**REVIEW**

**Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria**

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Bacteria generate and attach countless glycan structures to diverse macromolecules. Despite this diversity, the mechanisms of glycoconjugate biosynthesis are often surprisingly similar. The focus of this review is on the commonalities between lipopolysaccharide (LPS) and glycoprotein assembly pathways and their evolutionary relationship. Three steps that are essential for both pathways are completed by membrane proteins. These include the initiation of glycan assembly through the attachment of a first sugar residue onto the lipid carrier undecaprenyl pyrophosphate, the translocation across the plasma membrane and the final transfer onto proteins or lipid A-core. Two families of initiating enzymes have been described: the polyprenyl-P N-acetylhexosamine-1-P transferases and the polyprenyl-P hexosamine-1-P transferases, represented by *Escherichia coli* WecA and *Salmonella enterica* WbaP, respectively. Translocases are either Wzx-like flippases or adenosine triphosphate (ATP)-binding cassette transporters (ABC transporters). The latter can consist either of two polypeptides, Wzt and Wzm, or of a single polypeptide homolog to the *Campylobacter jejuni* PglK. Finally, there are two families of conjugating enzymes, the N-oligosaccharyltransferases (N-OTases), best represented by C. jejuni PglB, and the O-OTases, including *Neisseria meningitidis* PglL and the O antigen ligases involved in LPS biosynthesis. With the exception of the N-OTases, probably restricted to glycoprotein synthesis, members of all these transmembrane protein families can be involved in the synthesis of both glycoproteins and LPS. Because many translocation and conjugation enzymes display relaxed substrate specificity, these bacterial enzymes could be exploited in engineered living bacteria for customized glycoconjugate production, generating potential vaccines and therapeutics.

**Keywords:** flippase/glycosylation/ligase/lipopolysaccharide/oligosaccharide/transferase

**Introduction**

Glycoconjugates are essential components of all living cells. Their sugar moieties, covalently attached to proteins or lipids, fulfill diverse cellular functions ranging from warranting structural integrity and stability to signaling and intercell communication (Helenius and Aebi 2001). Bacteria produce multiple glycoconjugates, including lipopolysaccharides (LPS), peptidoglycan, glycoproteins, capsules, teichoic acids and exopolysaccharides. In many cases, these molecules are synthesized by similar pathways (Figure 1). Nucleotide-activated sugars serve as substrates for different glycosyltransferases, which perform the sequential addition of monosaccharides onto a lipid carrier. Once assembled, the glycolipid intermediate is flipped across the plasma membrane. In many cases, the glycans are polymerized, which can take place in the cytoplasmic or periplasmic compartment. Finally, the carbohydrates are detached from the lipid carrier and assembled en bloc into their final structures.

In this review, we discuss the similarities of LPS and bacterial en bloc glycosylation pathways. Remarkable parallels can be drawn at every step of the assembly processes. Furthermore, many of the proteins involved in these pathways are homologous, suggesting close evolutionary connections. Although we will focus on the similarities between the assembly steps of these two classes of glycoconjugates, we will occasionally relate these processes to other bacterial and eukaryotic pathways. Finally, from a biotechnological point of view, we indicate how these commonalities could be exploited for the generation of novel glycoengineered structures that may have applications as vaccines and therapeutics.

**Lipopolysaccharides**

LPS constitutes an abundant and well-studied molecule in Gram-negative bacteria. It is the main component of the outer leaflet of the outer membrane, being anchored to the membrane by the lipid A moiety widely known as endotoxin (Beutler 2002; Heumann and Roger 2002; Trent et al. 2006; Freudenberg et al. 2008). Extended outward from the membrane is a core oligosaccharide followed by the O antigen, a polymer of short oligosaccharide repeats. LPS acts as a barrier, protecting the bacteria against adverse environmental factors. In pathogenic Gram-negative bacteria, the O antigen
constitutes a major virulence factor, shielding the cells from components of the immune system such as the complement system (Lerouge and Vanderleyden 2002). Sometimes, glycan structures on LPS or lipooligosaccharides (LOSs) mimic human epitopes. O antigens of Helicobacter pylori contain Lewis antigens which are also present on a variety of human cells and interact with dendritic cells of the human immune system, resulting in modulation of immune responses (Bergman et al. 2004). Similarly, LOS of Campylobacter jejuni displays the structure of human gangliosides (Yuki et al. 1993). In rare cases, C. jejuni infections activate immune responses against these structures, leading eventually to an autoimmune attack (Rinaldi and Willson 2008).

Different LPS biosynthesis pathways have been described which mainly diverge in the O antigen translocation and polymerization mechanisms and the proteins required for these steps (Raetz and Whitfield 2002). In all pathways, lipid A-core and the O antigen substructures are independently assembled at the cytoplasmic side of the inner membrane. Subsequently, these molecules are separately translocated over the plasma membrane to face the periplasm. Next, the O antigen ligase WaaL covalently joins lipid A-core and O antigen (Raetz and Whitfield 2002). The mature LPS is finally actively transported to the cell surface (Sperandio et al. 2009). Two main O antigen biosynthetic pathways have been described. In the Wzy-independent pathway, the sugars are sequentially assembled onto the lipid carrier at the cytoplasm and the polymer is translocated across the membrane using an ABC transporter. In the other major pathway, O antigen subunits are assembled in the cytoplasm and translocated across the inner membrane via a flipase of the Wzx family. In the periplasm, the polymerase Wzy in conjunction with the chain length regulator Wzz polymerizes O antigen repeating units before they are transferred by WaaL.

A detailed review concerning LPS biosynthesis is available (Raetz and Whitfield 2002).

**Protein glycosylation**

Glycoproteins, once believed to be exclusively present in eukaryotes, are now recognized as common components of bacteria and archaea. There are two distinct mechanisms of glycoprotein biosynthesis. Sugars can be attached directly onto carrier proteins by cytoplasmic glycosyltransferases. In bacteria, flagellins and some adhesins are frequently glycosylated through this mechanism (Benz and Schmidt 2001; Logan 2006; Gross et al. 2008). Alternatively, glycan chains can be assembled on a lipid carrier previous to their transfer en bloc onto protein acceptors. In this case, oligosaccharyltransferases (OTases) are required to accomplish the final glycosylation step (Szymanski et al. 1999; Wacker et al. 2002; Kelleher and Gilmore 2006). This pathway, which resembles the N-glycosylation system in the endoplasmic reticulum of eukaryotes, can lead in bacteria to both, N- and O-linked glycosylation (Young et al. 2002; Faridmoayer et al. 2007). In some bacteria, this modification was described for only a single protein. For example, certain Pseudomonas aeruginosa strains glycosylate the major subunit of the type IV pilin (Castric 1995), and S-layer proteins are targeted by the glycosylation machinery in a number of Gram-positive bacteria such as Paenibacillus alvei and Geobacillus stearothermophilus (Steiner et al. 2008; Zarschler et al. 2010). In contrast, in C. jejuni, Neisseria spp. and recently Bacteroides fragilis, general glycosylation systems have been described in which the pre-assembled glycans are transferred onto multiple acceptor proteins (Szymanski et al. 1999; Fletcher et al. 2009; Ku et al. 2009; Vik et al. 2009).

In most cases, the physiological significance of bacterial en bloc glycosylation systems remains unclear. Because the bacteria with best-studied glycosylation systems are human pathogens, potential roles of glycosylation in virulence have been investigated. A glycosylation-deficient P. aeruginosa mutant strain produced pili of similar number and appearance as the wild-type strain but was less efficient in colonizing a mouse model (Smedley et al. 2005). Similarly, in C. jejuni, inability to glycosylate resulted in fewer adherence and invasion of host cells and reduced colonization of mice and chickens (Szymanski et al. 2002; Karlyshev et al. 2004). Glycosylation can have an impact on the activity of single proteins. One C. jejuni glycoprotein, VirB10, is a component of the type IV secretion system, required for DNA uptake. It was shown that glycosylation of VirB10 is necessary for full natural competence (Larsen et al. 2004). Nevertheless, a role for glycosylation in virulence is not established in all cases. A connection between type IV pilin glycosylation and pathogenicity was not confirmed in Neisseria spp. (Marceau et al. 1998; Power et al. 2007; Virji 2009). An alternative function for bacterial glycoproteins in the host system was shown for the C. jejuni glycoproteins. The N-acetylgalactosamine-rich glycans were found to interact with macrophage galactose-type lectins present on immature human dendritic cells and certain macrophages, altering their cytokine production (van Sorge et al. 2009).

However, bacterial protein glycosylation is not an exclusivity of pathogens. As mentioned, the intestinal symbiont B.
fragilis has a general en bloc glycosylation system (Fletcher et al. 2009) and deep sea vent epsilon proteobacteria such as Sulfurovum and Nitratiruptor contain putative OTase genes (Nakagawa et al. 2007). However, the presence of an OTase homolog does not warrant that the glycosylation process takes place in a given organism. The release of free plasmidic glycans as a mechanism to allow for osmolarity balance was proposed as an alternative role of members of this enzyme family (Nothaft et al. 2009). The broad distribution of glycosylation systems suggests that this form of protein modification likely has important functions in the natural environment of the organism and possibly improves the stability or activity of certain polypeptides, and it is tempting to speculate that glycans could be involved in intra- or interspecies communication, as the example of the C. jejuni glycans interacting with human lectins suggests.

Stepwise comparison of bacterial LPS biosynthesis and en bloc protein glycosylation

Lipid carrier

In most bacteria, oligo- and polysaccharides are generally assembled onto undecaprenyl phosphate (UndP) via a pyrophosphate linker. The lipid carrier of these glycans is therefore undecaprenyl pyrophosphate (UndPP). This is true not only for en bloc protein glycosylation and O antigen synthesis, but also for peptidoglycan, exopolysaccharide and teichoic acid biosynthesis (Higashi et al. 1967; Wright et al. 1967; Leigh and Coplin 1992; Feldman et al. 2005; Faridmoayer et al. 2007; Weidenmaier and Peschel 2008). Group 1 and 4 capsules are thought to be assembled onto a glycerol phosphate molecule (Whitefield 2006). Interestingly, the carrier for the synthesis of arabino-galactan in Mycobacteria sp. is decaprenyl pyrophosphate, being one isoprene unit shorter than UndPP (Wolucka et al. 1994). Eukaryotic N-glycosylation requires a similar polisoprenoid carrier for glycan assembly, dolichyl pyrophosphate (DolPP), which is located in the membrane of the endoplasmic reticulum. DolPP consists of some more isoprene units and has an α-saturated isoprene unit (Chojnacki and Dallner 1988).

The energy-rich pyrophosphate bond between the carrier lipid and the glycan is generated during the enzymatic transfer of a sugar–phosphate onto UndP by an initiating glycosyltransferase, using nucleotide-activated sugar as the donor (Bugg and Brandish 1994). UndPP is released after the en bloc transfer of the glycan. One phosphate group is subsequently removed by a phosphatase and the polyprenyl-P is transported back to the cytoplasmic leaflet of the membrane to be reused as a carrier for glycan assembly (El Ghachi et al. 2005; Tatar et al. 2007; Touze et al. 2008; Valvano 2008).

Several, but not all the membrane proteins involved in glycoconjugate synthesis seem to favor lipid carriers of a specific length, cis–trans conformation or α-saturation status (Schenk et al. 2001). Preference for a specific carrier suggests that the transmembrane portions of these enzymes interact with the lipid. However, the Neisseria meningitidis O'Tase PgIL, which naturally uses UndPP as a carrier for O-glycosylation, is able to transfer in vitro sugars attached to farnesyl pyrophosphate (Faridmoayer et al. 2008). This lipid is considerably shorter and has an opposite isomeric conformation than UndPP, indicating that for this case the role of the carrier may be limited to providing a platform to assemble and translocate the glycan, placing the substrate in the proper distance to the enzyme.

Initiating enzymes

The first step during glycan assembly for en bloc protein glycosylation and LPS biosynthesis is carried out by polyprenyl-phosphate glycosyl-1-phosphate transferases. Using UDP-sugars, these enzymes form a pyrophosphate bridge between the lipid carrier at the cytoplasmic face of the plasma membrane and the sugar residue. Based on primary sequence similarity and membrane topology, initiating enzymes are grouped into two unrelated protein families. Prototypes for the two classes of initiating enzymes are the Escherichia coli WecA and the Salmonella enterica WbaP. LPS and glycoprotein biosynthesis pathways do not differ in the use of their initiating enzyme. Instead, both pathways can be initiated by either a WecA-like or a WbaP-like protein.

WecA Family: Polyprenyl-P N-Acetyllhexosamine-1-P Transferases. Transferring N-acetyllhexosamine-1-phosphates onto polyprenyl-phosphate lipids, initiating enzymes belonging to this class are called polyprenyl-P N-acetyllhexosamine-1-P transferases (PNPTs) (Price and Momany 2005). The E. coli WecA is a prototype of this protein family and acts as an undecaprenyl-phosphate N-acetyllhexosamine-1-phosphate transferase, generating undecaprenyl pyrophosphate-N-acetyllglucosamine (UndPP-GlcNAc) (Meier-Dieter et al. 1992). It is encoded in the cluster for the synthesis of enterobacterial common antigen (ECA). The UndPP-GlcNAc intermediate is the acceptor for further glycosyltransferases attaching an N-acetyl-o-mannosaminuronic acid (ManNAcA) and a 4-acetamide-4,6-dideoxy-D-galactose (Fuc4NAc) residue to build the ECA trisaccharide subunit Fuc4NAc1-4ManNAc1-4ManNAcAβ1-4GlcNAc (Lugowski et al. 1983). This glycolipid intermediate is transported to the periplasmic face of the inner membrane where the glycan is polymerized and attached onto either diacylglycerol or lipid A-core as the final acceptors or cyclized and released as free circular oligosaccharide into the periplasm. The resulting three types of ECA are named ECAαPG, ECAβPS and ECAγCYC, respectively (Kuhn et al. 1988).

Many Enterobacteriaceae use their WecA in addition for LPS biosynthesis. Glycosyltransferases required for the second step in the synthesis of the O antigen compete with the ECA producing glycosyltransferases for their common acceptor UndPP-GlcNAc. E. coli serotypes O16 (most laboratory strains), O8 (Figure 2F) and O9, Klebsiella pneumoniae serotypes O3, O5 and S. enterica serovar Borreze are examples for strains using WecA for both ECA and LPS biosynthesis (Stevenson et al. 1994; Yao and Valvano 1994; Raetz and Whitefield 2002). However, Yersinia enterocolitica serotype O3 encodes besides WecA a second WecA-like protein, WbcO, in its LPS outer core gene cluster (Anderson et al. 2000). WbcO initiates the assembly of the outer core by
attaching an N-acetylfucosamine (FucNAc) residue via a
diphosphate linkage to UndP.

Outside the family of Enterobacteriaceae, other bacteria,
although lacking the eca gene cluster use WecA-like enzymes
in the biosynthesis of a variety of glycans. LPS production in
H. pylori (Figure 2H) is dependent on WecA (Hug et al.
2010). The P. aeruginosa WecA homolog WbpL is involved in
LPS A-band and B-band synthesis as well as in pilin
glycosylation (Rocchetta et al. 1998). This requirement of the
same enzyme in LPS and glycoprotein synthesis gives the
clearest demonstration of how closely these pathways are con-
nected with each other (Figure 2C). WbpL exhibits relaxed
sugar specificity, either using FucNAc for LPS B-band and
glycoprotein synthesis or using GlcNAc for LPS A-band
assembly (Rocchetta et al. 1998). Examples for members in
Gram-positive bacteria are Bacillus subtilis TagO which

Fig. 2. Key enzymes are shared between LPS and glycoprotein biosynthesis pathways. The schematic representation shows LPS and glycoprotein biosynthesis pathways of eight selected bacteria. Initiating enzymes are shown in a rhomboid shape, translocating enzymes as ovals and the conjugating enzymes as octagons. The color of these membrane proteins signifies the enzyme family they belong to. PHPTs are purple, PNPTs blue, Wzx-flipases red, two-component ABC transporters orange, single polypeptide ABC transporters yellow, ligases and O-OTases turquoise and N-OTases green. (A) S. enterica LPS, (B) Neisseria spp.
O-glycosylation, (C) P. aeruginosa PAO1 LPS and O-glycosylation, (D) N-glycosylation in eukaryotes, (E) P. alvei O-glycosylation, (F) E. coli O8/O9 LPS,
(G) C. jejuni N-glycosylation and (H) H. pylori LPS. Except for the N-OTases, all enzyme families have members in glycoprotein and also LPS biosynthesis pathways.
initiates teichoic acid synthesis using UDP-GlcNAc as a substrate (Soldo et al. 2002) and *Streptococcus mutans* RgpG, required for the synthesis of a rhamnose–glucose polysaccharide (Yamashita et al. 1999). Another related bacterial enzyme is MraY, an initiating glycosyltransferase essential for peptide-glycan synthesis (Ikeda et al. 1991). MraY links the N-acetylmuramic acid (MurNac)-pentapeptide to UndP by forming a pyrophosphate linkage. Eukaryotic cells also contain one PNPT. The first step in the eukaryotic N-glycosylation pathway is the formation of a pyrophosphate linkage between GlcNAc-1-P and DolP at the cytoplasmic side of the ER membrane (Figure 2D). Required for this enzymatic reaction is the dolichyl-P N-acetyleneosamine-1-P transferase GPT (Lehman 1991).

PNPTs are integral membrane proteins with multiple membrane-spanning domains. They have an unusually high isoelectric point around 10, are inhibited by the UDP-GlcNac analog tunicamycin and require Mg$^{2+}$ or Mn$^{2+}$ for activity (Price and Momany 2005). Product formation is believed to be achieved via a double displacement mechanism, generating an intermediate of HexNAc-1-P covalently bound to the enzyme (Heydanek et al. 1969; Brandish et al. 1996; Price and Momany 2005). The catalytic site of these proteins is expected to be situated in the cytoplasmic compartment where the nucleotide-activated HexNAc substrate and the phosphate group of the lipid acceptor are located. Whereas the enzymes are thought to contain five cytoplasmic domains, conserved motifs and residues located in the second and third cytoplasmic loops are thought to be mechanistically important (Bouhss et al. 1999; Amer and Valvano 2002; Price and Momany 2005; Lehrer et al. 2007). The fifth and largest cytoplasmic domain is less conserved and is believed to determine substrate specificity (Anderson et al. 2000; Amer and Valvano 2001; Price and Momany 2005). Especially the eukaryotic transferase, GPT, shows no primary sequence similarity in this domain. However, the role of these C-terminal domains in substrate recognition will have to be confirmed with future experiments.

Experiments for the evaluation of acceptor lipid specificities led to the surprising conclusion that eukaryotic PNPTs, as exemplified by a GPT from pig brain, used DolP as an acceptor but was unable to transfer a GlcNAc-1-P onto UndP, whereas the inverse result was obtained for the *E. coli* WecA (Mankowski et al. 1975; Rush et al. 1997). PNPTs from the extreme thermophilic bacterium *Thermotoga maritima*, a WecA and an MraY analog, proved less specific toward the lipid acceptor. *T. maritima* WecA accepted UndP analogs with a minimum length of 35 carbons, but also DolP with about 60% activity (Al-Dabbagh et al. 2008). An interesting challenge for future research is to determine if putative PNPT domains interacting with polyyneryl structures do exist.

**WbaP Family: Polyyneryl-P Hexose-1-P Transferases.** Glycan assembly is in many cases initiated by a polyyneryl-P hexose-1-P transferase (PHPT). Enzymes belonging to this family commonly generate an UndPP-hexose linkage. Although conducting analogous functions, PNPTs and PHPTs are evolutionarily unrelated, differing in primary sequences and topologies. PHPTs are involved in the biosynthesis of a variety of glycoconjugates including LPS, capsule, exopolysaccharide and glycoprotein (Jiang et al. 1991; Rubens et al. 1993; Stevenson et al. 1996; Linton et al. 2005). However, homologous enzymes are not known in eukaryotes.

The PHPT prototype is the *S. enterica* WbaP, a galactosyl-1-P transferase required for O antigen assembly (Figure 2A) (Jiang et al. 1991). Its glycosyltransferase activity is localized in a cytoplasmic domain which is followed by a transmembrane domain at the C-terminus (Wang et al. 1996; Saldias et al. 2008). Unlike the other regions of WbaP, this glycosyltransferase domain is conserved between PHPTs and is sufficient for the PHPT activity. Additional domains fused to either its C- or N-terminal ends are found in many members of the WbaP family. These extra domains are often not related and account for various activities. The N-terminus of *S. enterica* WbaP contains four transmembrane domains and adds the T-function, proposed to assist in the release of the reaction product from the enzyme (Wang et al. 1996) or simply to support membrane interaction (Saldias et al. 2008). Whereas the C-terminal part of *S. enterica* WbaP is sufficient for *in vivo* function, the N-terminus is required for activity *in vitro* (Saldias et al. 2008). A periplasmic loop connecting the N-terminus with the glycosyltransferase domain is important for the regulation of the O antigen chain length (Saldias et al. 2008). This domain likely interacts with the O antigen polymerase Wzy.

PHPTs constitute another link of the evolutionary chain between LPS and protein glycosylation biosynthetic pathways. Besides the described role in O antigen biosynthesis, members of this family are involved in both N- and O-linked protein glycosylation. The initiating glycosyltransferases in the general protein glycosylation machineries of *C. jejuni* and *N. meningitidis* or *N. gonorrhoeae* are homologous to PHPTs (Figure 2G and B, respectively) (Power et al. 2000; Linton et al. 2005). However, the sugar residues attached onto UndPP by these enzymes are not hexoses. Instead, a 2,4-diacetamido-2,4,6-trideoxyglucose, also named DATDH or bacillosamine, is transferred by *C. jejuni* PglIC (Young et al. 2002; Glover et al. 2006). *N. meningitidis* and *N. gonorrhoeae* require either PglB or PglB2 for glycoprotein synthesis. Both are initiating enzymes and homologs of WbaP. *Neisseria* strains having the PglB homolog initiate glycan assembly with a DATDH residue. Unlike *C. jejuni* PglIC, whose similarity to WbaP is restricted to the conserved glycosyltransferase sequence, the *Neisseria* PglB has in addition an acetyltransferase domain at the C-terminus, involved in the biosynthesis of the DATDH residue (Power et al. 2000). In about half of the *Neisseria* spp., the pgIB gene was disrupted with the insertion of 2 kb DNA (Power et al. 2003). This insertion resulted in the exchange of the PglB acetyltransferase domain with another domain homologous to carbamoyl phosphate synthases. The modified enzyme was designated PglB2 (Kahler et al. 2001). A novel sugar residue, glyceramido acetamido trideoxyhexose or GATDH, is synthesized and transferred by PglB2 (Chomot-Rooke et al. 2007).

Other protein glycosylation pathways prime glycan assembly with more conventional PHPTs. S-layer protein glycosylation in *P. alvei* and *G. stearothermophilus* involve the galactose-1-P transferases *WsfP* and *WsaP*, respectively.
Many prokaryotes. Although evidence indicating that clusters that also encode the Wzy polymerase (Raetz and the transport of UndPP-linked glycans across the plasma C- and N-termini located in the cytoplasm (Mazur et al. 2005; synthesis, revealed 12 transmembrane domains with theduction, and PssL, involved in exopolysaccharide pro-
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UndPP-glycan transporters
After assembly, UndPP-glycans are translocated across the plasma membrane, a process requiring specialized transporter polypeptides. Bacteria apply different unrelated strategies to accomplish this task. However, these strategies can be used for both LPS synthesis and protein glycosylation. Two major UndPP-glycan transporters have been studied, the Wzx flippases and the ABC transporters. A third transporter, known as the synthase, has been reported.

Wzx Flippases. Wzx is a putative UndPP-glycan flippase in many prokaryotes. Although evidence indicating that Wzx-like polypeptides function as the flippases required for the transport of UndPP-linked glycans across the plasma membrane is strong, this has yet to be directly demonstrated. The gene encoding Wzx is usually located in the O antigen clusters that also encode the Wzy polymerase (Raetz and Whitfield 2002). In E. coli, its mutation leads to the accumulation of UndPP-O subunits in the cytoplasmic compartment (Liu et al. 1996). However, additional unidentified components might be required for efficient flipping of the glycolipids.

Wzx-like polypeptides are multi-membrane spanning inner membrane proteins involved in the biosynthesis of many O antigens (Figure 2A and C), but also group 1 and 4 capsules, teichoic acids and exopolysaccharides, and they form part of the protein glycosylation machineries of many bacteria (Liu et al. 1996; Stevenson et al. 1996; Whitfield 2006; Gonzalez et al. 2008). Examples for Wzx-dependent flipping of UndPP-glycans in protein glycosylation pathways are the O-glycosylation systems present in P. aeruginosa (Figure 2C), Neisseria spp. (Figure 2B) and B. fragilis (Faridmoayer et al. 2007; Fletcher et al. 2009). Membrane topology determinations using LacZ and PhoA fusions to truncated Rhizobium leguminosarum PssL, involved in exopolysaccharide production, and S. typhimurium Wzx, required for O antigen bio-
synthesis, revealed 12 transmembrane domains with the C- and N-termini located in the cytoplasm (Mazur et al. 2005; Cunneen and Reeves 2008). Although flippases from different bacteria share low sequence identity, they all contain the Pfam family domain Polysac_synth PF01943 (Cunneen and Reeves 2008). Carbohydrates transported by Wzx proteins are usually short oligosaccharides of complex and often branched structures. Substrate specificity is surprisingly low, allowing the exchange of Wzx genes from different bacteria (Feldman et al. 1999). The deletion of E. coli wzxLPS does not completely abolish flipping of lipid-linked O antigen subunits. Other translocases, such as WzxE involved in ECA biosynthesis, can partially complement an O antigen flippase deficiency. Nevertheless, some selectivity has been reported toward the first sugar residue connected to the lipid (Marolda et al. 2004). Following translocation, the oligosaccharides are commonly polymerized by Wzy polymerases in conjunction with Wzz chain length regulators (Raetz and Whitfield 2002). Although Wzx proteins can translocate foreign O antigens, the presence of their cognate Wzy or Wzz proteins in the same bacteria inhibits such a cross complementation (Marolda et al. 2006). Based on this observation, it was hypothesized that protein complexes are formed in vivo, holding together the O antigen biosynthesis machinery for efficient assembly. Although such interactions probably occur in vivo, these proteins can work independent of each other in vitro where Wzy and Wzz were functional in the absence of Wzx (Woodward et al. 2010).

In eukaryotic N-glycosylation, the transmembrane flipping was proposed to be accomplished by Rft1, a polypeptide remotely related to Wzx (Figure 2D) (Helenius et al. 2002). Rft1 may not be the sole component required for this function as the absence of Rft1 had no effect on in vitro DolPP-glycan translocation in yeast microsomes (Rush et al. 2009). However, in vitro experiments may not represent cellular conditions. Thus, how Dol-PP-bound glycans are translocated across the ER membrane is still a matter of controversy (Helenius et al. 2008).

ABC Transporters. The second strategy for UndPP-glycan translocation involves ABC transporters. The hydrolysis of ATP is thought to provide the energy for the passage across the plasma membrane (Raetz and Whitfield 2002). Most O antigen ABC transporters consist of two polypeptides. The ATPase domain is contained in Wzd, whereas Wzm has multiple membrane-spanning sequences believed to build a channel in the inner membrane (Zhang et al. 1993; Raetz and Whitfield 2002). Wzm proteins are poorly conserved in the primary sequence but are predicted to feature similar secondary structures (Raetz and Whitfield 2002). Wzt–Wzm transporters are involved in O antigen flipping in many bacteria, as well as in group 2 capsule and teichoic acid biosynthesis pathways (Lazarevic and Karamata 1995; Raetz and Whitfield 2002; Whitfield 2006). Classical two-component ABC transporters also appear to be involved in S-layer protein glycosylation in P. alvei (Figure 2E) (Zarschler et al. 2010).

Deletion of either wzt or wzm is toxic in E. coli O8 and O9a. Conditional mutants constructed in strains only allowing O antigen synthesis when mannose is added to the growth medium accumulated long O polysaccharides in the cytoplasm (Cuthbertson et al. 2005). ABC transporters involved in group 2 capsule synthesis are, however, not essential for cell viability (Pavelka et al. 1994; Pigeon and Silver 1994).

Unlike Wzx flippases, ABC transporters generally move long glycan chains rather than short complex oligosacchar-
ides. Therefore, no polymerization is required after translo-
cation. Wzm proteins are not specific toward the polysaccharide and can translocate foreign O antigens, although efficiency might be reduced (Cuthbertson et al. 2005). In E. coli O8 and
O9a, the glycan chains appear to be fully synthesized and elongation terminated before passage through the putative Wzm channel (Figure 2F). Polysaccharide elongation is stopped by chain length terminators, modifying the nonreducing end of O antigens, for example, by methylation in *E. coli* O8 or by adding a methyl group after prior phosphorylation in *E. coli* O9a (Clarke et al. 2004). *E. coli* Wzt contains a C-terminal domain important to establish a specific contact for these modified glycan caps, allowing translocation only for O polysaccharides with a given terminal structure and modification (Cuthbertson et al. 2005, 2007). However, not all Wzt transporters have this stringent substrate specificity. The *K. pneumoniae* O2a Wzt polypeptide is restricted to the conserved ATPase domain (Kos et al. 2009). It does neither recognize O antigen structures nor cappings and can translocate various UndPP-glycans. Not surprisingly, no capping mechanism modifies the end of the glycan chain. In *K. pneumoniae* O2a, translocation requires ongoing O chain synthesis and the chain length is determined by the ABC transporter (Kos et al. 2009).

Another interesting example of the evolutionary connections between LPS synthesis and protein glycosylation are the two closely related ABC transporters that participate in the general N-glycosylation system in *C. jejuni* and LPS biosynthesis in *H. pylori*. Single polypeptides, named PglK or Wzk, respectively, are necessary and sufficient for the translocation of the UndPP-glycan substrates across the plasma membrane (Figure 2G and H) (Alaimo et al. 2006; Hug et al. 2010). PglK activity is dependent on ATP hydrolysis and the Walker motifs typical for ABC transporters are located at its C-terminus (Alaimo et al. 2006). Walker motifs are also present in Wzk. PglK and Wzk have no apparent structural requirements toward the glycan moiety of the substrates. They can accomplish the translocation of diverse glycans and complement each other in role in glycoprotein or LPS biosynthesis, respectively. Both enzymes are able to complement Wzx mutations (Alaimo et al. 2006; Hug et al. 2010). These two transporters are closely related to MsbA, the flippase of the lipid A-core and it is tempting to speculate that they evolved after a duplication event of the *msbA* gene (Hug et al. 2010).

Synthase. The synthase WbbF accomplishes two functions in the LPS biosynthesis pathway of *S. enterica* serovar Borreze (Keenleyside and Whitfield 1996). It contains a glycosyltransferase domain, processively elongating the O antigen through stepwise addition of sugar residues. Furthermore, WbbF is required for the translocation of the O chain to the periplasm. Other synthases are known in exopolysaccharide and capsule biosynthesis pathways, but *S. enterica* serovar Borreze provides the only known example in LPS biosynthesis (Raetz and Whitfield 2002).

Conjugating enzymes

The final step in LPS and *en bloc* glycoprotein biosynthesis is the transfer of the oligo- or polysaccharide from the UndPP carrier onto the lipid A-core or the protein acceptor. The glycan chains are attached onto the defined hydroxyl groups (O-linkage) on terminal sugar residues of the lipid A-core by a WaaL ligase, or on serine, threonine or tyrosine residues of carrier proteins by an O-OTase. In N-glycosylation the covalent linkage is built over an amino group (N-linkage) on selected asparagine residues by an N-OTase. Ligases and O-OTases are evolutionarily related, whereas N-OTases probably have a different origin.

O Antigen Ligases. O antigen ligases are encoded by the *waaL* genes, which are often but not always located in the cluster for the outer-core synthesis (Raetz and Whitfield 2002). WaaL polypeptides have multiple membrane-spanning domains and share low primary sequence similarity (Raetz and Whitfield 2002). They belong to the pfam04932 family and contain the Wzy_C domain. Membrane topology calculations predict a large periplasmic loop, which was confirmed experimentally with LacZ and PhoA fusions to the *E. coli* WaaL (Perez et al. 2008).

The general role of O antigen ligases is the detachment of a polysaccharide chain from UndPP, cleaving a phosphate–sugar bond, and subsequently binding the glycan to the lipid A-core, forming a glycosidic bond (Figure 2A, C, F and H). However, substrates as well as acceptors of ligases from different strains are structurally different.

Ligase activity seems to be independent of the substrate glycan structure (Raetz and Whitfield 2002). For example, the O antigen ligases of *P. aeruginosa* and *Porphyromonas gingivalis* each transfer two structurally different O antigens onto the lipid A-cores (Abeyrathne et al. 2005; Rangarajan et al. 2008). Furthermore, LPS variants containing glycans derived from ECA and colanic acid pathways instead of conventional O antigens were found in *E. coli* (Kiss et al. 1978; Meredith et al. 2007), and the *H. pylori* WaaL can transfer a heptasaccharide from *C. jejuni* *in vitro* (Hug et al. 2010). Bacteria could take advantage of this relaxed substrate specificity and alter their LPS structure according to environmental conditions, as it was shown in the example of the colanic acid-LPS (Meredith et al. 2007).

Specificity of ligases toward acceptor structures on the contrary is very strict and even differences of single sugar residues can abolish ligation (Heinrichs et al. 1998). Based on complementation experiments in *S. enterica*, it was suggested that additional factors besides WaaL were involved in determining this specificity toward the acceptor structure (Kaniuk et al. 2004). As the diphosphate linkage between carrier lipid and the O antigen is present in all ligation reactions, this structure is likely recognized in the putative WaaL catalytic center containing conserved positively charged amino acids (Perez et al. 2008). Further investigations on lipid structure requirements for substrates and acceptors will add valuable information toward the O antigen ligation mechanism.

Recently, functional WaaL proteins from *P. aeruginosa* and *H. pylori* were purified and ligase activities were demonstrated *in vitro* (Abeyrathne and Lam 2007; Hug et al. 2010). Curiously, although there is not a clear ATP-binding domain, the *P. aeruginosa* ligase was suggested to require ATP in an *in vitro* assay (Abeyrathne and Lam 2007). However, the *H. pylori* homolog functioned regardless of ATP addition (Hug et al. 2010), and mutations in *E. coli* ligase sequences which resemble common ATP-binding or hydrolysis domains
had no inhibitory effect on in vivo ligation, suggesting that ATP is not needed for the ligation reaction in these bacteria (Perez et al. 2008). The in vitro activity of purified ligases expressed in heterologous strains suggests that, at least in some strains, WaaL is indeed the only polypeptide required and sufficient for the ligation reaction. Purification of functional WaaL enzymes is hopefully the first step toward their crystallization and the elucidation of the catalytic mechanism.

O-Oligosaccharyltransferases. Like O antigen ligases, O-OTases attach glycans onto the hydroxyl groups on acceptor molecules, in this case on side chains of amino acid residues in protein acceptors. The predicted membrane topologies of the established P. aeruginosa 1244 PilO (Figure 2C) and the Neisseria PglL (Figure 2B) O-OTases are comparable with O antigen ligases (Power et al. 2006; Qutyan et al. 2007). Importantly, the Wzy_C motif found in WaaL ligases is also present in the O-OTases characterized so far. WaaL and O-OTases are therefore evolutionarily connected. With the current knowledge, it is not possible to determine based on amino acid sequences whether a polypeptide functions as an O-OTase or rather as a ligase. Consequently, it is required to experimentally confirm the activity of potential O-OTase candidates.

The glycans naturally transferred by the P. aeruginosa 1244 and Neisseria O-OTases are short oligosaccharides (Stimson et al. 1995; Castric et al. 2001). In a natural occurring link between protein glycosylation and LPS synthesis, the P. aeruginosa pilin glycan consists of a single O antigen subunit (Figure 2C) (Castric et al. 2001). However, other glycans can be attached onto the protein acceptors by these O-OTases when offered as substrates linked to UndPP, including even peptido-glycan subunits in the case of PglL (DiGiandomenico et al. 2002; Faridmoayer et al. 2007; Faridmoayer et al. 2008). Structurally no requirements were found for the substrate recognition of PilO and PglL, except that the P. aeruginosa PilO substrates have a limited maximum length of around 10 sugar residues (Faridmoayer et al. 2007; Faridmoayer et al. 2008).

Initial experiments concerning the substrate lipid specificity suggested that some O-OTases, unlike other enzymes using UndPP-glycans as substrates, do not interact with the lipid moiety of the substrate. Intriguingly, in an in vitro assay N. meningitidis PglL transferred an oligosaccharide linked to farnesyl pyrophosphate (Faridmoayer et al. 2008).

In P. aeruginosa, the pilin subunit PilA remains the only protein substrate of PilO identified to date. The enzyme has stringent acceptor requirements as the glycan is attached only to the C-terminal serine residue (Virji et al. 1996; Comer et al. 2002). Surprisingly, however, investigations using monoclonal antibodies raised against the Neisseria glycans identified several more glycoproteins in N. meningitidis and N. gonorrhoeae in addition to the pili subunit PilE (Ku et al. 2009; Vik et al. 2009). In many of these proteins, the glycosylation site seems to be located in a region of low complexity, rich in serine, alanine and proline residues. However, the glycosylation site of pilin, the most prominent Neisseria glycoprotein, does not follow this pattern (Vik et al. 2009). Thus, how PglL recognizes its substrates remains unclear. The O-OTase mechanism is particularly puzzling, because PglL appears to have relaxed specificity toward both moieties of its glycolipid substrate, and also toward the protein acceptor sequence.

Other WaaL-like polypeptides putatively acting as O-OTases are WsfB and WsaB, which are believed to attach polysaccharides onto S-layer proteins in P. alvei (Figure 2E) and G. stearothermophilus, respectively (Steiner et al. 2008; Zarschler et al. 2010). In G. stearothermophilus, serine and threonine residues are glycosylated, whereas the glycans in P. alvei are attached onto tyrosine residues (Schaffer et al. 2002; Zarschler et al. 2010). Another interesting case was reported about Aggregatibacter actinomycetemcomitans, which was found to use a single WaaL enzyme to ligate O antigens and in addition to glycosylate the adhesin EmaA (Tang and Mintz 2010).

In other strains, O-glycosylation of bacterial proteins may involve unrelated O-OTases. B. fragilis contains a general glycosylation system targeting several proteins at serine or threonine residues in a DS/TA/I/L/V consensus sequence (Fletcher et al. 2009). The glycosylation locus contains a wxx flippase gene, suggesting that glycosylation occurs en bloc in the periplasmic compartment; however, no O-OTase has been identified to date. Another curious glycoprotein is the type IV pilin in P. aeruginosa strain 5196. The pilin subunits are modified with arabinofuranose oligomers at several sites in the polypeptide. This glycan is not related to the O antigen of this strain, but it is similar to the arabinogalactan present in the mycobacteria cell wall (Voisin et al. 2007; Kus et al. 2008). An en bloc transfer by the putative O-OTase TfpW was suggested because mutant strains without this predicted multimeric-spanning protein produced nonglycosylated pilin subunits. Further evidence for the assembly of the glycan onto UndPP and its subsequent translocation over the inner membrane will be required to confirm TfpW as a novel O-OTase. Interestingly, en bloc O-glycosylation is not known in eukaryotes and genes encoding O antigen ligases and the related O-OTases seem to be restricted to prokaryotes.

N-Oligosaccharyltransferases. Protein N-glycosylation is best studied in eukaryotes (Heinemann and Aebl 2001). The eukaryotic oligosaccharyltransferase (OST) is in most cases built by a complex of proteins. The catalytic subunit is comparable with O antigen ligases (Stimson et al. 1995; Castric et al. 2001). In a natural occurring link between protein glycosylation and LPS synthesis, the P. aeruginosa pilin glycan consists of a single O antigen subunit (Figure 2C) (Castric et al. 2001). However, other glycans can be attached onto the protein acceptors by these O-OTases when offered as substrates linked to UndPP, including even peptido-glycan subunits in the case of PglL (DiGiandomenico et al. 2002; Faridmoayer et al. 2007; Faridmoayer et al. 2008). Structurally no requirements were found for the substrate recognition of PilO and PglL, except that the P. aeruginosa PilO substrates have a limited maximum length of around 10 sugar residues (Faridmoayer et al. 2007; Faridmoayer et al. 2008).

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The topology of PglB contains multiple transmembrane and three larger periplasmic domains (Li et al. 2010). N-OTases share a highly conserved WWDYG motif located in the large C-terminal periplasmic domain (Wacker et al. 2002). Single mutations in this sequon (W458A, D459A) render PglB inactive (Wacker et al. 2002). Crystal structures of the soluble C-terminal parts of PglB and an archaeal N-OTase indicated a putative catalytic center around the WWDYG and two additional motifs, D/MxxK/I/MxxKMW/W/I/K and xxD (Maita et al. 2010). The PglB-like N-OTases are not homologous to the bacterial O-OTases. Furthermore, they are not known to be involved in LPS biosynthesis, and N-linkages connecting O antigens to lipid A-cores have not been identified to date. Therefore, the N-OTases are the only class of enzymes involved in en bloc protein glycosylation without any known counterparts in LPS biosynthesis.

Like the O-OTases, C. jejuni PglB is able to transfer a variety of glycan structures (Feldman et al. 2005). However, there seem to be a few restrictions. The presence of an acetoxy group at position C-2 of the reducing-end sugar residue was shown to be required for transfer, whereas a β1-4 linkage between the two proximal sugars seems not to be tolerated (Wacker et al. 2006; Chen, Glover, et al. 2007).

Eukaryotic and prokaryotic N-OTases require the consensus acceptor sequence NxS/T, where x can be any amino acid but proline (Bause 1983; Nita-Lazar et al. 2005). However, C. jejuni PglB requires in addition a negatively charged amino acid at position −2 and recognizes therefore the extended acceptor sequence D/ExNxS/T (Kowarik, Young, et al. 2006). Interestingly, whereas the oligosaccharide in eukaryotic and archael protein glycosylation systems is assembled on the DolPP carrier, the bacterial N-OTase PglB uses UndPP-linked glycans as substrates and failed to transfer DolPP-bound sugars in vitro (Chen, Weerapana, et al. 2007).

Bacteria seem to have found an additional alternative mechanism to N-glycosylate proteins. The adhesin HMW1 in H. influenzae is N-glycosylated with hexose residues at multiple locations, mostly consistent with the eukaryotic NxS/T sequon (Gross et al. 2008). Unexpectedly, the cytoplasmic glycosyltransferase HMW1C is responsible for the transfer of the hexose residues using nucleotide-activated sugars as substrates and not only attaching them onto asparagine residues of the polypeptide chain, but also creating hexose-dimers at a few locations (Grass et al. 2010). This novel N-glycosyltransferase has no homology with STT3- or WaaL-like enzymes, but resembles O-GlcNAc transferases instead.

**Evolutionary connections**

The enzymes involved in LPS biosynthesis were identified decades ago. On the contrary, the discovery of bacterial protein glycosylation systems is relatively recent. Presently, several protein glycosylation pathways are well established, making analogous steps to LPS biosynthesis evident. Sequence analysis showed that O antigen synthesis and protein glycosylation pathways actually share homologous components. Evolutionary connections can be found at almost every step of the LPS and glycoprotein biosynthetic pathways.

It appears that to build a pathway for either LPS or glycoprotein assembly, a pool of possible enzymes for each biosynthetic step is encoded in the bacterial metagenome. Evolutionary selection involving both vertical and horizontal gene transfer has generated a multitude of glycoconjugate assembly pathways, optimized for the particular needs of the individual bacterial species. Many enzymes for the synthesis of nucleotide-activated sugars as well as glycosyltransferases of many bacteria have common evolutionary origins. Also the transmembrane enzymes, including initiating glycosyltransferases, translocases and conjugating enzymes, have members of all families represented in the synthesis of both glycoconjugates, LPS and glycoproteins, with the exception that N-OTases are presently only known in protein glycosylation pathways. The use of different transmembrane enzymes for LPS and glycoprotein biosynthesis in some model pathways is schematically illustrated in Figure 2.

Bacteria follow several strategies to separate the various glycoconjugate synthesis pathways. Often, only one glycan is assembled onto UndPP. In C. jejuni and in Neisseria spp., the UndPP-oligosaccharide is exclusively transferred onto acceptor proteins, as these bacteria produce LOS instead of LPS and therefore omit UndPP assembly of O antigens (Preston et al. 1996). The opposite is found in H. pylori where no en bloc glycosylation system seems to be present and UndPP is instead the carrier in the LPS pathway (Hug et al. 2010). However, other bacteria such as P. aeruginosa and A. actinomycetemcomitans use one type of UndPP-glycans as substrates for both types of glycoconjugates (Faridmoayer et al. 2007; Tang and Mintz 2010). Furthermore, P. aeruginosa and also P. gingivalis produce two different UndPP-O antigens, initiating glycan assembly with different monosaccharides (Rocchetta et al. 1999; Rangarajan et al. 2008). The same initiating sugar is used for the assembly of different glycan structures in many Enterobacteria, as WecA is used for the synthesis of ECA and LPS (Raetz and Whitfield 2002). It is possible that in these cases a regulatory switch favors the expression of either one or the other glycoconjugate according to the environmental conditions. Alternatively, both polysaccharides could be expressed simultaneously, and in that scenario, secondary glycosyltransferases would compete for the UndPP-GlcNAc substrate.

The variety and diversity of prokaryotic glycan structures are remarkable, indicating a great evolutionary freedom to develop new glycans. Horizontal gene transfer is a prokaryotic mechanism which further supports the creation of novel glycans and glycoconjugates. The roles of prokaryotic glycans are not universal, and their structures are adapted to benefit the individual species in its environment. In eukaryotes, however, accurate N-glycosylation is essential for protein-folding control (Helenius and Aebi 2001), which reduces the success of newly appearing glycan structures drastically and thereby slows down evolution of eukaryotic N-glycans.

**Applications in glycoengineering**

The analogies and homologies between O antigen biosynthetic pathways and bacterial protein glycosylation invite the exploitation of these pathways for the generation of novel
recombinant glycoconjugates in living bacteria. This “glycoengineering” approach imitates the natural mixing and matching of enzymes for glycoconjugate synthesis. As genes required for the biosynthesis of a given UndPP-glycan are usually located in a distinct cluster, they can often be easily amplified and transferred into other bacteria with better conditions for large-scale glycoconjugate production, for example, into E. coli. Using this strategy, chimeric LPSs consisting, for example, of E. coli lipid A-core and H. influenzae O antigen were generated (Phillips et al. 2000). Similarly, diverse unrelated O antigens were transferred onto protein acceptors when UndPP-O antigen biosynthesis genes were co-expressed with genes encoding OTase and acceptor protein (Feldman et al. 2005; Faridmoayer et al. 2008). These novel glycoengineering techniques can produce harmless bacteria exposing recombinant LPS on their surfaces and as such being possibly applicable as live vaccines, and similarly, recombinant glycoproteins could be produced to be employed as conjugate vaccines.

Immune responses directed toward microbial polysaccharides, in particular O antigens and capsules, are frequently preventing or clearing colonization and infection of bacterial pathogens (Jones 2005). However, especially for the immunization of young infants, free glycans are insufficient vaccines. Instead, bacterial glycans must be covalently attached onto appropriate protein carriers to constitute potential vaccines able to provide long-term protection (Mond et al. 1995; Jones 2005). The efficacy of this approach is best exemplified with the successful conjugate vaccines against H. influenzae type b and S. pneumoniae (Jones 2005).

Presently, the production of these conjugate vaccines requires intricate synthetic chemistry for obtaining and activating the polysaccharides and attaching them onto protein carriers (Bernardes et al. 2009). Using traditional procedures, vaccine costs are unaffordable in developing countries. Furthermore, considerable variations occur from batch to batch (Bernardes et al. 2009). Therefore, novel approaches like the above-described glycoengineering technique are needed to allow the global use of conjugate vaccines for the prevention of bacterial infections. Although first glycoproteins produced by engineered living bacteria are immunogenic (J. Iwashkiw et al., in preparation), it remains to be examined if they are protective against bacterial infections. The first clinical study analyzing such recombinant glycoproteins as vaccines is currently underway (www.glycovaxyn.com).

Considering the tremendous diversity of glycan structures present in bacterial glycoconjugates, it is logical to speculate that any desired glycan structure can be assembled onto the UndPP carrier. Customized glycans could be designed and produced by cells engineered to express the enzymes required for the synthesis of nucleotide-activated sugars along with the right set of glycosyltransferases. Customized sugars may include human sugar epitopes, which may be required to assure the activity or stability of recombinant human proteins, or for the creation of glycan-based immunomodulatory therapeutics. However, many hurdles may have to be overcome before the in vivo glycoengineering approach can be successful. It is not clear if the yields of bacterially produced humanized glycoproteins will be competitive with those obtained from engineered eukaryotic cells such as yeast or mammalian cell cultures. Moreover, the specificities of glycosyltransferases might not always be as strict as expected, especially as the conditions in the host cells are not identical to the natural environment of the enzymes. Furthermore, it has been proposed that the glycosyltransferases often physically interact in vivo to efficiently assemble oligo- and polysaccharides (Kos and Whittfeld 2010). Based on this idea, some glycosyltransferases may display impaired activity or specificity in the absence of their partner enzymes. This could result in heterogeneity of the produced glycan. However, some human epitopes are naturally mimicked by bacteria. For example, C. jejuni LOS contains the human epitope GM1, H. pylori expresses Lewis antigens on its LPS and E. coli O86 the blood group B antigen epitope (Andersson et al. 1989; Yuki et al. 1993; Aspinall et al. 1996). Furthermore, human gangliosides and Lewis antigens were produced by engineered bacteria co-expressing selected glycosyltransferases originating from various species (Fort et al. 2005; Dumon et al. 2006; Ilg et al. 2010). These examples demonstrate the feasibility of recombinant glycan production in living bacteria. However, to date, no recombinant glycans were assembled on UndPP and subsequently transferred onto protein acceptors in the same bacterial cells. It remains to be demonstrated that such a strategy can successfully be applied for the generation of fully customized glycoproteins.

In a short term, the synthesis of glycoproteins containing human glycans may be pursued with a mixed approach, combining glycosylation in living bacteria with in vitro techniques. This is exemplified in a recent glycoengineering work (Schwarz et al. 2010). Glycoproteins containing the C. jejuni heptasaccharide were obtained from recombinant E. coli cells, whereupon the glycan moiety except the reducing-end sugar was removed in vitro. A eukaryotic glycan was subsequently attached at the glycosylation site by transglycosylation. As with an all-in vivo glycoengineering approach, this technique ensures a defined glycan–protein linkage, because the conjugation step is controlled by bacterial OTases with specific acceptor site requirements. The combination of in vivo glycoprotein production with in vitro addition of terminal sugars is another variation of the glycoengineering technique which can produce humanized glycoconjugates (Hug et al., in preparation).

Besides the manipulation of the glycan moiety, it is also possible to customize the protein acceptor when employing the C. jejuni N-OTase. Because the glycosylation sequon is known to be D/E/NX/S/T, any polypeptide sequence can be glycosylated if engineered to contain this consensus sequence (Kowarik, Young, et al. 2006). However, for in vivo glycosylation, the acceptor protein must be directed to the periplasm, but alternatively cytosolicly expressed proteins can be glycosylated in vitro (Kowarik, Numao, et al. 2006).

Concluding remarks

LPS and protein glycosylation in bacteria follow a similar general biosynthetic pathway. Many of the different steps are catalyzed by homologous enzymes. Besides the fascinating
implications of their evolutionary connections, the analogies and homologies between these pathways have an enormous biotechnological potential. Further investigations are needed to allow the exploitation of these systems for industrial applications, but it is clear that the combination of LPS and glyco-protein systems could lead to a new era in glycoengineering.

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**Conflict of interest statement**

None declared.

**Abbreviations**

ABC transporter, ATP-binding cassette transporter; ATP, adenosine triphosphate; DATDH, 2,4-diacetamido-2,4,6-trideoxyglucose; DolPP, dolichyl pyrophosphate; ECA, enterobacterial common antigen; FucNAc, N-acetylfucosamine; GATDH, glyceraldehyde acetamido trideoxyhexose; GPT, dolichyl-P-N-acetylmuramyl-P-glucosaminyl-1-P-transferase; LOS, lipooligosaccharide; LPS, lipopolysaccharide; ManNAcA, N-acetyl-D-mannosaminuronic acid; MurNAc, N-acetylmuramic acid; OST, eukaryotic oligosaccharidyltransferase; OTase, oligosaccharidyltransferase; PHPT, polypreynl-P-hexosamine-1-P-transferase; PNPT, polypreynl-P-N-acetylmuramyl-P-glucosaminyl-1-P-transferase; UndP, undecaprenyl phosphate; UndPP, undecaprenyl pyrophosphate; UndPP-GlcNAc, undecaprenyl pyrophosphate-ferase; UndP, undecaprenyl phosphate; UndPP, undecaprenyl saccharide; LPS, lipopolysaccharide; ManNAcA, N-acetylmuramyl-P-glucosaminyl-1-P-glucosaminyl-1-P-transferase; FucNAc, trideoxyglucose; DolPP, dolichyl pyrophosphate; ECA, enterobacterial common antigen; FucNAc, trideoxyglucose; DolPP, dolichyl pyrophosphate; ECA, enterobacterial common antigen.

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