The C-terminal Domain of the *Escherichia coli* WaaJ Glycosyltransferase Is Important for Catalytic Activity and Membrane Association

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The *waaJ* gene encodes an α-1,2-glycosyltransferase involved in the synthesis of the outer core region of the lipopolysaccharide of some *Escherichia coli* and *Salmonella* isolates. WaaJ belongs to glycosyltransferase CAZy family 8, characterized by the GT-A fold, a DXD motif, and by retention of configuration at the anomeric carbon of the donor sugar. Detailed kinetic and structural information for bacterial family 8 glycosyltransferases has resulted from studies of *Neisseria meningitidis* LgtC. As many as 28 amino acids could be deleted from the C terminus of LgtC without affecting its *in vitro* catalytic behavior. This C-terminal domain has a high ratio of positively charged and hydrophobic residues, a feature conserved in WaaJ and some other family 8 representatives. Unexpectedly, deletion of as few as five residues from the C terminus of WaaJ resulted in substantially reduced *in vivo* activity. With deletions of 15 residues or less, activity was only detected when levels of expression were elevated. *In vivo* activity was detected after the removal of 20 amino acids, regardless of expression levels. Longer deletions (20 residues and greater) compromised the ability of WaaJ to associate with the membrane. However, the reduced *in vivo* activity in enzymes lacking 5–12 C-terminal residues also reflected a dramatic drop in catalytic activity *in vitro* (a 294-fold decrease in the apparent $k_{cat}/K_{m}$). Deletions removing 20 or more residues resulted in a protein showing no detectable *in vitro* activity. Therefore, the C-terminal domain of WaaJ plays a critical role in enzyme function.

The outer leaflet of the Gram-negative outer membrane contains lipopolysaccharide (LPS) as a major component. LPS consists of three structural domains as follows: lipid A, the core oligosaccharide region (core OS), and O antigen. In the Enterobacteriaceae, the core OS can be further conceptually divided into the inner core, containing Kdo residues and a heptose backbone, and the outer core, composed of hexoses and acetamido sugars (Fig. 1). Lipid A-core OS is formed by the combined activity of Lpx* enzymes synthesizing lipid A, which then becomes the acceptor for the Waa* glycosyltransferase enzymes involved in the further synthesis of the inner and outer core OS regions (reviewed in Ref. 1). Once formed, the lipid A-core OS is transported by MsbA to the periplasmic face of the inner membrane (reviewed in Ref. 2). O antigen is synthesized separately and added to core OS by the action of the putative ligase, WaaL, prior to translocation of completed LPS to the outer leaflet of the outer membrane. LPS plays a variety of roles in the virulence of bacterial pathogens and is essential for outer membrane integrity and the viability of most Gram-negative bacteria (1).

*Escherichia coli* isolates produce one of five core OS types: K-12, R1, R2, R3, and R4 (reviewed in Ref. 3), and there are at least two core OS types in *Salmonella* isolates (4). The backbone of the inner (lipid A proximal) core OS is typically conserved, and the various core types primarily arise from differences in inner core substitution and the structure of the part of the outer core that provides the attachment site for O antigen. The genetic basis for these differences has been described (4, 5).

Glycosyltransferases play diverse critical roles in the biology of prokaryotes and eukaryotes. However, bacterial enzymes have provided some influential models to assess glycosyltransferase structure and function because of the relative ease of their manipulation. The outer core OS biosynthesis glycosyltransferases in *E. coli* provide an interesting collection of related enzymes to examine principles of substrate (UDP-sugar) and linkage specificity. These enzymes transfer hexose residues to a lipid A-core acceptors in reactions that, unlike O antigen biosynthesis, do not involve polyisoprenyl-linked donors (1). WaaJ is required for the addition of an α-1,2-glucose to the outer core OS in *R3 E. coli* (6) and *Salmonella enterica* serovar Typhimurium (7), which becomes the point of attachment for O antigen (Fig. 1) (8, 9).

According to the CAZy classification system, WaaJ belongs to glycosyltransferase family 8, a family with a relatively solid foundation of structure-function data. Although the nature of the UDP-sugar donors and *in vivo* acceptors differ widely, this group of enzymes is characterized by the GT-A structural fold, a DXD motif that binds a catalytically important divalent metal ion, and retention of configuration at the anomeric carbon of the donor sugar. Solved structures are available for the family 8 glycosyltransferase representatives, *Neisseria meningitidis*
WaaJ Glycosyltransferase Function

FIGURE 1. Schematic structure of the outer core of E. coli R3 LPS (8). The known Waa* enzymes involved in core OS biosynthesis are indicated.

LgtC (10, 11) and rabbit glycogenin (12). N. meningitidis LgtC is an α-(1,4)-galactosyltransferase that has been subject to detailed kinetic characterization using a variety of synthetic acceptor substrates (11, 13). LgtC follows an ordered bi bi mechanism, with binding of UDP-Gal preceding binding of the acceptor (11). UDP-sugar donor binding is achieved with the participation of the metal ion and is thought to be followed by a conformational change that forms the binding site for the acceptor. The donor anomer is positioned at an opening in the binding pocket to be accessible to attack by the acceptor sugar nucleophile. After the reaction occurs, the Gal-linked product is released first, followed by UDP.

Although N. meningitidis LgtC and E. coli R3 WaaJ both belong to glycosyltransferase family 8, they exhibit limited sequence similarity, utilize different UDP-sugar donor substrates, and catalyze different linkages of the donor sugar to their respective acceptors. They do, however, share a C-terminal domain with predominantly hydrophobic and positively charged residues (Fig. 2). This region (25 amino acid residues) was deleted to obtain derivatives of LgtC that did not aggregate and were amenable to crystallization (10, 11). The resulting protein was active with soluble synthetic acceptors. It was speculated that the predominantly hydrophobic and positively charged residues in the deleted domain contributed to aggregation and, furthermore, that the C-terminal domain might play a role in associating LgtC with the membrane. However, the precise function of this C-terminal domain has not been investigated directly, and this represents the objective of this work. The work presented here conclusively demonstrates the importance of the C-terminal 20 amino acids of WaaJ in membrane association, in vivo function, and in vitro catalytic activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The E. coli F653 R3 prototype is a derivative of serotype O111 with a complete core OS and an undeﬁned defect in O antigen biosynthesis (14, 15). E. coli CWG350 is a waaA::aacC1 (gentamycinr) mutant derived from F653 (6). WaaJ derivatives were expressed in E. coli TOP10 (F– mcrA Δ(mrr-hsdRMS-merBC) f80lacZM15 ΔlacX74 deoR recA1 araD139 Δ(ara-λeva)7697 galU galK rpsL (Str r) endA1 nupG) and E. coli BL21 (DE3) (B F– hsdS r (F r m H r) gal dcm ompT (ADE3)); both were purchased from Novagen.

DNA Methods and Plasmid Construction —The plasmids used in this work are described below, and the primers used in their construction are listed in supplemental Table I. Plasmid pWQ272 is a pBAD18 derivative (16) containing the coding sequence for a His6-WaaJ WT fusion protein that was moved from the previously reported pET-based pWQ155 plasmid (6). PCR amplification was used to generate constructs encoding proteins with deletions of 5, 10, 15, 20, 30, and 37 residues from the C terminus (designated His6-WaaJ5 through His6-WaaJ37, respectively). The forward primer (ML18) anneals upstream of the coding sequence with the AGG-3’ in the primer forming part of the AGGA ribosome-binding site located nine nucleotides upstream of the ATG initiation codon. The reverse primers (ML19, ML20, ML23, ML26, ML24, and NK5) all introduced new termination (TAA) codons at the appropriate positions. PCR-generated products were digested with the appropriate restriction endonucleases at sites incorporated into the primer sequences and cloned in pBAD18 as pWQ273 (encoding His6-WaaJ5), pWQ274 (His6-WaaJ10), pWQ276 (His6-WaaJ15), pWQ277 (His6-WaaJ20), pWQ278 (His6-WaaJ25), and pWQ279 (His6-WaaJ30), respectively. Plasmid pWQ275 (encoding His6-WaaJ37) was created by introducing a stop codon through QuickChange (Stratagene) mutagenesis of pWQ272 template using primers CF24 and CF25. All DNA primers were synthesized by Sigma Genosys, and all of the constructs were conﬁrmed to be error-free by sequencing at the Guelph Molecular Supercenter (University of Guelph).

Complementation Experiments to Assess in Vivo Activity of His6-WaaJ Derivatives—WaaJ functionality was determined by electrotransformation of E. coli CWG350 with plasmids encoding the various WaaJ derivatives. Cultures of transformed bacteria were grown overnight at 37 °C in LB containing 100 µg/ml ampicillin, and 0.1-ml aliquots were used to inoculate 5-ml cultures of the same medium supplemented with 0, 0.002, or 0.02% l-arabinose to induce expression from the pBAD promoter in pBAD18 (16). After growth at 37 °C for 5 h, SDS-proteinate K whole-cell lysate samples were made following the procedure of Hitchcock and Brown (17). The samples were then separated by electrophoresis using 4–12% gradient NuPAGE gels (Invitrogen). Electrophoresis was carried out at 150 V for 75 min. The gels were silver-stained using standard methods (18). The extent of complementation was determined by scanning the gels using a Bio-Rad GS-800 calibrated densitometer and determining the relative amounts of the two major bands with QuantityOne software.

Overexpression and Purification of WaaJ—His6-WaaJ derivatives were overexpressed in E. coli TOP10 cells. A 250-ml culture was grown at 37 °C until it reached an A600 of ~0.5, and gene expression was then induced with l-arabinose at a ﬁnal concentration of 0.02%. After 3 h at 37 °C, the cells were collected by centrifugation (10,000 × g, 4 °C, 15 min). The pellet was resuspended in 8 ml of 50 mM Tris, pH 7.5, and one-half of a Mini-EDEA-free protease inhibitor tablet (Roche Applied Science) was added. The suspension was then stored at −20 °C until use. The frozen cell pellet was thawed and supplemented with 300 mM NaCl, 5 mM MgCl2, 15 mM imidazole, and one-half of a protease inhibitor tablet. The cell suspension was sonicated on ice, and unbroken cells were removed from the lysate by centrifugation (20,000 × g, 4 °C, 10 min). The resulting cell-free supernatant was subjected to ultracentrifugation (112,000 × g, 4 °C, 1 h). The clarified supernatant was mixed with 1 ml of His-Select nickel afﬁnity gel resin slurry (Sigma), and one-half of a protease inhibitor tablet was added. After batch binding at 4 °C for 1 h, the mixture was packed into a gravity column. The resin was washed twice with 5 ml of 50 mM Tris, pH 7.5, 300 mM
NaCl, 5 mM MgCl₂, 30 mM imidazole, 10% glycerol. The protein was eluted from the resin with four 1-ml washes using the same buffer containing 500 mM imidazole; the majority of the protein eluted in the first two fractions. The protein-containing fractions were concentrated and the buffer exchanged using a PD10 column (GE HealthCare) equilibrated with 50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, and 50% glycerol, according to manufacturer’s procedure. The protein was eluted with 3.5 ml of the same buffer, yielding a sample of >95% purity, based on SimplyBlue (Invitrogen) staining. The protein was dispensed in 0.2-ml aliquots for single use, and total protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as the standard. The enzyme was stable in this form at 4 °C for periods of up to 2 weeks. Storage at −20 °C offered no additional stability, and enzyme samples thawed after storage at −80 °C showed significant loss of activity.

Subcellular Localization of His₆-WaaJ Derivatives—Subcellular localization studies were performed by expressing the various constructs in E. coli CWG350. Preparation of the cell-free lysates followed the protocols used for enzyme purification. After the ultracentrifugation step, the soluble fraction was collected, and the membrane pellet was washed twice with 2 ml of 50 mM Tris-HCl, pH 7.5, and resuspended in the same buffer. The fraction volumes were adjusted to facilitate direct comparison of the amount of membrane protein corresponding to a given amount of soluble protein. Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The Western immunoblots were developed using HisProbe H3 mouse anti-His₆ primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-mouse alkaline phosphatase-conjugate secondary antibody (Jackson ImmunoResearch, Montreál, Quebec, Canada). Nitro blue tetrazolium (from Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (from Roche Applied Science) were used as substrates to develop the Western blots. The bands were quantified by densitometry using a Bio-Rad GS-800 calibrated densitometer with QuantityOne software.

In Vitro Determination of the Activity of His₆-WaaJ Derivatives—The activity of the various constructs was determined based on the transfer of radioactivity from UDP-[³⁵S]Glc (305.9 mCi/mmol; PerkinElmer Life Sciences) to an acceptor comprising the LPS isolated from E. coli CWG350. To achieve the appropriate donor concentrations, nonradioactive UDP-Glc was added from stock. Activity was compromised by concentrations of ethanol (from the radioactive donor) above 6% (v/v) ethanol (data not shown); all reactions contained <3% (v/v) ethanol. The LPS (3453 g/mol calculated molecular weight) was purified as described previously (6) by the phenol/chloroform/petroleum ether method (19). The isolated LPS was frozen and lyophilized. Working stocks were stored as 2 or 5 mg/ml aqueous solutions at −20 °C. To determine the precise LPS content in the samples, Kdo content was determined (20). Recognizing there is heterogeneity in Kdo content in the isolated R3 LPS (6, 8), the measured Kdo values were entirely consistent with the calculated values for a homogeneous sample containing (on average) two Kdo residues (data not shown). For reactions, the LPS stock was thawed, vortexed to resuspend any precipitated LPS, and then placed at 55 °C for at least 10 min to redissolve the LPS. After this, the LPS stayed in solution at room temperatures or higher. Aliquots of the LPS stock were then added to a reaction buffer containing Tris, pH 7.5, and EDTA. The UDP-glucose and MgCl₂ were then added. Incubation of LPS with MgCl₂ for extended periods led to precipitation of the substrate. However, under the conditions and time course employed, no precipitation was detected. Final reaction conditions after the addition of WaaJ were 1–145 μM UDP-Glc, 2–750 μM LPS, 100 mM Tris, pH 7.5, 0.4 mM EDTA, 5 mM MgCl₂, and 200–900 nM WaaJ in a final volume of 0.1 ml. The WaaJ concentration was adjusted to ensure that it was always at least 5-fold below the lowest substrate concentration. To test the effect of phosphatidylethanolamine (E. coli lipopolysaccharide type 5, Sigma; 700 g/mol calculated molecular weight) on WaaJ activity, a 6 mg/ml (8.57 mM) stock solution in 25% (v/v) methanol/ water) was added to the LPS solution before heating to 55 °C; it was present at 2.23 mM in the final 100-μl reaction volume. This followed a method reported previously (21, 22).

Reactions were initiated by the addition of enzyme. Twenty-microliter samples were removed and transferred to 1 ml of ice-cold 2 M acetic acid. Although dilute acetic acid is known to release polysaccharide from lipid A, no such release was observed during the course of the reaction as long as all fractions were kept on ice. Incorporation of radioactivity into LPS was measured using a filter-binding assay. A cellulose filter (0.45 μm, 25 mm; MicroSep Cellulosic, Osmonics Inc.) was pre-washed with 1 ml of 2 M acetic acid, and the 1-ml stopped sample was then added. The filter was washed three times with 1 ml of 2 M acetic acid and left to air-dry. Each filter was then placed in a scintillation vial with 4 ml of EcoLite (MP, Irvine, CA) scintillation mixture, and the samples were quantified using a Beckman Coulter LS 6500 multipurpose scintillation counter. Data were derived from (at least) triplicate samples. Prism 4 (GraphPad) was used to calculate the rates of reaction for WaaJ at each UDP-glucose and LPS concentration from the linear fit of the 0-, 0.5-, and 1-min time points. In each case, less than 20% substrate conversion had occurred. However, assays over a longer time course established that the reaction was linear to at least 50% substrate conversion (corresponding to 4–5 min for His₆-WaaJ) at saturation of both substrates. The rates at each substrate concentration were then fit to the Michaelis-Menten equation to determine values for kₐ and kₘ. Because this was a stopped assay, these should be considered as apparent values for kₐ and kₘ.

RESULTS

Similarities Shared by LgtC and WaaJ—As might be anticipated for proteins that both belong to glycosyltransferase family 8, LgtC and WaaJ primary sequences share some highly conserved domains (Fig. 2A), despite the fact that the overall sequence identity (22%) and similarity (43%) are limited. When secondary structure predictions are taken into consideration, there is evident similarity in overall architecture. As a result, when WaaJ is threaded over the solved LgtC structure (Fig. 2B), there is extensive overlap. The base structure for LgtC lacks the 25 C-terminal amino acid residues because their presence resulted in aggregation problems that prevented crystallization (11). This region is therefore not included in the threading...
experiment. The sequences of \textit{WaaJ} and \textit{LgtC} predict no transmembrane helices. However, given that their acceptors are nascent lipid A-core OS molecules, membrane association of the enzyme might be beneficial for its activity. Although the truncated \textit{LgtC} derivative was active with synthetic acceptors, Persson \textit{et al.} (11) proposed that native \textit{LgtC} might be tethered to the membrane by interactions between positive and aromatic amino acids of this C-terminal region with the hydrophobic and negatively charged regions of the inner membrane phospholipids. Alignment of the C-terminal domains of \textit{LgtC} and other known and predicted family 8 members, including several core biosynthesis (\textit{Waa}-prefix proteins (5)), demonstrates the conserved presence of a region enriched in positively charged and aromatic amino acids (Fig. 3; Table 1).
Overexpression and Subcellular Localization of His<sub>6</sub>-WaaJ Derivatives—The results from the complementation experiments could reflect altered association of the truncated proteins with the membrane where the acceptor (lipid A-core OS) resides. Analysis of the cellular distribution of His<sub>6</sub>-WaaJ indicated that ~55% of the full-length protein was associated with the membrane fraction (Fig. 5). Removal of five residues resulted in an ~10% reduction in membrane-associated protein. Deletions of 5–15 residues (i.e. within the predicted terminal α-helix) had no significant effect on this distribution. In contrast, C-terminal truncations exceeding 15 residues led to a progressive loss of protein from the membrane fraction and a corresponding increase in the soluble protein pool (Fig. 5). The His<sub>6</sub>-WaaJ<sup>ΔC20</sup> truncation deletes the entire final α-helix and linker region, as well as part of the penultimate α-helix (Fig.

### Table 1

**Analysis of positively charged and aromatic amino acid content of representative bacterial glycosyltransferases from CAZy family 8**

| Protein | No. of amino acids compared | Percent aromatic + positive | Relative enrichment in C-terminal domain |
|---------|-----------------------------|-----------------------------|----------------------------------------|
| EcWaaJ  | 72                          | 39                          | 1.6                                    |
| EcWaaT  | 71                          | 35                          | 1.4                                    |
| EcWaaO  | 74                          | 35                          | 1.8                                    |
| EcWaaL  | 73                          | 39                          | 1.6                                    |
| EcWaaW  | 79                          | 38                          | 1.7                                    |
| EcWaaS  | 74                          | 40                          | 2.1                                    |
| RelgtA  | 73                          | 32                          | 1.7                                    |
| NmLgtC  | 68                          | 35                          | 1.5                                    |
| HpHP0159| 88                          | 40                          | 1.8                                    |
| HpHP1416| 85                          | 35                          | 1.8                                    |

<sup>a</sup> C-terminal region is aligned in Fig. 3.

<sup>b</sup> Remaining polypeptide after C-terminal domain is excluded.

**FIGURE 4. In vivo activity of His<sub>6</sub>-WaaJ and its C-terminal truncation derivatives.** Plasmids encoding the various derivatives were used to transform E. coli CW350<sup>::aarC1</sup> cells. A, shows silver-stained polyacrylamide gels with LPS samples prepared as whole-cell lysates from cultures undergoing different inducing conditions. B, extent of complementation (i.e., full-length chain extension of CW350 LPS) was determined by densitometry. The data (average ± S.D.) reflects three independent experiments. C, shows the amount of His<sub>6</sub>-WaaJ and its derivatives produced in whole-cell lysates under the induction conditions used for the complementation experiment. Protein was detected by Western immunoblotting using antibodies recognizing the His<sub>6</sub> tag.

(queues)
The WaaJ protein was subject to varying but limited proteolysis in some lysates (for example, see the soluble His<sub>6</sub>-WaaJ<sup>Δ20</sup> in Fig. 5A), and the extent was considerably reduced by addition to the lysates of protease inhibitor tablets (data not shown). The OmpT protease was implicated as being responsible for a significant amount of the observed degradation, as use of <i>E. coli</i> BL21 (DE3) as the expression host substantially reduced the proteolysis. OmpT targets pairs of basic residues (23). There are three Lys-Lys pairs within the last 37 amino acids of WaaJ, located 37, 33, and 18 amino acids from the C terminus, respectively (Fig. 2). Based on the apparent molecular weight of the proteolytic product from His<sub>6</sub>-WaaJ<sup>Δ20</sup> observed by SDS-PAGE (Fig. 5A), most of the cleavage appears to occur at the site located 37 amino acid residues from the C terminus.

**Effect of WaaJ C-terminal Truncations on in Vitro Kinetic Behavior**—The <i>in vivo</i> complementation data showed that activity of enzyme was significantly compromised by even the shortest C-terminal truncation. The progressively reduced activity was apparent in WaaJ derivatives whose membrane association was similar, suggesting that the deletion was also having an effect on the catalytic properties of the enzyme. Any truncation had a marked effect on the <i>in vitro</i> behavior of WaaJ. Removal of 20 residues (His<sub>6</sub>-WaaJ<sup>Δ20</sup>) or more rendered the proteins inactive <i>in vivo</i> (Fig. 4, A and B) and <i>in vitro</i> (data not shown). This is not a result of global misfolding of truncated His<sub>6</sub>-WaaJ derivatives, as there is no significant difference between His<sub>6</sub>-WaaJ<sup>WT</sup> and His<sub>6</sub>-WaaJ<sup>Δ20</sup> spectra in circular dichroism experiments (data not shown).

Apparent <i>in vitro</i> kinetic parameters were determined for each of the active shorter deletions. The enzyme followed typical Michaelis-Menten kinetics (data not shown). Although C-terminal truncation had at most a modest effect on <i>k<sub>cat</sub>/K<sub>m,UDP-Glc</sub></i> removal of only five amino acids caused a 3-fold increase in the value for <i>K<sub>m</sub>LPS</i> compared with His<sub>6</sub>-WaaJ<sup>Δ12</sup>, resulting in an overall 294-fold decrease in <i>k<sub>cat</sub>/K<sub>m</sub>LPS</i> (Table 2). The activity of His<sub>6</sub>-WaaJ<sup>Δ10</sup> showed a further decrease in <i>k<sub>cat</sub></i> and a 2-fold increase in <i>K<sub>m</sub>LPS</i>, resulting in an overall 109-fold decrease in <i>k<sub>cat</sub>/K<sub>m</sub>LPS</i> (relative to His<sub>6</sub>-WaaJ<sup>WT</sup>). Interestingly, His<sub>6</sub>-WaaJ<sup>Δ12</sup> exhibited the same <i>k<sub>cat</sub></i> as His<sub>6</sub>-WaaJ<sup>Δ10</sup>, although the <i>K<sub>m</sub>LPS</i> increased another 3-fold to give a 294-fold decrease in <i>k<sub>cat</sub>/K<sub>m</sub>LPS</i> compared with His<sub>6</sub>-WaaJ<sup>WT</sup>. His<sub>6</sub>-WaaJ<sup>Δ10</sup> exhibited a very low level of glucosyltransferase activity (5% of the full-length protein in fixed end-point assays). However, the lack of
solubility of the LPS acceptor at the high concentrations (>750 μM) necessary to generate a Michaelis-Menten plot prevented determination of the kinetic parameters.

**DISCUSSION**

Relatively few retaining enzymes containing the GT-A fold have undergone detailed in vitro kinetic analysis. Some belong to other CAZy families, such as bovine α-galactosyltransferase (24, 25), a representative of family 6. *N. meningitidis* LgtC is a logical comparison for *E. coli* WaaJ, because both are family 8 enzymes that add a single sugar onto the growing core OS chain (13). Although rabbit glycochenin is also assigned to family 8, it performs a multiple self-glucosylation reaction that renders it mechanistically distinct from other examples (reviewed in Ref. 26).

The starting hypothesis for this work was that the C-terminal domain, and its characteristic enrichment in positively charged and aromatic amino acids, played a role in associating the enzyme with the membrane. This was tested by examining the properties of precise C-terminal deletions. Longer deletions (greater than 15 residues) certainly altered the cellular distribution of the enzyme, with a substantial increase of enzyme in the soluble fraction. This effect implicated the terminal α-helical domain in membrane association. Whether this domain interacts directly with membrane phospholipids or with other enzymes in what may be a multienzyme complex for core OS biosynthesis is currently unknown. Some previous literature reported higher in vitro activity for various glycosyltransferases such as WaaI from *Salmonella* in the presence of phospholipids (21, 22, 27, 28). Experiments were performed comparing the activity of His6-WaaJWT in the presence and absence of 3:1 (w/w) phosphatidylethanolamine/LPS, but no difference in rate was observed (data not shown).

Surprisingly, the catalytic activity of the enzyme was already substantially compromised by C-terminal truncations. This effect was entirely unanticipated because LgtC is fully active with soluble synthetic acceptors after removal of as many as 28 residues from the C terminus (13). At basal expression levels (growth in LB with no inducer added), only the His6-WaaJWT protein fully complemented the *E. coli* CWG350 waaJ::aacC1 null mutation. Elevating the level of expression of the His6-WaaJ variants provided some detectable activity up to His6-WaaJ315. This was consistent with an overall reduction in the in vitro catalytic activity beginning in His6-WaaJ35 and leading to a complete loss of activity with deletions of 20 amino acids or more.

Sequential deletions in the C-terminal domain had a very limited effect on the *Kₘ,UDP-Glc* (Table 2) but led to a significant increase in *Kₘ,UDP-LPS*. This altered in vitro activity correlates well with the in vivo complementation results. In the LgtC crystal structure, the last loop forming the UDP-sugar binding pocket is located 55 amino acids from the C terminus of the native protein (11). The secondary structure features of this region are well conserved in the predicted WaaJ structure (Fig. 2) and would be located a substantial distance (20 amino acids toward the N terminus) from the longest deletion studied here. It is therefore expected that the donor-binding pocket would be largely unaffected by C-terminal truncation, and the kinetic data are consistent with this contention. It is not possible to directly compare substrate *Kₘ* values for LgtC and WaaJ. The WaaJ reactions described in this paper were determined using the LPS acceptor substrate, whereas the LgtC reactions were performed using shorter synthetic acceptor substrates. It has been established that the LgtC *Kₘ,UDP-Gal* values are dependent upon the identity of the synthetic substrate; LgtC-19 exhibited *Kₘ,UDP-Gal* values of 4.4 and 30 μM with FCHASE-Lac (13) and Lac (29) acceptors, respectively.

The properties of the truncated proteins indicate that the final α-helix plays a major role in catalysis (Table 2). Increasing truncation of the WaaJ C terminus resulted in a successive decrease in *kₘ*. Decreases of 6- and 16-fold in *kₘ* values were observed with His6-WaaJ35 and His6-WaaJ315, respectively. The truncations also led to a dramatic progressive rise in *Kₘ,UDP*. Of particular note, the His6-WaaJ16 and His6-WaaJ12 enzymes have essentially identical *kₘ* values but differ dramatically in their respective *Kₘ,UDP*. The end points for these deletions lie in the N-terminal third of the final α-helix. The loss of 10 amino acids might be sufficient to disorder this helix and compromise its role in catalysis, whereas the additional loss of an aromatic amino acid and a hydrophobic aliphatic amino acid would further affect LPS acceptor binding. Whatever the precise role the C-terminal α-helix plays in interactions with LPS lipid A-core acceptor, this requirement seems to be lost in LgtC when synthetic FCHASE-linked acceptors, or lactose, are used (10, 13). Attempts were made to utilize FCHASE-Lac or FCHASE-α-Gal acceptors for His6-WaaJWT, but unfortunately, no transfer of Glc from UDP-Glc donor was detected with His6-WaaJWT and His6-WaaJ310 under the reaction conditions tested (data not shown). The reasons for this remain unclear, but they serve to further emphasize subtle differences between glycosyltransferases in the same family.

In summary, the observed loss of LPS affinity with successive C-terminal truncation is still consistent with the suggestion by Persson et al. (11) that the C-terminal α-helices of family 8 transferases are involved in critical interactions with the negatively charged, hydrophobic membrane lipids. However, these interactions are not the sole arbiter of membrane association nor is the role of the C-terminal domain confined to a simple localization process. The comparison of data obtained for WaaJ and the published information for the influential LgtC model underscore the difficulty in making generalized assumptions regarding catalytic and donor/acceptor-binding states among different glycosyltransferases, even within the same family of enzymes.

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