Glycosylation is one of the most common post-translational modifications of proteins across all kingdoms of life. Diverse monosaccharides and polysaccharides can be attached to a range of amino acid residues generating N-glycosylation, O-glycosylation, C-glycosylation, N-glycosylation, S-glycosylation, as well as P-glycosylation. The functions of the eukaryotic glycosylation system during protein folding in the endoplasmic reticulum (ER) and Golgi are well-studied. Increasing evidence in the recent decade has demonstrated the presence of oligosaccharyltransferases (OSTs) in bacteria and archaea. In particular, the oligosaccharyltransferase (PglB) of *Escherichia coli*.

This strategy can be applied to the development of glycoconjugate vaccines using engineered host. For instance, UDP-GalNAc, a sugar donor for O-linked glycosylation, is cleaved by GTs into UDP-β-phosphate and GalNAc. The generated UDP-β-phosphate accepts the proton from threonine while the GalNAc moiety forms a glycosidic bond with the hydroxyl oxygen of threonine.

Multiple bacterial protein glycosylation mechanisms have been reported in recent years in bacterial species from the most diverse genera and demonstrated that protein glycosylation affects flagellar formation, motility, virulence, and adhesion to host cells. For example, *PglB*, a major OST for N-linked glycosylation, has been found in *Campylobacter jejuni*, *Wolinella succinogenes*, and *Desulfovibrio desulfuricans*. The deletion of protein glycosylation lo-
2 Structure and Mechanism of Bacterial N-Linked Glycosylation Systems

*C. jejuni* is a foodborne pathogen of humans and animals, and is primarily associated with poultry and domestic animals [22]. The bacterium possesses genes that encode an N-linked glycosylation system, located on the single chromosomal *pgl* gene cluster [23].

2.1 N-Linked Glycosylation Mediated by the *pgl* Gene Cluster of Campylobacter *jejuni*

Protein glycosylation has been described in all domains of life. For example, studies have demonstrated that N-linked glycosyltransferases (GTs) are present in prokaryotes and have high similarity to the eukaryotic STT3 family [23,24]. Furthermore, the discovery of N-linked OSTs in archaebacteria and bacteria increases the understanding of the mechanism of action of OSTs and their potential application [25]. Well-characterized prokaryotic OSTs, oligosaccharyltransferase (AglB) in archaea and PglB in eubacteria, catalyze the transfer of oligosaccharides to the amide moiety of asparagine (N).

The mechanism of N-linked glycosylation is conserved in bacterial species and has been well-characterized in *C. jejuni*, which possesses a 17-kb *pgl* gene cluster that encodes proteins for synthesizing and transferring oligosaccharides (Fig. 1A). The process of N-linked glycosylation in *C. jejuni* could be divided into two steps, namely oligosaccharide synthesis, and transfer (Fig. 1B). Oligosaccharide synthesis begins with modification of UDP-GlcNAc to UDP-2,4-diacetamidoglucosamine (UDP-diNAcBac) by glycosyltransferases: PglF, PglE, and PglD, followed by partial transfer of UDP-diNAcBac by PglC onto undecenol diphosphate. The oligosaccharyltransferase (PglA, PglJ, and PglH enzymes) enzymes subsequently catalyze the formation of a hexasaccharide by the fusion of five units of GalNAc. LLO synthesis is completed by PglI, which adds a branched glucose residue to the hexasaccharide [21,26–28]. The LLO is then flipped across the inner membrane into the periplasm by PglK [29,30], and transferred to the substrate asparagine of the -D/E/NXS/T-consensus by PglB, which is the final key step in N-linked glycosylation [31].

2.2 Mechanism of Substrate Peptide and LLO Binding by Campylobacter *jejuni* PglB

The mechanism of bacterial N-linked glycosylation by OSTs has been investigated in detail by determining the structures of the AglB enzyme of *Archaeoglobus fulgidus* and the PglB enzymes of *N. meningitidis*, *C. jejuni*, and *C. lari*. The full-length structures of AglB and PglB comprise an N-terminal transmembrane domain and a C-terminal periplasmic domain (Fig. 2A). The C-terminal domain of the AglB protein of *Ar. fulgidus* and the PglB enzyme of *C. jejuni* share a high structural similarity, with both proteins containing a central core (CC) domain and an inserted β-structure. In particular, the catalytic sites of the WWDYG motif are conserved in the AglB and PglB enzymes of *Ar. fulgidus* and *C. jejuni*, respectively [32,33]. However, the isolated C-terminal domains of AglB and PglB lack catalytic activity and are unable to bind the acceptor peptide, suggesting that the N-terminal transmembrane domain is essential for catalytic activity. The full-length structure of the PglB protein of *C. lari* complexed with a hexapeptide has been solved at 3.4 Å resolution. The N-terminal transmembrane domain of the PglB protein of *C. lari* comprises 13 transmembrane helices, an external EL1 helix, and a long external EL5 loop (Fig. 2B). The structure of the complex also highlighted the specificity of the DQNATF substrate motif and demonstrated that the WWD motif of the periplasmic domain of PglB forms three hydrogen bonds with the β-hydroxyl group of the +2 Thr residue of the hexapeptide (Fig. 2C) [34].

Interestingly, the long external EL5 loop is partially ordered, and 25 residues of the EL5 loop are disordered in the electron density map [34]. However, a study demonstrated that EL5 contains a conserved tyrosine residue (Tyr293), and the Y293A mutation leads to an almost complete loss of catalytic activity, confirming that the aromatic side chain of Y293 is essential for catalysis [35]. Moreover, LLO binding induces dynamic changes in the EL5 loop, re-
Fig. 1. Mechanism of N-linked glycosylation mediated by the PglB Oligosaccharyltransferase (OST) of C. jejuni. (A) The 17 kb protein glycosylation locus pgl of C. jejuni. N-linked glycosylation in C. jejuni is cooperatively accomplished by the pgl gene cluster. PglB is the core factor for the glycosylation process. (B) Schematic diagram depicting N-linked glycosylation in C. jejuni. The first step of glycosylation involves oligosaccharide synthesis in the cytoplasm, which is mediated by glycosyltransferases (GTs), including PglF/E/D, PglC, PglA/J/H, PglI, and PglK. The transfer of oligosaccharides to the periplasm is carried out by PglK. Subsequently, PglB transfers the hexasaccharide to the asparagine residue of the substrate with the sequence D/E-X-N-X-S/T.
Fig. 2. The structure of the PglB enzyme of *C. jejuni* and the ternary complex of PglB bound to a substrate DQNATF peptide and an lipid-linked oligosaccharide (LLO) analog. (A) Schematic diagram of the PglB enzymes of *C. lari* and *C. jejuni* and the AglB protein of *Ar. fulgidus*. All the enzymes possess a transmembrane domain and a periplasmic domain, while the AglB protein of *Ar. fulgidus* comprises two more peripheral domains, P1 and P2. (B) Crystal structure of the PglB protein of *C. jejuni* (PDB ID: 3RCE). The PglB enzyme of *C. jejuni* comprises 13 transmembrane helices, EL1, and EL5. EL1 is a periplasmic helix and EL5 is a periplasmic loop located between the transmembrane helices. The periplasmic EL1 and EL5 domains are considered to be important for peptide recognition and LLO binding. (C) Crystal structure of the ternary complex of *C. jejuni* PglB with a substrate DQNATF peptide and an LLO analog (PDB ID: 5OGL). The substrate peptide and the LLO analog in the ternary complex are situated opposite to each other in the platform formed by the transmembrane and periplasmic domains, and the distance between the asparagine residue and LLO analog is 6 Å. The WWD motif in the periplasmic domain is important for peptide binding, and forms three hydrogen bonds with the β-hydroxyl group of the +2 Thr residue of the bound hexapeptide. The structure of the EL5 monomer comprises N-EL5 and C-EL5, which are essential for peptide and LLO binding, of which the E319 residue of C-EL5 and Y293 of N-EL5 are particularly important. Color scheme: transmembrane domain (green), periplasmic domain (gray), substrate peptide (cyan), LLO analog (blue), N-EL5 (purple), and C-EL5 (red).
Thr-Asp^{156} motifs in PglB, are considered to be pivotal for coordinating the metal ions based on the proximity of their side chains [34,39]. The PglB protein of C. lari possesses a conserved short loop with a DGGK motif in the periplasmic domain of all C. sp., which mediates the binding of the substrate peptide and LLO analog [37,40].

3. Mechanism of Bacterial O-Linked Glycosylation and Receptor Sequence Recognition

O-linked glycosyltransferases (GTs) transfer a GlcNAc moiety from UDP-GlcNAc to the hydroxyl oxygen of a serine or threonine residue [41,42]. O-linked glycosylation has been reported to be important for fundamental cellular processes, including transcription, signal transduction, metabolism, subcellular localization, and immune response [43]. It has been observed that the pathway for the O-glycosylation of proteins in B. cepacia affects the growth of the bacterium. Non-glycosylated proteins have been shown to not fully utilize multiple carbon sources, and are easy to be broken down [44]. Furthermore, both O-antigen ligases (WaaL ligases) and O-OSTs harbor an evolutionarily conserved Wzy_C domain, but differ concerning the lipid A-core and amino acid residues for glycan recognition (Fig. 3A) [45,46]. The mechanism of action and function of the O-linked GTs, PilO from P. aeruginosa, and PglL of N. meningitidis are well studied. Both enzymes share very little sequence identity in their glycan recognition region and acceptor proteins.

3.1 PilO, the O-Linked Glycosyltransferases (GTs) of Pseudomonas aeruginosa

The PilO enzyme of P. aeruginosa comprises a transmembrane domain that anchors to the periplasmic membrane, and a periplasmic core domain that catalyzes the transfer of glycans to pilin via its Wzy_C domain, but differ concerning the lipid A-core and amino acid residues for glycan recognition (Fig. 3A) [47]. Analysis of the transmembrane helix of PilO revealed that the transmembrane region includes residues 1-266, and the residues include nine transmembrane helices. Residues 267-461 of PilO have some amino acids that are less hydrophobic and form four transmembrane domains, in which there are two periplasmic loops and two cytoplasmic loops. A subsequent BLAST search of the Wzy_C structural domain of PilO (residues 275-325) revealed that residues 281-301 are conserved and have no homologous sequences. In order to investigate whether this region affects glycosylation, mutations were introduced at different positions. Residues 281-287 (RDVLW) and 287 (RDVLW) were deleted in the first mutant, while residue R281 was mutated to alanine in the second mutant (pR281A). The results demonstrated that mutations in these regions prevented substrate glycosylation, and that these residues are necessary for glycosyltransferases (GT) activity. In addition, other catalytic residues that play a role in the glycosylation of pilin were deleted, and 1-322, 1-430, and 1-453 truncates were constructed. The results demonstrated that none of the truncates exhibited GT activity. Altogether, these findings suggested that residues 267-461 are important for the GT activity of the enzyme [47]. PilO exhibits non-specificity to substrates, only recognizing the C-terminal serine residue of the target peptides, which makes it a potential candidate for glycoprotein vaccine development [48]. Interestingly, the PilO enzyme transfers short glycan chains to an acceptor serine residue, while the PglL enzyme transfers polysaccharides [49].

3.2 O-Linked OST of Neisseria meningitidis PglL and PglL Receptor Recognition Sequences

N. meningitidis is a gram-negative bacterium that causes meningitis and is a common commensal in the human respiratory tract. The genes and phenotype of N. meningitidis have been reported to undergo in vivo alteration within a very short time to evade the host immune system and enhance virulence [50]. The O-linked protein glycosylation system affects polysaccharide diversity owing to selective gene expression. It has been reported that ST-11 strains of N. meningitidis express 7 to 21 different glycans, resulting from the diversity and variation of pgl genes [51]. The PglL enzyme of N. meningitidis contains multiple consecutive transmembrane domains and two periplasmic domains (Fig. 3A). The PglL enzyme has been demonstrated to be responsible for attaching UndPP-glycan to pilin (PilE), and Ser63 is one of the glycosylation sites on PilE [52,53] (Fig. 3B). The glycosyltransferase (GT) activity of PglB transfers 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) to undecaprenol diphosphate [49,54]. The PglA and PglE GTs subsequently add two monosaccharide units to the side chain of the sugar moiety. The trisaccharide is finally flipped by the PglF GT and transferred to the serine residue of its substrate by PglL (Fig. 3B). The final recognition motif of PglL on the exotoxin A (EPA) protein of Pseudomonas aeruginosa was initially identified as WPGNNTSAGV^{66}, which was optimized to an 8-residue-long sequence (WPAAASAP) referred to as the minimum optimal O-linked recognition (MOOR) motif [55]. AniA, an outer membrane nitrite reductase, is glycosylated at serine^{373} and serine^{378} residues of the L^{358}SDTAYAGNAAAPAASAAPAASASEK^{387} peptide by PglL. The sequence contains a repetitive AASAP motif with the glycosylated serine residue at the middle of the residue stretch. However, subsequent studies with the AASAS^{385} motif, which contains two serine residues that were not glycosylated by PglL [56]. These results suggested that the AASAP peptide might be the optimum peptide sequence for PglL.

However, full-length structures of the PglL/PilO enzymes or PglL/PilO enzymes bound to the substrate peptide complex have not been solved to date. These structures are necessary for further studies on elucidating the mechanism of peptide binding and glycan transfer by PglL/PilO.
3.3 PglL, the O-Linked OST of Vibrio Cholerae

*V. cholerae* is a gram-negative pathogen causing cholera, a water-borne diarrheal disease in animals and humans [57]. Bioinformatics analysis identified the VC0393 gene, which encodes O-OTases in the O1 E1 strain of *V. cholerae*, and is referred to as PglLVC. When the foreign substrate protein DsbA1 was introduced, the glycosylation site by PglLVC was mapped as VQTSVPDASAASAPAGLVEQNYTVLANPIPQQQAGK [58]. PglLVC glycosylates multiple proteins of *V. cholerae*, including the outer membrane proteins OmpA and OmpU, the periplasmic chaperones SurA-like and FkpA, and the DegP chaperone/protease. Using DegP as a representative candidate for identifying glycosylation sites, a study demonstrated that the peptides 47KVTPAVSVIAGE58 and 371SLHQGLSGAE380 are modified by glycosylation with a diNACBac moiety at a single site [59]. However, the specific structural model is unknown thus far.

3.4 GmaR, the O-Linked Glycosyltransferase (GT) of Gram-Positive Listeria monocytogenes

O-linked glycosylation systems have also been detected in gram-positive bacteria, including *L. monocytogenes*, *Clostridium difficile*, *Staphylococcus aureus*, and *Streptococcus agalactiae* [13,60]. The flagellin protein of *L. monocytogenes* is modified by the addition of O-linked N-acetylglucosamine (GlcNAc) moieties at serine and threonine residues, which is regulated by the GmaR GT [61,62]. GmaR is a bifunctional protein in which one hand is a temperature sensor that controls the temperature-dependent transcription of flagellar motility genes by interacting with MogR via conformational changes [63,64]; the other hand is a GT-2 family GT that glycosylates the flagellin protein FlaA. Unlike the PilO or PglL enzymes that transfer oligosaccharides to the substrate peptides, GmaR only transfers a mono-GlcNAc to serine and threonine residues of FlgA. The secondary structure of GmaR comprises an N-terminal domain for GT activity, a C-terminal domain that binds MogR for temperature sensing, and a linker region. Small-angle X-ray scattering (SAXS) analysis revealed that the apo GmaR protein adopts a multidomain shape, and the binding of Mg ions induces conformational changes in GmaR so that the protein adopts an elongated and relatively open conformation. The linker region is essential for binding Mg ions and donor substrate induces conformational changes in GmaR so that the protein adopts an elongated and relatively open conformation. The linker region is essential for binding Mg ions and donor substrate induces conformational changes in GmaR so that the protein adopts an elongated and relatively open conformation.

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**Fig. 3. Structure of enzymes and mechanism of bacterial O-linked glycosylation.** (A) Structure of the WaaL protein of *E. coli*, PilO enzyme of *P. aeruginosa*, and the PglL protein of *N. meningitidis*. The Wzy_C domain is shared among the three species of bacteria. (B) Schematic diagram depicting the mechanism of O-linked glycosylation in *N. meningitidis*. The PglB, PglA, and PglE enzymes transfer UDP-DATDH and monosaccharides to undecaprenol diphosphate, and PglF flips the oligosaccharides from the cytoplasm to the periplasm. The LLO is finally transferred by PglL to a serine residue of the substrate.
Table 1. Biological polysaccharide conjugate vaccine.

<table>
<thead>
<tr>
<th>Pathogen/Antigens</th>
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<tr>
<td><em>Shigella flexneri</em> 2a /polysaccharide component of the <em>Shigella flexneri</em> 2a O-antigen</td>
<td>PgIBcJ</td>
<td>N-linked</td>
<td>exotoxin protein A of <em>Pseudomonas aeruginosa</em> (EPA)</td>
<td>Access to vaccine safety and tolerability data.</td>
<td>Phase 1 (Complete)</td>
</tr>
<tr>
<td><em>Shigella Flexyn</em> 2a /polysaccharide component of the <em>Shigella flexneri</em> 2a O-antigen</td>
<td>PgIBcJ</td>
<td>N-linked</td>
<td>exotoxin protein A of <em>Pseudomonas aeruginosa</em> (EPA)</td>
<td>Testing the ability of vaccines to induce an immune response.</td>
<td>Phase 2 (Complete)</td>
</tr>
<tr>
<td><em>Shigella</em> /polysaccharide component of the <em>Shigella flexneri</em> 2a O-antigen</td>
<td>PgIBcJ</td>
<td>N-linked</td>
<td>exotoxin protein A of <em>Pseudomonas aeruginosa</em> (EPA)</td>
<td>To demonstrate the safety and reactogenicity of the vaccine alone or in combination with an adjuvant (aluminum hydroxide).</td>
<td>Phase 1 (Complete)</td>
</tr>
<tr>
<td><em>Shigella</em> /polysaccharide component of the <em>Shigella</em> O1 lipopolysaccharide</td>
<td>PgIBcJ</td>
<td>N-linked</td>
<td>exotoxin protein A of <em>Pseudomonas aeruginosa</em> (EPA)</td>
<td>First-in-human data on safety and immunogenicity in infants was obtained.</td>
<td>Phase 2 (Complete)</td>
</tr>
<tr>
<td><em>E. coli</em> Tetravalent /Oligosaccharide</td>
<td>PgIBcJ</td>
<td>N-linked</td>
<td>exotoxin protein A of <em>Pseudomonas aeruginosa</em> (EPA)</td>
<td>This study aims to assess the safety and efficacy of a Tetravalent <em>E. coli</em> vaccine in inducing an immune response and reducing the incidence of urinary tract infections.</td>
<td>Phase 1 (Complete)</td>
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<td><em>Klebsiella Pneumoniae</em> Tetravalent /O-polysaccharide (OPS)</td>
<td>PgILNm</td>
<td>O-linked</td>
<td>cholera toxin B subunit (rCTB)</td>
<td>Obtain first-in-human data on its safety and immunogenicity in healthy adults.</td>
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<tr>
<td><em>New Pneumococcal</em> /Capsular polysaccharides (CPS)</td>
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4. Vaccine Development Using Bacterial Oligosaccharyltransferases (OSTs)

Bacterial lipopolysaccharide (LPS) comprises a conserved lipid A moiety, a core oligosaccharide chain, and a variable polysaccharide chain [65]. Variable polysaccharide chains are crucial for antigenicity, and polysaccharides are ideal vaccine targets to enhance the immune protection of vaccines [66]. Therefore, the development of novel vaccines using glycans conjugated to protein is an efficient strategy for enhancing the immunoprotective effect of the vaccine. Glycoconjugate vaccines have attracted considerable attention for their novel vaccine design during the past three decades since glycoproteins can enhance immunogenicity and induce T-cell-dependent immune responses against numerous pathogens, including Salmonella Typhi, Streptococcus pneumoniae, and Neisseria meningitidis [67,68]. Current chemical conjugation methods allow the continuous production of recombinant vaccines, especially against pathogenic targets, but the production of glycoconjugate vaccines is complex and expensive. For example, a chemical glycoconjugate vaccine against *F. tularensis* requires purification of the receptor CRM197 protein from *C. diphtheriae*. LPS was then extracted from pathogenic *F. tularensis* for endotoxin lysis, further purification, and chemical activation [69].

To overcome the challenges of glycoconjugate vaccine production, a one-step purification of glycoconjugate vaccines produced by a bioengineered bacterial host, especially using the PglB or PglL oligosaccharyltransferases (OSTs), has been employed in bacterial vaccine design. *E. coli* lacks the pathways for synthesizing N-linked polysaccharide proteins; consequently, glycoengineering with *E. coli* generally involves the introduction of three components: a glycan plasmid for the production of the lipid-linked polysaccharide, a plasmid for the carrier protein, and a plasmid encoding an OST (Fig. 4) [70].

4.1 N-Linked Glycoprotein Conjugate Vaccines

PglB<sub> CJ </sub> can transfer the natural *C. jejuni* polysaccharide, the antigenic capsular polysaccharide of Gram-positive bacteria, and the antigenic lipopolysaccharide of Gram-negative bacteria to the specific recognition sequence of the substrate [71]. There are currently many recombinant vaccines produced by the *C. jejuni* N-glycosylation system; for example, Williams AJ et al. [72] used PglB<sub> CJ </sub> to produce a sugar-conjugated vaccine against Enterotoxigenic *E. coli* in non-pathogenic *E. coli*. These successfully induced an O-antigen polysaccharide-specific response in mice, producing bactericidally active IgG antibodies [72]. In addition, Huttner et al. [73] describe the early development of a conjugate vaccine against extraenteric pathogenic *E. coli* (ExPEC), from creation in the laboratory to testing in a large first-in-human phase Ib trial (Table 1). Recombinant vaccine candidates against *B. pseudomallei* were developed by utilizing the O-polysaccharide (OPS II) of *B. pseudomallei*, along with the PglB enzyme of *C. jejuni* and the appropriate protein carrier (AcrA) of the *C. jejuni*. This recombinant vaccine produced an IgG immune response during intranasal challenge and protected against *B. pseudomallei* [74]. A glycoconjugate vaccine candidate was developed against *Actinobacillus pleuropneumoniae* by combining a fragment of the Apx toxin with a conserved glycan, which can potentially confer enhanced protection against all serotypes of *Act. pleuropneumoniae* [75]. Shigella sp are gram-negative pathogen that causes severe diseases in humans. A new recombinant glycoconjugate vaccine, (EPA-2a), produced by combining *S. flexneri* 2a with the EPA carrier protein of *P. aeruginosa* using the PglB OST of *C. jejuni* enhanced specific protection against *S. flexneri* 2a [76]. Subsequent trials showed that the vaccine elicited antibodies in a rat model and were safe and immunogenic. [77]. Moreover, Riddle et al. [78] verified the safety and bactericidal activity of the vaccine through phase I clinical trials, and induced functional antibodies, which provided a reference for further production of this technology (Table 1).

Although PglB<sub> CJ </sub> has been successfully used in some vaccine production, there are limitations in the production reaction. The sugar chain that can be recognized by *C. jejuni* PglB has an acetyl group at the C<sub>2</sub>-N position of the reducing end and forms a stable hydrogen bond with the carrier protein. If the C<sub>2</sub>-N acetyl group at the reducing end is missing or the β-(1→4) glycan is missing in the two monosaccharides before the reducing end, the glycosylation efficiency will be reduced [79,80]. For example, *Salmonella enterica* sv. *Typhimurium* LT2 and *E. coli* capsular polysaccharide K30 lack the C<sub>2</sub>-N acetyl group at the reducing end, so the signal of glycosylation cannot be detected on the substrate. This limits the selection of antigenic polysaccharides and hinders the large-scale production of recombinant vaccines. While Ihssen J et al. [81] discovered that mutations in the key amino acids (N311V, Y77H, and S80R) in wild-type *C. jejuni* PglB enhanced glycosylation efficiency, the underlying mechanism remains unclear. Furthermore, these mutations may also result in non-specific glycosylation of PglB [81].

4.2 O-Linked Glycoprotein Conjugate Vaccines

Compared to N-linked glycosylation, there is a greater flexibility of glycan structures and protein acceptors in O-linked glycosylation. Faridmoayer et al. [49] demonstrated that in engineered *E. coli* and *Salmonella* cells, PgL can transfer any sugar carried by undecaprenyl pyrophosphate (UndPP) to the pliin substrate. Considering whether the lipid carrier was a limiting factor for PgL, the authors established the authors established a glycosylation system in vitro using purified PgL, pliin, and chemically synthesized lipid farnesyl pyrophosphate (FarPP) with penta saccharides. The results indicated that synthetic lipid carriers could also transfer polysaccharides to the periplasm and localize polysaccharides around PgL. PgL is also broadly
OTase-dependent glycosylation is used as an example. Oligosaccharyltransferase, carrier protein, and polysaccharide are expressed in the host and assembled in the periplasm. Purple: plasmid encoding OST; Blue: plasmid with carrier protein sequence; Red: a glycan plasmid for the production of the lipid-linked polysaccharide.

Salmonella enteritidis (SE) is a zoonotic pathogen. Li M et al. [82] constructed the PglL plasmid, recombinant Pseudomonas aeruginosa exotoxin A (rEPA), and cholera toxin B subunit (CTB) plasmids into S. enteritidis strains. It was detected that when PglL was expressed, rEPA and CTB could undergo glycosylation. The results provide the basis for the SE glycoprotein biosynthesis [82]. A conjugate vaccine against S. enterica serovar Paratyphi A produced by the co-expression of PglL and the CTB4573 carrier protein in vivo evokes a protective and specific immune response against the bacterium [83]. A polysaccharide conjugate vaccine against Klebsiella pneumoniae serotype O2 strain was developed by transfecting a K. pneumoniae waaL mutant with a vector encoding PglL and a recombinant cholera toxin B subunit, and the vaccine conferred effective protection in a murine model (Table 1) [84,85]. Brucellosis is a common human and animal infectious disease worldwide, endangering human health. There is currently no effective vaccine against this disease. Huang et al. [86] designed a vaccine by replacing the Brucella O-antigen polysaccharide (OPS) with the low-pathogenic Yersinia enterocolitica serotype O:9 (YeO9) OPS via an O-linked glycosylation system. As a result, specific immunity was successfully induced, effectively defending against brucellosis and avoiding large-scale cultivation of pathogenic strains [86].

In order to further mass-produce biological conjugate vaccines against B. abortus, Li S et al. [87] established a new vaccine engineering production process in 2023. The OPS gene cluster was divided into five separate fragments and then reassembled, and subsequently expressed in E. coli. The final product is glycosylated by PglL. A series of experiments proved that the vaccine can induce a strong immune response, which induces the production of specific antibodies [87]. Other glycosyltransferases in the O-linked glycosylation system have also been used in vaccine development in recent years. For example TIfPM from Moraxella osloensis. Knoot CJ et al. [88] found that TIfPM-derived bioconjugates can trigger specific antibody IgG production in mice and are immunogenic. Because a variety of long-chain polysaccharides can be transferred to the substrate protein carrier, the shortest recognition glycosylation sequence is identified. This provides new options for the production of new sugar-conjugated vaccines in the future [88].

Another glycosyltransferase, PglS, can transfer many different types of bacterial glycans, including those with glucose at the reducing end. For example, Feldman et al. [89] successfully developed a bivalent K1/K2 Klebsiella pneumoniae biological conjugate vaccine with immunogenicity and effectiveness through PglS. Vaccines protect mice against infection by hypervirulent K. pneumoniae (hvKp). Although in vivo work suggests vaccine efficacy against hvKp in the lung, further studies are needed to assess efficacy against hvKp in other sites including the liver, blood, and meninges (Table 1) [89]. Harding CM et al.
[90] utilized PglS to successfully produce sugar-conjugated vaccines with glucose polysaccharides at the reducing end, thereby overcoming the limitations imposed by the structural requirements of polysaccharides. Furthermore, PglS demonstrated the ability to glycosylate various vaccine carrier proteins. Inoculating mice with the polyvalent pneumococcal bioconjugate vaccine resulted in the production of antisera that effectively killed bacteria in vitro [90]. Group B Streptococcus (GBS) is a leading cause of neonatal infections and invasive diseases in nonpregnant adults worldwide. Duke et al. [91] expressed GBS types Ia, Ib, and III in recombinant E. coli and studied the generation of bioconjugate vaccines using PglS conjugation with the carrier protein Pseudomonas aeruginosa exotoxin A (EPA). This technology provides a basis for the subsequent development of multivalent GBS bioconjugate vaccines [91]. These results support our expansion to future sugar-conjugate vaccines.

5. Conclusions

Prokaryotic glycosylation systems are simpler than eukaryotic systems as protein glycosylation is achieved by a single OST. The transfer of an N-linked or O-linked OST, a substrate protein, and a plasmid for glycans synthesis to E. coli is therefore sufficient for the production of glycoconjugate proteins. To date, the structure and mechanism of N-linked glycosylation by the PglB OST of C. jejuni have been studied in depth and applied to the design of glycoconjugate vaccines against F. tularensis, B. pseudomallei, A. pleuropneumoniae, and S. flexneri. O-linked glycosylation allows a broader variability of substrate amino acid residues, including serine, threonine, and tyrosine. However, the structure and mechanism of LLO binding by the PiLO protein of P. aeruginosa and the PglL enzyme of N. meningitidis remain unelucidated, which necessitates further research on bacterial O-linked glycosylation mechanisms and their application. Although sugar-conjugated vaccines have been expressed and produced by engineering bacteria, it is still difficult to address the problems of exogenous plasmids, re-expressed and produced by engineering bacteria, it is still replication. Although sugar-conjugated vaccines have been expressed and produced by engineering bacteria, it is still difficult to address the problems of exogenous plasmids, re-expressed and produced by engineering bacteria, it is still replication. Although sugar-conjugated vaccines have been expressed and produced by engineering bacteria, it is still difficult to address the problems of exogenous plasmids, re-expressed and produced by engineering bacteria, it is still replication. Although sugar-conjugated vaccines have been expressed and produced by engineering bacteria, it is still difficult to address the problems of exogenous plasmids, re-expressed and produced by engineering bacteria, it is still replication. Although sugar-conjugated vaccines have been expressed and produced by engineering bacteria, it is still difficult to address the problems of exogenous plasmids, re-expressed and produced by engineering bacteria, it is still replication.


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