Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays

Matias A Musumeci, Amirreza Faridmoayer², Yasuharu Watanabe³, and Mario F Feldman¹

Department of Biological Sciences, Alberta Glycomics Centre, University of Alberta, CW405 Biological Sciences Building, Edmonton, AB, Canada T6G 2E9

Received on September 12, 2013; revised on September 24, 2013; accepted on September 24, 2013

Bacterial O-Oligosaccharyltransferases (O-OTases) constitute a growing family of enzymes that catalyze the transfer of a glycan from a lipid carrier to protein acceptors. O-OTases are inner membrane proteins that display limited sequence similarity, except for the Wzy_C signature domain also present in a predicted periplasmic loop of the WaaL ligase, the enzyme responsible for transferring the O antigen to the lipid A core. The mechanism of O-OTase-dependent glycosylation is poorly understood. In this work, conserved amino acid residues in the O-OTases were replaced with alanine in PgIL, the O-OTase of Neisseria meningitidis. The activity of wild-type PgIL and its mutant derivatives were analyzed in vivo in engineered Escherichia coli cells, and in in vitro assays. We identified two additional sites of pilin glycosylated exclusively by PgIL in E. coli. Both sites are modified with phosphoglycerol (PG) by different enzymes in Neisseria gonorrhoeae and Neisseria meningitidis. Limited proteolysis experiments revealed a conformational change that is triggered upon interaction of the C-terminal region of PgIL with the lipid-linked oligosaccharide (LLO) substrate. These experiments showed that Q178 and Y405 are required for optimal function, whereas H349 is essential for activity and plays a critical role in the interaction with LLO. The equivalent His residue is also essential for WaaL activity, which suggests a common mechanism for both enzymes, and supports the hypothesis that O-glycosylation and lipopolysaccharide (LPS) synthesis are evolutionarily related. These results contribute to the elucidation of the mechanism of O-OTases, which are promising targets for novel antibiotics and present an enormous potential for glycoengineering novel vaccines and therapeutics.

Keywords: bacteria / limited proteolysis / O-oligosaccharyltransferases / O-linked glycosylation

Introduction

O-linked glycosylation is a widespread protein modification in bacteria. Initially, pilins and flagellins were thought to be the only targets for glycosylation. However, it is now clear that many bacterial species contain general O-glycosylation systems responsible for the modification of multiple proteins (Iwashkiw et al. 2013). Some of these O-glycosylation systems are required for pathogenesis and, therefore, constitute promising targets for novel antimicrobials (Iwashkiw et al. 2012). O-glycosylation can occur by the action of glycosyltransferases in the bacterial cytoplasm. This is the mechanism employed for O-glycosylation of flagellins and adhesins (Twine et al. 2009; Asakura et al. 2010). Alternatively, proteins can be modified via an oligosaccharyltransferase (OTase)-dependent mechanism. In this pathway, the O-oligosaccharyltransferases (O-OTases) are responsible for the transfer of the glycan from its undecaprenyl pyrophosphate (UndPP) carrier to serine or threonine residues in their protein targets. The glycosylation process starts at the cytoplasmic side of the inner membrane, where specific sugars are assembled onto the UndPP carrier. The lipid-linked oligosaccharide (LLO) is then flipped to the periplasm, where the attachment of the sugars to the proteins is mediated by the O-OTase (Iwashkiw et al. 2013).

O-OTases have been described in Neisseria meningitidis, Neisseria gonorrhoeae, Vibrio cholerae, Burkholderia thailandensis, Acinetobacter baumannii, Acinetobacter baylyi and some Pseudomonas aeruginosa strains (Castric 1995; Voisin et al. 2007; Fletcher et al. 2009; Schulz et al. 2013). The most studied O-OTases are PgIL from Neisseria spp. and PilO from Pseudomonas aeruginosa (Castric 1995). The O-OTase PgIL has been identified as the enzyme responsible for the transfer of glycans to pilin (Power et al. 2006; Faridmoayer et al. 2007), among other protein acceptors.

The pilin proteins are structural components of the type IV pilus, which plays a key role in adhesion of the bacteria to host cells (Morand et al. 2001). Pilin is glycosylated at S63 with a
variable trisaccharide, whose structure is dependent upon the absence or presence of several glycosyltransferases (Stimson et al. 1995; Marceau et al. 1998; Borud et al. 2010). In addition, the pilins of N. meningitidis are subject to other post-translational modifications including the addition of phosphocholine (PC) (Weiser et al. 1998; Warren and Jennings 2003) and phosphoglycerol (PG) (Stimson et al. 1996), whereas the closely related pilus of N. gonorrhoeae was found to be modified through the addition of PC and phosphoethanolamine (PE) (Hegge et al. 2004). PC and PE were not found to have a significant effect on adhesion, transformation, pilation or twitching motility (Forest et al. 1999). However, PG was determined to play a role in the detachment of the pilus from bacterial aggregates, allowing for colonization of new sites (Forest et al. 1999). The addition of PC and PE is mediated through the activity of the enzymes PptA and PptB, respectively (Warren and Jennings 2003). The residues within pilin to which the unusual PG post-translational modifications are added via PptB have been identified as S69 in N. gonorrhoeae and S93 in N. meningitidis. The S69 has thus been found with all the three modifications, as this is the location PC and PE modifications have previously been identified (Weiser et al. 1998; Warren and Jennings 2003; Hegge et al. 2004). Although pilin is the predominant O-glycosylated protein in N. gonorrhoeae, it has been shown that other glycoproteins exist, including several periplasmic lipoproteins (Vik et al. 2009). However, acceptor glycosylation motifs have not been identified, with the exception of low complexity regions (LCRs) rich in serine, proline and alanine (Ku et al. 2009; Vik et al. 2009).

All O-OTases appear to contain a conserved predicted periplasmic loop that is also present in the WaaL O antigen ligases (Power et al. 2006). WaaL ligases also transfer a glycan from the UndPP carrier, but the acceptor is the lipid A core instead of the UndPP-linked heptasaccharide (CjLLO). We have previously shown that PglL transfers the UndPP-linked heptasaccharide (CjLLO) to the S63 residue of PilE within the fragment 52LNHGEPGNNTSAG65 in engineered E. coli strains (Faridmoayer et al. 2008). In optimized expression conditions (see "Materials and methods" section), PglL activity resulted in three distinct bands that reacted with the antipilin and anti-glycan antibody, which complicated the analysis of the activity of the different PglL variants. We hypothesized that these extra bands corresponded to additional glycoforms of pilin (Figure 2A). The existence of the second and third glycosylated sites in pilin has not been previously reported in N. meningitidis. To demonstrate that these bands were indeed the result of pilin glycosylation, glycosylated pilin was purified by immobilized metal affinity chromatography followed by lectin affinity chromatography, and the slowest migrating bands were cut from the gel and digested as indicated in the "Materials and Methods" section. PglL glycosylates N. meningitidis pilin at three sites in E. coli in vivo

We have previously shown that PglL transfers the C. jejuni heptasaccharide to the S63 residue of PilE within the fragment 52LNHGEPGNNTSAG65 in engineered E. coli strains (Faridmoayer et al. 2008). In optimized expression conditions (see "Materials and methods" section), PglL activity resulted in three distinct bands that reacted with the antipilin and anti-glycan antibody, which complicated the analysis of the activity of the different PglL variants. We hypothesized that these extra bands corresponded to additional glycoforms of pilin (Figure 2A). The existence of the second and third glycosylated sites in pilin has not been previously reported in N. meningitidis. To demonstrate that these bands were indeed the result of pilin glycosylation, glycosylated pilin was purified by immobilized metal affinity chromatography followed by lectin affinity chromatography, and the slowest migrating bands were cut from the gel and digested as indicated in the "Materials and Methods" section. PglL glycosylates N. meningitidis pilin at three sites in E. coli in vivo

We have previously shown that PglL transfers the C. jejuni heptasaccharide to the S63 residue of PilE within the fragment 52LNHGEPGNNTSAG65 in engineered E. coli strains (Faridmoayer et al. 2008). In optimized expression conditions (see "Materials and methods" section), PglL activity resulted in three distinct bands that reacted with the antipilin and anti-glycan antibody, which complicated the analysis of the activity of the different PglL variants. We hypothesized that these extra bands corresponded to additional glycoforms of pilin (Figure 2A). The existence of the second and third glycosylated sites in pilin has not been previously reported in N. meningitidis. To demonstrate that these bands were indeed the result of pilin glycosylation, glycosylated pilin was purified by immobilized metal affinity chromatography followed by lectin affinity chromatography, and the slowest migrating bands were cut from the gel and digested as indicated in the "Materials and Methods" section. PglL glycosylates N. meningitidis pilin at three sites in E. coli in vivo

Results

Analysis of the role of O-OTases-conserved amino acids in vivo

The alignment of the sequences of the O-OTases from N. meningitidis, V. cholerae, A. baylyi, B. thailandensis and Ralstonia pickettii revealed the existence of conserved amino acid residues in these enzymes (Figure 1A). These amino acids could be either embedded in the membrane or facing the periplasm (Figure 1B). To evaluate the role of some of these conserved residues in the activity of the enzyme, the amino acids Q178, N180, G316, G318, H349, H400, E404 and Y405 of PglL were mutated to alanine and the activity of the mutant enzymes were analyzed in vivo. Escherichia coli CLM24 cells were co-transformed with plasmids expressing the O-OTase variants and the N. meningitidis pilin acceptor protein in the presence of the genes required for the synthesis of Campylobacter jejuni UndPP-linked heptasaccharide (CjLLO). The CLM24 strain lacks the WaaL ligase and it has been previously employed to study the glycan specificity of PglL (Faridmoayer et al. 2008). Whole-cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by western blot using the antibodies SM1 (monoclonal, antipilin) and HR6 (anti-C. jejuni heptasaccharide). Additional bands corresponding to forms of pilin with slower electrophoretic mobility were observed for each PglL variant, except H349A (Figure 2A). Interestingly, glycosylation with wild-type PglL showed four bands. The lower one presumably corresponds to unglycosylated pilin. The three upper bands reacted with both antipilin and anti-glycan antibodies, suggesting that these correspond to glycosylated forms of pilin. A similar glycosylation pattern was observed with the mutants G316A and G318A. The other PglL variants exhibited an intermediate pattern, showing only some of the hypothetical glycosylation bands. Exploiting the His-Tag added to the C-terminus of PglL, the expression of the PglL variants was analyzed by western blot employing anti-His antibodies (Figure 2B). The pilin protein acceptor also has a C-terminal tag, and therefore, it was reactive to the antibody. Although all the variants of PglL were expressed, mutants Q178A, N180A, G316A, G318A and H349A showed lower expression levels than the wild-type enzyme. An antibody against RNA polymerase showed that identical amounts of protein were loaded (Figure 2C). This indicates that the different levels of pilin visualized between the lanes are the consequence of stabilization of pilin by glycosylation and that the differences in the intensities in the bands corresponding to PglL reflect variable expression or more likely, reduced stability of the mutants.
Fig. 1. Identification of conserved amino acids in the O-OTases. (A) Alignment of the O-OTases of *N. meningitidis* (PgL Nm), *B. thailandensis* (PgL Bt), *R. picketti* (PgL Rp), *A. baylyi* (PgL Ab) and *V. cholerae* (PgL Vc). Highly conserved amino acids are indicated in black boxes. (B) Model of PgL of *N. meningitidis* highlighting the conserved amino acids mutated to alanine. The model was constructed with the server TMHMM server v 2.0 and plotted with the software TMRPres2d (Kall et al. 2004).
are the additional glycosylation sites. To identify the exact two consecutive serine residues in each peptide can be glycosylated by PglL.

The MS/MS results indicated that S69 or S70 and S93 or S94 were glycosylated by PglL, and site-directed mutagenesis of this residue abolished the highest of the three glycoforms as expected, leaving only two glycosylation sites within the rest of the protein (Figure 4A and B, Lane 3). If S69 was the glycosylation site, the mutant S63AS69A should be glycosylated within the 91MASSN95 peptide. Unexpectedly, the double-mutant S63AS69A and the double-mutant S63AS70A were still doubly glycosylated (Figure 4A). These results suggested that both serine residues could be indistinctly acting as the acceptors for the glycan in the absence of the adjacent serine. Consequently, the triple mutant S63AS69AS70A was created, which contained only one glycosylation site within the 91MASSN95 peptide and, hence, was glycosylated only once (Figure 4A), indicating that no other serine residue within the pilin accounted for the doubly glycosylated pilin forms seen in the double mutants. A control containing pilin (pAMF16) and pEXT20 (no PglL) was included to ensure that additional bands were not due to processing variants of pilin. The western blot employing the antipilin antibodies showed that the quantity of tri-glycosylated pilin is significantly smaller than the mono or di-glycosylated forms; however, the signal corresponding to the multiglycosylated pilin are amplified when visualized with anti-glycan (Figure 4).

The second glycopeptide observed by MS/MS, 91MASSN95, also contained adjacent serine residues. To determine the exact glycan attachment site within this peptide, mutagenesis of serine residues S93 and S94 was performed, obtaining similar result (Figure 4B). These results demonstrate that PglL can attach a glycan chain to either S93 or S94, as is also the case for S69 and S70.

**In vitro glycosylation assay**

The experiments described in the previous section “The two consecutive serine residues in each peptide can be glycosylated by PglL” demonstrated that in *E. coli* cells, wild-type PglL transfers the *C. jejuni* heptasaccharide to three serine residues of pilin (S63, S69 or S70, and S93 or S94). This information led us to conclude that the band patterns obtained for mutants Q178A, N180A, E404A and Y405A in Figure 2A are indicative of reduced glycosylation activity in vivo. H349 was inactive in vivo. To assess the impact of each mutation on the activity of PglL in a quantitative manner, analysis of glycosylation was performed in vitro employing identical amounts of purified wild-type PglL and its mutant derivatives. This in vitro glycosylation assay allowed us to rule out that the lower activity seen for some of the mutants was due to differences in expression levels or stability of the enzyme. For this assay, we selected one mutant in each region of PglL. Versions Q178A, H349A and Y405A were purified as described in (Musumeci, Hug et al. 2013). Purified pilin was employed as glycan acceptor. The other component of the reaction, CjLLO, was extracted from SCM6 *E. coli* cells as described in the “Materials and methods” section. The *Escherichia coli* SCM6 strain was chosen because it lacks the WaaL enzyme that transfers oligosaccharides from the UndPP lipid carrier onto lipid A, which increases the LLO yields (Feldman et al. 2005). This strain also lacks the LPS and enterobacterial common antigen initiating...
GlcNAc transferase WecA (Alaimo et al. 2006), eliminating the presence of contaminant glycans in the CjLLO preparations. The purified PglL variants were co-incubated with the pilin substrate in the presence of different concentrations of CjLLO for 2 h at 30°C. As in previous experiments, in these in vitro conditions, wild-type PglL glycosylated almost 100% of the pilin, but only at S63 (Figure 5A). Glycosylation at the other sites only occurs in vivo (Faridmoayer et al. 2008). In agreement with the in vivo data, the H349A mutant did not show any glycosylation activity. Variants Q178A and Y405A exhibited reduced activity in in vitro conditions. The intensities of glycosylated and nonglycosylated bands of pilin were quantified and the percentage of glycosylated pilin was calculated as described by (Musumeci, Hug et al. 2013). The glycosylation yields were plotted at each concentration of CjLLO employed (Figure 5B). The wild-type PglL displayed the highest glycosylation percentage in all the assayed conditions. In agreement with the in vivo data, the mutants Y405A and Q178A exhibited reduced glycosylation levels, suggesting a role for these amino acids in PglL function.

PglL undergoes a conformational change upon binding of the CjLLO substrate

To study the interaction between PglL and the CjLLO substrate and to determine whether the mutant enzymes exhibited folding deficiencies, we decided to employ the limited proteolysis technique. Limited proteolysis experiments have been widely employed to probe conformational features of proteins. This approach relies on the fact that unfolded or partially unfolded proteins are more susceptible to degradation by proteases (Fontana et al. 2004). The method can be used to study the domain organization of a protein or to infer the presence of stable sub-domains (Fontana et al. 2004). Limited proteolysis has been applied to evaluate the stability of different mutants of the C. jejuni N-OTase PglB (Jaffee and Imperiali 2011). Purified PglL was incubated with CjLLO extract to promote enzyme-substrate interaction. Next, proteinase K was added (0.06 mg/mL) during 0, 5, 15, 30, 60, 180 and 300 min at room temperature. The reaction was stopped by adding PMSF 5 mM in 1X SDS–PAGE loading buffer and further freezing at −80°C. The same procedure was followed with a control containing extract from E. coli SCM6.
cells transformed with the empty pACYC plasmid. Thus, the only difference between these two conditions is the presence or absence of the CjLLO. The degradation patterns of wild-type PgL in both conditions were obtained by western blot analysis with anti-His antibodies (Figure 6A). The expected molecular weight of PgL is 65 kDa. However, at time 0, PgL is visualized as a band of \( \approx 50 \) kDa (Figure 6A, left panel). Abnormal electrophoretic mobility is common for proteins containing multiple transmembrane domains (Rath et al. 2009). Some low levels of proteolysis were detected, probably due to the activity of endogenous \textit{E. coli} proteases. After 60 min of incubation with the protease in the absence of the CjLLO, PgL was almost completely degraded. However, an intense immunoreactive band migrating at around 30 kDa was observed almost exclusively in the presence of CjLLO. This band was intense and stable for at least 180 min. MS/MS analysis of this material demonstrated that this band corresponds to the region encompassing the amino acids R287 to the C-terminus, including the largest predicted periplasmic loop and most of the conserved amino acids (Figure 1B). This result can be explained by a conformational change of PgL that occurs upon binding of the CjLLO, resulting in stabilization of the C-terminal domain of the enzyme. Alternatively, this fragment could be the result of steric blockage of the proteolytic sites by a component present in the CjLLO extract. To analyze this possibility, an identical experiment using trypsin instead of proteinase K was performed. A similar C-terminal fragment stabilized by the presence of CjLLO was detected (Figure 6A, middle panel). A control without protease was included (Figure 6A, right panel) to rule out degradation from endogenous proteases. The observation that two proteases with different target sites produce a similar stabilization effect supports the hypothesis of a

Fig. 4. Determination of the additional glycosylation sites of \textit{N. meningitidis} pilin through site-directed mutagenesis. Western blot of purified His-tagged pilin variants after in vivo glycosylation. The wild-type and mutant pilin were probed with antipilin (left-hand) and anti-glycan (right-hand) antibodies. The anti-pilin antibody binds all the four forms of the glycosylated pilin, with the anti-glycan only binding mono, di and tri-glycosylated species. (A) Glycosylation of Ser mutants of pilin at the glycosylation site 69SEIK73 by wild-type PgL. (B) Glycosylation of Ser mutants of pilin at the glycosylation site 91MASSN96 by wild-type PgL.

Fig. 5. Study of the activity of mutant and wild-type PgLs in vitro. (A) Western blot showing different levels of glycosylation of pilin at increasing concentrations of the substrate CjLLO. The same concentrations of wild-type, Y405A and Q178A PgL variants were used in all the reactions. The PgL mutant H349A did not show activity in the assayed conditions. Monoclonal antipilin antibody was used. (B) Percentage of glycosylation of pilin at each CjLLO concentration assayed displayed by the PgLs wild-type (circles), Y405A (triangles) and Q178A (squares). The glycosylation percentage was calculated from the total intensity resulting of sum of nonglycosylated and the glycosylated bands. The Odyssey software was employed to quantify the respective bands. The values were calculated from two or three independent measurements. One of the three or two western blot analyses is shown as representative of each PgL variant.
conformational change in PglL occurring upon binding of the CjLLO. This hypothesis is further supported by the demonstration that mild-acid treatment of the extract to hydrolyze the pyrophosphate linkage of the CjLLO eliminated the stabilizing effect (Figure 6B).

Interaction between PglL and its CjLLO substrate is affected by mutation of H349

The limited proteolysis experiments suggested that the binding of CjLLO triggers a folding of the C-terminal region of PglL. We analyzed whether this process also occurs for the PglL mutants Q178A and Y405A, which exhibited reduced activity, and for the inactive variant H349A. The same experimental procedure was applied to the purified mutant enzymes (Figure 6C). Q178A and Y405A showed a similar behavior compared with the wild-type PglL, i.e. the formation of a stable C-terminal fragment in the presence of the CjLLO (Figure 6C). However, the mutant H349A exhibited a degradation pattern insensitive to the presence or absence of CjLLO indicating that this mutant could not bind the glycolipid substrate (Figure 6C). Interestingly, addition of the protein substrate, pilin, did no stabilize any of the PglL variants, suggesting that the folding of the C-terminal domain of PglL only occurs with the CjLLO substrate (data not shown). Interestingly, in the absence of substrate, all the mutants displayed degradation patterns similar to those of the wild-type PglL, as shown in Figure 6A (left pannel) and Figure 6C, indicating that the stability in vitro of PglL is not affected by these mutations.

Discussion

We previously reported the reconstitution of N. meningitidis pilin glycosylation in E. coli where the glycan attachment site, S63, remained constant in Neisseria and E. coli systems.
MA Musumeci et al.

(Faridmoayer et al. 2008). In this work, two additional glycosylation sites that are only glycosylated in E. coli were identified. Prediction of O-glycosylation sites to date has had little success with the exception of the Bacteroides spp., where a number of glycoproteins were found to have conserved D-S/T-X, where X is any amino acid with one or more methyl groups (Fletcher et al. 2009). Vik et al. identified several glycoproteins in N. gonorrhoeae and noticed that its O-OTase recognizes LCRs containing serines, alanines and prolines. LCRs are also recognized by the O-OTase from A. baumannii (Iwashkiw et al. 2012).

However, the two novel sites of pilin recognized by PglL in our experimental conditions are not part of LCRs, indicating that the protein recognition mechanisms of PglL are complex and possibly involve specific three-dimensional motifs. It is remarkable that the two novel sites identified contain two consecutive serine residues and that both residues in each site can serve as the glycan attachment point. None of the novel glycosylation sites has been found glycosylated in the native host. Instead, in N. meningitidis S93 is modified with PG by the enzyme PptB. This modification has several potential selective advantages for the bacterium, including transmission to new hosts and colonization of new sites in the same host, thus avoiding nutrient exhaustion and possibly favoring escape from the local immune surveillance (Chamot-Rooke et al. 2011). In N. gonorrhoeae, the site S69 is modified with PE or phosphocholine (PC). A major question that now arises concerns the mechanisms of pilus modification within Neisseria and the relationships between pilus modification processes. It appears that glycosylation and PG addition in N. meningitidis, and modification with PE in N. gonorrhoeae, recognize the same structural determinants and that in the absence of one modification, the other takes place at the same residue. Supporting this idea, in N. gonorrhoeae the disruption of the O-OTase resulted in the replacement of the glycan chains with PC and PE in two glycoproteins (Anonsen et al. 2012). This phenomenon resembles the relationship between glycosylation and phosphorylation in eukaryotes, where it is accepted that there exists a “yin-yang” relationship between serine O-glycosylation and serine phosphorylation (Hart et al. 1995). It is puzzling that these sites are recognized by PglL only in vivo. In vitro, PglL only glycosylated pilin at S63. It is tempting to speculate that these differences between in vivo and in vitro conditions are the result of some sort of coordination between translocation, folding and glycosylation at the bacterial periplasm.

The alignment of the O-OTases identified to date revealed the existence of conserved amino acid among the members of this family of enzymes (Figure 1A). PglL topology modeling predicted that all these conserved residues are facing or embedded in the periplasmic space, where the reaction takes place (Figure 1B). To investigate their role, conserved amino acids were replaced with alanine in PglL. We measured the activity of the mutant enzymes in vivo, and the ones exhibiting reduced glycosylation levels were also tested in vitro. We also studied the interaction of the PglL variants with an LLO substrate employing limited proteolysis. Limited proteolysis experiments are powerful tools to study protein folding and enzyme-substrate interaction (Fontana et al. 2004). This approach becomes valuable to study conformational changes in enzymes where NMR or crystallography is extremely difficult, as for many proteins with multiple transmembrane regions such as PglL. A conformational change upon incubation with CjLLO resulting in the stabilization of the C-terminal region was evident for the wild-type enzyme (Figure 6A). This region includes a large portion of the putative periplasmic loop that contains the Wzy_C domain and most of the conserved amino acids in O-OTases as determined by MS/MS.

In vitro assays, the activity can be measured independently of the level of expression of each PglL variant. All the assayed PglL variants retained a certain level of activity, with the exception of the mutant H349A, which was inactive, both in vivo and in vitro (Figure 2A). The activity of the G316A and G318A mutants were similar to the wild-type PglL in vivo. However, the expression levels of these enzymes were lower than the wild-type, suggesting a destabilization of the overall structure of the enzyme (Figure 2B). Thus, residues G316 and G318 could be required to maintain the structural integrity of the enzyme. The variants Q178A, N180A, E404 and Y405 showed reduced activity in vivo with respect to the wild-type PglL (Figure 2A). Q178A and Y405 were purified and their reduced activity was confirmed in vitro (Figure 5). Similarly, these two mutants were stabilized by addition of CjLLO, suggesting that they can bind the glycolipid substrate (Figure 6C). Because these two mutants never accomplish 100% efficiency, even at high concentration of CjLLO substrate, we propose that Q178 and Y405 play an important, although nonessential, role in catalysis. The lack of pure LLO substrates and other factors such as the multiple transmembrane domains of PglL complicate an accurate calculation of kinetic parameters of PglL and affinity or dissociation constants.

O-OTases and WaaL O antigen ligases share several common features (Power et al. 2006; Perez et al. 2008; Musumeci, Hug et al. 2013). They have been predicted to contain a similar transmembrane organization, with a large periplasmic loop containing the signature domain Wzy_C. Indeed, the reaction catalyzed by PglL and WaaL is very similar, as they both recognize LLOs and transfer the glycans to hydroxyl groups, either in a protein or in a sugar. As mentioned, PglL H349A was inactive in in vivo and in vitro conditions. Interestingly, mutation of the equivalent histidine residue (H300) to alanine in WaaL enzymes of different bacteria rendered WaaL inactive (Schild et al. 2005; Abeyrathe and Lam 2007; Perez et al. 2008). It has been proposed that this conserved histidine could interact with the phosphate groups of UndPP in WaaL ligases (Perez et al. 2008). Our limited proteolysis experiments suggest that H349 cannot bind the UndPP-linked substrate and therefore support a role of this amino acid in the interaction with its substrate. We have shown that PglL activity is independent of the glycan structure and that the lipid carrier can be substituted by a nucleotide, as PglL transferred the monosaccharide diBacNac (bacillosamine) employing UDP-bacillosamine as substrate (Musumeci, Hug et al. 2013). Therefore, the interaction of PglL with the phosphates of the UndPP-glycan may be absolutely critical to the reaction. It is also possible that H349 plays a critical role in modulating the conformational change triggered by the binding of the glycan substrate.

The binding of the CjLLO to PglL occurred in the absence of the protein substrate. It is also possible that a conformational change that takes place upon binding of the glycolipid is
required for the efficient binding of the protein acceptor. This theory is supported by studies based on predictions of structures of substrate peptide which have proposed that the protein substrates bind to PglL with an induced fit mechanism, where local structural conformation could be key to the enzyme-substrate interaction (Schulz et al. 2013). Clearly, these models require additional experimental support. The study of proteins containing multiple transmembrane domains such as PglL is extremely challenging. The identification of amino acids involved in substrate binding and catalysis and the demonstration of the conformational changes that occur during the reaction constitute an important step toward the elucidation of the mechanism of O-OTases. This fascinating family of enzymes present an enormous potential for the customized synthesis of glycoconjugates of biotechnological interest and that constitute logical targets for novel antimicrobials agents.

**Material and methods**

**Alignment of O-OTases**

The sequences of the O-OTases known to date from *N. meningitidis* MC 58 (gi 341867160), *V. cholerae* O1 El Tor N16961 (gi 15640420), *B. thailandensis* E264 (gi 83721181), *Acinetobacter* sp. ADP1 (gi 50083393) and *R. pickettii* 12J (gi 187927579) were aligned by using ClustalX 2.0.11. Site-directed mutagenesis

The polymerase chain reaction (PCR) primers used for site-directed mutagenesis are listed in Table 2. Mutated codons are underlined in the oligonucleotide sequences. PCR was carried out using Pfu Turbo polymerase (Fermentas, ON, Canada) and pAMF10 or pAMF15 as template unless otherwise stated according to the method described by (Fisher and Pei 1997). Mutagenesis was confirmed by DNA sequencing. The expression of the PglL and PilE mutants was confirmed by western blot analysis using anti-His and antipilin monoclonal (SM1) antibodies, respectively.

**In vivo glycosylation assay and expression analysis**

Pilin glycosylation reactions were carried out in the *E. coli* strain CLM24 transformed with three plasmids containing the pilin protein acceptor, a PglL variant and the CjLLO biosynthetic machinery as described in (Gebhart et al. 2012). After 4 h induction, volumes of cell lysate equivalent to 0.15 OD were loaded in 15% polyacrylamide gels to perform SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane and pilin glycosylation or PglL expression were detected by western blot analysis using HR6/SM1 and anti-His antibodies, respectively. Loading controls were performed by using antibodies anti-subunit α of *E. coli* RNA polymerase. Assays were performed in duplicate.

**Purification of glycosylated pilin**

*Neisseria meningitidis* MC58 pilin (PilE) glycosylated with the *C. jejuni* heptasaccharide was produced in *E. coli* SCM3 transformed with plasmid containing the CjLLO biosynthetic machinery as described in (Gebhart et al. 2012). After 4 h induction, volumes of cell lysate equivalent to 0.15 OD were loaded in 15% polyacrylamide gels to perform SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane and pilin glycosylation or PglL expression were detected by western blot analysis using HR6/SM1 and anti-His antibodies, respectively. Loading controls were performed by using antibodies anti-subunit α of *E. coli* RNA polymerase. Assays were performed in duplicate.

---

**Table I.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F- g80dlacZ M15 (lacZYA-argF169 deoR recA1 endA1 hsdR17 (rK− mK+)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CLM24</td>
<td>W3110 lacking WaaL ligase</td>
<td>Feldman et al. (2005)</td>
</tr>
<tr>
<td>SCM3</td>
<td>Sφ874 ΔwaaL</td>
<td>Miguel Valvano (University of Western Ontario)</td>
</tr>
<tr>
<td>SCM6</td>
<td>Sφ874 ΔwaaL, ΔwecA</td>
<td>Miguel Valvano (University of Western Ontario)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMLBAD</td>
<td>Cloning and expression vector, arabinose-inducible, TpR</td>
<td>Lefebvre and Valvano (2002)</td>
</tr>
<tr>
<td>pEXT20</td>
<td>Cloning and expression vector, IPTG-inducible, AmpR</td>
<td>Dykxhoorn et al. (1996)</td>
</tr>
<tr>
<td>pACYCpglBmut</td>
<td>Carries <em>C. jejuni</em> pgl containing mutations W458A and D459A in PglB, CmR</td>
<td>Wacker et al. (2002)</td>
</tr>
<tr>
<td>pJHCV32</td>
<td>Expresses the O7 antigen cluster from <em>E. coli</em>, TetR</td>
<td>Marolda et al. (1999)</td>
</tr>
<tr>
<td>pAMF10</td>
<td>C-10 His-tagged PglL cloned in pEXT20, AmpR</td>
<td>Faridmoayer et al. (2007)</td>
</tr>
<tr>
<td>pAMF14</td>
<td>C-6 His-tagged PilE cloned in pMLBAD, TpR</td>
<td>Faridmoayer et al. (2007)</td>
</tr>
<tr>
<td>pAMF16</td>
<td>C-10 His-tagged PilE cloned in pMLBAD, TpR</td>
<td>Faridmoayer et al. (2007)</td>
</tr>
</tbody>
</table>
overnight at 4°C in 50 mM Tris containing 250 mM imidazole. Protein solutions were dialyzed washed with the same buffer to remove unbound proteins. Bound proteins were eluted from the column using Buffer 2 containing 20 mM imidazole and nickel-nitrilotriacetic acid agarose column (Qiagen) previously equilibrated with Buffer 2. Unbound proteins were removed by eluted with Buffer 3 containing 0.5 M d-galactose. 3), and applied to an SBA-Agarose column (Vector Labs) equi-
mM NaCl, 1 mM dithiothreitol (DTT), and 0.8% DDM (Buffer g, 2) containing 20 mM. The solution was applied to nickel-nitrilotriacetic acid agarose column (Qiagen) previously equilibrated with Buffer 2 containing 20 mM imidazole and washed with the same buffer to remove unbound proteins. Bound proteins were eluted from the column using Buffer 2 containing 250 mM imidazole. Protein solutions were dialyzed overnight at 4°C in 50 mM Tris–HCl, pH 8.5, containing 10 mM NaCl, 1 mM dithiothreitol (DTT), and 0.8% DDM (Buffer 3), and applied to an SBA-Agarose column (Vector Labs) equi-
librated with Buffer 3. Unbound proteins were removed by washing the column with Buffer 3 and bound proteins were eluted with Buffer 3 containing 0.5 M d-galactose.

### Western blot analysis for MS analysis

Western blot analyses were carried out using standard methods. The presence of proteins on nitrocellulose membranes was detected using the following antibodies. HR6 or R12 was used to detect C. jejuni LLO. SM1 monoclonal antipilin or poly-
clonal anti-his (Rockland Labs) was used to detect pilin. Mouse monoclonal anti-RNA polymerase α-subunit from E. coli was used in the loading controls (Neoclon). Anti-mouse and anti-rabbit antibodies were IR-DYE labeled and supplied by LI-COR Biosciences (NE). Western blots were visualized using the Odyssey imaging system (LICOR Biosciences, Lincoln, Nebraska, USA) or ECL western blot detection reagents (GE Healthcare).

### In-gel pilin digestion

In-gel digestion of pilin was carried out by following a protocol of Shevchenko et al. (1996) with slight modifications (Shevchenko et al. 1996). In brief, purified glycosylated pilin was run on a 15% SDS–PAGE gel and stained with Coomassie blue. Protein bands corresponding to glycosylated pilin were cut and transferred to 1.5 mL microfuge tubes. Pilin was reduced by the addition of 10 mM DTT in 50 mM ammonium bicarbonate, and thiol groups were alkylated with 50 mM iodoacetamide in 50 mM ammonium bicarbonate, and thiol groups were alkylated with 50 mM iodoacetamide in 50 mM ammonium bicarbonate. The gel pieces were washed with water and alternately dehydrated and rehydrated by incubation with acetonitrile (100%) and acetonitrile-ammonium bicarbonate (NH₄HCO₃) 1:1 (v/v) respectively. Subsequently, pilin was digested with proteinase K (Roche), thermolysin (Calbiochem) or porcine trypsin (Promega), as indicated. Peptides eluted from the gel pieces were desalted by Zip-TipC18 (Millipore) according to the protocol of the supplier. Desalted peptides were dried by SpeedVac, dissolved in 0.1% formic acid (v/v) in water and used for MS analyses.

### Nano-liquid chromatography-electrospray ionization MS and MS/MS analysis

Peptides obtained from protease hydrolysis were analyzed using a hybrid quadrupole orthogonal acceleration time-of-flight (TOF) mass spectrometer, Q-TOF Premier (Waters, Manchester, UK.), equipped with a nanoACQUITY ultra performance liquid chromatography system (Waters Manchester, UK). Briefly, 2 μL of the peptide solution was injected into a microprecolumn C18
cartridge that was connected to a 75 μm (inside diameter) by 150-mm Atlantis dC18 column (Waters). Solvent A consisted of 0.1% formic acid and 1% acetonitrile in water and solvent B consisted of 0.1% formic acid and 1% water in acetonitrile. After a 2-min trap wash in the precolumn with solvent A at a flow rate of 10 μL/min, peptides were separated by using a solvent gradient and electrospayed into the mass spectrometer at a flow rate of 300 nL/min. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide ions. The instrument was calibrated, and data acquisition was performed as described in (Wang et al. 2007). The data analysis was carried out using MassLynx (Waters; MassLynx, v. 4.1).

In vitro glycosylation assay

The PglL variants and the protein acceptor pilin were purified as described by (Musumeci, Hug et al. 2013). The Escherichia coli strain SCM6 was transformed with pACYCpglBmut plasmid or pACYC base plasmid, 1 L of these cells were grown at 30°C until OD600 = 1.0 and CjLLO or UndPP was extracted, respectively, following the published procedure. The concentration of CjLLO was estimated by the phenol-sulphuric method using galactose as standard, considering that CjLLO has one DATDH and six residues of GalNAc. The extraction from E. coli SCM6 cells transformed with the base plasmid pACYC (lacking the biosynthetic pathway of CjLLO) was used as blank. The in vitro glycosylation assays were performed in duplicate or triplicate as described by (Musumeci, Hug et al. 2013; Musumeci, Ielmini et al. 2013).

Limited proteolysis experiments

Aliquots of 15 μM of purified PglL were incubated on ice for 20 min with 1 μL of CjLLO organic extraction (CjLLO 120 μM) to promote interaction enzyme-substrate. The organic extraction of CjLLO was performed from SCM6 E. coli strains as in the previous section “In vitro glycosylation assay” above. Next, proteinase K (0.06 mg/mL final concentration, resuspended in Tris 50 mM, pH 7.5, CaCl2 10 mM) or trypsin (0.04 mg/mL final concentration) was added and the degradation was carried out at room temperature. A control without added protease was included. In the three cases, the degradation reaction was quenched after 0, 5, 15, 30, 60, 180 and 300 min by adding 5 mM PMSF, 1× SDS–PAGE loading buffer containing SDS 0.2% (w/v) and further freezing at −80°C. In parallel, 15 μM of purified PglL were incubated with 1 μL of a negative control (i.e. without CjLLO substrate), which was obtained from SCM6 E. coli cells transformed with the pACYC base plasmid, as in the section “In vitro glycosylation assay” above. After incubation of 20 min on ice, these samples were incubated with proteinase K following the same procedure described formerly. Finally, the samples were loaded in a 10% tricine SDS–PAGE gels and western blot analyses using anti-His antibodies were performed to visualize the degradation pattern. Duplicates or triplicates were performed.

Funding

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Alberta Glycomics Center.

Acknowledgements

We thank Markus Aebi (ETH Switzerland) and Michael Koomey (University of Oslo) for kindly supplying us with antibodies. We thank the members of the laboratory for critical reading of the manuscript and Amy McLeod for technical assistance.

Conflict of interest

None declared.

Abbreviations

CjLLO, Campylobacter jejuni heptasaccharide linked to Undecaprenyl pyrophosphate; DATDH, 2,4-diamino-2,4,6-trideoxy-D-glucose; DDM, n-dodecyl-β-D-maltoside; DTT, dithiothreitol; ECL, electrochemiluminescent; GlcNAc, N-Acetyl Glucosamine; IPTG, isopropyl-β-D-1-tiogalactopiranosido; LCRs, low complexity regions; LLO, lipid-linked oligosaccharide; LPS, lipopolysaccharide; MS, mass spectrometry; NMR, nuclear magnetic resonance; OD, optical density; O-Tases, O-Oligosaccharyltransferases; OTAse, oligosaccharyltransferase; PC, phosphorylcholine; PCR, polymerase chain reaction; PE, phosphethanolamine; PG, phosphoglycerol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SM1, monoclonal anti-piIE of N. meningitides; TOF, time-of-flight; UDP, Uridine diphosphate; UndPP, Undecaprenyl pyrophosphate.

References


Castric P. 1995. pilO, a gene required for glycosylation of Pseudomonas aerugi-


Dykxhoorn DM, St Pierre R, Linn T. 1996. A set of compatible tac promoter ex-


