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Escherichia coli and *Pseudomonas aeruginosa* lipopolysaccharide O-antigen ligases share
similar membrane topology and biochemical properties

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Running Title: Membrane topology of WaaL proteins

Summary

WaaL is an inner membrane glycosyltransferase that catalyzes the transfer of O-antigen polysaccharide from its lipid-linked intermediate to a terminal sugar of the lipid A-core oligosaccharide, a conserved step in lipopolysaccharide biosynthesis. Ligation occurs at the periplasmic side of the bacterial cell membrane, suggesting the catalytic region of WaaL faces the periplasm. Establishing the membrane topology of the WaaL protein family will enable understanding its mechanism and exploit it as a potential antimicrobial target. Applying oxidative labelling of native methionine/cysteine residues, we previously validated a topological model for *Escherichia coli* WaaL, which differs substantially from the reported topology of the *Pseudomonas aeruginosa* WaaL, derived from the analysis of truncated protein reporter fusions. Here, we examined the topology of intact *E. coli* and *P. aeruginosa* WaaL proteins by labeling engineered cysteine residues with the membrane-impermeable sulfhydryl reagent polyethylene glycol maleimide (PEG-Mal). The accessibility of PEG-Mal to targeted engineered cysteine residues in both *E. coli* and *P. aeruginosa* WaaL proteins demonstrates that both ligases share similar membrane topology. Further, we also demonstrate that *P. aeruginosa* WaaL shares similar functional properties with *E. coli* WaaL and that *E. coli* WaaL may adopt a functional dimer conformation.

Introduction

The Gram-negative bacterial cell envelope comprises an outer and an inner membrane separated by the periplasmic space. The lipopolysaccharide (LPS), a glycolipid made of lipid A, core oligosaccharide (core), and O-antigen (OAg) polysaccharide (Valvano, 2011), is a predominant component of the outer membrane. LPS helps maintain the stability and relative impermeability of the outer membrane. The lipid A provides a scaffold for the assembly of the core *via* sequential sugar transfer reactions. The lipid A-core is further remodelled by chemical modifications that provide bacteria with resistance to cationic antimicrobial peptides and hydrophobic antibiotics, contributing to bacterial evasion of innate immune recognition and intrinsic antimicrobial resistance (Needham and Trent, 2013).

OAg are polymers of sugar repeating units that vary greatly in chemical composition, structure, and antigenicity across individual strains and bacterial species (Knirel, 2011). Bacteria expressing OAg resist innate immune attack by host macrophages and serum complement (Pluschke and Achtman, 1984, Joiner, 1988). OAg are independently assembled as undecaprenyl-pyrophosphate (Und-PP)-linked saccharides and ligated to the lipid A-core (Valvano, 2011) resulting in the release of Und-PP, which is recycled into Und-P by a poorly characterized pathway. Und-P is essential for the biosynthesis of other surface polymers such as capsules and cell wall peptidoglycan (Valvano, 2011). All these processes occur in the inner membrane and require the activities of membrane-embedded proteins that participate in various key steps of lipid A-core and OAg assembly (Valvano, 2011). LPS export from the inner to the outer membrane and the proper insertion of LPS in the outer leaflet of the outer membrane require Lpt proteins, which form a trans-cell envelope multiprotein complex spanning the inner and outer membranes (Okuda *et al.*, 2016, Sperandio *et al.*, 2017).

Und-PP-linked OAg appearing on the periplasmic side of the membrane become ligated to preformed lipid A-core (Valvano, 2011). The membrane protein WaaL catalyzes the ligation step that joins the OAg to the rest of the LPS. WaaL can specifically recognize one or more terminal sugars of the lipid A-core acceptor (Feldman *et al.*, 1999), but shows no specificity for the structure of the lipid-linked saccharide substrate (Heinrichs *et al.*, 1998c). Therefore, despite different OAg can be recombinantly expressed in *Escherichia coli* distinct WaaL proteins cannot cross-complement an *E. coli* K-12 *waaL* mutant (Valvano *et al.*, 2011). The periplasmic location of the ligation reaction suggests that potential inhibitory molecules need not enter into the bacterial cytosol and could evade membrane drug efflux mechanisms, which reduces the efficacy of conventional antibiotics. Elucidating the ligation mechanism is a critical step prior to the rational design of novel ligase inhibitors.

The best-studied ligases to date are the *E. coli* and *Pseudomonas aeruginosa* WaaL proteins (herein WaaL_{Ec} and WaaL_{Pa}, respectively) (Abeyrathne *et al.*, 2005, Abeyrathne and Lam, 2007, Islam *et al.*, 2010, Pan *et al.*, 2012, Pérez *et al.*, 2008, Ruan *et al.*, 2012). Both proteins have 12 transmembrane helices (TMHs) and a characteristic large periplasmic loop (PL5). Conceivably, WaaL activity involves amino acids exposed to the periplasmic space where they could interact with donor and acceptor molecules (Pérez *et al.*, 2008, Ruan *et al.*, 2012). A conserved arginine residue critical for ligation, R215 in WaaL_{Ec}, is present in the preceding short periplasmic loop PL4 (Pérez *et al.*, 2008). We proposed a structural model for the WaaL_{Ec} PL5 in which arginine-288 (R288) and histidine-338 (H338), two conserved residues that are needed for efficient ligation, are exposed to the solvent in a spatial arrangement that suggests direct interactions with substrate molecules (Pérez *et al.*, 2008). The critical H338 residue is part of a conserved motif

(H[NSQ]X₉GX₂[GTY]) spanning the C-terminal end of PL5 and the periplasm-membrane boundary of the TMH-X (Ruan *et al.*, 2012).

Structural evidence from the analysis of the WaaL-catalyzed linkage between the acceptor sugar residue at the lipid A-core and the OAg residue linked to Und-PP, together with biochemical data, suggest WaaL proteins are metal-independent inverting glycosyltransferases (Ruan *et al.*, 2012). Therefore, WaaL proteins should have common features, including a similar topological location of functionally relevant amino acid residues. Both WaaL_{Ec} and WaaL_{Pa} have relatively similar *in silico* predicted membrane topologies (Fig. 1A and B). However, the experimental determination of the WaaL_{Pa} topology using truncated protein derivatives arising from a deletion/reporter fusion analysis revealed striking differences with WaaL_{Ec} (Islam *et al.*, 2010). While experimental data and *in silico* predictions agree with the topology of the N- and C-terminal regions of WaaL_{Pa} (spanning TMH-I to TMH-III and TMH-IX to TMH-XII, respectively), major differences appear in the central region of the protein spanning TMH-IV to TMH-VIII (Fig. 1B). These TMHs in the experimental model had no concordance with the predicted boundaries for each of the corresponding TMHs in the *in-silico* model (Fig. 1A and B). A striking difference between the two models was the presence in the experimental model (WaaL_{Pa}-Del/Fus in Fig. 1A and B) of a large cytosolic loop between amino acid residues at positions 153-182 (CL3), which contained highly hydrophobic residues commonly present in TMH segments. Further, the predicted conserved essential arginine residue R212 (corresponding the R215 in WaaL_{Ec}) was buried in TMH-VIII of the WaaL_{Pa}-Del/Fus model, but exposed to the PL4 in the *in-silico* model (Fig. 1A and B). Intriguingly, the experimentally assigned TMH-VIII in WaaL_{Pa}-Del/Fus also contained three positively charged residues in tandem (arginine-231, lysine-232, and lysine-233) (Islam *et al.*, 2010), which would be highly unusual residues to be

embedded in the lipid bilayer. In this work, we have comparatively evaluated the topology of WaaL_{Ec} and WaaL_{Pa} by a scanning cysteine accessibility mutagenesis (SCAM) strategy using the membrane-impermeable sulfhydryl reagent polyethylene glycol maleimide (PEG-Mal). Our experiments demonstrate that both proteins share identical topology. Further, examining the *in vitro* ligation reaction using purified WaaL_{Pa}, we demonstrate that ligation occurs in the presence of EDTA, suggesting it cannot require ATP, as we previously observed for WaaL_{Ec} (Ruan *et al.*, 2012), and we also provide evidence that WaaL_{Ec} forms functional dimers. We conclude that WaaL_{Ec} and WaaL_{Pa} proteins are archetypes of a family of metal-independent, membrane embedded glycosyltransferases sharing similar topology and functional properties.

Results

E. coli and *P. aeruginosa* WaaL proteins lacking native cysteines remain functional and both require a conserved arginine residue

To investigate the topology of WaaL_{Ec} and WaaL_{Pa}, we first expressed both proteins as C-terminal FLAG-10xHis-tag fusions under the control of exogenous L-arabinose from a pBAD24 vector derivative (Table 1). Protein expression was detected by Western blot using an anti-FLAG monoclonal antibody, and it was optimal in the presence of 0.2% L-arabinose (Fig. S1). A cysteine less version of WaaL_{Ec} (WaaL_{Ec}C-less) was constructed by sequential site-directed replacements to alanine of each native cysteine residue in the native protein at positions 29, 54, 203, and 225. We also replaced by alanine a cysteine residue at position 428, which originated from the FLAG-tag fusion construction. Further, we generated WaaL_{Ec} versions with a single

cysteine at position 29 (WaaL_{Ec}C29) and 428 (WaaL_{Ec}C428), whereby the other native cysteine residues were replaced by alanine. Additional single-cysteine replacements were made at selective sites.

Similarly, we constructed a cysteine less version of WaaL_{Pa} (WaaL_{Pa}C-less) by replacing the native cysteine residue 114 and the Cys-403 residue originated from the FLAG-tag fusion by alanine. By replacing each cysteine residue individually, we also generated versions of WaaL_{Pa} with single cysteines at positions 114 (WaaL_{Pa}C114) and 403 (WaaL_{Pa}C403). Under induction with 0.2% L-arabinose, all the mutant derivatives of WaaL_{Ec} or WaaL_{Pa} were well expressed in *E. coli* DH5 α (Fig. 2B and 3, upper panels, respectively) with apparent molecular masses of 50 kDa and 47 kDa, respectively. The parental FLAG-10xHis-tag fusions proteins WaaL_{Ec} and WaaL_{Pa} were also expressed with similar apparent molecular masses, respectively (Fig. S1).

The cysteine less version of WaaL_{Ec} and all the single cysteine-alanine substitutions did not affect OAg ligation, as determined by the ability of each mutant protein to support O7 LPS surface expression in *E. coli* SCM3 (pJHCV32) (Fig. 4A). This *E. coli* K12 strain has a chromosomal deletion that eliminates the OAg and colanic capsule synthesis gene clusters and contains also a deletion of the *waaL* gene (Pérez *et al.*, 2008), while the cosmid pJHCV32 encodes the O7 lipopolysaccharide antigen gene cluster (Valvano and Crosa, 1989). The WaaL G286C mutant showed an apparently slight decrease in ligase activity, which could not be attributed to reduced protein expression and may be due to reduced efficiency of the enzyme.

Likewise, the cysteine less version of WaaL_{Pa} and most of the single cysteine substitutions restored OAg LPS surface expression in the *P. aeruginosa* PAO1*waaL*::Gm^R mutant to levels comparable to those in the parental PAO1 strain (Fig. 4C). The replacements R147C, L155C, and S187C showed a small reduction in ligase function in comparison to the parental WaaL_{Pa},

while the S169C and G282C replacements had a stronger effect since OAg in the LPS was not detected by silver staining (Fig. 4C, upper panel). To determine if the reduced ligase activity observed by OAg complementation was due to different levels of protein expression, we isolated total membranes and detected the WaaL_{Pa} proteins by immunoblot. This experiment demonstrated that all the single Cys replacements are expressed at WaaL_{Pa} parental levels, except for the G282C in which the protein level is reduced (Fig. 4C, bottom panel). We used a more sensitive approach to determine the ligase activity of the single Cys mutants based on immunoblot with the anti-O5 monoclonal antibody (MF15-4). This experiment shows that only the G282C replacement is affected in its ability to restore OAg ligation, while all the other replacements supported OAg ligation (Fig. 4C, middle panel). A G282A replacement mutant also exhibited the same phenotype as G282C (Fig. S2), suggesting that the G282 residue in WaaL_{Pa} is important for ligase function either by being involved in the reaction or by maintaining the local conformation of a putative catalytic fold. Together, we conclude that, except for WaaL_{Pa}G282C the cysteine less versions of WaaL_{Ec} and WaaL_{Pa}, as well as their single cysteine replacement derivatives remain functional.

Because the Arg-212 residue in WaaL_{Pa} is the counterpart of Arg-215 in WaaL_{Ec}, which is required for ligase function (Pérez *et al.*, 2008, Ruan *et al.*, 2012), we generated a WaaL_{Pa} derivative with an alanine replacement at this site (WaaL_{Pa}R212A). This mutant protein lost the ability to complement the ligase function in the PAO1*waaL*::Gm^R ligase defective mutant (Fig. 4B). The loss of function phenotype is not due to defects in protein expression since the WaaL_{Pa}R212A was produced at wild type levels (Fig. 4B, bottom panel). The results indicate that similarly to Arg-215 in WaaL_{Ec}, the corresponding Arg-212 residue in WaaL_{Pa} is also critical for ligation.

Labeling of Cys residues with PEG-Mal supports the predicted topological model of WaaL_{Ec}

Our previous study using GFP as a topology probe and trypsin digestion determined a C-terminus inside, a large periplasmic loop PL5 and a small adjacent periplasmic loop PL4 for the *E. coli* K-12 WaaL, which is consistent with the predicted topology proposed by Perez et al. (Pérez *et al.*, 2008) and with a study using oxidative labeling of Met and Cys followed by mass spectroscopy (Pan *et al.*, 2012). In this study we used the substituted-cysteine accessibility method (SCAM) for mapping WaaL topology in their native state. This approach is based on the introduction of single Cys residues at various positions in a membrane protein followed by chemical modification with a membrane impermeable thiol-specific reagent, polyethylene glycol maleimide (PEG-Mal), either before or after disrupting cell membrane integrity by detergent treatment (Fig. 2A).

The results show that WaaL_{Ec}C-less could not be labelled by PEG-Mal in the absence or presence of SDS, or in the presence of N-ethylmaleimide (NEM), a low molecular weight, membrane permeable sulfhydryl-reactive compound that blocks PEGylation (see *Experimental Procedures* and Fig. 2A), and sodium dodecylsulfate SDS (Fig. 2B), providing a negative control for the PEGylation assay. In contrast, residue C29 neither was accessible to PEG-Mal in the absence of SDS nor could be blocked by NEM, but it was labelled by PEG-Mal in the presence of SDS (Fig. 2B), indicating that C29 is embedded within a TMH, as we previously demonstrated by oxidative labelling (Pan *et al.*, 2012). The residue C428 was inaccessible to PEG-Mal in the absence of SDS, but accessible to NEM and PEG-Mal in the presence of SDS (Fig. 2B), as expected for a residue facing the cytosol, which is consistent with the C-terminus inside topology of WaaL_{Ec} (Pan *et al.*, 2012, Pérez *et al.*, 2008).

We then examined the accessibility of residues I162, T176 and T190, which were predicted to reside in the periplasmic PL3 loop, TMH-VI, and the cytoplasmic CL3 loop of WaaL_{Ec}, respectively (Fig. 1). The counterpart of this region in the model of WaaL_{Pa} proposed by Islam *et al.* (2010) exhibits a very different topology (Fig. 1). The WaaL_{Ec} I162C residue was accessible to PEG-Mal in the absence of SDS, but blocked by NEM and labeled by PEG-Mal in the presence of SDS (Fig. 2B), denoting a periplasmic location. Residue T176C was not accessible to PEG-Mal in the absence of SDS, could not be blocked by NEM and could be labeled by PEG-Mal in the presence of SDS (Fig. 2B), indicating the residue forms part of the TMH-VI. Residue T190C was consistently not labeled by PEG-Mal in the presence of SDS (Fig. 2B), so we could not precisely localize its location. This suggest the residue may be sequestered into a local secondary structure configuration that makes it inaccessible to PEG-Mal, as we have shown for other membrane proteins (Furlong *et al.*, 2015).

Residues T214 and A216 flank R215, a crucial residue for the WaaL_{Ec} function (Pérez *et al.*, 2008, Ruan *et al.*, 2012). Both T214C and A216C were weakly PEGylated in the presence of SDS (Fig. 2B). Further, T214C was also labeled by PEG-Mal in the absence of SDS and blocked by NEM (Fig. 2B), indicating this residue faces the periplasmic space. In contrast, residue A216C was not accessible to PEG-Mal in the absence of SDS, but PEGylation was blocked by NEM (Fig. 2B), suggesting A216 is at the periplasmic border of TMH8. Together, these results support the prediction that R215 is located in a short loop exposed to the periplasm (Pérez *et al.*, 2008, Ruan *et al.*, 2012). Residue G286C was accessible to PEG-Mal, both in the absence and presence of SDS, and labeling was blocked by NEM (Fig. 2B), indicating that G286 is located in periplasm, consistent with the prediction that G286 is a residue in PL5. Together, the results

from PEGylation accessibility of the single-Cys mutants are consistent with the previously predicted topology of WaaL_{Ec} (Fig. 5A).

PEG-Mal labelling of Cys residues in P. aeruginosa WaaL validates a topological model similar to that of E. coli WaaL

As shown in Fig. 3, WaaL_{Pa}C-less was not labeled at any condition providing a negative control for the PEGylation assays. Residue C114 of WaaL_{Pa} was not labeled by PEG-Mal in the presence of SDS (Fig. 3), so we could not assign a location for this residue. Residue C403 was inaccessible to PEG-Mal in the absence of SDS, weakly PEGylated with SDS, but PEGylation was blocked by NEM in the presence of SDS (Fig. 3). This is consistent with a C-terminus inside topology of WaaL_{Pa}. We then examined 10 residues predicted *in silico* to be located in TMH-V (R147), the PL3 loop (L155, L157, T158 and G161), TMH-VI (N165, S169, A170 and A178), and in the CL3 loop (S187) (Fig. 1). These same 10 residues were assigned to TMH-VI (R147), CL3 (L155, L157, T158, G161, N165, S169, A170 and A178), and TMH-VII (S187) in the model proposed by Islam *et al.* (2010) (Compare Fig. 5B and C). The single cysteine substitutions of the 10 residues were labeled by PEG-Mal in the presence of SDS (Fig. 3). Residue R147C was inaccessible to PEG-Mal in the absence of SDS and unblocked by NEM (Fig. 3), indicating that R147 is embedded in a TMH. Residues L157C, T158C and G161C were accessible to PEG-Mal in the absence of SDS and also blocked by NEM (Fig. 3), indicating they are exposed to the periplasm. L155C was not accessible to PEG-Mal in the absence of SDS, but accessible to PEG in the presence of SDS (Fig. 3), indicating a location near the periplasmic border of the TMH. N165C, S169C, A170C and A178C were not accessible to PEG-Mal in the absence of SDS and not blocked by NEM (Fig. 3), indicating they are part of the TMH. S187C

was not accessible to PEG-Mal in the absence of SDS, but PEGylation was blocked by NEM (Fig. 3), indicating that S187 is in cytoplasm. The results demonstrate that the topology of WaaL_{Pa} resembles that of WaaL_{Ec} (Fig. 5A and B).

Further, residues S211 and T213 flank R212, a counterpart of the functional residue R215 in the PL4 of WaaL_{Ec}. Both WaaL_{Pa} S211C and T213C residues were labeled by PEG-Mal in the presence of SDS (Fig. 3). S211C was also PEGylated in the absence of SDS and accessible to NEM (Fig. 3), indicating this residue faces the periplasm. On the contrary, T213C was inaccessible to PEG-Mal in the absence of SDS but accessible to NEM (Fig. 3), suggesting a location in the periplasmic borders closer to TMH. These data demonstrate that R212 is also part of short periplasmic loop PL4, in contrast to the model by Islam *et al.* (2010) (Fig. 5C), in which S211, R212 and T213 are located in TMH-VIII.

We also examined six residues spanning the segment between L251 and V309, which are predicted to be located in PL5 and its borders (Fig. 5B). All the single-Cys substitutions of these residues were accessible to PEG-Mal in the presence of SDS (Fig. 3). Residue V255C was also labeled by PEG-Mal in the absence of SDS and labeling blocked by NEM (Fig. 3), indicating a periplasmic location (Fig. 5B). L251C was not accessible to PEG-Mal in the absence of SDS, but PEGylation in SDS was blocked by NEM (Fig. 3), indicating it is located at the periplasmic borders closer to the C-terminus of TMH-IX. P253C was not accessible to PEG-Mal in the absence of SDS and not blocked by NEM (Fig. 3), also suggesting this residue may be located near the border of TMH-IX (Fig. 5C). Residue G282C was accessible to PEG-Mal in the absence of SDS and labeling blocked by NEM (Fig. 3), indicating a periplasmic location within PL5 (Fig. 5B). Both L307C and V309C were not accessible to PEG-Mal in the absence of SDS and not blocked by NEM (Fig. 3), indicating they are embedded within TMH-X. Therefore, the

experimental results obtained by PEG-Mal labeling of these six residues agree with both the *in silico* model for WaaL_{Pa} (Fig. 1B) and the topology proposed by Islam *et al.* (2010) (Fig. 1C). Together, our experimental data demonstrate that the topology predictions by Islam *et al.* (2010), which were based on deletion-fusion experiments are not accurate to delineate the topology of the central portion of WaaL_{Pa} (Figs. 1 and 5C) since they are not supported by PEG-Mal labeling of intact protein derivatives. Based on our results, we conclude that both WaaL_{Pa} and WaaL_{Ec} share a similar membrane topology, as shown in Fig. 5A and B.

WaaL_{Pa} exhibits similar functionality as WaaL_{Ec}

Previous work reported that WaaL_{Pa} required ATP hydrolysis for ligation, while this was not the case for the WaaL_{Ec} protein (Han *et al.*, 2012, Ruan *et al.*, 2012). To assess the WaaL_{Pa} functionality we purified this protein and performed an *in vitro* ligase assay, as previously described (Ruan *et al.*, 2012). Initial experiments using LPS ligation substrates containing a mixture of Und-PP-linked O-polysaccharides (including A-band homopolysaccharide and B-band heteropolysaccharide) and LPS core prepared from PAO1*waaL*::Gm^R and mixed them with purified WaaL_{Pa} did not result in ligation products that were detectable by silver staining. *P. aeruginosa* produces two forms of lipid A-core. One of them consists of a capped core in which an L-rhamnose is attached to a terminal glucose (glucose-I) by WapR, while the other (uncapped core) contains L-rhamnose attached to glucose-II by MigA (Kocincova and Lam, 2011). From these two core forms, only the capped core is a substrate for ligation with O-antigen polysaccharide. To increase the proportion of capped core we constructed pXR93, overexpressing WapR under the control of the *lac* promoter. The strategy of preparing the ligation substrates from the *P. aeruginosa* strain PAO1*waaL*::Gm^R(pXR93) expressing the

WapR protein allowed us to detect LPS products by silver staining (Fig. 6). The results indicated that 37°C was a suitable temperature for the ligation reaction in contrast to 22°C and 30°C (Fig. 6, Lanes 3-5). The addition of IPTG showed increased detection of ligation products (Fig. 6, Lanes 5-8). However, 10 mM ethylenediaminetetraacetic acid (EDTA), a concentration that could chelate divalent cations including Mg^{2+} and disrupt the ATPase hydrolysis, did not inhibit the reaction (Fig. 6, Lane 9). This indicated that, as in the *in vitro* ligase assay for WaaL_{Ec}, WaaL_{Pa} is also an ATP hydrolysis-independent, metal ion-independent glycosyltransferase (Ruan *et al.*, 2012).

Ligation results in inversion and is specific for the lipid A core substrate

Structural data on LPS of many different species revealed a β -linkage of the sugar from the OAg unit attached to the terminal lipid A-core acceptor site. Interestingly, this sugar is also the one proximal to Und-PP, which arises from the diphosphate nucleotide sugar precursors in the α -configuration employed in the initiation reaction by enzymes of the WecA and WbaP families (Ruan *et al.*, 2012, Valvano, 2011), indicating WaaL is an inverting glycosyltransferase (Ruan *et al.*, 2012). To gather additional evidence for this mechanism we examined the reactivity of chemically characterized *E. coli* lipid A-core forms with a monoclonal antibody specific to β -O-linked *N*-acetylglucosamine (β -O-GlcNAc). For this experiment, we employed LPS from different *E. coli* strains (Fig. 7). W3110 has a lipid A-core with a terminal β -linked-GlcNAc, which results from the synthesis of a truncated O-antigen unit consisting of Und-PP-GlcNAc (Feldman *et al.*, 1999). CLM35, a strain with *wecA* and *wzx* double deletion, produces lipid A-core devoid of GlcNAc (Marolda *et al.*, 2006b). The LPS from CLM24, a strain with *waaL* deletion contains a mixture of lipid A-core and Und-PP-GlcNAc (Feldman *et al.*, 2005), while

CLM24(pXR1) contains the plasmid pXR1 expressing *E. coli* WaaL and therefore producing lipid A-core with a terminal GlcNAc residue (Fig. 7A). The strain F632 is an *E. coli* R2 chemotype bacterium producing a lipid A-core with a terminal α -linked-GlcNAc (Leipold *et al.*, 2007). While the LPS preparations looked similar by silver staining, only those from W3110 and CLM24(pXR1) reacted with the anti- β -O-GlcNAc antibody (Fig. 7A), confirming that the terminal GlcNAc attached to K-12 LPS core is in β -configuration. The absence of a positive signal with the antiserum in the F632 LPS shows that the antiserum cannot detect GlcNAc in the α -configuration. These results provide additional support to the notion that WaaL is an inverting glycosyltransferase.

Another property of the ligase is its specificity for the terminal core acceptor site, which was determined by genetic experiments (Heinrichs *et al.*, 1998a, Heinrichs *et al.*, 1998b, Heinrichs *et al.*, 1998c). We took advantage of the *in vitro* ligation assay to determine *in vitro* if the WaaL_{Ec} can ligate O-antigen substrates from different bacterial species. We used four LPS preparations containing lipid A-core and endogenously made Und-PP-linked O-antigen substrates (Fig. 7B). They included LPS from strain CLM35 (Core_{Ec}) containing lipid A-core plus Und-PP-GlcNAc, LPS from strain CLM24(pMF19) (LPS_{Ec}) containing lipid A-core plus Und-PP-OAg, LPS from strain PAO1waaL::Gm^R(pXR93) (LPS_{Pa}) containing lipid A-core core plus Und-PP-OAg (from *P. aeruginosa*), and lipid A-core from strain K56-2waaL_{Bc}::pGPQTp (LPS_{Bc}) containing lipid A-core plus Und-PP-OAg (from *Burkholderia cenocepacia*). We mixed purified WaaL_{Ec} with different combination of the LPS preparations as described in *Experimental Procedures*. Our results show that LPS preparations from *P. aeruginosa* (LPS_{Pa}) and *B. cenocepacia* (LPS_{Bc}) enriched for Und-PP-linked O antigen result in the production of *E. coli* lipid A-core with the ligated O-polysaccharides from the respective species (Fig. 7B, Lane 5, and 7C, Lane 6,

respectively), as efficient as LPS preparation from *E. coli* (LPS_{Ec}) (Fig . 7B, Lane 6). From these experiments, we conclude WaaL lacks specificity of the Und-PP-linked OAg.

E. coli WaaL forms functional oligomers

Purified *E. coli* and *P. aeruginosa* WaaL preparations typically show variable amounts of larger products in SDS-polyacrylamide gel electrophoresis (PAGE) that react with the anti-FLAG antibody suggesting the presence of multimeric forms of the protein samples (Fig. S1) likely due to incomplete denaturation since heating at 100°C in SDS precludes detection of polytopic membrane proteins in SDS-PAGE (Abeyrathne and Lam, 2007, Lehrer *et al.*, 2007, Marolda *et al.*, 2004, Pérez *et al.*, 2008, Ruan *et al.*, 2012, Vinés *et al.*, 2005, Tatar *et al.*, 2007). However, these larger forms could also occur by dimerization. To investigate if WaaL can form functional dimers, we reasoned that constructs lacking ligase function if expressed singly could revert to functional enzyme forms when expressed jointly. To explore this possibility, we used WaaL_{Ec} functionally defective constructs that exhibited observable protein expression, but could potentially restore ligation if jointly expressed. These included WaaLΔN2TM, a derivative of WaaL_{Ec} with a deletion removing amino acid residues from Leu-2 to Asn-71 and eliminating TMHs I and II, WaaL-R288A and WaaL-R215A (Ruan *et al.*, 2012). All these recombinant proteins, expressed from pBAD24 in the presence of L-arabinose were detected by immunoblot at comparable levels (Fig. 8A, left panel). Similarly, WaaLΔN2TM expressed from pBAD33, a plasmid that is compatible with the pBAD24 (Guzman *et al.*, 1995), was also readily detected by immunoblot (Fig. 8A, right panel). We first confirmed that the recombinant WaaL proteins function *in vivo* as expected. For this we transformed the *E. coli* SCM3(pJHCV32) with various plasmids encoding each of the WaaL proteins and examined the production of O7

polysaccharide. Fig. 8B, left panel, shows that no O7 polysaccharide was produced in the presence of WaaL Δ N2TM, WaaL-R288A, and WaaL-R215A. In contrast, O7 polysaccharide was detected in SCM3(pJHCV32) bacteria expressing the parental WaaL and WaaL_{R215K}, as expected from previous results showing that a positive charged amino acid is required at position 215 of *E. coli* WaaL (Pérez *et al.*, 2008, Ruan *et al.*, 2012). Next, we co-expressed each of these proteins with WaaL Δ N2TM, which was expressed from pBAD33. The results revealed that O7 polysaccharide was produced by co-expression of WaaL Δ N2TM/WaaL-R288A or WaaL Δ N2TM/WaaL-R215A (Fig. 8, right panel), indicating that the reversal of the ligase function could originate from protein-protein interactions between WaaL Δ N2TM and WaaL-R288A, or between WaaL Δ N2TM and WaaL-R215A.

We also investigated WaaL oligomerization by chemical crosslinking. The *E. coli* WaaL and WaaL_{C-less} proteins with FLAG and 10xHis tags were purified from *E. coli* JM105v, as described previously (Ruan *et al.*, 2012). The membrane-permeable crosslinker dithiobis (succinimidylpropionate) (DSP) was used, which crosslink molecules whose respective lysine residues come within 12.0 Å, the spacer length of the crosslinker (Smith *et al.*, 2011). Extensive crosslinking of WaaL was apparent by anti-His immunoblot. A portion of each sample was treated with the reducing agent dithiothreitol (DTT) to cleave the crosslinking. The results show that WaaL_{Ec} forms oligomeric species of higher apparent mass in the presence of DSP, while the monomeric protein is efficiently recovered by addition of DTT (Fig. 8C,). To investigate if dimerization involves disulfide bond formation, we also performed crosslinking experiments with the WaaL_{C-less} protein. The results show that native cysteines are partially involved in dimerization, as crosslinking was less efficient with the cysteine less protein (Fig. 8C).

Therefore, the genetic reconstitution experiments and biochemical crosslinking data indicate WaaL can form oligomers and is likely a functional dimer *in vivo*.

Discussion

Using a SCAM strategy based on PEGylation, we show here the topologies of *E. coli* and *P. aeruginosa* WaaL proteins are highly similar. The WaaL_{Pa} topology model we constructed differs from that previously reported in the literature, which was based on a dual-reporter fusion approach (Alexeyev and Winkler, 1999) and resulted in a library of truncated versions of WaaL_{Pa} C-terminally fused to reporter β -galactosidase and alkaline phosphatase enzymes for determining the cytoplasmic or periplasmic location of each fusion end point, respectively (Islam *et al.*, 2010). Reporter fusions are commonly used to map the topology on membrane proteins, but the strategy is not devoid of pitfalls, especially considering that the topogenic signals for membrane protein insertion depend from both amino acid and membrane lipid compositions (Bogdanov and Dowhan, 1999). Indeed, the dual-reporter fusion strategy for WaaL_{Pa} did not accurately predict TMHs and loops boundaries in the central region of the protein. This could most likely be caused by disruption of TMH-TMH interactions leading to alternative topologies of the truncated WaaL segment included in the fusion. Similar observations were made for TMH-I of the *E. coli* α -ketoglutarate permease, which was not detected by a PhoA fusion owing to the presence of positively charged residues in other TMH domains that were required to neutralize the negatively charged residues in TMH-I, facilitating its membrane insertion (Seol and Shatkin, 1993). Alternatively, truncated TMH domains may form helical hairpins that result in the mislocalization of the reporter protein (Cassel *et al.*, 2008).

SCAM is also not free of drawbacks, as residues in certain locations may become inaccessible to sulfhydryl labeling (Bogdanov *et al.*, 2010). We noted that the cysteine replacement at position 190 in WaaL_{Ec} (T190C) could not be labeled under any of the conditions tested. This residue, presumably located in the CL3, is flanked by a tyrosine residue at one end and two lysine residues at the other end, suggesting the possibility of a local secondary structure stabilized by salt bridges between the tyrosine and the lysine residues, which would prevent the access of PEG-Mal by steric hindrance. Local secondary structure preventing access of PEG-Mal was noted before in the TMH-V of membrane protein WcaJ, which does span the membrane and forms a hairpin helix around a proline and a serine residue (Furlong *et al.*, 2015). Similarly, the native cysteine residue at position 114 of WaaL_{Pa} could not be labeled. This residue is predicted to be embedded in TMH-IV, and lack of labeling with PEG-Mal in this case could arise from the absence of ionization of the thiol group within a strong hydrophobic environment (Bogdanov *et al.*, 2010). However, despite the difficulties with the localization of these specific residues, the overall topology of WaaL_{Pa} derived from PEG-Mal SCAM agrees with that of WaaL_{Ec}. Indeed, the critical residues for ligase function in both proteins, Arg-215 in WaaL_{Ec} and Arg-212 in WaaL_{Pa} are orientated towards the periplasmic space within a short PL4 region. This is consistent for a protein that requires access to substrates in the periplasmic space.

Further, our *in vitro* ligation experiments demonstrated that the WaaL_{Pa}-catalyzed ligation occurs in the presence of high concentration of the metal divalent ion chelator EDTA, indicating that the reaction is metal cation independent, which also rules out an involvement of ATP hydrolysis, as previously reported (Abeyrathne and Lam, 2007). Therefore, we conclude that WaaL_{Pa} has the same general properties of previously characterized ligases such as those from *E.*

coli K-12 (Ruan *et al.*, 2012), *E. coli* O86 (Han *et al.*, 2012), and the *Helicobacter pylori* (Hug *et al.*, 2010), displaying similar membrane topology and biochemical function.

In a previous study, we provided evidence suggesting the WaaL_{Ec} is an inverting glycosyltransferase (Ruan *et al.*, 2012). This means the glycosyl bond formed by the ligation reaction has an inverted anomeric configuration with respect to the donor substrate (Lairson *et al.*, 2008). The substrate for the ligase reaction is the Und-PP-linked proximal sugar, which in turn is the product of the initiation reaction resulting in the formation of a phosphoanhydride bond without a change in the anomerization of the sugar backbone structure (Valvano, 2011). Indeed, the diphosphate nucleotide sugar precursors for the initiation reaction, such as UDP-Gal, UDP-Glc and UDP-GlcNAc are all in α -configuration (Murazumi *et al.*, 1979, Weisgerber and Jann, 1982, Olsthoorn *et al.*, 2000). In this work, we provide additional evidence for inversion of anomerization upon ligation by showing that a single GlcNAc residue ligated to *E. coli* K-12 lipid A-core reacted with an anti- β -O-GlcNAc antibody, confirming that this residue is in the β -configuration.

Several cytoplasmic membrane proteins implicated in the assembly of cell surface polysaccharides form oligomeric structures (Collins *et al.*, 2017, Liston *et al.*, 2015, Whitfield and Trent, 2014). Further, the MraY protein that initiates cell wall peptidoglycan synthesis forms dimers both in detergent micelles and in the membrane (Chung *et al.*, 2013). To probe whether WaaL can form oligomers *in vivo*, we employed a genetic reconstitution strategy by co-expressing an N-terminally truncated WaaL_{Ec} version that contains intact functional residues in PL4 and PL5 regions with an intact WaaL_{Ec} with single substitutions in functional amino acids. This experiment resulted in the restoration of O-antigen synthesis, which was not possible with any of these proteins when expressed individually. Oligomerization of WaaL_{Ec} was

independently supported by chemical cross-linking analyses, suggesting this protein adopts a functional oligomeric form in the membrane.

Together, this study demonstrates that WaaL_{Ec} and WaaL_{Pa} proteins share similar topology and functionality, indicating that O-antigen ligases have conserved features consistent with their function at the periplasmic side of the membrane and pointing out to a common glycosyl transfer mechanism.

Experimental Procedures

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. Bacteria were cultured at 37°C in a Luria-Bertani (LB) medium supplemented with ampicillin (100 µg ml⁻¹), tetracycline (20 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹) for *E. coli*, or with tetracycline (100 µg ml⁻¹), gentamicin (30 µg ml⁻¹) for *P. aeruginosa*, or with 0.2% (w/v) L-arabinose, when appropriate.

Construction of strains and plasmids

P. aeruginosa genomic knockout mutants PAO1*waaL*::Gm^R was constructed for complementation. The *waaL* gene of the *P. aeruginosa* strain PAO1 was amplified from genomic DNA by PCR using forward primer 5368 and reverse primer 5369 (Table S1), and cloned into pEX18Tc vector, followed by inserting a gentamicin-resistance cassette into *Sa*I site within the *waaL* gene, producing the insertional mutant PAO1*waaL*::Gm^R. This construct was transformed into PAO1 by electroporation, and transformants plated on LB + 100 µg ml⁻¹ of

tetracycline. *waaL* knockout mutants were selected by subsequent plating on 5% sucrose agar plates containing 30 µg ml⁻¹ of gentamicin. For complementation experiments, the PAO1 *waaL* gene was subcloned from pXR14, pXR23, and all pXR23 and pJM plasmids with single cysteine substitution mutants by digesting with *EcoRI* and *HindIII*, and ligating it into pUCP26, also digested with the same restriction enzymes. The *wapR* gene of PAO1 was amplified from the genomic DNA by PCR using forward primer 7049 and reverse primer 7050 (Table S1), and the amplicon digested with *EcoRI* and *PstI* and ligated to pUCP26, which was also digested with the same restriction enzymes, yielding plasmid pXR93.

The *E. coli waaL*_{ΔN2TM}, which carries a deletion eliminating amino acids Leu-2 to Asn-71, was amplified from pXR1 using primers 5995 and 5730 (Table S1), and the amplicon digested with *EcoRI* and *PstI* was ligated to pBADFLAG, which was also digested with the same restriction enzymes, yielding plasmid pXR39. This strategy deletes the complete WaaL encoding sequence and allows replacement with the truncated one. To generate pXR76, encoding *E. coli waaL*_{ΔN2TM} in pBAD33, both pXR39 and pBAD33 were digested with *ClaI* and *HindIII*, and the 2.7 Kb insert was ligated to the 4-Kb fragment from pBAD33. All replacements were verified by DNA sequencing.

Site-directed mutagenesis

pXR1, encoding a C-terminal FLAG followed by 10xHis-tagged *E. coli* WaaL, was used as a DNA template for site-directed mutagenesis to construct pXR36, encoding cysteine-less *E. coli* WaaL. pXR36 was then used as a DNA template for site-directed mutagenesis to construct *E. coli* WaaL mutants with single cysteine replacements. pXR14, encoding a C-terminal FLAG followed by 10xHis-tagged *P. aeruginosa* WaaL, was used as a DNA template for site-directed

mutagenesis to construct pXR23, encoding cysteine-less *P. aeruginosa* WaaL. pXR23 was then used as a DNA template for site-directed mutagenesis to construct *P. aeruginosa* WaaL mutants with single cysteine replacements. The Cys-428 codon in *E. coli* WaaL and Cys-403 codon in *P. aeruginosa* WaaL do not exist in the native gene, but from the construction of FLAG-tag. pXR14 was also used as a DNA template for site-directed mutagenesis to construct *P. aeruginosa* WaaL-R212A. Table S1 of supplementary data shows the DNA sequences of all primers used for mutagenesis. *Pfu* polymerase was used in PCR amplification for site-directed mutagenesis. The PCR products were digested overnight with 1 U *DpnI* at 37°C, and then introduced into *E. coli* DH5α competent cells by transformation. Ampicillin-resistant colonies were screened. All substitutions were confirmed by DNA sequencing.

Sulfhydryl Labeling

Overnight cultures of DH5α cells containing the appropriate plasmids (Table 1) supplemented with 100 µg ampicillin ml⁻¹ were diluted in LB medium (5 ml) to an OD₆₀₀ of 0.02. When the OD₆₀₀ reached around 0.6, protein expression was induced with 0.2% (w/v) L-arabinose. After a 2.5-h induction, the OD₆₀₀ for each culture was measured and adjusted to 0.5 with LB medium. Then 2 ml of each adjusted culture was centrifuged at 16,000 x g for 1 min, washed twice with 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM HEPES [pH6.8], 5 mM MgCl₂) and re-suspended in 0.2 ml of the same buffer, which is referred to as ‘0.2 ml cell suspension’ as below.

For protein expression controls, the 0.2 ml cell suspension were added 0.1 mg ml⁻¹ lysozyme and 25 µg ml⁻¹ DNaseI, incubated at 37°C for 20 min, and then mixed with protein loading dye for SDS-PAGE on 14% acrylamide gels. For sulfhydryl labeling with Polyethyleneglycol

maleimide (PEG-Mal, 5 KDa, Sigma), each of the 0.2 ml cell suspension was incubated with 5 mM EDTA at room temperature for 10 min (+PEG-Mal) or 0.2% (w/v) SDS at 37°C for 5 min (+SDS/+PEG-Mal), and then incubated with 1 mM PEG-Mal at room temperature for 1 h. Samples were quenched with 45 mM DTT for 10 min. Then the samples were treated with lysozyme and DNaseI before SDS-PAGE as mentioned above. For PEG-Mal labeling in the presence of N-ethylmaleimide (NEM, Sigma), each of the 0.2 ml cell suspension was incubated with 5 mM NEM at room temperature for 30 min (+NEM/+SDS/+PEG-Mal), and centrifuged at 16,000 x g for 1 min. Pellets were washed twice with the HEPES buffer, and re-suspended in 0.2 ml of the same buffer. Samples were then incubated with 2% (w/v) SDS at 37°C for 5 min and with 1 mM PEG-Mal at room temperature for 1 h, and quenched with 45 mM DTT for 10 min. Then the samples were treated as mentioned above for SDS-PAGE.

Purification of WaaL proteins and in vitro ligase assay

The *E. coli* and *P. aeruginosa* WaaL proteins with FLAG and 10xHis tags were purified from *E. coli* JM105v as described previously (Ruan et al. 2012). LPS was prepared as described previously (Marolda *et al.*, 2006a). In the *in vitro* ligase assay for WaaL_{Pa}, the LPS from PAO1*waaL::Gm^R*(pXR93) expressing WapR in the absence or presence of IPTG (0-1 mM) contains both substrates for the reaction. The *in vitro* ligase assay for WaaL_{Pa} was performed using 6 µl of LPS and purified *P. aeruginosa* WaaL (1 µM) in 50 mM phosphate buffer at pH 7.0 in 60 µl reaction volume, and the mixture was incubated at 22°C, 30°C or 37°C for 25 h, and terminated by adding 30 µg ml⁻¹ proteinase K at 60°C for 20 min. The sample was deproteinized by adding 60 µl of hot phenol at 70°C for 15 min and centrifuged to separate the phenol phase.

The upper aqueous phase was collected and analyzed by Tricine-SDS-PAGE. The *in vitro* ligase assay for WaaL_{Ec} was processed as described previously (Ruan *et al.*, 2012).

LPS and protein analysis

LPS samples were separated on 14% (w/v) Tricine-SDS-PAGE and the gels stained with silver nitrate (Marolda *et al.*, 2006a). For Western blot to detect β -O-linked N-acetylglucosamine, samples in the polyacrylamide gel were transferred to nitrocellulose membranes and reacted with O-GlcNAc Monoclonal Antibody (IgM) (Covance) at a 1:1000 dilution. For O-antigen detection anti-O5 antigen monoclonal antibody MF15-4 (Antibodies-online) was used at a 1:10,000 dilution. Alexa Fluor® 680 Goat Anti-Mouse IgM (Invitrogen) or IRDye® 800CW Goat anti-Mouse IgG (LI-COR) were used as secondary antibodies. For protein analysis, 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 anti-FLAG M2 monoclonal Antibody (Sigma) at a 1:5000 dilution or anti-His (Sigma) at a 1:10,000 dilution. IRDye® 800CW Goat anti-Mouse IgG (LI-COR) or Alexa Fluor® 680 Goat anti-mouse immunoglobulin G (IgG) (Invitrogen) were used as secondary antibodies. Reacting bands were detected by fluorescence with an Odyssey infrared imaging system (Li-cor Bioscience, Lincoln, NE).

Topology prediction

The membrane topology of WaaL proteins was assessed by TMHMM (Sonnhammer *et al.*, 1998) and graphically displayed using Textopo (Beitz, 2000).

Chemical cross-linking

The *E. coli* WaaL and WaaLC-less proteins with FLAG-His tags were purified from *E. coli* as described previously (Ruan *et al.*, 2012). Cross-linking was performed using dithiobis[succinimidyl propionate] (DSP) following manufacturer's instructions (Thermo Scientific). DSP was prepared as a 25 mM solution in DMSO and mixed with purified protein (in PBS) to a 5 mM final concentration. The mix was incubated for 45 min at room temperature. The reaction was stopped by adding the quenching solution (50 mM Tris-HCl pH 7.5) and incubated for 15 min. To cleave DSP, DTT was added at 50 mM final concentration and incubated at 37°C for 30 min. Samples were separated on 12.5% SDS-PAGE, using a loading buffer without DTT. Samples in the polyacrylamide gel were transferred to nitrocellulose membranes and incubated overnight with anti-His (Sigma) monoclonal antibody and IRDye® 800CW Goat anti-Mouse IgG (LI-COR) was used as secondary antibody. Reacting bands were detected by an Odyssey infrared imaging system (Li-cor Bioscience, Lincoln, NE).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table 1. Strains and plasmids

Strain or plasmid	Relevant properties ^a	Source/reference
<i>E. coli</i>		
CLM24	W3110, $\Delta waaL$	(Feldman <i>et al.</i> , 2005)
CLM35	W3110, $\Delta wecA \Delta wzx$	(Marolda <i>et al.</i> , 2006b)
DH5 α	F ⁻ $\Phi 80 lacZM15 endA recA hsdR(r_k^- m_k^+) supE$ <i>thi gyrA relA</i> $\Delta(lacZYA-argF)$ U169	Laboratory stock
F632	<i>E. coli</i> R2, R-LPS mutant of O100:K? (B):H2	(Heinrichs <i>et al.</i> , 1998a)
SCM3	SØ874, $\Delta waaL$	(Pérez <i>et al.</i> , 2008)
SØ874	<i>lacZ trp</i> $\Delta(sbcB-rfb) upp rel rspL$	(Neuhard and Thomassen, 1976)
W3110	<i>E. coli</i> K-12 <i>rph-1</i> IN(<i>rrnD-rrnE</i>)1	Laboratory stock
JM105v	JM105 derivative with a spontaneous mutation resulting in a truncated lipid A-core OS	Laboratory stock
<i>P. aeruginosa</i>		
PAO1	Prototypical laboratory strain (from L. Pasador)	(Holloway <i>et al.</i> , 1979)
PAO1 <i>waaL::Gm^R</i>	PAO1, $\Delta waaL$, Gm ^R	This study
<i>B. cenocepacia</i>		
K56-2	Cystic fibrosis clinical isolate	Laboratory stock
K56-2 <i>waaL::pGPΩTp</i>	K56-2, <i>waaL::pGPΩTp</i>	(Ortega <i>et al.</i> , 2009)
Plasmids		
pBADFLAG	pBAD24; for FLAG-tagged C-terminal fusions; arabinose inducible, Ap ^R	(Saldías <i>et al.</i> , 2008)
pBAD33	pBAD18 containing the pBR322-compatible replication origin from pACYC 184, Cm ^R	(Guzman <i>et al.</i> , 1995)
pEX18Tc	Tc ^R , <i>oriT⁺</i> , <i>sacB⁺</i> , gene replacement vector with multi cloning site from pUC18	(Hoang <i>et al.</i> , 1998)
pJHCV32	<i>wb(rfb)</i> _{EcO7} cosmid, Tc ^R O7 ⁺	(Valvano and Crosa, 1989)
pUCP26	pUC18-derived broad-host-range vector, Tc ^R	(West <i>et al.</i> , 1994)
pXR1	pBADFLAG, <i>E. coli waaL</i> _{FLAG-10xHis} , Ap ^R	(Ruan <i>et al.</i> , 2012)
pXR4	pXR1, <i>E. coli waaL</i> _{R215A} , Ap ^R	(Ruan <i>et al.</i> , 2012)
pXR5	pXR1, <i>E. coli waaL</i> _{R215K} , Ap ^R	(Ruan <i>et al.</i> , 2012)
pXR7	pXR1, <i>E. coli waaL</i> _{R288A} , Ap ^R	(Ruan <i>et al.</i> , 2012)
pXR14	pBADFLAG, <i>P. aeruginosa waaL</i> _{FLAG-10xHis} , Ap ^R	(Ruan <i>et al.</i> , 2012)
pXR39	pXR1, <i>E. coli waaL</i> _{ΔN2TM} ; <i>E. coli waaL</i> _(L2-N71) deleted; Ap ^R	This study
pXR76	pBAD33, <i>E. coli waaL</i> _{ΔN2TM} , Ap ^R	This study
pXR36	pXR1, <i>E. coli waaL</i> _{Cys-less} , Ap ^R	This study
pXR33	pXR1, <i>E. coli waaL</i> _{C29} , from <i>E. coli waaL</i> _{C54A/C203A/C225A/C428A} , Ap ^R	This study
pXR38	pXR1, <i>E. coli waaL</i> _{C428} , from <i>E. coli</i> <i>waaL</i> _{C29A/C54A/C203A/C225A} , Ap ^R	This study
pXR52	pXR36, <i>E. coli waaL</i> _{I1162C} , Ap ^R	This study
pXR53	pXR36, <i>E. coli waaL</i> _{T176C} , Ap ^R	This study
pXR54	pXR36, <i>E. coli waaL</i> _{T190C} , Ap ^R	This study

807	pXR55	pXR36, <i>E. coli</i> waaL _{T214C} , Ap ^R	This study
808	pXR56	pXR36, <i>E. coli</i> waaL _{A216C} , Ap ^R	This study
809	pXR43	pXR36, <i>E. coli</i> waaL _{G286C} , Ap ^R	This study
810	pXR23	pXR14, <i>P. aeruginosa</i> waaL _{Cys-less} , from	
811		<i>P. aeruginosa</i> waaL _{C114A/C403A} , Ap ^R	This study
812	pXR22	pXR14, <i>P. aeruginosa</i> waaL _{C114} , from	
813		<i>P. aeruginosa</i> waaL _{C403A} , Ap ^R	This study
814	pXR21	pXR14, <i>P. aeruginosa</i> waaL _{C403} , from	
815		<i>P. aeruginosa</i> waaL _{C114A} , Ap ^R	This study
816	pXR65	pXR23, <i>P. aeruginosa</i> waaL _{R147C} , Ap ^R	This study
817	pXR45	pXR23, <i>P. aeruginosa</i> waaL _{L155C} , Ap ^R	This study
818	pXR46	pXR23, <i>P. aeruginosa</i> waaL _{L157C} , Ap ^R	This study
819	pXR66	pXR23, <i>P. aeruginosa</i> waaL _{T158C} , Ap ^R	This study
820	pXR67	pXR23, <i>P. aeruginosa</i> waaL _{G161C} , Ap ^R	This study
821	pXR68	pXR23, <i>P. aeruginosa</i> waaL _{N165C} , Ap ^R	This study
822	pXR47	pXR23, <i>P. aeruginosa</i> waaL _{S169C} , Ap ^R	This study
823	pXR69	pXR23, <i>P. aeruginosa</i> waaL _{A170C} , Ap ^R	This study
824	pXR48	pXR23, <i>P. aeruginosa</i> waaL _{A178C} , Ap ^R	This study
825	pXR49	pXR23, <i>P. aeruginosa</i> waaL _{S187C} , Ap ^R	This study
826	pXR50	pXR23, <i>P. aeruginosa</i> waaL _{S211C} , Ap ^R	This study
827	pXR51	pXR23, <i>P. aeruginosa</i> waaL _{T213C} , Ap ^R	This study
828	pXR70	pXR23, <i>P. aeruginosa</i> waaL _{L251C} , Ap ^R	This study
829	pXR71	pXR23, <i>P. aeruginosa</i> waaL _{P253C} , Ap ^R	This study
830	pXR72	pXR23, <i>P. aeruginosa</i> waaL _{V255C} , Ap ^R	This study
831	pXR44	pXR23, <i>P. aeruginosa</i> waaL _{G282C} , Ap ^R	This study
832	pXR73	pXR23, <i>P. aeruginosa</i> waaL _{L307C} , Ap ^R	This study
833	pXR74	pXR23, <i>P. aeruginosa</i> waaL _{V309C} , Ap ^R	This study
834	pXR88	pXR14, <i>P. aeruginosa</i> waaL _{R212A} , Ap ^R	This study
835	pXR75	pUCP26, <i>P. aeruginosa</i> waaL, Tc ^R	This study
836	pXR79	pUCP26, <i>P. aeruginosa</i> waaL _{Cys-less} , from	
837		<i>P. aeruginosa</i> waaL _{C114A/C403A} , Tc ^R	This study
838	pXR89	pUCP26, <i>P. aeruginosa</i> waaL _{R212A} , Tc ^R	This study
839	pXR93	pUCP26, <i>P. aeruginosa</i> wapR, Tc ^R	This study
840	pXR95	pUCP26, <i>P. aeruginosa</i> waaL _{N165C} , Tc ^R	This study
841	pJM7	pUCP26, <i>P. aeruginosa</i> waaL _{R147C} , Tc ^R	This study
842	pJM8	pUCP26, <i>P. aeruginosa</i> waaL _{L155C} , Tc ^R	This study
843	pJM9	pUCP26, <i>P. aeruginosa</i> waaL _{L157C} , Tc ^R	This study
844	pJM10	pUCP26, <i>P. aeruginosa</i> waaL _{T158C} , Tc ^R	This study
845	pJM11	pUCP26, <i>P. aeruginosa</i> waaL _{G161C} , Tc ^R	This study
846	pJM12	pUCP26, <i>P. aeruginosa</i> waaL _{N165C} , Tc ^R	This study
847	pJM13	pUCP26, <i>P. aeruginosa</i> waaL _{S169C} , Tc ^R	This study
848	pJM14	pUCP26, <i>P. aeruginosa</i> waaL _{A170C} , Tc ^R	This study
849	pJM15	pUCP26, <i>P. aeruginosa</i> waaL _{A178C} , Tc ^R	This study
850	pJM16	pUCP26, <i>P. aeruginosa</i> waaL _{S187C} , Tc ^R	This study
851	pJM17	pUCP26, <i>P. aeruginosa</i> waaL _{S211C} , Tc ^R	This study
852	pJM18	pUCP26, <i>P. aeruginosa</i> waaL _{T213C} , Tc ^R	This study
853	pJM19	pUCP26, <i>P. aeruginosa</i> waaL _{L251C} , Tc ^R	This study
854	pJM20	pUCP26, <i>P. aeruginosa</i> waaL _{P253C} , Tc ^R	This study
855	pJM21	pUCP26, <i>P. aeruginosa</i> waaL _{V255C} , Tc ^R	This study
856	pJM22	pUCP26, <i>P. aeruginosa</i> waaL _{G282C} , Tc ^R	This study
857	pJM23	pUCP26, <i>P. aeruginosa</i> waaL _{L307C} , Tc ^R	This study
858	pJM24	pUCP26, <i>P. aeruginosa</i> waaL _{V309C} , Tc ^R	This study
859	pJM25	pXR79, <i>P. aeruginosa</i> waaL _{G282A} , Tc ^R	This study
860			
861			

862 **a.** Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance;
863 Gm^R, gentamicin resistance.

Figure legends

Fig. 1. Topology of WaaL proteins from *E. coli* and *P. aeruginosa*.

A. Cartoon representation of *in silico* topology models of WaaL proteins from *E. coli* and *P. aeruginosa* (WaaL_{Ec}-TMHMM and WaaL_{Pa}-TMHMM, respectively), which were obtained with TMHMM (Sonnhammer *et al.*, 1998). Also shown is a cartoon representation of the topology model of *P. aeruginosa* WaaL (WaaL_{Pa}-Del/Fus) obtained from the analysis of *phoA-lacZα* dual-reporter fusions (Islam *et al.*, 2010). Numbers next to each predicted transmembrane helix indicate the position of amino acid residues at the predicted boundaries between soluble and membrane (TMH) embedded regions. TMHs are indicated by roman numerals. Shaded TMHs in WaaL_{Pa}-Del/Fus denote helices that show major differences with in the WaaL_{Pa}-TMHMM model. PL, periplasmic loop; CL, cytoplasmic loop. Purple stars symbolize the location of known functional residues in WaaL_{Ec} (Pérez *et al.*, 2008, Ruan *et al.*, 2012) and WaaL_{Pa} (Abeyrathne and Lam, 2007), which were identified previously and also in this study (R212 in WaaL_{Pa}).

B. Linear representation of the topological models in A. The numbers indicate the position of the amino acid residues in WaaL_{Ec} (419 amino acids) and WaaL_{Pa} (401 amino acids). Concordant TMH segments in the three models are indicated in black. TMH segments IV, V, VI, VII and VIII in WaaL_{Pa}-Del/Fus (light gray) differ from those predicted *in silico* for both *E. coli* and *P. aeruginosa* WaaL proteins.

Fig. 2. Sulfhydryl labeling of *E. coli* WaaL cysteine-replacement mutants.

A. Schematic description of the sulfhydryl labeling test by PEGylation. Predicted western blot profiles of WaaL polypeptides with single cysteine replacements based on the accessibility of the residue depending on its location and the treatment with NEM, SDS or both. The expected profiles denote residues located in the periplasm, in periplasmic/TMH borders, embedded in the TMH, in TMH/cytoplasmic borders, or exposed to the cytoplasm. The structures of PEG-Mal and NEM are also indicated.

B. *E. coli* DH5 α producing plasmid-encoded Cys-substituted variants of WaaL_{Ec} were grown at 37°C in 5 ml of cultures to mid-exponential phase and processed as explained in *Experimental Procedures*. 15- μ l aliquots were separated by SDS-PAGE and immunoblotted using the anti-FLAG monoclonal antibody. Modification of *E. coli* WaaL by PEG-Mal causes a shift in the WaaL band of ~20 kDa. M, Dual Color Precision Plus Protein standards (Bio-Rad). No PEGMal. Control experiment in HEPES buffer only. A sample from *E. coli* expressing the parental WaaL (WaaL_{Ec}) was loaded as an expression control.

PEGMal. Bacterial cell suspensions were treated with 1 mM PEG-Mal and no SDS.

NEM/SDS/PEGMal. Cell suspensions were first treated with 5 mM NEM and then with 1 mM PEG-Mal in the presence of 2% SDS.

SDS/PEGMal. Cell suspensions were treated with 1 mM PEG-Mal in the presence of 2% SDS.

Fig. 3. Sulfhydryl labeling of *P. aeruginosa* WaaL cysteine-replacement mutants.

E. coli DH5 α producing plasmid-encoded Cys-substituted variants of WaaL_{Pa} were grown and processed as indicated in the legend to Fig. 2.

No PEGMal. Control experiment in HEPES buffer only. A sample from *E. coli* expressing the parental WaaL (WaaL_{Pa}) was loaded as an expression control.

PEGMal. Bacterial cell suspensions were treated with 1 mM PEG-Mal and no SDS.

NEM/SDS/PEGMal. Cell suspensions were first treated with 5 mM NEM and further treated with 1 mM PEG-Mal in the presence of 2% SDS

SDS/PEGMal. Cell suspensions were treated with 1 mM PEG-Mal in the presence of 2% SDS.

Fig. 4. Cysteine less and single-cysteine substitutions of WaaL_{Ec} and WaaL_{Pa} do not affect ligase function.

A. Silver-stained SDS-polyacrylamide gel showing the LPS profile of *E. coli* SCM3(pJHCV32) cells expressing parental WaaL_{Ec}, pBADFLAG plasmid control, WaaL_{Ec}C-less, and various single cysteine replacement WaaL_{Ec} mutants, as indicated.

B. Silver-stained SDS-PAGE showing the LPS profile of *P. aeruginosa* PAO1 as a positive control, and the profiles of *P. aeruginosa* PAO1*waaL*::Gm^R with no plasmid, pUCP26 vector control, and plasmids expressing WaaL_{Pa}, WaaL-R212A and WaaL_{Pa}C-less. Lower panel shows immunoblot using anti-His antibody of total membrane fraction (Mem) of *P. aeruginosa* PAO1*waaL*::Gm^R with no plasmid, pUCP26 vector control, and plasmids expressing WaaL_{Pa}, WaaL-R212A and WaaL_{Pa}C-less.

C. Silver-stained SDS-PAGE showing the LPS profile of *P. aeruginosa* PAO1*waaL*::Gm^R expressing parental WaaL_{Pa}, no plasmid, pUCP26 vector control, and plasmids expressing WaaL_{Pa}C-less and WaaL_{Pa}N165C. Middle panel shows the immunoblot of extracted LPS using anti-Oantigen antibody. Protein expression was tested in total membrane (Mem) fractions using anti-His antibody (lower panel).

Fig. 5. Topological models of WaaL_{Ec} and WaaL_{Pa} displayed with Textopo (Beitz, 2000).

The *E. coli* WaaL and *B. P. aeruginosa* WaaL topologies deduced from PEGylation experiments in this study were compared to the *P. aeruginosa* WaaL topology based on dual-reporter fusions (Islam *et al.*, 2010). PL, periplasmic loop; CL, cytoplasmic loop. TMHs are indicated in roman numerals. Residues in black squares denote TMH location. Purple diamonds indicate functional residues that when replaced by alanine abolish ligation. Methionine residues accessible by oxidation in WaaL_{Ec} are indicated by blue squares with black letters. Residues in blue circles are exposed to the periplasmic space, while residues in red circles are exposed to the cytosol. White residues in blue square background were experimentally positioned at the TMH-periplasmic border. The C-terminal residues of WaaL_{Ec} and WaaL_{Pa} protein (N419 and P401, respectively), as well as the additional residues of the FLAG-10xHis tag peptides are also indicated. To better emphasize the differences between the two WaaL_{Pa} models the location of the residues determined by PEGylation in *P. aeruginosa* WaaL (revised topology) has been maintained in the *P. aeruginosa* WaaL model.

A. Topology of WaaL_{Ec}.

B. Revised topology of WaaL_{Pa} based on cysteine accessibility experiments using the intact protein.

C. WaaL_{Pa} topology based on dual-reporter fusions using truncated proteins (Islam *et al.*, 2010).

Fig. 6. *In vitro* ligase assay for *P. aeruginosa* WaaL. LPS produced *in vivo* by the

PAO1*waaL*::Gm^R expressing *P. aeruginosa* WaaL (Lane 1) or WapR (Lane 2). The *in vitro* ligation assay using purified WaaL_{Pa} mixed with extracted LPS from PAO1*waaL*::Gm^R was

tested at 22°C (Lane 3), 30°C (Lane 4), or 37°C (Lane 5) . Product of *in vitro* ligation incubated at 37°C using purified *P. aeruginosa* WaaL mixed with LPS from PAO1*waaL*::Gm^R expressing WapR at different IPTG concentrations (Lane 6-8). Product of *in vitro* ligation in the presence of 10 mM EDTA mixed with LPS from PAO1*waaL*::Gm^R expressing WapR.(Lane 9). Samples were analyzed by SDS-PAGE followed by silver staining. The resolved O-antigen products are indicated by the dashed rectangle.

Fig. 7. Properties of the ligation reaction

A. The chemical structures of the core oligosaccharide of the various LPS types used in these experiments are indicated in the respective boxes. LPS samples were examined by silver staining and by immunoblot with a monoclonal antibody against β -O-linked N-acetylglucosamine (anti- β -O-GlcNAc).

B. The *in vitro* ligation of K-12 core with *P. aeruginosa* PAO1 O-antigen by purified WaaL_{Ec} was performed as described in *Experimental Procedures*. Lanes: 1, PAO1, LPS produced *in vivo* by the PAO1 *waaL*::Gm^R(pXR75) strain expressing WaaL_{Pa}; 2, Core_{Ec}+ LPS_{Pa}, negative control for the *in vitro* ligation assay consisting of a mixture of LPS from CLM35 (Core_{Ec}) and from PAO1*waaL*::Gm^R(pXR93) prepared in the presence of 0.2 mM IPTG (LPS_{Pa}); 3, Core_{Ec}+WaaL_{Ec}, negative control for the *in vitro* ligation assay with purified WaaL_{Ec} and *E. coli* LPS (Core_{Ec}); 4, LPS_{Pa}+WaaL_{Ec}, *in vitro* ligation assay with WaaL_{Ec} and LPS_{Pa}; 5, Core_{Ec}+LPS_{Pa}+WaaL_{Ec}, *in vitro* ligation assay with WaaL_{Ec} mixed with Core_{Ec} and LPS_{Pa}; 6, LPS_{Ec}+WaaL_{Ec}, *in vitro* ligation assay with WaaL_{Ec} and LPS from CLM24(pMF19) (LPS_{Ec}) expressing K-12 O-antigen; 7, CLM24(pMF19/pXR1) O-antigen production *in vivo*.

C. *In vitro* ligation of K-12 core with *B. cenocepacia* K56-2 O-antigen by purified WaaL_{Ec}. *In vitro* ligation reactions were carried out as described in panel B. Lanes: 1, K56-2, LPS produced *in vivo* by parental *B. cenocepacia* K56-2; 2, *waaL*_{Bc}::pGPΩTp, LPS produced *in vivo* by the *B. cenocepacia* K56-2 *waaL*_{Bc}::pGPΩTp ligase deficient mutant; 3, Core_{Ec}+LPS_{Bc}, negative control for the *in vitro* ligation assay consisting of a mixture of LPS from CLM35 (Core_{Ec}) and from K56-2 *waaL*_{Bc}::pGPΩTp (LPS_{Bc}); 4, Core_{Ec}+WaaL_{Ec}, negative control for the *in vitro* ligation assay with purified WaaL_{Ec} and *E. coli* LPS (Core_{Ec}); 5, LPS_{Bc}+WaaL_{Ec}, *in vitro* ligation assay with WaaL_{Ec} and LPS_{Bc}; 6, Core_{Ec}+LPS_{Bc}+WaaL_{Ec}, *in vitro* ligation assay with WaaL_{Ec}, Core_{Ec}, and LPS_{Bc}.

Fig. 8. Reconstitution of *E. coli* ligase function from two inactive WaaL ligases co-expressed in the same bacterial strain and WaaL oligomerization.

A. Expression of *E. coli* WaaL constructs in intact cells detected by immunoblot with the anti-FLAG antibody. The constructs were expressed in the presence of 0.2% (w/v) L-arabinose, as described in *Experimental Procedures*.

B. O7 antigen production by *E. coli* SCM3(pJHCV32) expressing single (left panel) or double (right panel) combinations of WaaL constructs, expressed from, pBAD24, pBAD33 or both, as indicated by the cartoons above each lane.

C. Oligomerization analysis of purified WaaL and WaaLC-less after treatment with the DSP crosslinker (5mM) and under reducing conditions (DTT). Samples were processed for SDS-PAGE and Western blotting with anti-His antibody. WaaL forms protein complexes that are not present when incubated with DTT or weakly formed in the C-less version. In non-reducing conditions, a monomeric, a dimeric form (**) and multimeric forms (****) were visible.