Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(11): 305
https://doi.org/10.31083/j.fbl2811305

Review

Advances in Bacterial Oligosaccharyltransferase Structure Elucidation and Potential Application to Glycoconjugate Vaccine Design

Riye Lu[1], Pengwei Li[2,3], Li Zhu[4,*], Ming Xian Chang[2,3,*], Songying Ouyang[1,*]

[1]Key Laboratory of Microbial Pathogenesis and Interventions of Fujian Province University, the Key Laboratory of Innate Immune Biology of Fujian Province, Biomedical Research Center of South China, Key Laboratory of OptoElectronic Science and Technology for Medicine of the Ministry of Education, College of Life Sciences, Fujian Normal University, Fuzhou, 350117, China
[2]State Key Laboratory of Freshwater Ecology and Biotechnology, Key Laboratory of Aquaculture Disease Control, Institute of Hydrobiology, Chinese Academy of Sciences, 430072 Wuhan, Hubei, China
[3]College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, 100049 Beijing, China
[4]State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, 100071 Beijing, China
*Correspondence: jewly54@126.com (Li Zhu); mingxianchang@ihb.ac.cn (Ming Xian Chang); ouyangsy@fjnu.edu.cn (Songying Ouyang)
Academic Editor: Sukmook Lee
Submitted: 22 December 2022 Revised: 2 August 2023 Accepted: 23 August 2023 Published: 28 November 2023

Abstract

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, 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 *Campylobacter jejuni* and oligosaccharyltransferase (PglL) enzyme of *Neisseria meningitidis* are the most characterized OSTs that catalyze bacterial N-linked glycosylation and O-linked glycosylation, respectively. Glycoprotein administered as glycoconjugate vaccines have been shown to be effective prophylactic to protect against numerous pathogenic bacteria. The chemical synthesis of glycoproteins is complex and expensive, which limits its application to the development of glycoconjugate vaccines. However, studies have demonstrated that the biosynthesis of glycoproteins is realizable by transferring PglB, a plasmid encoding a substrate protein, or PglL, a plasmid encoding genes for glycan synthesis to *Escherichia coli.* This strategy can be applied to the development of glycoconjugate vaccines using engineered host *E. coli*. This review summarizes the structure and mechanism of action of the bacterial OSTs, PglB and PglL, and discusses their potential application to glycoconjugate vaccine design.

Keywords: oligosaccharyltransferases; PglB; PglL; glycoconjugate vaccine

1. Introduction

Oligosaccharyltransferases (OSTs) catalyze the transfer of oligosaccharides to substrate proteins via a glycosidic bond by a process termed protein glycosylation, which is necessary for protein trafficking, folding, stability, cellular signaling, and self/non-self-interaction [1]. N-linked and O-linked glycosylations are the most well-characterized types of protein glycosylation in eukaryotes [2]. N-linked glycosylation is catalyzed by OSTs that attach a lipid-linked oligosaccharide (LLO) to the acceptor asparagine of the Asn-X-Thr/Ser motif, where X represents any amino acid except proline [3,4]. Mammalian OSTs that catalyze N-linked glycosylation reactions are multi-subunit complexes comprising DAD1 (Ost2p), N33/Tusc3 or MagT1 (Ost3p and Ost6p), OST48 (Wbp1p), ribophorin I (Ost1p), ribophorin II (Swp1p), and STT3A or STT3B (Stt3p) [5]. STT3A-OSTs catalyze the co-translational glycosylation of proteins as they emerge from the translocon into the lumen of the endoplasmic reticulum (ER), while STT3B-OSTs catalyze post-translational glycosylation after polypeptide synthesis is complete [6]. O-linked glycosylation reactions use more saccharide donors (GalNAc, Fuc, GlcNAc, Man, Glc, Xyl, and Gal) and substrate residue receptors (serine, threonine, tyrosine, hydroxylysine, and hydroxyproline) than other glycosylation reactions. O-linked glycosylation is initiated by distinct glycosyltransferases (GTs) [2,7]. GTs comprise an N-terminal catalytic domain for binding and glycosylating substrates, and a C-terminal lectin domain for binding GalNAc [8]. 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 [9].

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 [10,11]. PglB, a major OST for N-linked glycosylation, has been found in *Campylobacter jejuni*, *Wolinella succinogenes*, and *Desulfovibrio desulfuricans* [12,13]. The deletion of protein glycosylation lo-

cus (*pgl*) gene clusters has been shown to weaken bacterial virulence, adherence, and invasion in human intestinal cells, in addition to impairing temperature sensitivity and antibiotic resistance [14,15]. PglL, a ubiquitous O-OST for O-linked glycosylation [16], is found in several bacterial species, including *Ralstonia solanacearum*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae*. PglL is capable of catalyzing the O-linked glycosylation of pilin proteins, and the lack of $PglL_{Rs}$ (from *R. solanacearum*) affects biofilm formation and virulence during bacterial infections [17]. The disruption of O-linked glycosylation in *Burkholderia* results in defective biofilm formation and reduces the activity of siderophores [18,19].

Therefore, the glycosylation systems of pathogenic bacteria are key factors for regulating bacterial invasion and adherence by modulating the interactions between glycans and host cells; however, these processes also depend on the level of host immunity to pathogenic bacteria. In the last few decades, novel glycoprotein vaccines have been designed by transferring a foreign bacterial glycosylation system to bacteria lacking glycosylation machinery, mostly *E. coli*. This approach enhances antibody production and the protective effect of vaccines against numerous antigenic species [20,21]. This review discusses and highlights the structures and mechanisms of bacterial N-linked and O-linked glycosylation systems, with particular emphasis on the PglB and PglL enzymes. More importantly, this paper also discusses that the unique characteristics of OST are expected to become an important tool for the development of new sugar vaccines for glycoengineering in the future.

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 - $D/E_XN_XS/T$ -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. gonorrhoeae, 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-

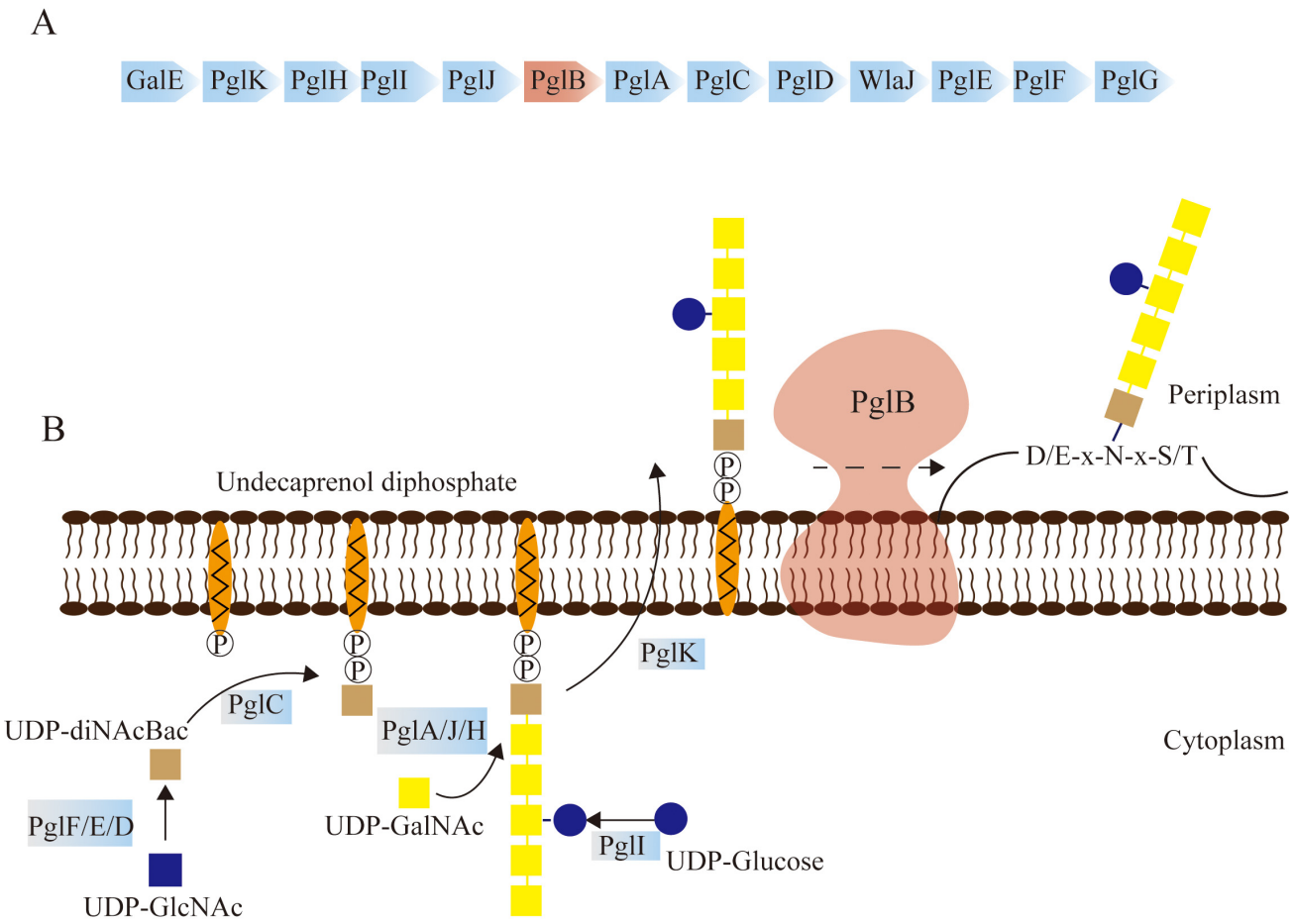

Fig. 1. Mechanism of N-linked glycosylation mediated by the PglB Oligosaccharyltransferase (OST) of *C. jejuni*. (A) The 17 kb protein glycosylation locus pgl of *C. jejuni*. N-linked glycosylation in *C. jejuni* is cooperatively accomplished by the pgl gene cluster. PglB is the core factor for the glycosylation 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/ExNxS/T.

sulting in movements in the transmembrane domain, which progressively induces the formation of an open conformation of EL5 [36]. In order to obtain further insights into the mechanism of LLO transfer and the function of the EL5 loop, Napiórkowska *et al*. [37] solved the ternary complex of PglB bound to an acceptor DQNATF peptide and a synthetic nonhydrolyzable (ωZZZ)-PPC-GlcNAc LLO analog. The ternary structure revealed that the acceptor peptide and the LLO analog were bound to two cavities at opposite sides of the PglB enzyme, and the shortest distance between the peptide and the LLO was approximately 6 Å. The structure revealed that the acceptor peptide interacted with the WWD motif of the periplasmic domain. In addition, E319 of C-EL5 and D56 are located close to the carboxamide group of the acceptor asparagine, and mutations in D56 and E319 are reported to impair the catalytical activity of PglB [34,37]. Analyses of the interactions of the LLO analog revealed that Y293 of N-EL5 formed a hydrogen bond with the oxygen atom of the second pyrophosphate, while R375 formed a hydrogen bond with the oxygen atom of the first pyrophosphate, which explained the complete abolishment of catalytic activity of PglB in the Y293A mutant [37]. Napiórkowska *et al*. [38] solved a new ternary complex of PglB in which the distance between the substrates and LLO analog is significantly closer (approximately 3.4 Å). The ternary complex comprises PglB, Dab-containing peptides, and a synthetic ωZZZ-PP-GlcNAc LLO analog. Structural analysis demonstrated that the reduction in the distance between the substrate and LLO analog was attributed to the fact that the pyrophosphate and GlcNAc moieties shifted closer to the catalytic center [38].

Metal ions are essential for enzymatic activity, and it has been reported that Zn^{2+} and Mg^{2+} metal ions are bound to the catalytic center of AglB and PglB, respectively. DXD (Asp-X-Asp) motifs such as Gly-X-Asp^{47} and Asp-His-His^{163} in AglB, and the Thr-Asn-Asp^{56} and Asp-

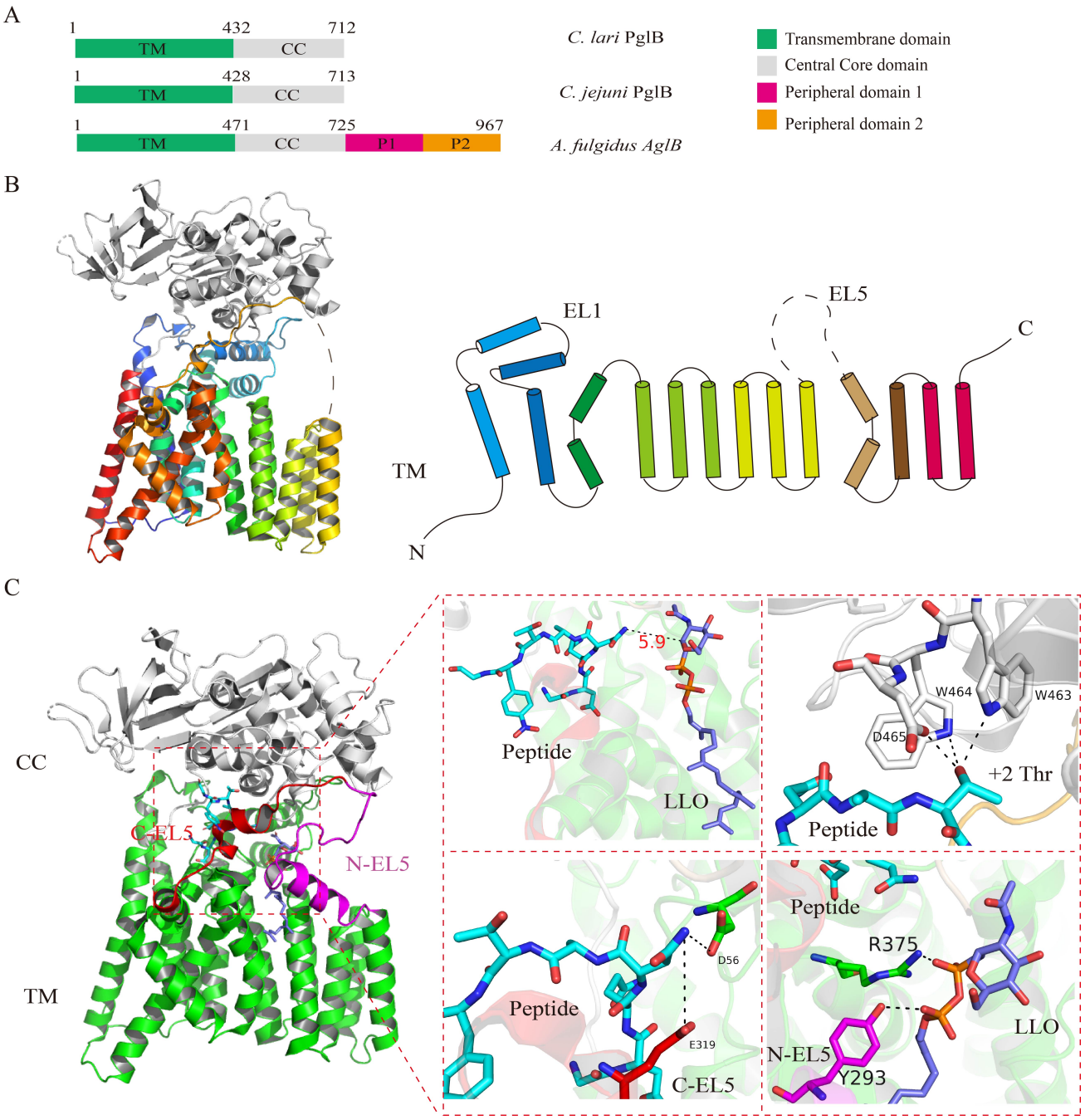

Fig. 2. The structure of the PglB enzyme of *C. jejuni* and the ternary complex of PglB bound to a substrate DQNATF peptide and an lipid-linked oligosaccharide (LLO) analog. (A) Schematic diagram of the PglB enzymes of *C. lari* and *C. jejuni* and the AglB protein of *Ar. fulgidus*. All the enzymes 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 reception (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 (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 hydrophilic 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 (^{281}RDVLWRD^{287}) 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 glycosyltranserases (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 aeruginos*a was initially identified as ^{57}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 $_{358}$LSDTAYAGNGAAPAASAPAASAPAASASEK$_{387}$ 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 $_{381}$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.

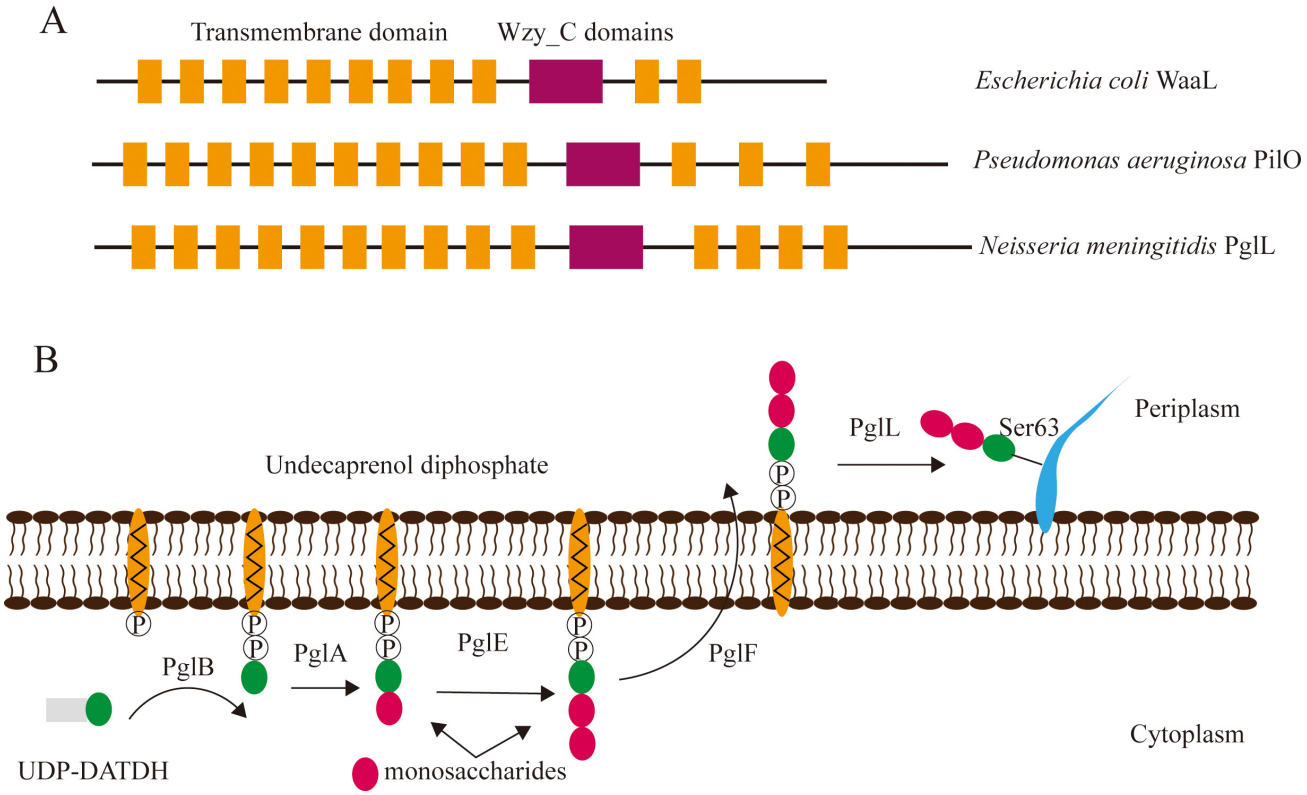

Fig. 3. Structure of enzymes and mechanism of bacterial O-linked glycosylation. (A) Structure of the WaaL protein of *E. coli*, PilO enzyme of *P. aeruginosa*, and the PglL protein of *N. meningitidis*. The Wzy_C domain is shared among the three species of bacteria. (B) Schematic diagram depicting the 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.

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 $PglL_{Vc}$. When the foreign substrate protein DsbA1 was introduced, the glycosylation site by PglLvc was mapped as VQTSVPADSAPAASAAAAPAGLVEGQNYTVLANPIPQQQAGK [58]. $PglL_{Vc}$ 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 ^{47}KVTPAVVSIAVE^{58} and ^{371}SLHQGLSGAE^{380} 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^{2+} 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^{2+} and the donor substrate, and the N-terminal ^{83}DXD^{85} motif is critical for metal-dependent enzymatic activity [60].

Table 1. Biological polysaccharide conjugate vaccine.

Pathogen/Antigens	Glycosyltransferase	Linked	Substrate protein	Efficacy results	Clinical development phase
Shigella flexneri 2a /polysaccharide component of the *Shigella flexneri* 2a O-antigen	$PglB_{Cj}$	N-linked	exotoxin protein A of *Pseudomonas aeruginosa* (EPA)	Access to vaccine safety and tolerability data.	Phase 1 (Complete)
*Shigella Flexyn*2a /polysaccharide component of the *Shigella flexneri* 2a O-antigen	$PglB_{Cj}$	N-linked	exotoxin protein A of *Pseudomonas aeruginosa* (EPA)	Testing the ability of vaccines to induce an immune response.	Phase 2 (Complete)
Shigella /polysaccharide component of the *Shigella flexneri* 2a O-antigen	$PglB_{Cj}$	N-linked	exotoxin protein A of *Pseudomonas aeruginosa* (EPA)	To demonstrate the safety and reactogenicity of the vaccine alone or in combination with an adjuvant (aluminum hydroxide).	Phase 1 (Complete)
Shigella /polysaccharide component of the *Shigella* O1 lipopolysaccharide	$PglB_{Cj}$	N-linked	exotoxin protein A of *Pseudomonas aeruginosa* (EPA)	First-in-human data on safety and immunogenicity in infants was obtained.	Phase 1 Phase 2 (Complete)
E. coli Tetravalent /*Oligosaccharide*	$PglB_{Cj}$	N-linked	exotoxin protein A of Pseudomonas aeruginosa (EPA)	This study aims to assess the safety and efficacy of a Tetravalent *E. coli* vaccine in inducing an immune response and reducing the incidence of urinary tract infections.	Phase 1 (Complete)
Klebsiella Pneumoniae Tetravalent /O-polysaccharide (OPS)	$PglL_{Nm}$	O-linked	cholera toxin B subunit (rCTB)	Obtain first-in-human data on its safety and immunogenicity in healthy adults.	Phase 1 Phase 2 (Complete)
New Pneumococcal /Capsular polysaccharides (CPS)	PglS	O-linked	exotoxin protein A of Pseudomonas aeruginosa (EPA)	Assess the safety and immunogenicity of a bioconjugate investigational vaccine compared to the control group (Pneumovax23).	Phase 1 (Complete)

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 CRM_{197} 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

$PglB_{Cj}$ 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 $PglB_{Cj}$ 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 pleuropnuemoniae* 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 ratmodel 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 $PglB_{Cj}$ 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_2-N position of the reducing end and forms a stable hydrogen bond with the carrier protein. If the C_2-N acetyl group at the reducing end is missing or the β-(1$\rightarrow$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_2-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, PglL can transfer any sugar carried by undecaprenyl pyrophosphate (UndPP) to the pilin substrate. Considering whether the lipid carrier was a limiting factor for PglL, the authors established the authors established a glycosylation system *in vitro* using purified PglL, pilin, and chemically synthesized lipid farnesyl pyrophosphate (FarPP) with pentasaccharides. The results indicated that synthetic lipid carriers could also transfer polysaccharides to the periplasm and localize polysaccharides around PglL. PglL is also broadly

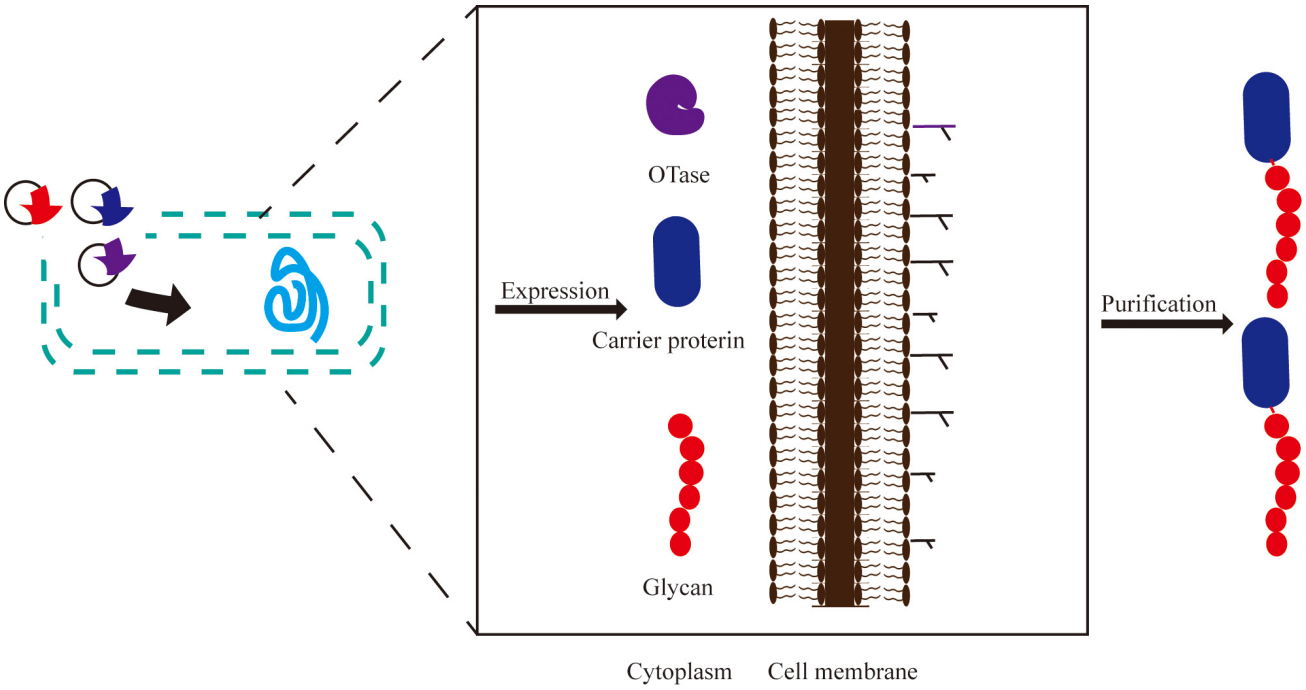

Fig. 4. Engineering bacteria to produce glycoproteins. OTase-dependent glycosylation is used as an example. Oligosaccharyltransferase, carrier protein, and polysaccharide are expressed in the host and assembled in the periplasm. Purple: plasmid encoding OST; Blue: plasmid with carrier protein sequence; Red: a glycan plasmid for the production of the lipid-linked polysaccharide.

selective for vectors. The flexibility of PglL is likely to play a role in the production of future glycoconjugate vaccines [44]. Therefore, it has a higher application potential in the development of new conjugate vaccines. *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 TfpM from *Moraxella osloensis*. Knoot CJ *et al.* [88] found that TfpM-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, recombinant expression, and host cell modification. Shibata *et al*. [92] encountered these problems when expressing the cell wall polysaccharide of *Streptococcus* mutans in *E. coli*. For example, the expressed glycan only has the rhamnose backbone and lacks side chain residues. They speculated that another genetic modification may be required for glucose side chain formation in recombinant expression. The deletion experiment of genes involved in the assembly of rhamnose synthesis infers that the genes play a role in the process of rhamnose assembly in a certain order, but the method to modify them in the host body has not been confirmed [92]. These issues still await further research.

Author Contributions

Conceptualization, SO, MXC and LZ; methodology, SO, MXC and LZ; software, PL and RL; validation, SO; formal analysis, PL and RL; data curation, PL and RL; writing —original draft preparation, PL and RL; writing review and editing, MXC, and LZ; visualization, PL and RL; supervision, SO, MXC and LZ; project administration, SO, MXC and LZ; fund acquisition, SO, LZ. All the authors have read and approved the final version of the manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This study was supported by the National Key Research and Development Project of China (grant number: 2021YFC2301403, 2021YFC2102100), the National Natural Science Foundation of China (grant numbers: 82225028, 82172287, 32271507 and U20A20361), and the High-level personnel introduction grant of Fujian Normal University (grant number: Z0210509).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Eichler J. Protein glycosylation. Current Biology: CB. 2019; 29: R229–R231.

[2] Schjoldager KT, Narimatsu Y, Joshi HJ, Clausen H. Global view of human protein glycosylation pathways and functions. Nature Reviews. Molecular Cell Biology. 2020; 21: 729–749.

[3] Kelleher DJ, Gilmore R. An evolving view of the eukaryotic oligosaccharyltransferase. Glycobiology. 2006; 16: 47R–62R.

[4] Taguchi Y, Yamasaki T, Ishikawa M, Kawasaki Y, Yukimura R, Mitani M, *et al*. The structure of an archaeal oligosaccharyltransferase provides insight into the strict exclusion of proline from the N-glycosylation sequon. Communications Biology. 2021; 4: 941.

[5] Aebi M. N-linked protein glycosylation in the ER. Biochimica et Biophysica Acta. 2013; 1833: 2430–2437.

[6] Lu H, Fermaintt CS, Cherepanova NA, Gilmore R, Yan N, Lehrman MA. Mammalian STT3A/B oligosaccharyltransferases segregate N-glycosylation at the translocon from lipid-linked oligosaccharide hydrolysis. Proceedings of the National Academy of Sciences of the United States of America. 2018; 115: 9557–9562.

[7] Joshi HJ, Narimatsu Y, Schjoldager KT, Tytgat HLP, Aebi M, Clausen H, *et al*. SnapShot: O-Glycosylation Pathways across Kingdoms. Cell. 2018; 172: 632–632.e2.

[8] Gill DJ, Clausen H, Bard F. Location, location, location: new insights into O-GalNAc protein glycosylation. Trends in Cell Biology. 2011; 21: 149–158.

[9] Hurtado-Guerrero R. Recent structural and mechanistic insights into protein O-GalNAc glycosylation. Biochemical Society Transactions. 2016; 44: 61–67.

[10] Iwashkiw JA, Vozza NF, Kinsella RL, Feldman MF. Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. Molecular Microbiology. 2013; 89: 14–28.

[11] Lu Q, Li S, Shao F. Sweet Talk: Protein Glycosylation in Bac-

terial Interaction with the Host. Trends in Microbiology. 2015; 23: 630–641.
[12] Nothaft H, Liu X, McNally DJ, Szymanski CM. N-linked protein glycosylation in a bacterial system. Methods in Molecular Biology (Clifton, N.J.). 2010; 600: 227–243.
[13] Li H, Debowski AW, Liao T, Tang H, Nilsson HO, Marshall BJ, *et al*. Understanding protein glycosylation pathways in bacteria. Future Microbiology. 2017; 12: 59–72.
[14] Howard SL, Jagannathan A, Soo EC, Hui JPM, Aubry AJ, Ahmed I, *et al*. Campylobacter jejuni glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. Infection and Immunity. 2009; 77: 2544–2556.
[15] Terra VS, Mauri M, Sannasiddappa TH, Smith AA, Stevens MP, Grant AJ, *et al*. PglB function and glycosylation efficiency is temperature dependent when the pgl locus is integrated in the Escherichia coli chromosome. Microbial Cell Factories. 2022; 21: 6.
[16] Schulz BL, Jen FE, Power PM, Jones CE, Fox KL, Ku SC, *et al*. Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. PLoS One. 2013; 8: e62768.
[17] Elhenawy W, Scott NE, Tondo ML, Orellano EG, Foster LJ, Feldman MF. Protein O-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. Glycobiology. 2016; 26: 301–311.
[18] Oppy CC, Jebeli L, Kuba M, Oates CV, Strugnell R, Edgington-Mitchell LE, *et al*. Loss of *O*-Linked Protein Glycosylation in *Burkholderia* cenocepacia Impairs Biofilm Formation and Siderophore Activity and Alters Transcriptional Regulators. MSphere. 2019; 4: e00660–19.
[19] Willcocks SJ, Denman C, Cia F, McCarthy E, Cuccui J, Wren BW. Virulence of the emerging pathogen, *Burkholderia pseudomallei*, depends upon the *O*-linked oligosaccharyltransferase, PglL. Future Microbiology. 2020; 15: 241–257.
[20] Schön K, Lepenies B, Goyette-Desjardins G. Impact of Protein Glycosylation on the Design of Viral Vaccines. Advances in Biochemical Engineering/biotechnology. 2021; 175: 319–354.
[21] Naegeli A, Aebi M. Current Approaches to Engineering N-Linked Protein Glycosylation in Bacteria. Methods in Molecular Biology (Clifton, N.J.). 2015; 1321: 3–16.
[22] Igwaran A, Okoh AI. Human campylobacteriosis: A public health concern of global importance. Heliyon. 2019; 5: e02814.
[23] Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, *et al*. N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science (New York, N.Y.). 2002; 298: 1790–1793.
[24] Shrimal S, Gilmore R. Oligosaccharyltransferase structures provide novel insight into the mechanism of asparagine-linked glycosylation in prokaryotic and eukaryotic cells. Glycobiology. 2019; 29: 288–297.
[25] Igura M, Kohda D. Selective control of oligosaccharide transfer efficiency for the N-glycosylation sequon by a point mutation in oligosaccharyltransferase. The Journal of Biological Chemistry. 2011; 286: 13255–13260.
[26] Baker JL, Çelik E, DeLisa MP. Expanding the glycoengineering toolbox: the rise of bacterial N-linked protein glycosylation. Trends in Biotechnology. 2013; 31: 313–323.
[27] Jarrell KF, Ding Y, Meyer BH, Albers SV, Kaminski L, Eichler J. N-linked glycosylation in Archaea: a structural, functional, and genetic analysis. Microbiology and Molecular Biology Reviews: MMBR. 2014; 78: 304–341.
[28] Ishiwata A, Taguchi Y, Lee YJ, Watanabe T, Kohda D, Ito Y. N-Glycosylation with synthetic undecaprenyl pyrophosphate-linked oligosaccharide to oligopeptides by PglB oligosaccharyltransferase from *Campylobacter jejuni*. Chembiochem: a European Journal of Chemical Biology. 2015; 16: 731–737.
[29] Alaimo C, Catrein I, Morf L, Marolda CL, Callewaert N, Valvano MA, *et al*. Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. The EMBO Journal. 2006; 25: 967–976.
[30] Perez C, Gerber S, Boilevin J, Bucher M, Darbre T, Aebi M, *et al*. Structure and mechanism of an active lipid-linked oligosaccharide flippase. Nature. 2015; 524: 433–438.
[31] Mohanty S, Chaudhary BP, Zoetewey D. Structural Insight into the Mechanism of *N*-Linked Glycosylation by Oligosaccharyltransferase. Biomolecules. 2020; 10: 624.
[32] Igura M, Maita N, Kamishikiryo J, Yamada M, Obita T, Maenaka K, *et al*. Structure-guided identification of a new catalytic motif of oligosaccharyltransferase. The EMBO Journal. 2008; 27: 234–243.
[33] Maita N, Nyirenda J, Igura M, Kamishikiryo J, Kohda D. Comparative structural biology of eubacterial and archaeal oligosaccharyltransferases. The Journal of Biological Chemistry. 2010; 285: 4941–4950.
[34] Lizak C, Gerber S, Numao S, Aebi M, Locher KP. X-ray structure of a bacterial oligosaccharyltransferase. Nature. 2011; 474: 350–355.
[35] Lizak C, Gerber S, Zinne D, Michaud G, Schubert M, Chen F, *et al*. A catalytically essential motif in external loop 5 of the bacterial oligosaccharyltransferase PglB. The Journal of Biological Chemistry. 2014; 289: 735–746.
[36] Lee HS, Im W. Transmembrane motions of PglB induced by LLO are coupled with EL5 loop conformational changes necessary for OST activity. Glycobiology. 2017; 27: 734–742.
[37] Napiórkowska M, Boilevin J, Sovdat T, Darbre T, Reymond JL, Aebi M, *et al*. Molecular basis of lipid-linked oligosaccharide recognition and processing by bacterial oligosaccharyltransferase. Nature Structural & Molecular Biology. 2017; 24: 1100–1106.
[38] Napiórkowska M, Boilevin J, Darbre T, Reymond JL, Locher KP. Structure of bacterial oligosaccharyltransferase PglB bound to a reactive LLO and an inhibitory peptide. Scientific Reports. 2018; 8: 16297.
[39] Matsumoto S, Shimada A, Nyirenda J, Igura M, Kawano Y, Kohda D. Crystal structures of an archaeal oligosaccharyltransferase provide insights into the catalytic cycle of N-linked protein glycosylation. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110: 17868–17873.
[40] Barre Y, Nothaft H, Thomas C, Liu X, Li J, Ng KKS, *et al*. A conserved DGGK motif is essential for the function of the PglB oligosaccharyltransferase from *Campylobacter jejuni*. Glycobiology. 2017; 27: 978–989.
[41] Wells L, Whelan SA, Hart GW. O-GlcNAc: a regulatory post-translational modification. Biochemical and Biophysical Research Communications. 2003; 302: 435–441.
[42] Yang X, Qian K. Protein O-GlcNAcylation: emerging mechanisms and functions. Nature Reviews. Molecular Cell Biology. 2017; 18: 452–465.
[43] Chang YH, Weng CL, Lin KI. O-GlcNAcylation and its role in the immune system. Journal of Biomedical Science. 2020; 27: 57.
[44] Fathy Mohamed Y, Scott NE, Molinaro A, Creuzenet C, Ortega X, Lertmemongkolchai G, *et al*. A general protein *O*-glycosylation machinery conserved in *Burkholderia* species improves bacterial fitness and elicits glycan immunogenicity in humans. The Journal of Biological Chemistry. 2019; 294: 13248–13268.
[45] Hug I, Feldman MF. Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. Glycobiology. 2011; 21: 138–151.
[46] Harding CM, Nasr MA, Kinsella RL, Scott NE, Foster LJ, Weber BS, *et al*. Acinetobacter strains carry two functional oligosac-

charyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to O-glycosylation of multiple proteins. Molecular Microbiology. 2015; 96: 1023–1041.
[47] Qutyan M, Paliotti M, Castric P. PilO of *Pseudomonas aeruginosa* 1244: subcellular location and domain assignment. Molecular Microbiology. 2007; 66: 1444–1458.
[48] Qutyan M, Henkel M, Horzempa J, Quinn M, Castric P. Glycosylation of pilin and nonpilin protein constructs by *Pseudomonas aeruginosa* 1244. Journal of Bacteriology. 2010; 192: 5972–5981.
[49] Faridmoayer A, Fentabil MA, Haurat MF, Yi W, Woodward R, Wang PG, *et al*. Extreme substrate promiscuity of the Neisseria oligosaccharyl transferase involved in protein O-glycosylation. The Journal of Biological Chemistry. 2008; 283: 34596–34604.
[50] Omer H, Rose G, Jolley KA, Frapy E, Zahar JR, Maiden MCJ, *et al*. Genotypic and phenotypic modifications of *Neisseria meningitidis* after an accidental human passage. PloS One. 2011; 6: e17145.
[51] Børud B, Bårnes GK, Brynildsrud OB, Fritzsønn E, Caugant DA. Genotypic and Phenotypic Characterization of the *O*-Linked Protein Glycosylation System Reveals High Glycan Diversity in Paired Meningococcal Carriage Isolates. Journal of Bacteriology. 2018; 200: e00794–17.
[52] Bhat AH, Maity S, Giri K, Ambatipudi K. Protein glycosylation: Sweet or bitter for bacterial pathogens? Critical Reviews in Microbiology. 2019; 45: 82–102.
[53] Power PM, Seib KL, Jennings MP. Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. Biochemical and Biophysical Research Communications. 2006; 347: 904–908.
[54] Chamot-Rooke J, Rousseau B, Lanternier F, Mikaty G, Mairey E, Malosse C, *et al*. Alternative Neisseria spp. type IV pilin glycosylation with a glyceramido acetamido trideoxyhexose residue. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104: 14783–14788.
[55] Pan C, Sun P, Liu B, Liang H, Peng Z, Dong Y, *et al*. Biosynthesis of Conjugate Vaccines Using an O-Linked Glycosylation System. mBio. 2016; 7: e00443–16.
[56] Schulz BL, Jen FEC, Power PM, Jones CE, Fox KL, Ku SC, *et al*. Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. PloS One. 2013; 8: e62768.
[57] Silva AJ, Benitez JA. *Vibrio cholerae* Biofilms and Cholera Pathogenesis. PLoS Neglected Tropical Diseases. 2016; 10: e0004330.
[58] Gebhart C, Ielmini MV, Reiz B, Price NL, Aas FE, Koomey M, *et al*. Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in *Vibrio cholerae* and *Burkholderia thailandensis*. Glycobiology. 2012; 22: 962–974.
[59] Vorkapic D, Mitterer F, Pressler K, Leitner DR, Anonsen JH, Liesinger L, *et al*. A Broad Spectrum Protein Glycosylation System Influences Type II Protein Secretion and Associated Phenotypes in *Vibrio cholerae*. Frontiers in Microbiology. 2019; 10: 2780.
[60] Choudhary P, Badmalia MD, Ashish, Rao A. Shape-function insights into bifunctional O-GlcNActransferase of *Listeria monocytogenes* EGD-e. Glycobiology. 2021; 31: 275–287.
[61] Schirm M, Kalmokoff M, Aubry A, Thibault P, Sandoz M, Logan SM. Flagellin from *Listeria monocytogenes* is glycosylated with beta-O-linked N-acetylglucosamine. Journal of Bacteriology. 2004; 186: 6721–6727.
[62] Shen A, Kamp HD, Gründling A, Higgins DE. A bifunctional O-GlcNAc transferase governs flagellar motility through anti-repression. Genes & Development. 2006; 20: 3283–3295.
[63] Kamp HD, Higgins DE. A protein thermometer controls temperature-dependent transcription of flagellar motility genes in *Listeria monocytogenes*. PLoS Pathogens. 2011; 7: e1002153.
[64] Kamp HD, Higgins DE. Transcriptional and post-transcriptional regulation of the GmaR antirepressor governs temperature-dependent control of flagellar motility in Listeria monocytogenes. Molecular Microbiology. 2009; 74: 421–435.
[65] Rathinam VAK, Zhao Y, Shao F. Innate immunity to intracellular LPS. Nature Immunology. 2019; 20: 527–533.
[66] Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet MJ, *et al*. LPS-induced cytokine production in human monocytes and macrophages. Critical Reviews in Immunology. 2011; 31: 379–446.
[67] Adamo R. Glycoconjugate vaccines: classic and novel approaches. Glycoconjugate Journal. 2021; 38: 397–398.
[68] Del Bino L, Østerlid KE, Wu DY, Nonne F, Romano MR, Codée J, *et al*. Synthetic Glycans to Improve Current Glycoconjugate Vaccines and Fight Antimicrobial Resistance. Chemical Reviews. 2022; 122: 15672–15716.
[69] Cuccui J, Wren B. Hijacking bacterial glycosylation for the production of glycoconjugates, from vaccines to humanised glycoproteins. The Journal of Pharmacy and Pharmacology. 2015; 67: 338–350.
[70] Harding CM, Feldman MF. Glycoengineering bioconjugate vaccines, therapeutics, and diagnostics in *E. coli.* Glycobiology. 2019; 29: 519–529.
[71] Wacker M, Wang L, Kowarik M, Dowd M, Lipowsky G, Faridmoayer A, *et al*. Prevention of *Staphylococcus aureus* infections by glycoprotein vaccines synthesized in *Escherichia coli*. The Journal of Infectious Diseases. 2014; 209: 1551–1561.
[72] Williams AJ, Warfel KF, Desai P, Li J, Lee JJ, Wong DA, *et al*. A low-cost recombinant glycoconjugate vaccine confers immunogenicity and protection against enterotoxigenic *Escherichia coli* infections in mice. Frontiers in Molecular Biosciences. 2023; 10: 1085887.
[73] Huttner A, Gambillara V. The development and early clinical testing of the ExPEC4V conjugate vaccine against uropathogenic *Escherichia coli*. Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018; 24: 1046–1050.
[74] Garcia-Quintanilla F, Iwashkiw JA, Price NL, Stratilo C, Feldman MF. Production of a recombinant vaccine candidate against *Burkholderia pseudomallei* exploiting the bacterial N-glycosylation machinery. Frontiers in Microbiology. 2014; 5: 381.
[75] Passmore IJ, Andrejeva A, Wren BW, Cuccui J. Cytoplasmic glycoengineering of Apx toxin fragments in the development of Actinobacillus pleuropneumoniae glycoconjugate vaccines. BMC Veterinary Research. 2019; 15: 6.
[76] Kämpf MM, Braun M, Sirena D, Ihssen J, Thöny-Meyer L, Ren Q. *In vivo* production of a novel glycoconjugate vaccine against *Shigella flexneri* 2a in recombinant *Escherichia coli*: identification of stimulating factors for *in vivo* glycosylation. Microbial Cell Factories. 2015; 14: 12.
[77] Ravenscroft N, Braun M, Schneider J, Dreyer AM, Wetter M, Haeuptle MA, *et al*. Characterization and immunogenicity of a *Shigella flexneri* 2a O-antigen bioconjugate vaccine candidate. Glycobiology. 2019; 29: 669–680.
[78] Riddle MS, Kaminski RW, Di Paolo C, Porter CK, Gutierrez RL, Clarkson KA, *et al*. Safety and Immunogenicity of a Candidate Bioconjugate Vaccine against *Shigella flexneri* 2a Administered to Healthy Adults: a Single-Blind, Randomized Phase I Study. Clinical and Vaccine Immunology: CVI. 2016; 23: 908–917.
[79] Chen MM, Glover KJ, Imperiali B. From peptide to protein: comparative analysis of the substrate specificity of N-linked gly-

cosylation in *C. jejuni*. Biochemistry. 2007; 46: 5579–5585.
[80] Wacker M, Feldman MF, Callewaert N, Kowarik M, Clarke BR, Pohl NL, *et al*. Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103: 7088–7093.
[81] Ihssen J, Haas J, Kowarik M, Wiesli L, Wacker M, Schwede T, *et al*. Increased efficiency of *Campylobacter jejuni* N-oligosaccharyltransferase PglB by structure-guided engineering. Open Biology. 2015; 5: 140227.
[82] Li M, Liu E, Zhang W, Luo H, Li P. Biosynthesis of *Salmonella enteritidis* O antigen-based glycoproteins. Sheng Wu Gong Cheng Xue Bao. 2022; 38: 2377–2388.
[83] Sun P, Pan C, Zeng M, Liu B, Liang H, Wang D, *et al*. Design and production of conjugate vaccines against *S. Paratyphi A* using an O-linked glycosylation system in vivo. NPJ Vaccines. 2018; 3: 4.
[84] Peng Z, Wu J, Wang K, Li X, Sun P, Zhang L, *et al*. Production of a Promising Biosynthetic Self-Assembled Nanoconjugate Vaccine against *Klebsiella Pneumoniae* Serotype O2 in a General *Escherichia Coli* Host. Advanced Science (Weinheim, Baden-Wurttemberg, Germany). 2021; 8: e2100549.
[85] Zhang L, Pan C, Feng E, Hua X, Yu Y, Wang H, *et al*. Biosynthesis of polysaccharide conjugate vaccines against *Klebsiella pneumoniae* serotype O2 strains. Sheng Wu Gong Cheng Xue Bao. 2020; 36: 1899–1907. (In Chinese)
[86] Huang J, Pan C, Sun P, Feng E, Wu J, Zhu L, *et al*. Application of an *O*-Linked Glycosylation System in *Yersinia enterocolitica* Serotype O:9 to Generate a New Candidate Vaccine against *Brucella abortus*. Microorganisms. 2020; 8: 436.
[87] Li S, Huang J, Wang K, Liu Y, Guo Y, Li X, *et al*. A bioconjugate vaccine against *Brucella abortus* produced by engineered *Escherichia coli*. Frontiers in Bioengineering and Biotechnology. 2023; 11: 1121074.
[88] Knoot CJ, Wantuch PL, Robinson LS, Rosen DA, Scott NE, Harding CM. Discovery and characterization of a new class of O-linking oligosaccharyltransferases from the *Moraxellaceae* family. Glycobiology. 2023; 33: 57–74.
[89] Feldman MF, Mayer Bridwell AE, Scott NE, Vinogradov E, McKee SR, Chavez SM, *et al*. A promising bioconjugate vaccine against hypervirulent *Klebsiella pneumoniae*. Proceedings of the National Academy of Sciences of the United States of America. 2019; 116: 18655–18663.
[90] Harding CM, Nasr MA, Scott NE, Goyette-Desjardins G, Nothaft H, Mayer AE, *et al*. A platform for glycoengineering a polyvalent pneumococcal bioconjugate vaccine using *E. coli* as a host. Nature Communications. 2019; 10: 891.
[91] Duke JA, Paschall AV, Robinson LS, Knoot CJ, Vinogradov E, Scott NE, *et al*. Development and Immunogenicity of a Prototype Multivalent Group B *Streptococcus* Bioconjugate Vaccine. ACS Infectious Diseases. 2021; 7: 3111–3123.
[92] Shibata Y, Yamashita Y, Ozaki K, Nakano Y, Koga T. Expression and characterization of streptococcal rgp genes required for rhamnan synthesis in *Escherichia coli*. Infection and Immunity. 2002; 70: 2891–2898.