O-antigen synthesis at the point fucose would be added. The truncated LPS of the *wreE* mutant was visible just above LPS II (Fig. 3, small black arrowhead), and this same LPS profile was seen in *fcl* and *gmd* mutants (Fig. 3, lane 11; also data not shown).

Antigenicity. Additional information about the mutant LPSs was gained by probing immunoblots with monoclonal antibodies (MAb) JIM26 and JIM28 (17, 36). Although truncated LPS was resolved best by Tricine SDS-PAGE (Fig. 3), immunoblot staining was much more sensitive after Laemmli-type SDS-PAGE (Fig. 4). MAb JIM26 recognizes the *R. etli* CE3 O antigen (36) and bound the truncated LPSs from *wreB*, *wreM*β, *wreL*, *wreT*, *wreH*, and *wreS* mutants (Fig. 4B and data not shown). MAb JIM28 specifically requires the *R. etli* CE3 O-antigen terminal residue as part of its epitope (17, 43). Only the truncated LPS of the *wreM*β mutant was bound by MAb JIM28, suggesting that the terminal residue was present in the truncated O antigen of this strain (Fig. 4C).

Sugar compositions. The presence or absence of specific O-antigen sugars was investigated by sugar composition analysis of purified mutant LPSs (Table 2). As indicated on SDS-polyacrylamide gels (Fig. 3 and 4), the amount of the O-antigen sugars contributed by the truncated LPSs would be low compared to the amount of sugars of the wild-type LPS I and LPS II. To ensure that background noise or contamination was not mistaken as a low level of a given sugar in a mutant, an *lpcA* mutant was constructed (Table 1) and included as a negative control. In *lpcA* mutants, the galactose of the core region is not attached (44); thus, the O antigen cannot be attached to the truncated core, and the LPS of this mutant should lack the O-antigen sugars entirely (Table 2).

The *wreB* mutant contained all the O-antigen sugars in wild-

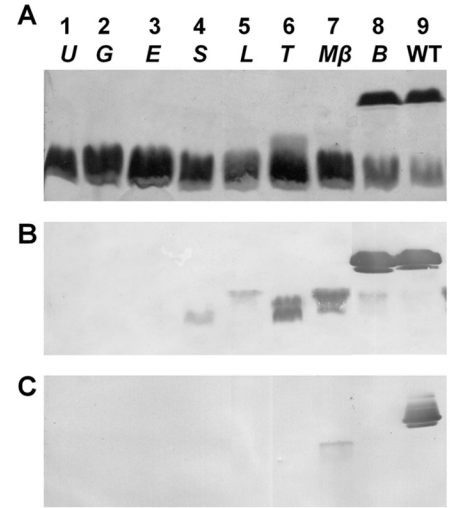

FIG 4 MAb binding to truncated LPSs from mutants with lesions in genes encoding the nine predicted glycosyltransferases. (A) 18% Laemmli SDS-polyacrylamide gel with LPS stained by silver-periodate. (B and C) LPS material from the gel shown in panel A was transferred to nitrocellulose and probed with MAb JIM26 (B) or MAb JIM28 (C). Lane 1, *wreU* mutant (CE113); lane 2, *wreG* mutant (CE358); lane 3, *wreE* mutant (CE470); lane 4, *wreS* mutant (CE551); lane 5, *wreL* mutant (CE518); lane 6, *wreT* mutant (CE522); lane 7, *wreM*β mutant (CE542); lane 8, *wreB* mutant (CE503); lane 9, WT (CE3). The positions of LPS bands are indicated to the right of the gel as follows: I, LPS I; T, truncated LPS; II, LPS II.

TABLE 2 O-antigen sugar contents relative to two core sugars in the mutants[a]

Strain or mutant[b]	Relative content of sugar[c]						
	QuiN	Fuc	3OMe6dTal	GlcA	Me_xFuc	Man	Gal
WT	0.47	2.67	2.62	2.85	0.63	1.53	1.00
wreU	ND[d]	ND	ND	NT[e]	ND	1.04	1.00
wreG	0.031	ND	ND	NT	ND	0.95	1.00
fcl	0.014	ND	ND	NT	ND	1.01	1.00
wreE	0.013	ND	ND	0.021	ND	0.94	1.00
wreS	0.010	0.021	ND	0.018	ND	0.92	1.00
wreT	0.030	0.142	ND	0.056	ND	1.01	1.00
wreH	0.025	0.115	0.020	0.029	ND	0.91	1.00
wreL	0.018	0.091	0.011	0.092	ND	1.33	1.00
*wreM*β	0.027	0.086	0.032	0.083	0.05	1.21	1.00
wreB	0.41	2.77	2.08	2.72	ND	1.47	1.00
lpcA	ND	0.002	ND	0.018	ND	1.00	0.03

[a] The GC peak areas were used directly to calculate the relative content of each sugar normalized to the GC peak area of Gal. The values for the negative-control *lpcA* mutant were normalized to mannose, because the *lpcA* mutation blocks LPS core synthesis before the addition of galactose. The internal standard sugar, Gal or Man, was set to a value of 1.00 to reflect its molar amount in the LPS core. Neutral and amino sugar compositions were determined as alditol acetates after acid hydrolysis of purified LPSs. GlcA contents were determined from separate analysis after mild acid hydrolysis, removal of lipid A, and conversion of sugars to trimethylsilyl-methylglycoside derivatives.

[b] The strains were as follows: WT, CE3; *wreU*, CE113; *wreG*, CE358; *wreE*, CE470; *fcl*, CE549; *wreS*, CE551; *wreT*, CE522; *wreL*, CE518; *wreM*β, CE542; *wreB*, CE503; and *lpcA*, CE532. Analysis of LPS from the *wreU* mutant strain CE566 exhibits the same absence of O-antigen sugars as in strain CE113 (data not shown).

[c] Abbreviations for O-antigen-specific sugars: QuiN, quinovosamine; Fuc, fucose; 3OMe6dTal, 3-*O*-methyl-6-deoxytalose; GlcA, glucuronic acid; Me_xFuc, di-*O*-methylfucose and tri-*O*-methylfucose. Abbreviations for core sugars: Man, mannose; Gal, galactose. Man is present in both the O antigen and the core, but its presence in the mutant LPSs is overwhelmingly due to LPS II.

[d] ND, not detected (i.e., ≤0.001 relative content of the sugar, normalized to galactose).

[e] NT, not tested.

type amounts except the terminal residue, as previously reported (17). Conversely, *wreU* mutants had undetectable levels of any O-antigen-specific sugar, including quinovosamine (QuiN) (Table 2). The other seven predicted glycosyltransferase mutants had low but detectable levels of QuiN, which correlated with the low abundance of their truncated LPS on gels. Mannose was present in all mutant LPSs, because it is a component of the core region (45); thus, a minor amount of mannose contributed by a truncated O antigen could not be distinguished from mannose contributed by the core region. Of those mutant LPSs that contained QuiN, fucose was detected in the truncated LPSs from *wreS*, *wreT*, *wreH*, *wreM*β, and *wreL* mutants, whereas it was not detected in *wreG* and *wreE* mutants. Furthermore, consistent with the O-antigen structure (Fig. 1), mutant LPSs that lacked QuiN (*lpcA* and *wreU* mutants) also lacked detectable levels of fucose. For LPSs that contained fucose, there was the potential to contain the rest of the O-antigen sugars. LPSs from the *wreH*, *wreM*β, and *wreL* mutants contained 3OMe6dTal, whereas this sugar was not detected in the LPSs from the *wreS* and *wreT* mutants. Only *wreM*β mutants contained detectable levels of the terminal residue (Table 2), in accord with the binding of its truncated LPS to MAb JIM28 (Fig. 4C).

There appeared to be a low, background level of glucuronic acid (GlcA) in all LPS preparations, even from the negative-control *lpcA* mutant. This GlcA contamination was not due to reagents, and it was not found in rinses of the column matrices. Mutant strains not expected to have GlcA—*wreE*, *wreS*, and *lpcA* mutants—had a fairly uniform amount of GlcA relative to the core sugars. GlcA in the *wreH* mutant was at or very close to this background level, but three mutants appeared to have relative amounts indicating true GlcA contents in their truncated LPSs, with the relative values in *wreL* and *wreM*β mutant LPS preparations being significantly higher than in the *wreT* mutant LPS preparations (Table 2).

Incorporation of [^{3}H]fucose into the truncated LPSs from *wreE* and *wreS* mutants. Of the sugars that may be unique to the *R. etli* CE3 O antigen (Fig. 1), only fucose is available commercially as the radioisotope-labeled portion of a nucleotide sugar (GDP-fucose in this case). This allows an alternative approach to demonstrate whether LPS from the *wreS* mutant differs from LPS from the *wreE* mutant by having a fucose residue. Cells of each strain were permeabilized and incubated with GDP-[^{3}H]fucose. Separation of the resulting fucose-labeled molecules by TLC revealed a band unique to the *wreS* mutant (Fig. 5), consistent with the presence of fucose in the truncated LPS of the *wreS* mutant and the absence of fucose in the truncated LPS of the *wreE* mutant.

DISCUSSION

In this study, nine ORFs predicted to encode glycosyltransferases were analyzed and determined to be necessary for the synthesis of the *Rhizobium etli* CE3 O antigen. None of these predicted enzymes have been tested by an activity assay; however, amino acid similarities with these particular glycosyltransferase families have tended to be valid predictions of function (46, 47). Together, the mutagenesis experiments suggest that O-antigen biosynthesis initiates with the activity of WreU and requires the sequential glycosyltransferase activities of WreG, WreE, WreS, WreT, WreH, WreMβ, WreL, and WreB (Fig. 6). The arguments for these proposed assignments are presented below.

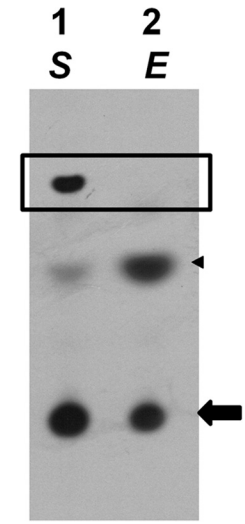

FIG 5 *In vitro* labeling of permeabilized cells with GDP-[^{3}H]fucose. Permeabilized cells were incubated with GDP-[^{3}H]fucose, and the resulting labeled molecules were extracted with chloroform-methanol-water (1:2:0.3), separated by TLC in the isobutyric acid–1.0 M ammonium hydroxide (5:3) solvent (39–41), and visualized by fluorography. A metabolite labeled with radioactive fucose that is present in the *wreS* mutant and absent in the *wreE* mutant is shown by a box around the relevant part of the gel. The positions of unincorporated GDP-fucose (black arrow) and fucose (small black arrowhead) are also indicated. Lane 1, *wreS* mutant (CE551); lane 2, *wreE* mutant (CE470).

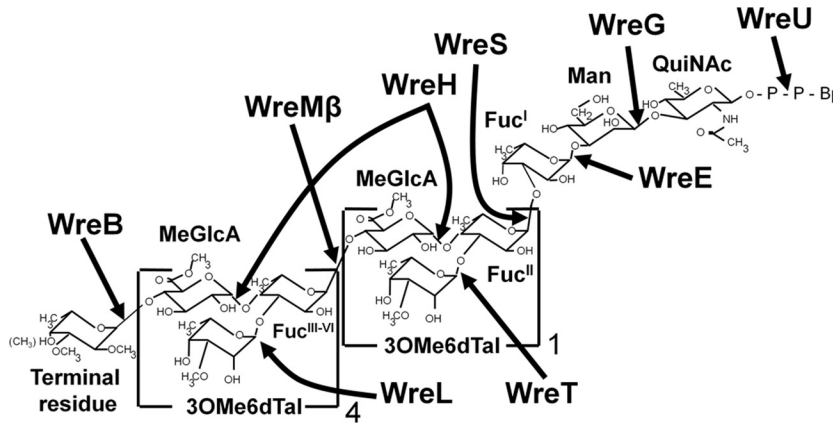

FIG 6 *R. etli* CE3 O-antigen biosynthesis model. Current data indicate a synthesis model in which WreU attaches *N*-acetyl-quinovosamine-1-phosphate (QuiNAc) to bactoprenol phosphate (BpP); WreG attaches mannose (Man) to QuiNAc; WreE attaches the first fucose (Fuc^{I}); WreS attaches the fucose in the first instance of the repeating unit (Fuc^{II}); WreMβ attaches the fucoses of the second, third, fourth, and fifth repeating units (Fuc$^{III–VI}$); WreT attaches the 3-*O*-methyl-6-deoxytalose (3OMe6dTal) in the first instance of the repeating unit; WreL attaches 3OMe6dTal of the remaining repeating units; WreH attaches the methyl glucuronate (MeGlcA) of each repeating unit; and WreB attaches the terminal residue. Abbreviation: P-P-Bp, bactoprenol pyrophosphate.

QuiNAc as the primer and WreU as the catalyst for its addition to BpP. Most of the mutants constructed in this study produced LPS with severely truncated O antigens, and the amount of the truncated LPS was severely decreased in these mutants relative to wild-type O-antigen-containing LPS I (Fig. 3). Thus, in the total LPS from any of these mutants, any O-antigen-specific sugar was present in a small amount relative to that of the core-specific sugars. As sugar analysis showed, QuiN was one of the sugars present in much smaller amounts than the known core sugars (Table 2). Previous chemical/genetic studies (48) indicated that QuiNAc was linked to a Kdo residue (Fig. 1), and biosynthetic/genetic studies (44, 45, 49, 50) provide strong evidence that this Kdo is part of the biosynthetic core of the LPS. It follows from our composition data and these previous conclusions that QuiNAc not only is part of the O antigen but is the first sugar added in O-antigen synthesis.

Based on sequence and mutation analyses in this study, the *wreU* gene likely encodes the glycosyltransferase that initiates O-antigen synthesis. The predicted protein sequence of WreU (188 amino acids [aa]) is annotated as a prenyl-phosphate-sugar-phosphotransferase (see Fig. S2 in the supplemental material), the type of reaction generally required for the initial reaction of O-antigen synthesis (1). In *Salmonella enterica*, WbaP (476 aa) is the initiator of O-antigen synthesis, where the C-terminal half of the protein functions as the undecaprenyl-phosphate-galactosephosphotransferase (5, 51). Alignment of the C-terminal portion of WbaP (aa 290 to 476) and WreU (aa 16 to 188) revealed a high degree of similarity between the protein sequences, including the relative location of a predicted transmembrane domain and putative binding sites for sugar nucleotides and prenyl-phosphate substrates (5) (data not shown). Like WbaP-deficient strains, mutants defective in *wreU* (strains CE113 and CE566) produced LPS lacking all O-antigen-specific sugars, including QuiN (Table 2). Thus, it is proposed that WreU transfers QuiNAc-1-phosphate to the BpP lipid carrier, thereby initiating O-antigen biosynthesis in *R. etli* CE3.

Addition of sugars of the adaptor region by WreG, WreE, WreS, and WreT. Because all mutant LPS samples had a large proportion of LPS II, which contains mannose, sugar composition data did not definitively identify the particular mutant unable to add mannose to QuiNAc. Mutants defective in either one of two glycosyltransferase genes, *wreG* or *wreE*, produced LPS containing QuiN but lacking fucose (Table 2). Differences between the two strains were revealed by Tricine SDS-PAGE (Fig. 3). The *wreE* mutant produced an LPS of low abundance whose mobility was barely resolved from that of LPS II, whereas any truncated LPS produced by the *wreG* mutant was not resolved from LPS II. This result suggests a shorter O antigen in the *wreG* mutant. Furthermore, the LPS profile of the *wreE* mutant on Tricine SDS-polyacrylamide gels was identical to those of *fcl* mutants, which as expected also lacked fucose. All of these data support the assignment of WreG as the glycosyltransferase responsible for the addition of mannose and WreE for the addition of the first fucose (Fuc^{I}).

Arguments stated in the introduction predict that the additions of the first two fucose residues (Fuc^{I} and Fuc^{II}) of the O antigen require two different glycosyltransferases. The *wreS* mutant produces a truncated LPS whose mobility is lower than that of the *wreE* mutant (Fig. 3), suggesting an O antigen having another sugar residue after QuiNAc and mannose. Supporting this inference is the presence of fucose in composition analysis and the ability to incorporate [^{3}H]fucose into the truncated LPS of the *wreS* mutant (Table 2 and Fig. 5), implicating WreS as the glycosyltransferase for the second fucose (Fuc^{II}). Interestingly, the genes proposed to be involved in the synthesis of GDP-fucose (*gmd* and *fcl*) and the attachment of the first two fucose residues (*wreE* and *wreS*) are located consecutively in the chromosomal genetic cluster (Fig. 2A).

After two fucose residues have been added, the next glycosyltransferase would catalyze the addition of either MeGlcA or 3OMe6dTal. The *wreT* mutant produced the next fastest-moving truncated LPS, which was found to contain QuiN and contain twice as much fucose/QuiN as the *wreS* mutant but lack 3OMe6dTal (Fig. 3 and Table 2). Because all of the other mutants not considered yet (*wreH*, *wreM*β, and *wreL* mutants) do have 3OMe6dTal, it is proposed that this residue is added after the second fucose and that WreT is the responsible enzyme. In order

to add 3OMe6dTal to the second fucose and not to the first one, it seems likely that this enzyme has a stringent requirement that the acceptor substrate has two fucose residues. As mentioned in the introduction, this would be the only point in the synthesis of this O antigen that an enzyme with this specificity could act. Hence, in terms of the biosynthetic mechanism, this first 3OMe6dTal is proposed to be the last residue of the adaptor region of the *R. etli* CE3 O antigen.

Addition of repeat-unit sugars by WreH, WreMβ, and WreL. If the repeat unit is constructed by first adding 3OMe6dTal to the second fucose residue, the next step must be the addition of MeGlcA (Fig. 1) (or possibly GlcA before it is methylated). Using mobility on SDS-polyacrylamide gels as an indication of relative LPS length, the *wreH* mutant produced a truncated LPS larger than that of the *wreT* mutant and smaller than that of the *wreM*β or *wreL* mutant (Fig. 3). WreH is therefore proposed as the glycosyltransferase that acts next after WreT. In support of this assignment, sugar analysis showed the presence of QuiN, fucose, and 3OMe6dTal in the truncated LPS of this strain (Table 2). Also consistent with this assignment is the fact that, in the *wreH* mutant LPS, GlcA content was at or barely above the background amount observed from mutants that should have no GlcA, such as *wreE*, *wreS*, and *lpcA* mutants. On the other hand, a considerably higher content of GlcA above this background was detected in the *wreT* mutant. A likely explanation is that while the WreH enzyme uses the first 3OMe6dTal residue as part of its substrate recognition and is therefore mainly blocked in the *wreT* mutant, WreH can link MeGlcA to Fuc^{II} at very low efficiency in the absence of 3OMe6dTal. Indeed, when purified *wreT* mutant LPS was overloaded on Tricine-SDS gels, a closely spaced ladder of bands that stained less and less intensely as size increased could be observed (Fig. 3 and data not shown). These bands would be consistent with very small amounts of O antigen having variable numbers of MeGlcA-Fuc- repeats. All of these considerations make it most likely that WreH is the glycosyltransferase that attaches MeGlcA as shown in Fig. 6.

The first fucose (Fuc^{I}) is attached by a 1,3-linkage to β-D-mannose, the fucose of the first repeat unit (Fuc^{II}) is linked by a 1,3-linkage to the first α-L-fucose, and the fucose of the second, third, fourth, and fifth repeat units (Fuc^{III-VI}) is transferred to α-D-MeGlcA of the previous repeat unit by a 1,4-linkage (Fig. 1) (2, 13). Due to these different acceptor substrates and linkages, it is expected that the addition of fucose in the latter repeating units (Fuc^{III-VI}) requires a glycosyltransferase other than WreE or WreS. The *wreM*β mutant produced one of the slowest-moving truncated LPSs (Fig. 3), and it contained detectable amounts of all the O-antigen sugars, including the terminal residue (Fig. 4C and Table 2). In order for the terminal residue to be present, the residue to which it is normally attached (MeGlcA) is also presumed to be present. Apparently, in the absence of further elongation in this mutant, the terminal residue can be attached, which is the function of WreB as previously reported (17). Interestingly, it appears that the truncated O antigen, consisting of one repeat unit, does not serve as an optimal substrate for WreB because the terminal residue is not attached to all truncated O antigens produced by the *wreM*β mutant, which explains the presence of the double-band pattern seen on Tricine gels and the relatively small amount of this sugar in composition data (Fig. 3 and Table 2). The capability to attach the terminal residue, combined with the observation that the *wreM*β defect results in a truncated LPS, implies that this mutant likely cannot extend the O antigen beyond the sugars of the first repeating unit. Thus, the fucose residues of the second, third, fourth, and fifth repeating units (Fuc^{III-VI}) are added by the WreMβ glycosyltransferase.

The truncated LPS from the *wreL* mutant was the slowest moving LPS on the SDS-polyacrylamide gel (Fig. 3), thereby implicating WreL as the next glycosyltransferase to act in O-antigen synthesis. Considering the high similarity in protein sequence between WreL and WreT (data not shown), it is a logical possibility that WreT adds 3OMe6dTal to the first repeat unit and WreL adds this sugar to the subsequent repeat units. LPS sugar compositions are consistent with this conclusion: LPS produced by the *wreL* mutant contained the same neutral and amino sugars as LPS produced by the *wreH* mutant, the fucose/QuiN ratio from the data in Table 2 is at least as high as that of the *wreH* mutant, and the net GlcA/QuiN ratio is much higher in the *wreL* mutant than in the *wreH* mutant. Consideration of these results, as well as the deduced activities of the other predicted glycosyltransferases, leads to the assignment of the role of WreL being the one indicated in Fig. 6. This assignment completes the set of activities needed to link the sugars together to obtain the *R. etli* CE3 O-antigen structure that has been determined by chemical analysis (13) (Fig. 1 and 6).

Significance of the low abundance of truncated LPSs. SDS-PAGE is a common approach to identifying mutants defective in genes required for the biosynthesis of the core and O-antigen regions of LPS (52–55). Such analysis in *R. etli* reveals a set of mutants producing stepwise truncations in the O antigen that, as noted above, greatly aid in deducing functional assignments of glycosyltransferases for each discrete step in O-antigen synthesis. However, all of the glycosyltransferase mutants, with the exception of the *wreB* mutant, produce a truncated LPS in much less abundance than LPS I of the wild type (Fig. 3). One possible explanation is that the wild-type glycosyltransferase enzymes form multisubunit O-antigen-synthesizing complexes, and the rate of synthesis is reduced when the remaining enzymes cannot form optimal complexes. Alternatively, the truncated O antigens may not be ideal substrates for the ABC transporter or the O-antigen ligase, such that the truncated O antigens are not readily translocated across the inner membrane and/or attached to the core and lipid A regions, respectively.

Summary. It is important to determine O-antigen structures and understand their biosynthesis because of their central role in mutualistic symbioses (e.g., *Rhizobium* [2, 16, 56, 57]) and pathogenic symbioses (58, 59). This study has identified nine putative glycosyltransferase genes within the known O-antigen genetic regions, created mutants with defects in each gene, performed analyses on the resulting mutant LPSs, and demonstrated each predicted glycosyltransferase to have a role in O-antigen synthesis. The data support a model (Fig. 6) specifying the respective function of each predicted glycosyltransferase. It is a working hypothesis that may not be correct in all respects, but all assignments are based on evidence that is fully consistent with the assignments. The value of the model is that it is comprehensive and makes specific predictions of biosynthetic activities to be tested in future experiments.

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