The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases*

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WaaL is a membrane enzyme that catalyzes a key step in lipopolysaccharide (LPS) synthesis: the glycosidic bonding of a sugar at the proximal end of the undecaprenyl-diphosphate (Und-PP) O-antigen with a terminal sugar of the lipid A-core oligosaccharide (OS). Utilizing an in vitro assay, we demonstrate here that ligation with purified Escherichia coli WaaL occurs without adenosine-5'-triphosphate (ATP) and magnesium ions. Furthermore, E. coli and Pseudomonas aeruginosa WaaL proteins cannot catalyze ATP hydrolysis in vitro. We also show that a lysine substitution of the arginine (Arg)-215 residue renders an active protein, whereas WaaL mutants with alanine replacements in the periplasmic-exposed residues Arg-215, Arg-288 and histidine (His)-338 and also the membrane-embedded aspartic acid-389 are nonfunctional. An in silico approach, combining predicted topological information with the analysis of sequence conservation, confirms the importance of a positive charge at the small periplasmic loop of WaaL, since an Arg corresponding to Arg-215 was found at a similar position in all the WaaL homologs. Also, a universally conserved H[NSQ][X8]GXX[GTY] motif spanning the C-terminal end of the predicted large periplasmic loop and the membrane boundary of the transmembrane helix was identified. The His residue in this motif corresponds to His-338. A survey of LPS structures in which the linkage between O-antigen and lipid A-core OS was elucidated reveals that it is always in the β-configuration, whereas the sugars bound to Und-PP are in the α-configuration. Together, our biochemical and in silico data argue that WaaL proteins use a common reaction mechanism and share features of metal ion-independent inverting glycosyltransferases.

Keywords: glycosyltransferase / lipopolysaccharide / membrane protein / O-antigen / oligosaccharidyltransferase

Introduction

Lipopolysaccharide (LPS), which contributes to the effective permeability barrier of the bacterial outer membrane (Nikaido 2003), consists of lipid A, core oligosaccharide (OS) and the O-polysaccharide or O-antigen (Valvano 2011). The lipid A-core OS is synthesized at the cytoplasmic side of the inner membrane and translocated across the membrane by the ATP-binding cassette (ABC) transporter MsbA (Doerrler et al. 2001; Doerrler and Raetz 2002). The O-antigen is independently synthesized as an undecaprenyl-diphosphate (Und-PP)-linked intermediate and translocated across the membrane by either ABC transporter-dependent or transporter-independent pathways (Valvano 2011). Und-PP-linked O-antigens at the periplasmic face of the inner membrane are ligated to a terminal sugar of the lipid A-core OS in a reaction that requires the membrane protein WaaL (Valvano 2011).

WaaL plays a key role in the formation of a complete LPS consisting of lipid A-core OS and O-antigen. Mutations in the waaL gene prevent the ligation of O-antigen molecules to lipid A-core OS, resulting in bacteria that produce LPS devoid of O-antigen polysaccharide (“rough” LPS) and also accumulate intracellular Und-PP-linked O-antigen molecules (Mulford and Osborn 1983; McGrath and Osborn 1991). Because of the role of O-antigen in host-pathogen interactions (Bengoechea et al. 2004; West et al. 2005; Murray et al. 2006; Duerr et al. 2009; Paixao et al. 2009; Saldias et al. 2009), combined with the periplasmic location of the ligation reaction, WaaL is an excellent target for developing inhibitory molecules that could serve as novel anti-virulence factors.

WaaL proteins share a similar predicted topological arrangement characterized by multiple transmembrane (TM) helices and a relatively large periplasmic loop close to the C-terminus (Schild et al. 2005; Pérez et al. 2008; Islam et al. 2010). Conceivably, amino acids exposed to the periplasmic face of the membrane are important for WaaL activity as they could interact with donor and acceptor molecules. Molecular modeling of the large periplasmic loop of the Escherichia coli
K-12 WaaL suggests that this region adopts a fold consisting of two pairs of almost perpendicular α-helices (Pérez et al. 2008). One α-helix of one pair contributes an arginine (Arg-288), whereas an α-helix in the other pair contributes a histidine (His-338), both of which are essential for the function of WaaL in vivo (Pérez et al. 2008). Furthermore, a highly conserved Arg-215 in the preceding short periplasmic loop is also required for O-antigen ligation in vivo. We have proposed that these positively charged amino acids could interact with Und-PP, the common molecule of the Und-PP-linked O-antigen substrates (Pérez et al. 2008).

The joining of lipid-linked glycans to other lipid or protein acceptor molecules is a key reaction for the biogenesis of glycoconjugates in all cell types. However, the mechanism of ligation is poorly understood, and despite involving the formation of a glycosidic bond, WaaL proteins bear no relationship with classical glycosyltransferases. Whereas the ligation reaction shows virtually no specificity for the type of O-antigen or the mechanism of O-antigen export (Raetz and Whitfield 2002), a requirement for a specific lipid A-core OS acceptor structure has been established in several model systems (Heinrichs et al. 1998; Abeyrathne et al. 2005; Schild et al. 2005). It remains unclear how WaaL recognizes the Und-PP-linked O-antigen and, in particular, which part of this substrate molecule participates in the enzymatic reaction. Two groups have published contradictory results regarding a requirement of adenosine-5′-triphosphate (ATP) hydrolysis for the ligation reaction. Abeyrathne and Lam (2007) reported that purified WaaL from Pseudomonas aeruginosa has ATPase activity and ATP hydrolysis is essential for the in vitro ligation reaction, while Hug et al. (2010) reported that the Helicobacter pylori WaaL does not require ATP.

In this work, we have used in vitro ligation assays to functionally investigate purified WaaL proteins. We show that ligation mediated by the E. coli K-12 WaaL occurs in the absence of divalent ions and ATP and that both E. coli K-12 and P. aeruginosa WaaL proteins lack ATP hydrolysis activity. Furthermore, a survey of the nature of glycosidic linkages catalyzed by diverse WaaL proteins reveals that they all are β-linkages. In silico analyses to determine the sequence conservation of residues and motifs on predicted periplasmic loops of all WaaL sequences published to date confirm the importance of a positive charge at the small periplasmic loop of WaaL, as these proteins have residues comparable with Arg-215 and His-338 of E. coli K-12 WaaL, which are also predicted to be located in the corresponding small and periplasmic loops. Accordingly, our results provide strong support to the notion that WaaL proteins use a common reaction mechanism of glycosidic bond formation, which is ATP hydrolysis-independent, metal ion-independent, and causes the inversion of the anomeric configuration of the donor sugar. Similar features are also found in metal-independent inverting glycosyltransferases.

**Results**

**Purified WaaL catalyzes the in vitro transfer of O7 antigen from Und-PP-O7 to the E. coli K-12 lipid A-core OS**

WaaL was purified by Ni²⁺-affinity chromatography as described in Materials and methods. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, we estimated that WaaL was purified to more than 90% homogeneity (Figure 1A). The purified protein migrated in the gel to an apparent molecular mass of 50 kDa and its identity was confirmed by mass spectrometry (data not shown). Prior to purification, a larger product was also detected (asterisk in Figure 1A), which is likely a WaaL oligomeric form due to the incomplete denaturing conditions (incubation at 43°C for 30 min) used to run the samples. Complete denaturation, including treatment of the samples by heating at 100°C in SDS, prevents the detection of membrane proteins in SDS-PAGE, as it has been previously observed with WaaL. (Abeyrathne and Lam 2007; Pérez et al. 2008) and various other proteins containing multiple TM helices (Marolda et al. 2004; Vinés et al. 2005; Lehrer et al. 2007; Tatar et al. 2007). Purified WaaL was used in a cell-free in vitro ligation assay. This assay employed a lipid A-core OS acceptor that was prepared from proteinase K-treated cell lysates of the E. coli K-12 strains SCM3 (awaaL) bearing pHJCV32 (Table I). The pHJCV32 plasmid confers the ability to synthesize O7 antigen (Valvano and Crosa 1989), which in the ΔwaaL strain remains Und-PP bound and therefore unligated to lipid A-core OS (Pérez et al. 2008). The LPS(SCM3)pJHCV32 preparation contains a mixture of Und-PP-O7 and lipid A-core OS, which could both be used in the in vitro ligation assay as donor and acceptor substrates, respectively. The reaction was terminated by proteinase K digestion and hot phenol extraction, and the bands corresponding to ligated lipid A-core OS were detected by silver staining (Figure 1B, left panel) and immunoblot using anti-core-OS and anti-O7-specific antibodies (Abs; Figure 1B, central and right panels, respectively). The positive control, corresponding to LPS produced in vivo from the ΔwaaL strain containing pHJCV32 (O7 antigen biosynthesis) and pXR1 (expressing a functional WaaL), revealed polymeric O7 antigen that was detectable by the three methods, indicating the O7 polysaccharide was covalently linked to lipid A-core OS (Figure 1B, lanes 1). In contrast, LPS prepared from the ΔwaaL strain containing pHJCV32 revealed polymeric O7 antigen formed in vivo that was not detected by silver staining or lipid A-core OS-specific Abs, as expected for unligated Und-PP-O7 polysaccharide (Figure 1B, lanes 2). The sample corresponding to the product of the in vitro reaction with purified WaaL had polymeric O7 antigen reacting with both Abs and also detectable by silver staining, confirming that in vitro ligation had occurred (Figure 1B, lanes 3). These bands did not appear in reactions containing only WaaL (Figure 1B, lanes 4). Ligation was detectable as early as 30 min of incubation at 37°C and pH 6, which is the normal pH in the periplasmic space, appeared to be more favorable for ligation (Supplementary data, Figure S1). Also, the reaction was linear over ligase concentrations ranging from 0.67 to 3.4 μM (data not shown).

A positively charged residue at position 215 is critical for ligation

Based on in vivo results indicating the importance of a positive charge at the Arg-215 periplasmic position (Pérez et al. 2008),
vitro ligations were carried out with 0.67 μg of purified WaaL; 3, product of in vitro ligation after 16 h incubation of parental WaaL; 2, LPS produced in vivo by SCM3(pJHCV32) in the absence of WaaL; 2, LPS produced in vivo by the SCM3(pJHCV32/pXR1) strain expressing WaaL. The in vitro ligations were carried out with 0.67 μg of purified WaaL in 50 mM phosphate buffer, pH 6.0, at 37°C. Samples were analyzed by SDS-PAGE followed by silver staining (left panel), and immunoblot with anti-lipid A-mAb (central panel) or polyclonal anti-O7-specific Abs (right panel).

**Fig. 1.** (A) Purification of K-12 WaaL. WaaL was purified as described in Materials and methods and fractions analyzed by SDS–PAGE and western blotting. M, Dual Color Precision Plus Protein standards (Bio-Rad); membranes, the membranes solubilized in 1% DDM; flow-through, the flow-through from the Ni²⁺-chelating column; wash, the sample from the final 1 mL of washing; purified WaaL, 10 μg of concentrated WaaL purified after Ni²⁺-affinity chromatography. Samples were mixed with an appropriate amount of protein loading dye and incubated at 43°C for 30 min before SDS–PAGE. The polyacrylamide gel was stained with Coomassie blue (Coomassie stain). The western blot was probed with anti-FLAG mAb (western blot). Purification by Ni²⁺-affinity chromatography resulted in a yield of ~1 mg WaaL per liter of culture. No additional protein bands were detected by Coomassie blue staining, although several contaminating bands were detected by silver staining. Asterisk indicates the position of a WaaL band detected by Coomassie blue staining, although several contaminating bands were detected by silver staining. (B) In vitro ligation assays. Lanes: 1, LPS produced in vivo by the SCM3(pHVC32/pXR1) strain expressing parental WaaL; 2, LPS produced in vivo by SCM3(pJHCV32) in the absence of WaaL; 3, product of in vitro ligation after 16 h incubation of purified WaaL, and LPS prepared from SCM3(pJHCV32); 4, 16 h incubation of purified WaaL, without LPS prepared from SCM3(pJHCV32). The in vitro ligations were carried out with 0.67 μg of purified WaaL, in 50 mM phosphate buffer, pH 6.0, at 37°C. Samples were analyzed by SDS–PAGE followed by silver staining (left panel), and immunoblot with anti-lipid A-core OS-specific mAb (central panel) or polyclonal anti-O7-specific Abs (right panel).

WaaL cannot catalyze ATP hydrolysis

Our standard ligation assay did not require exogenous addition of ATP, and sodium azide did not affect ligation. Two previous studies, however, reported opposing findings regarding the requirement of ATP hydrolysis for in vitro ligation (Abeyrathne and Lam 2007; Hug et al. 2010). We therefore examined the ability of WaaL to catalyze ATP hydrolysis in more detail. Since it could be possible that ATP is tightly bound to WaaL and sodium azide could not be able to inhibit a putative ATPase activity of this protein, we measured directly whether WaaL can catalyze ATP hydrolysis using a coupled pyruvate kinase (PK)–lactate dehydrogenase (LDH) assay (Vogel and Steinhart 1976). If WaaL catalyzes ATP hydrolysis, the released adenosine diphosphate (ADP) would be a substrate for the PK and induce oxidation of reduced nicotinamide dinucleotide (NADH) by LDH, giving rise to a significant decline in NADH levels as determined by the change in absorbance at 340 nm (A₃₄₀). For these experiments, we also purified the WaaL protein of P. aeruginosa as a control (WaaLₚₐ), since this protein was reported to have ATP hydrolysis activity (Abeyrathne and Lam 2007). The plot of A₃₄₀ values in a reaction with purified WaaL and WaaLₚₐ showed the same slope as that of the control (coupling system alone), which could be attributed to a small amount of contaminating adenosine diphosphate (ADP), a low level of spontaneous ATP hydrolysis or both (Figure 4A). In contrast, there was a pronounced reduction in A₃₄₀ values in the coupled reaction containing purified FhuC, a membrane ATPase from Staphylococcus aureus (Speziali et al. 2006) that was used as a positive control (Figure 4A). From these data, the rate of ATP hydrolysis by FhuC corresponded to an ATPase-specific activity of 50 nmol mg⁻¹ min⁻¹. Coincubation of WaaL with...
E. coli WaaL corresponding to Arg-215 and His-338 of the E. coli K-12 linkages and the ligases involved have conserved residues (Figure 5), a concentration of chelator that disrupts the ATPase presence of 10 mM ethylenediaminetetraacetic acid (EDTA; pH 6 or 7, with and without EDTA, showed identical pro hydrolysis assay (data not show). A time-course experiment at a concentration of the ATPase activity of purified WaaL proteins and the lack of a requirement for magnesium ions are an obligatory cofactor of the ATPase hydrolysis reaction (Vinogradov 2000). However, ligation products were found in the absence of added Mg2+ and in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA; Figure 5), a concentration of chelator that disrupts the ATPase hydrolysis assay (data not show). A time-course experiment at pH 6 or 7, with and without EDTA, showed identical profiles of ligation (Supplemental data, Figure S3). Together, the absence of the ATPase activity of purified E. coli K-12 and P. aeruginosa WaaL proteins and the lack of a requirement for Mg2+ in the ligation reaction demonstrate that ligation is an ATP-independent process.

WaaL homologs catalyze the formation of \( \beta \)-glycosyl linkages and the ligases involved have conserved residues corresponding to Arg-215 and His-338 of the E. coli K-12 WaaL.

The E. coli K-12 WaaL catalyzes the formation of \( \beta \)-N-acetyl glucosamine (GlcNAc)-(1→7) and \( \beta \)-glucose (Glc)-(1→7) linkages to the same terminal lipid A-core OS heptose (Feldman et al. 1999; Meredith et al. 2007) upon the ligation of \( \alpha \)-antigen and colanic acid, respectively (Table II). Because the GlcNAc and Glc residues in Und-PP-GlcNAc and Und-PP-Glc are in the \( \alpha \)-configuration (Murazumi et al. 1979; Weisgerber and Jann 1982), WaaL could be an inverting glycosyltransferase, which causes the inversion of stereochemistry at the anomic center of the donor substrate (Lairson et al. 2008). A survey of all available structures where the \( \alpha \)-antigen–lipid A-core OS linkage region has been elucidated reveals that the glycosidic bond is in the \( \beta \)-configuration (Table II). This suggested a common reaction mechanism for all WaaL proteins. To get additional clues supporting a common reaction mechanism for ligation, we investigated whether the critical residues identified in this and in a previous work (Pérez et al. 2008) are conserved in other WaaL homologs. First, we examined in more detail the WaaL homologs in the bacteria listed in Table II for which the chemical nature of the lipid A-core \( \alpha \)-antigen linkage is known. These proteins were aligned based on the boundaries of their predicted TMs, cytoplasmic loops and periplasmic loops, revealing that they all contained a short periplasmic loop with at least one Arg residue (analogous to Arg-215 in the E. coli K-12 WaaL). This loop is adjacent to the predicted large periplasmic loop of variable length, ranging from 55 to 100 amino acids, which contained in all cases a His residue corresponding to His-338 (Figure 6).

Second, we developed a script enabling us to associate sequence conservation with topological predictions (see Materials and methods) in all WaaL homologs in the databases. With this approach, we evaluated in silico the conservation of Arg-215 in the small, predicted periplasmic loop in all WaaL proteins. To get additional clues supporting a common reaction mechanism for ligation, we investigated whether the critical residues identified in this and in a previous work (Pérez et al. 2008) are conserved in other WaaL homologs. First, we examined in more detail the WaaL homologs in the bacteria listed in Table II for which the chemical nature of the lipid A-core \( \alpha \)-antigen linkage is known. These proteins were aligned based on the boundaries of their predicted TMs, cytoplasmic loops and periplasmic loops, revealing that they all contained a short periplasmic loop with at least one Arg residue (analogous to Arg-215 in the E. coli K-12 WaaL). This loop is adjacent to the predicted large periplasmic loop of variable length, ranging from 55 to 100 amino acids, which contained in all cases a His residue corresponding to His-338 (Figure 6).
residues are part of the motif H[NSQ]X9GXX[GTY] that is conserved in all WaaL proteins we examined (Supplementary data, Figure S5). Residues at positions 5 and 7 of this motif vary but they are always hydrophobic (Supplementary data, Figure S5). The motif spans the C-terminal end of the large periplasmic loop and the membrane boundary of the TM helix. The functional importance of the other residues in the H[NSQ]X9GXX[GTY] motif remains to be determined, but its strong conservation across WaaL proteins suggests it must play a critical functional or structural role. From these data, we conclude that at least two of the four residues we have found to be important for WaaL activity in vivo (Pérez et al. 2008) and in vitro, namely Arg-215 and His-338 are highly conserved in WaaL proteins. The predicted topological conservation of key functional residues and the conserved anatomic structure of the glycosidic linkage suggest that WaaL proteins utilize a common mechanism of reaction.

Discussion

The ligation reaction is not unique to LPS biogenesis. A similar reaction occurs for protein glycosylation in bacteria and eukaryotes, with the difference that the glycan moiety (analogous to Und-PP-O-antigen) is transferred from its lipid-linked diphosphate carrier to specific amino acids that differ depending on the type of glycosidic bond (N- vs O-linked). Therefore, it is not surprising that all of these proteins share common features. For instance, they have multiple TM helices and contain at least one relatively large soluble extracytoplasmic region. We demonstrated previously that the large extracytoplasmic loop of the E. coli K-12 WaaL contains several positively charged residues that are important for ligation (Pérez et al. 2008). Our in vitro ligation assay shows direct evidence for the first time that Arg-288, His-338 and Asp-389 are important for the ligation reaction. WaaL homologs differ in their specificity for the core acceptor but lack specificity for the Und-PP-glycan substrate (Heinrichs et al. 1998; Heinrichs et al. 1999). Molecular modeling predicted that Arg-288 and His-338 of the E. coli K-12 WaaL reside in two separate α-helices with their side chains facing each other, suggesting a putative catalytic or substrate-binding cleft where these conserved residues could interact with the phosphates of the Und-PP-glycan substrate (Pérez et al. 2008).

Furthermore, WaaL also has a predicted extracytoplasmic Arg (Arg-215 in the E. coli K-12 WaaL) that is crucial for ligation (Pérez et al. 2008). Our results showing that an Arg...
residue is conserved at the same position in all WaaL proteins examined suggest that this residue plays a critical functional role in the ligation reaction. Previous results in vivo showed that an R215K WaaL derivative could complement O-antigen ligation in a WaaL-defective mutant, whereas R215A, R215Q, R215H and R215E derivatives did not restore function (Pérez et al. 2008). Here, we show that the same occurs in vitro. Together, these observations raise the strong possibility that the Arg-215 is involved in binding a conserved moiety of the ligation reaction, such as the phosphate groups of the Und-PP-saccharide substrates. A precedent for such function was recently documented for the PglB oligosaccharyltransferase. Structural data on this protein reveal that Arg-375, a residue located in a small periplasmic loop, interacts with the phosphate groups of the Und-PP-saccharide substrate (Lizak et al. 2011).

Abeyrathne and Lam (2007) provided the first direct evidence that a purified WaaL was sufficient to catalyze O-antigen ligation and they also showed that the reaction requires ATP hydrolysis. The ligation reaction involves donor and substrate molecules located at the extra cytosolic side of the cellular membrane, a compartment that lacks ATP. We could not demonstrate ATP hydrolysis by the purified E. coli K-12 and P. aeruginosa WaaL proteins and the in vitro ligation reaction employing the E. coli WaaL took place without added Mg2+. Consistent with these biochemical data, previous efforts to mutate amino acids of regions in the E. coli K-12 showing a low level of similarity to Walker A and B motifs (Walker et al. 1982) and a palmate motif (Yamaguchi et al. 1993), similar to those in the P. aeruginosa WaaL, did not result in any mutant protein with compromised WaaL function that could be attributable to ATP hydrolysis (Pérez et al. 2008). Additional biochemical information show that: (i) adding exogenous Mg2+ -ATP was not required for ligation and did not enhance the enzyme activity, (ii) a general ATPase inhibitor had no effect on the ligation reaction, (iii) competition for ATP by coincubation with a known ATPase did not affect ligation; and (iv) ligation occurred in the presence of 10 mM EDTA, a metal divalent ion chelator. Ligase activity at this high concentration of EDTA also precludes the participation of a wide range of other divalent ions.

A TPase assays. (A) The ATPase assay was based on a coupling reaction with PK/LDH. Ten micrograms of purified WaaLEc (from E. coli K-12), WaaLPa (from P. aeruginosa PA01), or 20 µg of FhuC was used in a 200 µL reaction volume. The reaction was incubated at 37°C for 12 min monitoring the decrease in the absorbance of NADH at 340 nm. (B) Analysis of in vitro ligation reactions containing the E. coli K-12 WaaL (lanes 1), WaaL + FhuC (lanes 2) and FhuC (lanes 3). The reactions were performed and analyzed by silver staining or immunoblot with polyclonal anti-O7-specific Abs (anti-O7) as indicated in Figure 1A.

Fig. 4. ATPase assays. (A) The ATPase assay was based on a coupling reaction with PK/LDH. Ten micrograms of purified WaaLEc (from E. coli K-12), WaaLPa (from P. aeruginosa PA01), or 20 µg of FhuC was used in a 200 µL reaction volume. The reaction was incubated at 37°C for 12 min monitoring the decrease in the absorbance of NADH at 340 nm. (B) Analysis of in vitro ligation reactions containing the E. coli K-12 WaaL (lanes 1), WaaL + FhuC (lanes 2) and FhuC (lanes 3). The reactions were performed and analyzed by silver staining or immunoblot with polyclonal anti-O7-specific Abs (anti-O7) as indicated in Figure 1A.

Fig. 5. EDTA does not affect ligation in vivo. Lane 1, LPS produced in vivo by the SCM3(pHJC32/pXR1) strain expressing parental WaaL; 2, LPS produced in vivo by SCM3(pHJC32) in the absence of WaaL; 3, product of in vitro ligation assayed immediately after addition of purified ligase; 4, in vitro ligation after 23 h incubation of purified WaaL with LPS prepared from SCM3(pHJC32); 5, in vitro ligation after 23 h incubation of purified WaaL with LPS prepared from SCM3(pHJC32) plus 10 mM EDTA. The in vitro ligations were carried out with 0.67 μM of purified WaaL in 50 mM phosphate buffer, pH 6.0, at 37°C. Samples were analyzed by SDS–PAGE followed by silver staining.
### Table II. Anomeric status of the linkages catalyzed by WaaL proteins

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<th>Straina</th>
<th>Linkageb</th>
<th>Source or reference</th>
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<td>B. cenocepacia</td>
<td>β-QuINAc(1→7)-α-Hep</td>
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<td>Vibrio cholerae H11</td>
<td>β-QuINAc(1→4)-β-altro-heptulose</td>
<td>Vinogradov et al. (1992)</td>
</tr>
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<td>β-GlcNAc(1→3)-α-L-Rha</td>
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<td>Plesiomonas shigelloides O54</td>
<td>β-GlcNAc(1→4)-β-β-Glc</td>
<td>Niedziela et al. (2002)</td>
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</table>

*aUnless indicated in parenthesis the donor substrate is O-antigen.

*bThe terminal sugar acceptor in the lipid A-core OS is indicated in bold. Acyl, (S)-3-hydroxybutanoyl; Hep, 1-glycero-β-manno-heptose; Sug, 4-keto-hexosamine, 2-acetamido-2,6-dideoxy-ß-xylo-hex-4-ulopyranose.

in the reaction. Therefore, our data do not support a role for ATP hydrolysis in the ligation reaction catalyzed by WaaL and are consistent with the report that the H. pylori WaaL does not require ATP for ligation (Hug et al. 2010).

Since ATP hydrolysis is not required for O-antigen ligation, reaction mechanisms that equate WaaL with ATP-dependent enzymes like DNA and RNA ligases, as postulated by Abeyrathne and Lam (2007), are unlikely. Conceivably, all of these enzymes utilize a common mechanism to join the polysaccharide-building blocks to the acceptor molecule, which involves a glycosyl transfer reaction to the nucleophile oxygen of a hydroxyl substituent of the acceptor (Lairson et al. 2008). In our model, the Und-PP moiety in the ligation reaction would be comparable with the nucleotide diphosphate moiety of nucleoside substrates of classical glycosyltransferases, with the difference that instead of being a soluble molecule the Und-PP is membrane-embedded. In both situations (Und-PP-linked or nucleotide diphosphate-linked sugars), the leaving group upon glycosyl transfer is a diphosphate molecule and the proximal sugar to the distal phosphate is O-linked to the terminal sugar of the acceptor. The E. coli K-12 WaaL catalyzes the formation of a β-GlcNAc(1→7) linkage to a terminal lipid A-core OS heptose (Feldman et al. 1999). We show here that in the other cases where the nature of the glycosyl linkage imparted by the ligase has been investigated the glycans are always attached to the lipid A-core acceptor sugar by a β-linkage (Table II). The Und-PP-linked proximal sugar in the substrates results from the initiation reaction, which is catalyzed by membrane proteins that belong to two different protein families and involves the formation of a phosphoanhydride bond (Valvano 2011). Because the nature of this reaction, the sugar phosphate is transferred without breaking the glycosidic bond and, therefore, its anomeration cannot change. Consequently, the diphosphate nucleotide sugar precursors are all expected to be in α-configuration, and this has been experimentally demonstrated for uridine diphosphate (UDP)-Gal, UDP-Glc and UDP-GlcNAc (Murazumi et al. 1979; Weisgerber and Jann 1982; Olsthoorn et al. 2000). Therefore, we favor a model suggesting that WaaL proteins function as inverting glycosyltransferases. Inverting glycosyltransferases employ an ATP-independent reaction mechanism where nucleophilic attack by the acceptor hydroxyl group leads to an inversion of stereochemistry at the anomeric center of the donor substrate (Lairson et al. 2008). These enzymes use a catalytic base and a bound metal ion that stabilizes the leaving phosphate groups (Lairson et al. 2008). Recently, a precedent for a similar inverting reaction was documented for PgiB-mediated protein N-glycosylation, except that in this...
case Mg\(^{2+}\) ions are required (Lizak et al. 2011). Our results indicate that *E. coli* K-12 WaaL activity does not rely on EDTA-chelatable divalent ions and can proceed in the absence of Mg\(^{2+}\). A subset of inverting glycosyltransferases are metal ion-independent and use basic amino acids such as arginine, lysine or histidine residues to perform the same function as that of the metal ion (Chiu et al. 2004, 2007; Pak et al. 2006). We have also shown here and in a previous study (Pérez et al. 2008) that two arginine and a histidine residues in the *E. coli* WaaL enzyme are essential for ligation. From these, residues analogous to Arg-215 and His-338 are universally conserved in all WaaL protein homologs examined. The functional importance of the conserved His residue in the H[NSQ] X9GXX[GTY] motif was also established for the *V. cholera* (Schild et al. 2005) and the *P. aeruginosa* (Abeyrathne and Lam 2007) WaaL proteins. Therefore, it is reasonable to propose that the side chains of these conserved residues could have a role stabilizing the leaving phosphate groups of Und-PP upon cleavage of the sugar. In summary, we believe our data strongly support the notion that WaaL proteins share properties in common with metal ion-independent inverting glycosyltransferases.

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**Fig. 6.** Topological alignment of WaaL homologs for which the anomeric nature of the glycosidic linkage formed is known (corresponding to the bacteria listed in Table I). The alignment shows the TM helices (red square boxes), extracytoplasmic (out) and cytoplasmic (in) loops. The Arg corresponding to R215 in *E. coli* K-12 WaaL (K12) is found in all the other homologs (see also Supplementary data, Figure S4). The large extracytoplasmic loop also contains the conserved His corresponding to H338 of the K12 WaaL, which is located next to an asparagine (N) (see also Supplementary data, Figure S5). When found, the GXR motif corresponding to the region of Arg-288 of the K12 WaaL is also indicated. Underlined residues are those whose essential role has been demonstrated experimentally. Ecol, *E. coli* K-12; Sson, *Shigella sonnei*; Kpne, *Klebsiella pneumoniae*; Yent, *Yersinia enterocolitica*; Sari, *Salmonella enterica* Arizonae; Styp, *Shigella dysenteriae*; Vcho, *Vibrio cholerae*; Paer, *Pseudomonas aeruginosa* PA01; Bcen, *Burkholderia cenocepacia* K56-2; Pgin, *Porphyromonas gingivalis*; Sdys, *Shigella dysenteriae*; Vcho, *Vibrio cholerae*; Rsol, *Ralstonia solanacearum*.

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<table>
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<th>Yent</th>
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<td>TTANFQGVLGAIVHAILVP</td>
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Materials and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids are listed in Table I. Transformations were performed by electroporation (Dower et al. 1988). Bacteria grew at 37°C in a Luria-Bertani (LB) medium supplemented with ampicillin (100 µg mL⁻¹), tetracycline (20 µg mL⁻¹), spectinomycin (100 µg mL⁻¹) or 0.2% (w/v) β-arabinosone, when appropriate. For purification, WaaL was expressed in the E. coli strain JM105v, which carries a spontaneous and unmapped mutation resulting in the formation of a truncated LPS core OS. This strain was used to prevent potential binding of WaaL to lipid A-core OS containing the terminal sugar acceptor site.

Construction of strains and plasmids

pCM235, encoding a C-terminal FLAG followed by 5 × His-tagged WaaL, was used as a DNA template to construct pXR1, expressing WaaL with a 10 × His-tagged. Modified His-tagged WaaL, was used as a DNA template to construct pCM235, encoding a C-terminal FLAG followed by 5 × His-tagged WaaLPa, and ligated to pXR1, which was also digested with the same restriction enzymes. The resulting plasmid, encoding WaaLPa and ligated to pXR1, which was also digested with the same restriction enzymes, was introduced into DH5α strain JM105v, which carries a spontaneous and unmapped mutation resulting in the formation of a truncated LPS core OS. This strain was used to prevent potential binding of WaaL to lipid A-core OS containing the terminal sugar acceptor site.

LPS preparation

LPS was prepared as described previously (Marolda et al. 2006). Briefly, cells grew overnight on LB plates and suspended in phosphate-buffered saline, pH 7.2. After centrifugation, the cell pellet was resuspended in lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol and 1 M Tris–HCl, pH 6.8) and boiled for 10 min. Then, the sample was treated with proteinase K and extracted with hot phenol. The isolated LPS was quantified by the Bradford assay after establishing conditions of linearity based on ligoase concentration, pH, temperature and optimal buffer strength, consisted of the following components: LPS from SCM3(pJHCV32) containing both substrates for the reaction in 60 µL reaction volume corresponding to 10 µmol of Kdo equivalents and purified WaaL (0.67 µM) in 50 mM phosphate buffer. The reaction was incubated at 37°C for 16 h at pH 6.0 and terminated by adding 0.03 mg mL⁻¹ proteinase K (at 60°C for 20 min). The sample was deproteinized by adding 60 µL of hot phenol (70°C for 15 min) and centrifuged to separate the phenol phase. The upper aqueous phase was collected and run by tricine–SDS–PAGE.

ATPase assay

The ATPase assay was based on a coupling reaction with PK/LDH (Vogel and Steinhart 1976). In brief, 10 µg of purified WaaL or 20 µg of FhuC was used in a 200 µL reaction volume containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.4), 0.25 mM NADH, 1.25 mM phosphoenolpyruvate, 2 µL PK/LDH (Sigma Chemical Co., St Louis, Mo), 5 mM ATP and 2.5 mM MgCl₂. The reaction was incubated at 37°C for 10 min and ATP hydrolysis was monitored in a Cary 50 Microplate Reader (Varian Inc., Mississauga, Ontario, Canada) by determining the decrease in the absorbance of NADH at 340 nm.

Immunoblots–SDS-polyacrylamide gels were transferred onto nitrocellulose membranes, which were blocked with 10% Western Blocking Solution (Roche Diagnostics). Membranes were incubated overnight at 4°C with any of the following primary Abs: anti-FLAG M2 monoclonal Ab (mAb; Sigma Chemical Co.) at a 1:5000 dilution, anti-O7 polyclonal rabbit antiserum at a 1:1000 dilution, anti-core LPS WN1 222-5 mAb (HyCult Biotechnology, Plymouth Meeting, PA) at a 1:5000 dilution. Reacting bands were detected by fluorescence with an Odyssey infrared imaging system (Li-cor Biosciences, Lincoln, NE) using as secondary Abs either Alex Fluor® 680.
anti-mouse immunoglobulin G (IgG) (Invitrogen, Carlsbad, CA) or IRDye800CW affinity-purified anti-rabbit IgG Abs (Rockland Immunochemicals, Gilbert, PA), as appropriate.

Membrane topology predictions and in silico analysis of conserved motifs in periplasmic loops of WaaL proteins

Selected WaaL homologs were examined for predicted membrane topology using PolyPhobius (Käll et al. 2005) and manually aligned based on the boundaries of the TM helices and periplasmic loops. Also, for a more comprehensive analysis, 300 sequences annotated as O-antigen ligases were downloaded from NCBI in FASTA format. The sequences were stored in a single FASTA file, which was filtered and manipulated using a script developed in Python programming language, producing a separate FASTA output file for each sequence. Redundant sequences were eliminated. Subsequently, sequences were processed with PolyPhobius (Käll et al. 2005) to predict TM helices and periplasmic/cytoplasmic loops. Then, a heuristic algorithm was implemented in Python to evaluate the presence of a large periplasmic loop (larger than 30 amino acids) in each proposed topology. In those sequences that presented a large periplasmic loop, the existence of a small loop (less than 30 amino acids) preceding the large periplasmic loop was evaluated. Sequences resulting from these criteria were further evaluated for unpredictability and all sequences with more than 90% similarity were eliminated, resulting in a panel of unique WaaL sequences. The presence of an Arg residue in the predicted small periplasmic loop and additional motifs in the predicted large periplasmic loop of WaaL proteins was determined by a combination of Clustal analyses and motif discovery using the Meme and Mast programs from the Motif-Based Sequence Analysis Tools (http://meme.nbcr.net/meme4_6_1/intro.html). Significant motifs were extracted and aligned by Clustal.

Statistical analyses

When appropriate, data were statistically analyzed using GraphPad Prism, version 4.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

ABC, ATP-binding cassette; ADP, adenosine diphosphate; Arg, arginine; Asp, aspartic acid; ATP, adenosine-5’-triphosphate; DDM, dodecyl-β-β-maltoside; EDTA, ethylenediaminetetraacetic acid; Glc, glucose; GlcNAc, N-acetyl glucosamine; His, histidine; IgG, immunoglobulin G; Kdo, 2-keto-3-deoxy-octulosonic acid; LB, Luria-Bertani; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MOPS, 3-(N-morpholino)propanesulfonic acid; NADH, reduced nicotinamide dinucleotide; OS, oligosaccharide; PCR, polymerase chain reaction; PK, pyruvate kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TM, transmembrane; UDP, uridine diphosphate; Und-PP, undecaprenyl-diphosphate.

References


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