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New discoveries in bacterial *N*-glycosylation to expand the synthetic biology toolbox

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Historically, protein glycosylation was believed to be restricted to eukaryotes, but now is abundantly represented in all three domains of life. The first bacterial *N*-linked glycosylation system was discovered in the Gram-negative pathogen, *Campylobacter jejuni*, and subsequently transferred into the heterologous *Escherichia coli* host beginning a new era of synthetic bacterial glycoengineering. Since then, additional *N*-glycosylation pathways have been characterized resembling the classical *C. jejuni* system and unconventional new approaches for *N*-glycosylation have been uncovered. These include cytoplasmic protein modification, direct glycan transfer to proteins, and use of alternate amino acid acceptors, deepening our understanding of the vast mechanisms bacteria possess for protein modification and providing opportunities to expand the glycoengineering toolbox for designing novel vaccine formulations and protein therapeutics.

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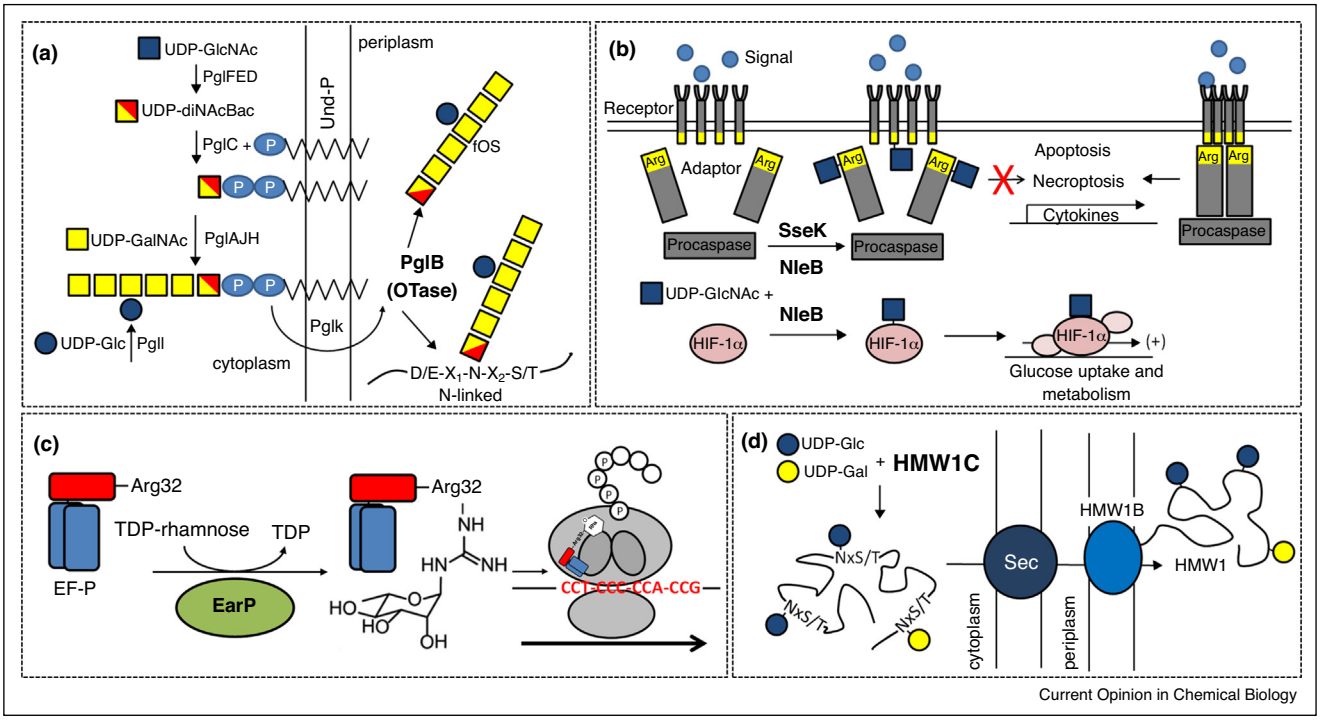
Introduction

Bacteria possess a diversity of protein glycosylation systems. The first and best characterized system is the asparagine (*N*)-linked protein glycosylation (*pgl*) operon encoding enzymes required to modify >80 proteins in the gastrointestinal pathogen, *Campylobacter jejuni* [1,2]. In *C. jejuni*, *N*-glycosylation influences bacterial adhesion and invasion *in vitro*, mouse and chicken colonization *in vivo*, proteolysis of surface proteins by gut proteases, DNA update through type IV secretion systems and recognition by innate and adaptive immune responses [3–7]. More recent studies have shown *N*-glycosylation of *C. jejuni* proteins also affects nitrate reductase activity, chemotaxis, nutrient transport, stress and antimicrobial resistance [1,8,9]. These *pgl* operons are found in all *Campylobacter* species [10] and other epsilon and delta proteobacteria [5], and require a membrane-bound oligosaccharyltransferase (OTase), PglB, related to the eukaryotic STT3 OTase [11]. The classical *N*-glycosylation pathway involves assembly of an oligosaccharide precursor on a lipid carrier that is subsequently flipped across the inner membrane and the sugars are transferred *en bloc* by PglB to the asparagine residue of the D/E-X_1-N-X_2-S/T consensus sequon where X_1, X_2 cannot be proline (Figure 1a, for review [4]). It is worth mentioning that although this sequon is optimal for the addition of the *N*-glycan, it is not absolutely required since low rates of glycosylation have been observed with partial glycosylation acceptor sequences [12–14]. Additional *N*-glycosylation systems have been discovered in gamma proteobacteria that modify one or few protein targets. To date, the bacterial speciality systems are atypical because they involve glycosyltransferases (GTases) that transfer a single nucleotide-activated sugar directly to a protein in the cytoplasm. These include *N*-glycosylation enzymes required for modification of asparagine residues of specific surface adhesins (Figure 1d), as first described in non-typeable *Haemophilus influenzae* [15,16] using the CAZy (Carbohydrate Active enZYmes database) GTase 41 family of GTases, HMW1C, and subsequently the *N*-glycosylating GTase designated NGT in *Actinobacillus pleuropneumoniae* [17,18]. Here, loss of glycosylation results in reduced adhesion to human epithelial cells [19]. In *H. influenzae*, HMW1C-mediated glycosylation is required for protein stability, folding and secretion of the HMW1 protein [20], and is important for colonization and pathogenesis [21,22]. Other functional HMW1C/NGT homologs have been studied in *Yersinia enterocolitica* [18], *Kingella kingae* and *Aggregatibacter aphrophilus* [23]. The specialized *N*-glycosylation systems also include GTases that attach *N*-acetylglucosamine (GlcNAc) or rhamnose (Rha) moieties onto guanidino groups of arginine residues (Figure 1b,c). While arginine GlcNAcylation is targeted to the host during infection to subvert the antimicrobial response, arginine rhamnosylation is used to rescue stalled ribosomes in certain bacteria. The initial focus of this review will be on these new arginine *N*-glycosylation systems followed by recent advances in the exploitation and structural characterization of the HMW1C/NGT and *C. jejuni N*-glycosylation pathways. It is worth mentioning that *O*-linked, *C*-linked and *S*-linked glycosylation pathways have been identified in bacteria for glycosylating a

Figure 1



Known pathways for bacterial *N*-linked protein glycosylation.
Panel **(a)**: The general *N*-linked protein glycosylation system of *C. jejuni*. An undecaprenyl-pyrophosphate (Und-PP)-linked heptasaccharide is assembled in the cytosol from nucleotide-activated donors. The complete Und-PP-heptasaccharide is flipped across the inner membrane into the periplasm by the ABC transporter, PglK. PglB (in bold), the central pathway enzyme, transfers the oligosaccharide *en bloc* to the amino group of asparagine in the protein consensus sequence, D/E-X_1-N-X_2-S/T (X_1 and X_2 not P), or releases it into the periplasm as free oligosaccharides, fOS (figure adapted from Nothaft *et al.* [4]). *Panel* **(b)**: Schematic of the function of the *E. coli*/*C. rodentium* (NleB, in bold) and *Salmonella* (SseK, in bold) type three secretion system effectors. The NleB/SseK proteins secreted into the host cytoplasm are GTases that specifically modify conserved arginine residues in the death domain (highlighted in yellow) of signaling adaptor and/or signaling receptor proteins with a single GlcNAc residue. GlcNAcylated proteins cannot form the membrane-associated death-inducing signalling complex resulting in loss of for example, caspase 8 activation and blockage of further downstream signalling that would normally lead to apoptosis or necroptosis. In addition, NleB1-mediated GlcNAcylation enhances HIF-1α activity thus increasing the expression of genes for glucose uptake and metabolism. *Panel* **(c)**: Schematic of EF-P activation by the EarP GTase (in bold). EF-P is activated by rhamnosylation at the conserved Arg32 located at the tip of the N-terminal domain (in red) by EarP using TDP-rhamnose as a substrate. Glycosylation-activated EF-P is then recruited to rescue stalled ribosomes at poly-proline stretches (figure adapted from Krafczyk *et al.* [47•]). *Panel* **(d)**: Transfer of sugars to proteins by the *H. influenzae* cytoplasmic GTase, HMW1C (in bold). Glucose (Glc) and in some cases galactose (Gal) are transferred from nucleotide-activated donors to the eukaryotic-like acceptor sequon N-X-S/T of high-molecular-weight adhesins (e.g. HMW1). The modified protein is secreted through the Sec translocation apparatus and through its dedicated outer-membrane translocator, for example, HMW1B, to which it remains tethered (figure adapted from Nothaft *et al.* [4]).

variety of proteins including flagellins, pilins, adhesins, or host proteins [24–27,28•]; however, these systems will not be discussed here.

Arginine GlcNAcylation

Arginine glycosylation is mediated by the bacterial effector proteins, SseK (up to three variants, SseK1, SseK2, and SseK3 exist in *Salmonella enterica* serovar Typhimurium) or the NleB orthologs (found in *Escherichia coli* and *Citrobacter rodentium*) that are injected into host cells via the bacterial type III secretion system [29,30]. Using UDP-GlcNAc as a donor substrate, these GTases covalently and irreversibly [31] attach GlcNAc onto the guanidino group of a conserved arginine residue on cell death-domain-containing adaptor proteins resulting in blockage of downstream signalling that would normally lead to apoptosis or necroptosis [32••,33–35]. By blocking cell death, NleB/SseK-catalyzed β-GlcNAcylation promotes enterocyte survival, prolongs attachment of the pathogen to the gut epithelium [36] or enhances survival in infected macrophages [34]. It is worth mentioning that some *E. coli* strains, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) possess two NleB orthologs, the enzymatically active NleB1 and the inactive, with yet unknown function, NleB2 variant. Interestingly, the latter one can bind but not modify certain target proteins [30,32••]. In contrast, *C. rodentium* possesses only one form which functions similarly to

NleB1, so it is unclear why EPEC and EHEC maintain both NleB orthologs [30]. Nevertheless, the arginine glycosylation event was unexpected since the guanidino group is a poor nucleophile at physiological pH based on partial delocalization of electrons on this moiety [32••]. SseK and NleB proteins are structurally similar, but differ slightly in the binding mode of the uracil group of UDP-GlcNAc [32••,37,38•,39•]. They all consist of a helix-loop-helix (HLH) domain, a lid domain, and a catalytic domain (Figure 2a). The lid-domain regulates the opening and closing of the active site in the catalytic domain and the HLH domain determines the substrate specificity [37]. A highly conserved His-Glu-Asn motif in the active site is important for enzyme catalysis and bacterial virulence. SseK/NleB proteins are retaining-glycosyltransferase type-A (GT-A)-family proteins, most likely displaying a front-face S_Ni mechanism for transferring the GlcNAc sugar. The active site glutamate residue in close proximity to the reactive anomeric carbon, essential for enzymatic activity, suggests a double-inversion mechanism where an enzyme–sugar intermediate is stabilized for the nucleophilic attack of the acceptor arginine side chain of the host substrates (Figure 2a) [38•]). Like all GT-A GTs, a conserved DXD metal-coordinating motif is necessary for ligand binding and enzymatic activity [37,38•]. A Trp-Arg motif in the acceptor substrate is also important for interaction with the SseK proteins, noting that this Arg is not the glycosylation site [37,38•].

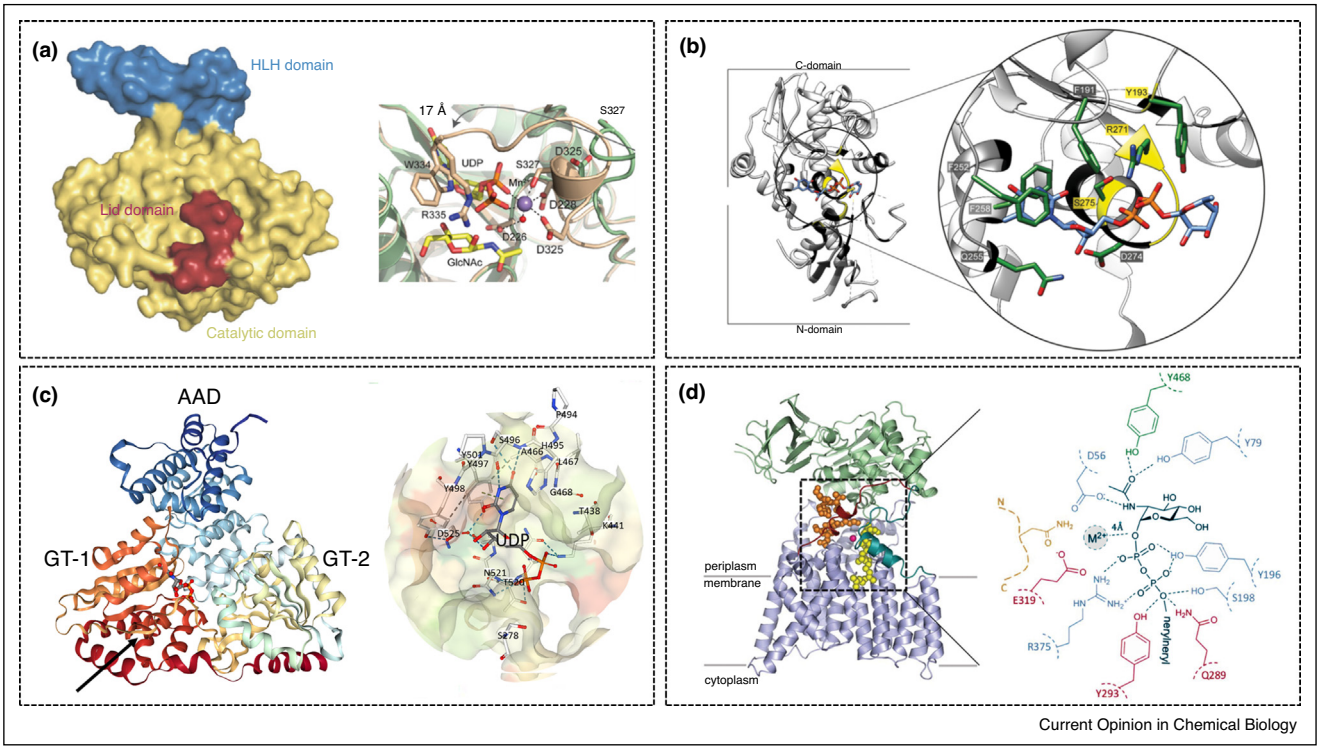
Targets for SseK/NleB GTs are host FADD TRADD, RIPK1, ensconsin (MAP7), TNFR1, TRAILR, HIF-1α (hypoxia-inducible factor 1α) and GAPDH [31,35,36,39•, 40–42]. However, *in vitro* glycosylation, cell culture experiments and *in vivo* mouse studies demonstrated that not every effector is targeting each protein [30,31,34,35]. The observed differences in substrate specificities could be explained by differences in the electrostatic surface charge distribution of the SseK/NleB proteins that could influence the active site accessibility of certain substrates [38•] or by different molecular dynamics of the HLH domain (i.e. between SseK1 that glycosylates and SseK2 that does not glycosylate FADD) [32••]. Moreover, different arginine-GlcNAcylation profiles could be a result of different experimental systems that were used in these studies (i.e. in *Salmonella* wildtype-infected cells versus ectopically expressed effector proteins) [31,39•]. Recently, NleB1 has also been implicated to modulate host glucose metabolism. NleB1-mediated arginine GlcNAcylation of HIF-1α, the master regulator of cellular O_2 homeostasis [41] enhances its transcriptional activity, thereby elevating the expression levels of genes involved in glucose metabolism. Indeed, mice infected with wild-type *C. rodentium* had significantly lower blood glucose levels compared to mice infected with the *C. rodentium nleB1* mutant [41] providing evidence for a causal link between pathogen infection and host glucose metabolism. In *Salmonella* or *E. coli*-infected humans, those NleB/SseK-mediated downstream effects could provide a new explanation for disease causation related to metabolic disturbances such as obesity and type 2 diabetes. Accordingly, arginine glycosylation systems provide a new target for intervention strategies [41] and novel GTase inhibitors have already been identified that prevent NleB1 glycosylation of TRADD and also inhibit *Salmonella enterica* replication in mouse macrophage-like cells without influencing mammalian *O*-GlcNAc GTases or host cell viability [43].

Arginine rhamnosylation

Reminiscent of the *E. coli*/*Salmonella* β-lysyl modification of Lys34 of the polyproline-specific bacterial translation elongation factor (EF-P), around 10% of bacteria from various phylogenetic groups encode an EF-P variant with a conserved arginine, Arg32 at the tip of the N-domain of EF-P, that in some cases has been shown to be modified with a rhamnose moiety [44••,45]. First described in 2015, this reaction is catalyzed by the novel inverting and very specific EF-P–arginine rhamnosyltransferase, EarP, using TDP-β-L-rhamnose as a substrate [44••,45,46]. Rather than directly influencing the conformation of the polypeptide to facilitate peptide-bond formation as has been shown for the EF-P Lys34 modification, Arg32-rhamnosylation has been proposed to function indirectly by interacting with and stabilizing the CCA-end of the P-site peptidyl-Pro-tRNA, thus stimulating Pro–Pro peptide bond formation to relieve ribosome stalling [44••,47•]. In *Shewanella oneidensis* and *Pseudomonas aeruginosa*, EF-P and its Arg32 modification are dispensable for cell viability, but are crucial for overall bacterial fitness and for pathogenicity. However, in *Neisseria meningitidis*, rhamnosylation of EF-P is essential probably since *N. meningitidis* contains more proline-codon stretches and/or EF-P activity is lower [48].

EarP structures from *N. meningitidis* [49] and *P. putida* (Figure 2b) [47•] revealed that these enzymes consist of two opposing Rossmann-fold domains separated by an inter-domain cleft constituting B-type inverting GTases (GT-B). This is structurally and phylogenetically unrelated to the SseK/NleB GTases described above indicating that both *N*-linked arginine glycosylation events are a product of convergent evolution. Independent adaptations leading to similar end products have also been observed for the biosynthesis of the same di-*N*-acetylbacillosamine reducing end sugar used in *Campylobacter N*-linked and *Neisseria O*-linked glycosylation [5]. Rhamnosylation of EF-P by EarP is expected to occur via an S_N2 reaction and most of the residues involved in the binding of TDP-Rha are conserved among EarP orthologs [47•,49]. TDP-Rha is coordinated within a cavity in the protein C-domain, a cage formed by aromatic residues (Phe185, Phe225, and Phe249 in $EarP_{Nm}$) that stack against the base of the nucleotide moiety [49]. The sugar ring of the nucleotide as well as the

Figure 2



GTase and OTase structures and functions.
Panel **(a)**: General domain architecture of SseK/NleB effector protein GTases. Left: the helix-loop-helix (HLH), catalytic and lid domains are depicted in blue, yellow and red, respectively. The UDP-GlcNAc binding site is hidden below the lid domain. Right: close-up of the residues that participate in UDP-GlcNAc interaction (from SseK3 as an example). The domain and binding site structures originally appeared in Park *et al.* [32••] and Esposito *et al.* [38•] and are reproduced with permission. Similar structures have been solved for *Salmonella* SseK1 and SseK2 and for NleB2 from *E. coli* O145:H28 [32••]. *Panel* **(b)**: Structure of the EarP GTase (EarP from *P. putida* as an example). Left: the domain architecture of $EarP_{Ppu}$ is shown; the TDP-Rha binding pocket in the C-domain is circled. Right: close-up of the TDP-Rha binding pocket; amino acid residues important for TDP-Rha positioning are labelled with a single-letter code. The $EarP_{Ppu}$ structure originally appeared in Krafczyk *et al.* [47•] and is reproduced with permission. Similarly, the structure for EarP from *N. meningitidis* has been solved by Sengoku *et al.* [49]. *Panel* **(c)**: The structure of the HMW1C GTase from *A. pleuropneumoniae* (ApHMW1C), now termed ApNGT, in complex with UDP is shown. Left: ApNGT consists of an N-terminal all α-domain (AAD) fold, a C-terminal GT-B fold that contains the GT-1 domain, the GT-2 domain, and an inter-domain region that connects GT-1 and GT-2 (indicated by an arrow), but lacks the tetratricopeptide repeat fold characteristic for the GT41 family. The GT-1 and GT-2 domains have a similar core structure and form the binding site for UDP-hexose at their interface; the UDP molecule is in stick representation. Right: One of two protomers obtained for the ApNGT–UDP crystal units is shown. Representative residues for ApNGT in the UDP binding pocket are indicated. Hydrogen-bonding interactions are shown with blue dashed lines; hydrophobic contacts are shown with grey dashed lines; pi interactions are shown with green dashed lines. The figure was generated from screenshot images from the RCSB PDB (rcsb.org) of PDB ID 3Q3H [59]. *Panel* **(d)**: Structure of the PglB OTase from *C. lari* with inhibitory Dab peptide and reactive LLO. Left: domain structure of PglB, the Dab peptide and the LLO bound within the active site are highlighted in orange and yellow, respectively. The N-terminal part of the external loop 5 (EL5) that binds the LLO and the C-terminal part that interacts with the acceptor peptide are in turquoise and in dark red, the divalent metal ion is in pink. Right: The divalent metal ion that stabilizes the pyrophosphate leaving group of the substitution reaction is coordinated by the glycosidic oxygen atom (rather than the negatively charged phosphate oxygens). The poor nucleophilicity of the carboxamide group of the acceptor asparagine might be affected by generating a reactive nucleophile through direct activation of the glycosidic oxygen by the metal ion that could subsequently allow glycan transfer without activation of the amide. The central D56 residue in the catalytic site of PglB forms hydrogen bonding interactions not only with the N-acetyl group of the LLO, but also with the peptide and the metal ion providing an explanation for the required acetyl group on the reducing end sugar for PglB to function. The PglB structure originally appeared in Napiorkowska *et al.* [79••] and is reproduced with permission.

diphosphate are further coordinated through multiple interactions with the protein, where in $EarP_{Ppu}$ the sugar itself seems to be surface exposed [47•,49]. Three negatively charged residues in the active site (i.e. Asp13, Asp17, and Glu273 in $EarP_{Ppu}$) in close proximity to the sugar are important for the catalytic activity and crucial for the stabilization of the positive charge of the acceptor guanidino group of EF-P Arg32 [47•,50••]. Interestingly, binding of Arg32 EF-P into the active site of EarP results in a structural change in the conserved TDP-β-L-Rha-binding loop and in a conformational change in the rhamnose ring (that would otherwise result in a direct steric

clash with the guanidino group of Arg32), to allow the inverting transfer of Rha to Arg32 [47•,49]. Although demonstrated earlier to be α-configured at the anomeric center, arginine rhamnosylation was later found to be configurationally labile with both α and β confirmations observed within EF-P of *P. aeruginosa* [51,52•]. Since EF-P is the only known substrate for EarP and *earP* mutants are more sensitive to antibiotics [45,48], these GTases are also novel targets for inhibitors with reduced side effects and lower risks for emergence of drug-resistant bacteria [49].

Glycoengineering

The discovery of the *C. jeuni* general *N*-glycosylation pathway, its functional transfer into *E. coli*, and the subsequent demonstration that PglB is able to transfer various glycans established a new era of bacterial glycoengineering (see Ref. [53]). Researchers now exploit the cytoplasmic (HMW1C/NGT-mediated) systems and take advantage of other OTases, GTases, and novel cloning and expression technologies.

Sialic acid modification of proteins is one approach to enhance the pharmacokinetic activity and serum half-life stability of industrially relevant glycoproteins. Keys *et al.* developed a biosynthetic pathway for site-specific polysialylation of recombinant proteins in the *E. coli* cytoplasm [54••]. By combining optimized NGT from *A. pleuropneumoniae*; LgtB, SynB, and a polysialyltransferase from *N. meningitidis*; and CstII from *C. jejuni*, the authors were able to achieve polysialylation of a cytoplasmically retained fragment of the autotransporter adhesin from *A. pleuropneumoniae*, an engineered GFP or a therapeutic DARPin [54••]. Similarly, Kong *et al.* recombinantly produced the specific *O*-antigen polysaccharide of *Shigella dysenteriae* type 1 on the surface (lipid A-linked) of *E. coli* K12 after transferring genes encoding GTases involved in the biosynthesis of these sugars [55]. To generate more stable and efficient glycoprotein production strains, *E. coli* K-12 has been 'glyco-recoded' by replacing major non-essential polysaccharide gene clusters with components for the biosynthesis of heterologous glycan structures and site-specific glycan conjugation to target proteins [56], resulting in higher glycosylation efficiencies when compared to plasmid encoded systems. Similarly, significant increases in glycoprotein yields could be achieved through chromosomal expression of the PglB OTase [57], by increasing intracellular glycan precursor pools through modifying central carbon metabolic pathways [58], and through alterations in growth rate and growth medium choices [59]. In addition, a PCR-free and ligase-free, recombination-based direct cloning and protein glycan coupling technology now allows the rapid engineering of glycoconjugate vaccines to keep abreast of quickly evolving bacterial pathogens [60].

To overcome limitations of recombinant expression of integral membrane OTase proteins, cell-free platforms developed for rapid synthesis and activity testing showed that soluble PglB proteins quantitatively added to *in vitro* activity assays achieved >75% glycosylation of the protein targets [61]. Other cell free systems decouple the production of glycoprotein synthesis components and target glycoprotein production to allow customizable single-pot glycosylation reactions [62•] and screening, characterization and optimization of glycosylation sequences for the underlying GTases [63]. GTases with different specificities or novel properties can also help to improve glycoprotein yields and potentially overcome substrate restrictions of other OTases and NGTs. For example, the *A. aphrophilus* NGT synthesizes glycopeptides with relaxed nucleotide-activated sugar donor selectivity [64]; *in vitro* testing of NGTs from four bacterial species revealed different preferences for the amino acids surrounding the *N*-glycosylation site [65] and also that NGTs might display different glycosylation specificities for native and non-native glycan acceptor peptides [66].

Although more and more specialized protein glycosylation systems are being discovered, their potential use for glycoengineering remains to be evaluated. The Arg-rhamnosylation system appears to be very specific concerning protein acceptors and sugar donors. Therefore, the use of this system for glycoengineering might be restricted. Arginine-GlcNAcylation occurs after injection of the GTase into host cells. A use in bioengineering would require expression of an appropriate (DD-domain) protein acceptor or exploitation of an already modified *E. coli* protein [31] that could subsequently be modified by other GTases, similar to what was described for the cytoplasmic NGT-system above. It is worth mentioning that Harding *et al.* [67••] recently described the use of an *O*-linking OTase, PglS, to generate a novel polyvalent pneumococcal bioconjugate vaccine in *E. coli* with polysaccharides containing glucose at the reducing end – a reaction no other natural OTase has catalyzed so far [67••]. For additional reviews describing technological developments in microbial glycoengineering see Refs. [53,68–70].

From structure to function

Structural information and understanding of the mechanistic details of biosynthetic pathway enzymes are important to further exploit the enzymes for glycoengineering or to develop enzyme-specific pathway inhibitors. In addition to the structural advances described for the new arginine *N*-glycosylation systems that have been the focus of this review, further progress has been made on the *N*-glycosylation pathways that are already being exploited. The structural and biochemical analysis of the cytoplasmic NGT from *A. pleuropneumoniae* showed that NGT is an inverting GTase of the GT41 family and, surprisingly, that NGT does not require a metal ion for catalysis [18,71] (Figure 2c, Table 1). For the *C. jejuni N*-glycosylation pathway, structures are available for the key PglB OTase [50••]; the PglD acetyltransferase [72]; the PglE aminotransferase

Table 1

Bacterial *N*-glycosylating enzymes

Host	GTase(GT)/OTase(OT)	CAzy	Mechanism	Sugar
P. putida and *N. meningitidis* (up to 10% of all bacteria across all genera)	EarP (GT)	GT104	GT-B Inverting S_N2 Metal-ion dependent	Rhamnose
Salmonella enterica *Escherichia coli* (EPEC/EHEC) *Citrobacter rodentium*	SseK/NleB (GT)	GT44 (GT88)	GT-A Retaining S_Ni Metal-ion dependent	GlcNAc
Epsilonproteobacteria: *Campylobacter* sp. Some *Helicobacter* sp. *Wolinella succinogenes* Deep-see vent bacteria [81] Deltaproteobacteria: *Desulfovibrio* sp.	PglB (OT)	GT66	GT-C Inverting S_N2 Metal-ion dependent	Various oligosaccharides
Pasteurellaceae and *Neisseriaceae*	HMW1C/NGT (GT)	GT41	GT-B Inverting S_N2? Metal-ion independent	Glc, Gal

[73]; the PglF dehydratase [74]; and the PglK flippase [75,76]. Ramirez *et al.* [77] recently solved the structure of *C. jejuni* PglH, a GT-B GTase catalyzing the transfer of exactly three α1,4 *N*-acetylgalactosamine (GalNAc) residues most likely following an S_N1-like mechanism. PglH contains an amphipathic 'ruler helix' that has a dual role of facilitating membrane attachment and glycan counting. Interestingly, the ruler helix contains three positively charged side chains that bind the pyrophosphate group of the lipid-linked oligosaccharide (LLO) substrate and thus limit the addition of GalNAc units to three [77]. Recently, the Locher group solved the ternary complex structure of the *C. lari* PglB OTase, first bound to a non-hydrolyzable LLO analog and a wildtype acceptor peptide [78••], and subsequently with a reactive LLO analog and an inhibitory (Dab) peptide [79••] accurately capturing the transition state of the glycosylation reaction and revealing how PglB interacts with a reactive LLO molecule [79••] (Figure 2d). To access the PglB active site, the glycan moiety of the LLO must thread under the disordered EL5 loop which then becomes ordered. Interestingly, reducing EL5 mobility decreases the catalytic rate of the OTase when full-size heptasaccharide LLO was provided, but not for a monosaccharide-containing LLO analog [78••]. In addition, a conserved DXXK motif has been shown to interact both with bound peptide and with the LLO [78••] confirming results of an earlier study that has predicted this motif in concert with EL1 and EL5 is essential for binding of the *C. jejuni* donor undecaprenyl-pyrophosphate and subsequent PglB-mediated *N*-glycosylation and free oligosaccharide release [80].

Conclusions

A variety of bacterial *N*-glycosylation pathways have been identified over the last two decades; however, these systems have so far only been observed in Gram-negative proteobacteria. Several advances have been made toward the use of these OTases/GTases in heterologous and cell-free systems to design both glycoconjugate vaccines and therapeutic proteins. In their native hosts, glycoprotein modification serves many biological functions and so the biosynthetic pathways are also attractive targets for novel intervention strategies. The bacterial protein glycosylation field continues to expand and to generate a wealth of tools that enables the engineering of glycan structures that were previously unimaginable, but already transforming our ability to produce novel glycoconjugates for the future.

Conflict of interest statement

Nothaft and Szymanski are both affiliated with VaxAlta Inc. and are developing glycoconjugate vaccines using bacterial expression systems.

Acknowledgements

This review is dedicated to Dr Patricia Guerry. It was in her laboratory that the *C. jejuni* *N*-linked protein glycosylation pathway was first discovered.

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Papers of particular interest, published within the period of review, have been highlighted as:

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- •• of outstanding interest

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