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The Surprising Structural and Mechanistic Dichotomy of Membrane-Associated Phosphoglycosyl Transferases

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Abstract

Phosphoglycosyl transferases (PGTs) play a pivotal role at the inception of complex glycoconjugate biosynthesis pathways across all domains of life. PGTs promote the first membrane-committed step in the *en bloc* biosynthetic strategy by catalyzing the transfer of a phospho-sugar from a nucleoside diphospho-sugar to a membrane-resident polyprenol phosphate. Studies on the PGTs have been hampered because they are integral membrane proteins, and often prove to be recalcitrant to expression, purification and analysis. However, in recent years exciting new information has been derived on the structures and the mechanisms of PGTs, revealing the existence of two unique superfamilies of PGT enzymes that enact catalysis at the membrane interface. Genome neighborhood analysis shows that these superfamilies, the polytopic PGT (polyPGT) and monotopic PGT (monoPGT), may initiate different pathways within the same organism. Moreover, the same fundamental two-substrate reaction is enacted through two different chemical mechanisms with distinct modes of catalysis. This review highlights the structural and mechanistic divergence between the PGT enzyme superfamilies and how this is reflected in differences in regulation in their varied glycoconjugate biosynthesis pathways.

INTRODUCTION

Glycoconjugates serve essential functions in all domains of life. In prokaryotes, diverse biomolecules, such as the bacterial peptidoglycan (PG) and archaeal S-layer glycoproteins afford mechanical stability to unicellular organisms in rapidly changing environments. Also,

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complex glycolipids, including the O-antigen component of the lipopolysaccharide (LPS) of Gram-negative bacteria, function to mediate interactions amongst cells and propagate deleterious pathogenic processes (1, 2). In eukaryotes, glycosylation is highly significant in numerous aspects of biology. For example *N*-linked protein glycosylation contributes to myriad functions from protein folding to intercellular communication (3). With these examples of the central biological roles of glycoconjugates in mind, understanding the molecular logic of the biosynthesis of these complex non-templated biomolecules is an important and rich area for investigation.

Glycoconjugate biosynthesis occurs via two general strategies - designated as sequential and *en bloc* (2). The sequential mechanism is commonly associated with assembly of smaller, less complex glycans than the *en bloc* strategy. This review will focus on enzymes that catalyze the first key step in *en bloc* glycan assembly and glycoconjugate biosynthesis. *En bloc* glycoconjugate assembly is initiated on the cytoplasmic face of cellular membranes, including the outer and inner membranes of Gram-positive and Gram-negative bacteria, respectively, and at the endomembrane system of eukaryotic cells. These sites of biosynthesis are advantageous, as glycoconjugates commonly must be integrated into cell-surface structures or secreted from cells for function. For example, the cross-linked peptidoglycan polymer is localized to the periplasmic space of Gram-negative bacteria and provides a thick outer layer coating the cellular membranes of Gram-positive bacteria. A signature feature of the early steps in glycoconjugate biosynthesis is the stepwise assembly of glycans on membrane-resident long-chain linear polyprenol phosphates (PrenPs), such as undecaprenol phosphate (UndP), which is the most common PrenP in bacteria (Figure 1) (4). These terminal secondary metabolites are highly conserved in glycan assembly pathways. To date, a complete understanding of the role of the amphiphilic, long-chain PrenPs, which range from seven to 20 or more isoprene units, is unclear. The simplest view that these "superlipids" (5) serve as membrane-associated anchors upon which to assemble glycans is certainly a part of the story (5, 6). However, unique effects of extended PrenPs on membrane order and pathway assembly and flux may also be pivotal (7, 8).

The first membrane-committed step in glycoconjugate biosynthesis is catalyzed by phosphoglycosyl transferases (PGTs) (Figure 1). This step is then followed by the action of a series of enzymes, acting at the membrane interface, which systematically elaborate the initial polyprenol diphosphate-linked carbohydrate (PrenPP-CHO). In recognition of the fact that PGTs catalyze the initial key step in the biosynthetic pathways in which they feature, these enzymes are often referred to as "priming glycosyl transferases". However, this is an imprecise term as the enzymes are not formally glycosyl transferases and in fact, they catalyze a very distinctive phospho-sugar transfer reaction. PGTs have also been classified based on the nature of their nucleoside diphospho-sugar (NDP-sugar) substrates (9) – namely, as the polyisoprenol-phosphate N-acetyl hexosamine-1-phosphate transferase (PNPT) and polyisoprenol-phosphate hexose-1-phosphate transferase (PHPT) families. However, discovery of additional PGTs with greater structural diversity and increasing knowledge of the breadth of substrate specificity have blurred the lines of this latter definition (10–12).

Membrane-associated pathways involving the PGTs lead to a variety of glycoconjugates (Figure 1). In bacteria, the diversity is extraordinary due both to the wide range of different glycoconjugate scaffolds that are assembled and also to the remarkable array of prokaryote-specific carbohydrate building blocks (13). In eukaryotes, although the glycans are less diverse in terms of carbohydrate composition (14), these pathways, including the *en bloc* strategy *N*-linked protein glycosylation pathway, are essential for innumerable cellular processes (3). In *en bloc* glycoconjugate assembly, there are two PGT superfamilies that mediate phospho-sugar transfer from an activated NDP-sugar to a membrane-resident PrenP on the cytoplasmic face of cellular membranes. Representative structures of PGT superfamily members published in the last ten years reveal functional core structures that are either polytopic, including multiple membrane- spanning domains, or monotopic and resident of a single leaflet of the lipid bilayer (Figure 1). The fundamental basis for the prominent structural dichotomy is still unknown however, it is now clear that it results in the adoption of different mechanistic and catalytic strategies as well as opportunities for differential regulation. These themes with be discussed herein.

PGT SUPERFAMILY TOPOLOGIES AND THE PATHWAYS TO WHICH THEY CONTRIBUTE

PGTs belong to two superfamilies, exhibiting strikingly different membrane-interaction modes of the functional cores (Figure 2A). The polytopic PGT (polyPGT) superfamily members are found in prokaryotes and eukaryotes. The common structural features of polyPGTs include a membrane-associated architecture comprising 10–11 transmembrane helices (TMHs) linked by soluble cytoplasmic loops that are involved in catalysis (15, 16). In contrast, the other PGT superfamily includes a monotopic functional core and thus is designated as the monotopic PGT (monoPGT) superfamily highlighting this topology. The monoPGT members are exclusively prokaryotic (17, 18). Extensive bioinformatic analysis reveals that the various monoPGT structures include a common monotopic functional core featuring a membrane-protein architecture that does not traverse the membrane but rather, engages *only* a single leaflet of the lipid bilayer *via* a re-entrant membrane helix (RMH) (12). The monoPGTs may simply comprise the signature functional core, as observed in the "small" monoPGT family exemplified by the *Campylobacter jejuni* PglC. Alternatively, the functional core may be elaborated at the N- or C-terminus (17). The most abundant monoPGT family, designated as the "large" monoPGT family, features an N-terminal extension that includes a series of four concatenated TMHs and a soluble cytoplasmic domain of unknown function. The topology of this monoPGT family was first elucidated experimentally by Valvano and coworkers for the *Escherichia coli* WcaJ (19). The third monoPGT family includes the "bifunctional" members, which are elaborated at either the N- or C-terminus with an additional functional domain. Recently, a sequence similarity network (SSN) analysis, representing over 38,000 non-redundant sequences, has been applied to the monoPGT superfamily. The analysis revealed the wide range of functional domains, including sugar-modifying enzymes, glycosyl transferases or regulatory domains, appended to the functional monoPGT core (17). Topology diagrams for representative polyPGTs (TarO and MraY also known as translocase 1) and the three families of monoPGTs including the minimal (PglC) and elaborated (PglB and CpsE) are illustrated in Figure 2A.

The study of bacterial PGTs and the pathways that they initiate is particularly informative as genes encoding glycoconjugate biosynthesis enzymes are often organized into operons that provide insight into entire pathways. Alternatively, genes that are not in a single operon have been annotated in the context of other pathway components that may be in different genome locations (Figure 2B). Analysis of selected pathways shows that the different PGT superfamilies may be represented in separate pathways within the same organism. For example, in *Staphylococcus aureus*, the polyPGT, TarO, acts at the beginning of the wall teichoic acid (WTA) pathway (20) and a small monoPGT, CapM, features at the initiation of capsular polysaccharide (CPS) assembly (21). In some pathways, the PGT genes are located adjacent to genes that encode enzymes related to the function of the PGT. For example, the small monoPGT gene *pglC* (*C. jejuni*) is found adjacent to the *pglF*/*pglE*/*pglD* gene cluster, which encodes enzymes that convert UDP-GlcNAc into UDP-*N,N'*-diacetylbacillosamine (UDP-diNAcBac), the prokaryote-specific UDP-sugar substrate of PglC. Intriguingly, in *N. meningitidis* and *N. gonorrhoeae*, the gene that encodes the monoPGT (*pglB* in *Neisseria*) also encodes a domain that catalyzes AcCoA-dependent acetylation of UDP-2'-acetamido-4'-amino-bacillosamine, the precursor of UDP-diNAcBac, the substrate of the monoPGT domain of PglB. Therefore, the *Neisseria* PglB is bifunctional and catalyzes the same transformations as PglD and PglC from *Campylobacter*. Alternatively, pathway genes may encode enzymes for the biosynthesis of elaborate PGT substrates such as the Park's nucleotide (UDP-MurNAc-pentapeptide) that is utilized by all bacterial MraYs for peptidoglycan biosynthesis. Such is the case with *murE*, *murF*, and *murD*, which are adjacent to *MraY*, in the *E. coli* pathway (Figure 2B) (22). By far the most common genome neighbors in the PGT-associated pathways are the glycosyl transferase (GT) genes (17). This is the case for *cpsF* and *cpsG* that are adjacent to the monoPGT gene *cpsE* from *S. pneumoniae* (23), *capL*, which is adjacent to *capM* in *S. aureus* (21) and *pglA*, which is adjacent to *pglC* in *C. jejuni* (24, 25). Some of the most important gene network components are those that are implicated in the regulation of glycoconjugate biosynthesis. Selected examples will be discussed in detail in a later section.

Ultimately, the extensive analysis of genomic data from different bacterial species and from genetic variants of the individual species, together with knowledge of the two PGT superfamilies that represent the hallmarks of PGT-dependent pathways will fuel new discovery. For example, informatics analysis will enable not only recognition of enzyme-interaction partnerships and regulation, but also provide the foundation for developing methods to predict the composition of glycoconjugates produced in selected pathways, and, potentially, the phenotypic effects of strain variation.

PHOSPHOGLYCOSYL TRANSFERASES – TWO ROUTES TO ONE DESTINATION

Historically, the first PGT superfamily to be defined included members showing the polytopic architecture. This was in part due to their importance as pharmacological targets and in part a result of the key role played by nucleoside natural product inhibitors in providing insight into the physiological significance of the enzymes (26). The core fold of the polytopic PGT superfamily (SCOP MraY-like family PF00953) includes members

typified by the *Clostridium bolteae* MraY (PDB 5JNQ) (27), which has ten TMHs in which the four N-terminal helices have an antiparallel topology and flank the six C-terminal helices which form two interlocking tandem repeats of three helices each (28). The *Homo sapiens* polyPGT known as UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosamine phosphotransferase (DPAGT1: PDB 6FWZ) (29, 30) shares the core fold of MraY with an excursion preceding the ultimate helix. Despite the low sequence identity between these prokaryotic and eukaryotic polyPGTs (16% identity; 29.7% similarity) their structural homology is clear (RMSD 3.3 Å). Thus far, members of the polyPGT superfamily have been shown to be dimeric (27–30) with a common interface (although the residue composition differs), supporting enzyme stability within each of the active protomers (29).

Elucidating how the polyPGT active site supports the catalytic mechanism has been of significant interest to gain insight into inhibitor design. As with all two-substrate enzymes, the polyPGTs may proceed through either the formation of a ternary complex, in which both substrates are concurrently bound to the enzyme, or a ping-pong mechanism, in which reaction of the first substrate releases the first product and creates a covalent intermediate that is subsequently acted upon by the second substrate. Originally, there were conflicting reports regarding the mechanism of the polyPGT-catalyzed reaction, with some of the original studies hampered by the difficulty of obtaining purified samples of the challenging integral membrane enzymes. However, in 2016, several key experiments with pure enzyme elegantly and convincingly demonstrated that the polyPGT reaction follows the ternary complex model (Figure 3A). Liu et al. (31) probed the mechanism through isotope exchange studies, in which *Bacillus subtilis* MraY was incubated with [$^{14}N_2$]-UDP-MurNAc-pentapeptide and [$^{15}N_2$]-UMP, in the presence and absence of the PrenP substrate, followed by LC/MS analysis. In the presence of the PrenP, both [$^{15}N_2$]-UDP-MurNAc-pentapeptide and [$^{14}N_2$]-UMP were observed, indicating that isotope exchange had occurred. However, these products were not detected in the absence of PrenP, thus supporting a ternary complex. Additionally, steady-state kinetic analysis fits to a random binding model where complex formation with both UDP-MurNAc-pentapeptide and PrenP substrates are required for UMP and Lipid I product release. Al-Dabbagh et al. (32) used isotopic-exchange studies, with [^{14}C]-labeled reactants and products, to draw similar conclusions. Furthermore, the potency of tunicamycin, the mureidomycins, and other bisubstrate analogs as inhibitors of polyPGTs supports the one-step, ternary-complex reaction mechanism (26).

In contrast to the polyPGTs, the monoPGT enzymes, typified by *Campylobacter concisus* PglC and *Salmonella enterica* WbaP differ dramatically in both structure and mechanism. The monoPGT enzymes share a core catalytic fold (11) where the signature structural motif is a α-helix-associated β-hairpin (designated the AHABh motif, PDB 5W7L) (12). The long β-hairpin of the AHABh motif is joined to two of the three amphipathic helices and to an extended loop structure and a double-twisted loop. These secondary structure elements make up the cytoplasmic module of the enzyme. At the N-terminus, two α-helices form a helix-break-helix motif comprising the RMH, which together with hydrophobic portions of the three amphipathic helices, make up the membrane-embedded module (thumbnail, Figure 1). The use of the RMH and amphipathic helices parallel to the membrane surface enforces

interaction with a single leaflet and hence the monotopic membrane topology for which the superfamily is named.

Perhaps it is not surprising that the difference in molecular architecture of the polytopic and monotopic PGT superfamilies is mirrored by a divergence in the enzymatic mechanisms. In the monoPGT superfamily the same fundamental two-substrate reaction is catalyzed through a covalent phosphoglycosyl intermediate formed on the carboxyl group of an active site aspartyl residue (Figure 3B). The aspartyl-phosphoglycosyl intermediate was positively identified via reductive cleavage with sodium [^{3}H]-borohydride (33), consistent with the known reactivity of the acyl-phosphate bond toward negatively charged nucleophiles (34). Moreover, in contrast to the findings in the case of the polyPGTs, Das et al. showed exchange of labeled 5'-[2-^{14}C]-UMP into substrate sugar donor UDP-diNAcBac in the absence of the acceptor UndP, providing further support for a Bi-Bi ping-pong mechanism (33). It is also noteworthy that the monoPGTs are not inhibited by nucleoside antibiotics such as tunicamycin (35) which are most effective against the enzymes exhibiting a ternary complex mechanism.

A CATALYTIC CONUMDRUM

Is there an evolutionary driving force for the recruitment or invention of two different scaffolds to catalyze the same chemical reaction? As discussed below, this dissimilarity in scaffold could allow for differential regulation of two pathways with a shared membrane-embedded substrate (36) (Figures 4A and 5A). Parenthetically, the linear PrenPs comprise a very low percentage of the lipidic fraction of membranes, approximately 0.1 %, creating the need for strict regulation to control use of the precious secondary metabolite (6, 37). Furthermore, the particular scaffold may, consequentially, be more suited to a Bi-Bi ping-pong versus ternary-complex mechanism. Previous bioinformatic and mutagenesis studies of polytopic family members MraY and WecA identified a conserved motif of aspartyl residues across the superfamily, which is essential for catalysis and was proposed to provide the Mg^{2+}-coordinating residues and nucleophile (Asp93, 94 and 231, respectively, *C. bolteae* numbering throughout) by analogy to prenyl transferases and terpene cyclases (38). Later, the *Aquifex aeolicus* structure of MraY (PDB 4J72) revealed that, of these residues, only Asp231 is within distance to coordinate to a bound Mg^{2+} ion (28). Overlay of the structure of MraY with that of human DPAGT1 (PDB 6FWZ, V264G variant (Figure 4A)) in complex with UDP-GlcNAc and Mg^{2+} ion shows that the other protein residue that coordinates to Mg^{2+} corresponds to Asn172 (29). Notably, these divalent cation-coordinating residues are two of only 12 amino acids conserved in all prokaryotic and eukaryotic sequences of the polyPGTs (38). The Mg^{2+} can act to neutralize the negative charge in the near-attack conformation and in the transition state. In ternary-complex formation, catalysis by approximation is expected to contribute significantly to reactivity and Mg^{2+} potentially plays a role in orienting the incoming UndP phosphoryl group with respect to the electrophilic phosphorus center (as judged by the relative positions in an overlay of UDP-GlcNAc and Mg^{2+} (PDB 6FWZ) and the branched chain acyl moiety of tunicamycin (PDB 6BW6) (Figure 4A, inset)). The co-positioning of two substrates is critical to the catalytic mechanism. Examination of the relative disposition of the polyprenyl moiety with respect to the electrophilic phosphate group of the activated sugar highlights catalytic residues.

In terms of catalytic strategy, such S_N2-type direct displacement reactions are enabled by an enzymatic general-acid catalyst activating the PrenP for attack, potentially with Lewis-acid activation of the departing UMP leaving group. Lys111 and Lys97, respectively, are positioned to potentially play these roles (Figure 4B). With a pK_a of 6.4, the UMP leaving-group protonation is expected to be facile.

In the monoPGTs, a mixed carboxylic–phosphoric anhydride intermediate kinetically and thermodynamically activates the phosphoryl group towards nucleophilic substitution, allowing the observed stepwise chemical transformation (33). In the second half-reaction, the electron-withdrawing aspartyl acyl group increases the electrophilicity of the phosphorus atom and lowers the energy of the transition state by stabilizing the developing negative charge on the aspartyl leaving group. The thermodynamic stability of such intermediates has been explained, in part, by protection of the active site from bulk solvent (39). Within the Asp93-Glu94 dyad (*C. concisus* PglC numbering), Asp has lower side-chain entropy than Glu (40) and this may be a driving force for its selection as the nucleophile, to attain greater catalytic efficiency (41). Additional orientation and activation of the attacking aspartyl carboxylate is afforded by positioning of Asp93 as the "cap" of a 3_{10} helix. A kink formed by Pro96 in concert with residues with low helical propensity (Ser91, Asp93, Glu94) distorts the N-terminal end of the helix (residues 92–96) into this 3_{10}-helix geometry. In such a helix, each turn, defined by the hydrogen-bonding partners, comprises ten atoms, making it more tightly-wound than a canonical α-helix. As a result, the catalytic Asp93-Glu94 dyad is brought into closer proximity than would be possible for two residues that are adjacent in primary structure in a typical α-helix (see Figure 5B for distances and comparison with a canonical α-helix). This proximity allows the proposed shift in coordination of the Mg^{2+} cofactor, facilitating the attack of the PrenP phosphoryl oxygen on the phosphoglycosyl aspartyl intermediate (Figure 5C). Moreover, participation of the Asp93 side-chain carboxylate oxygen in a helix-capping hydrogen bond, in addition to coordination of the catalytically-required Mg^{2+} ion, contributes to the nucleophilic reactivity of the non-coordinating oxygen via a disruption of resonance stabilization.

As it does for the polyPGTs, the Mg^{2+} cofactor in the monoPGTs can enhance electrophilicity at the phosphorus center by neutralizing the negative charge in the transition state and can serve to co-position the nucleophile and electrophile and to increase the acidity of the leaving group. The charge on the nucleophilic Asp carboxylate is shielded from the electrophilic center by the Mg^{2+} cofactor and side-chain functionality. Moreover, the divalent cation shields electrostatic repulsion of the attacking PrenP phosphate from the phosphorus center in the second half-reaction. Model studies using isotopic labeling on acetyl phosphate show that the Mg^{2+} ion favors P-O cleavage whereas with Ca^{2+}, C-O cleavage predominates (42). Thus, the metal ion cofactor is critical to both reactivity and selectivity of the second half reaction.

THE SCAFFOLD AS A STAGE FOR REACTION AT THE INTERFACE

The TMHs in the polyPGT scaffold culminate in loops that serve to form the active site, positioning the binding site for the water-soluble nucleotide-activated substrate at the interface for reaction with the membrane-resident PrenP substrate. Rotational and

translational calculation of the placement of DPAGT1 (PDB 6FWZ) in the membrane environment using the OPM server (43) places the electrophilic phosphoryl moiety of the bound UDP-GlcNAc coincident with the lipid headgroups (Figure 4A). This allows the co-positioning of this group with the nucleophilic PrenP phosphoryl moiety in the ternary complex without the energetically unfavorable removal of the PrenP prenyl chain from the hydrophobic lipid-tail environment (44, 45). In the monoPGTs, positioning of the Asp-Glu catalytic dyad on an amphipathic helix, likewise, positions the active site at the cytoplasmic interface with the membrane, estimated to be ~3Å from the membrane surface in *C. concisus* PglC (44) (Figure 5A). This "leave in place" strategy, describing the location of active sites, either at the interface or at a distance from the interface that does not necessitate extraction of the hydrophobic moiety of the membrane-resident substrate, is also utilized in other non-homologous monotopic enzymes such as WaaA (46) and PglH (47).

A difference in strategy for substrate colocalization is revealed by the analysis of the conservation of cationic residues within the mono- and polyPGT superfamilies. In the monoPGTs there is conservation of motifs containing arginine across all three families which act to frame and control the electrostatic environment of the active site. Mapping of the electrostatic charge onto the molecular structure *of C. concisus* PglC reveals numerous basic residues forming a positive "electrostatic funnel" poised for binding and orienting the negatively-charged phosphate-rich UDP-diNAcBac substrate (Figure 5D). Such cation-rich motifs are not revealed in the analysis of the polyPGTs and therefore, substrate binding may be more dependent upon colocation of these metabolites.

REGULATION - LEVELING THE PLAYING FIELD

Control of specific enzyme catalysts becomes more critical in pathways that share a common substrate. Indeed, studies using variants of enzymes in the outer membrane glycolipid pathway in *E. coli* suggest that there is competition for the pool of PrenP, wherein appropriate distribution among competing metabolic pathways is required to maintain cell shape (36). Examination of the steady-state kinetic constants for prototypes from the two PGT superfamilies highlights the necessity for control. A global approach utilizing omics data confirms that *in vitro* enzyme assays generally accurately reflect the maximal rates of enzyme-catalyzed reactions *in vivo* (48). A comparison of either k_{cat}, which defines the reaction rate of the PGTs under saturating substrate concentrations or of k_{cat}/K_m, which defines the reaction rate under low substrate conditions, finds that, unchecked, the monoPGT-catalyzed reaction would be faster than that of the polyPGT. For *C. jejuni* PglC k_{cat} is 3.7-fold greater than that of *B. subtilis* MraY and the k_{cat}/K_M^{UndP} is 50-fold greater (31, 33). Thus, differential regulation of the two pathways is critical, lest the rare PrenP substrate be preferentially partitioned into glycoconjugate pathways initiated by the monoPGT reactions. Having different scaffolds associated with each pathway facilitates such regulation. Using similar arguments, availability of sugar substrates could also affect pathway flux.

Glycoconjugate biosynthesis pathways may be regulated either at the transcriptional level or at the enzyme level via post-translational modification (see Table 1). Transcriptional regulation of genes encoding enzymes in glycoconjugate assembly is employed in numerous

pathways as an effective means of glycoconjugate regulation in response to environmental and cellular cues. Pathway regulation in bacteria is also contingent on a common pool of the essential, but limiting, PrenP lipid carrier substrate (36). In this context, the PGT-catalyzed step itself is reversible, therefore problematic sequestration of the PrenPP-linked substrates occurs from dysregulation of subsequent steps further downstream of the PGT reaction in the pathway. *In vivo* studies of wall teichoic acid biosynthesis in *B. subtilis*, initiated by the polyPGT TagO (homologous to *S. aureus* TarO) have shown that inactivation of later genes in the biosynthetic pathway leads to buildup of dead-end PrenPP-linked products, which is lethal because the PrenP is sequestered from the accessible pool (49). In contrast, suppressor mutations of the CpsE (large monoPGT) gene in the *Staphylococcus pneumoniae* CPS biosynthesis pathway, prevent buildup of PrenPP-linked products but results in reduction of CPS levels on the outer membrane (50, 51). The suppressor mutations occur in both the PGT core domain and the cytoplasmic domain of unknown function, emphasizing the functional importance of the cytoplasmic domain.

In another example from *E. coli*, the two-component system that controls biosynthesis of colanic acid, an exopolysaccharide (EPS), initiated by the monoPGT WcaJ, shows regulation at the transcriptional level. In this case, a transmembrane sensor domain (RcsC), a response regulator (RcsB), and a positive regulator (RcsA) function to modulate the transcription and subsequent expression of colanic acid biosynthetic genes (52–54). At 37 °C, RcsA protein levels are low, and thus colanic acid is not synthesized. However, at lower temperatures or when under stress, the RcsC sensor domain phosphorylates the RscB regulator, which in turn activates RscA, leading to upregulation of genes in the colanic acid biosynthetic pathway. In the yeast *Saccharomyces cerevisiae*, there is considerable evidence that the entire dolichol pathway is transcriptionally regulated at the *alg7* gene (55), which encodes a polyPGT. Also, the mammalian homolog of Alg7, DPAGT1, is regulated at the transcriptional level by the Wnt/β-catenin signaling pathway and upregulation of *dpagt1* gene transcription leads to increased *N*-linked protein glycosylation of E-cadherin (56)

Although there is no evidence of *direct* regulation of the polyPGT MraY or its orthologs, there are numerous examples of regulation of the downstream enzymes in peptidoglycan biosynthesis. In the Gram-negative exopolysaccharide pathway two-component systems, PASTA-eSTKs are integral membrane proteins with a cytoplasmic kinase domain fused to numerous PASTA receiver domains (57). Stimulated by Lipid II or free muropeptides, PASTA-eSTKs phosphorylate specific enzymes, modulating their respective activities. The action of PknB, a PASTA-eSTK from *Mycobacterium tuberculosis* includes modulation of flippase, uridyltransferase/acetyltransferase and export activities (58–63). *Listeria monocytogenes* PrkA, an *M. tuberculosis* PknB homolog, phosphorylates YvcK and GpsB (64, 65), which are involved in cell-wall homeostasis and assembly of class A penicillin-binding proteins, respectively. Phosphorylation of these targets in *M. tuberculosis* and *L. monocytogenes* lead to regulation of peptidoglycan at numerous steps and via processes including inhibition of activity and changes in cellular localization.

There are only a few definitive studies on systems showing *direct* regulation of glycoconjugate biosynthesis at the enzyme level, although this has been proposed in many cases. However, it is likely that regulation specifically at the PGT step would be

favorable as it would control use of a limited pool of PrenP substrate across numerous pathways. In fact, the exopolysaccharide biosynthesis tyrosine kinase superfamily (InterPro IPR005702) comprises bacterial protein kinases which specifically phosphorylate enzymes in the monoPGT-associated exopolysaccharide (EPS) biosynthetic pathways (see Table 1) (21, 66–68). Gram-negative bacteria may express one protein with both the receiver domain and the kinase domain, as exemplified by the *E. coli* tyrosine kinase Wzc. Alternatively, Gram-positive bacteria, such as *S. aureus*, have two distinct proteins acting as the intrinsic membrane receiver domain (CapA) and the tyrosine kinase (CapB). The targets of these kinases include the monoPGTs, CapM (21) and EpsE (68), as well as the sugar-modifying enzymes, UDP-Glc dehydrogenase (Ugd) (67), UDP-ManNAc dehydrogenase (CapO) (69), and UDP-GlcNAc dehydratase (CapE) (21), where phosphorylation activates or increases activity. In addition to the kinase, a complementary phosphatase is often encoded in the same operon, to fine-tune pathway activity. In the case of *S. aureus* CPS, the phosphatase CapC dephosphorylates both CapE and CapM (21). Notably, *in vitro* analyses of CapM also identified it as a substrate of the PASTA-eSTK PknB at a conserved threonine. This phosphorylation serves to negatively regulate CapM, based on the decrease in lipid I_{cap} production measured *in vitro*, allowing for cross-pathway regulation of peptidoglycan and capsular polysaccharide biosynthesis (21).

Together, the *S. aureus* CPS pathway employs positive and negative regulation of glycoconjugate biosynthesis at key steps throughout the pathway. These are the first membrane-committed, monoPGT-dependent steps and two sugar-modifying steps that control NDP-sugar substrate production. Such regulation allows for tight control of common substrate pools (PrenP and UDP-GlcNAc, respectively). Notably, these regulatory events in pathways producing essential cellular wall components or necessary virulence factors in bacteria may serve to control the pathogenesis of these organisms (70). Further, in commensal bacteria, regulation of CPS or EPS production may modulate their respective host-cell interactions (71).

CONCLUSIONS

In nature, the polyprenol phosphate phosphoglycosyl transferases carry out a strategic transformation at the inception of membrane-associated, *en bloc* glycoconjugate biosynthesis pathways. Until relatively recently, the polyPGTs, which are found in eukaryotes and prokaryotes, were believed to represent the principal enzymes catalyzing phospho-sugar transfer to PrenPs. However, biochemical and biophysical evidence has emerged revealing that the polyPGT superfamily is juxtaposed with a distinct monoPGT superfamily that is exclusive to prokaryotes. Therefore, two PGT superfamilies, the polyPGTs and monoPGTs, have evolved to carry out an identical chemical transformation using completely different structural scaffolds and chemical mechanisms. This evolutionary "sleight of hand" is particularly remarkable given the significant physical constraints on the transformation that they catalyze. Poly- and monoPGTs are integral membrane proteins that catalyze phospho-sugar transfer from a soluble negatively-charged NDP-sugar substrate to an amphiphilic linear polyprenol phosphate, which is most favorably positioned with the nucleophilic phosphate directly at the membrane interface. Extracting the PrenP from the

membrane represents a formidable task and therefore, both PGT superfamilies have simply elected a "leave in place" strategy. But, this is where the similarity ends.

The difference between the mechanisms, with intermediacy of a ternary complex with NDP-sugar substrate and PrenP versus a phosphoglycosyl aspartyl intermediate, leads to very different affinity profiles for inhibitory nucleoside natural product ligands. The exclusive occurrence of the monoPGTs in prokaryotes, coupled to the opportunities afforded by the dissimilar scaffolds, opens the way to design inhibitors that distinguish these PGTs, in ways that substrates do not. Moreover, each PGT superfamily makes use of the low-abundance, but highly-conserved PrenP substrate, which anchors the product facilitating subsequent steps at the membrane. However, the binding mode through potential interactions with a transmembrane versus a re-entrant membrane helix must differ. This dichotomy offers an embarrassment of riches for the study of evolutionary pressures on the scaffolds themselves and local interplay of these fascinating enzymes with the membrane environment and in the presence of the membrane-associated PrenP substrate.

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Abbreviations

CPS	capsular polysaccharide
EPS	exopolysaccharide
LPS	Lipopolysaccharide
PASTA-eSTKs	penicillin-binding protein and serine/threonine kinase associated domain-containing eukaryotic-like serine/threonine protein kinases
monoPGT	monotopic phosphoglycosyl transferase
PG	peptidoglycan
PGT	phosphoglycosyl transferase
polyPGT	polytopic phosphoglycosyl transferase
PrenP	polyprenol phosphate
RMH	reentrant membrane helix
SSN	sequence similarity network
TMH	transmembrane helix
UndP	undecaprenol phosphate
WTA	wall teichoic acid

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PERSPECTIVES

- The existence of structurally- and mechanistically-distinct PGT superfamilies at the inception of glycoconjugate biosynthesis raises important questions related to the evolution of each superfamily and the biological imperative of this dichotomy.
- There is a need for additional high-resolution structures of mono- and polyPGTs in native-like membrane environments with bound ligands to reveal aspects of catalysis and regulation and for structure-guided inhibitor design. The definition of the monoPGT superfamily as prokaryote-specific, offers opportunities for therapeutic targeting of monoPGTs in bacterial survival and virulence-associated pathways.
- Although the catalytic mechanisms of the two PGT superfamilies are divergent, both employ linear polyprenol phosphate substrates and a "leave-in-place" strategy for action at the interface with this membrane-embedded substrate, pointing to evolution-driven necessity for scaffold recruitment and the possibility of constraints imposed by co-evolution with the membrane environment.

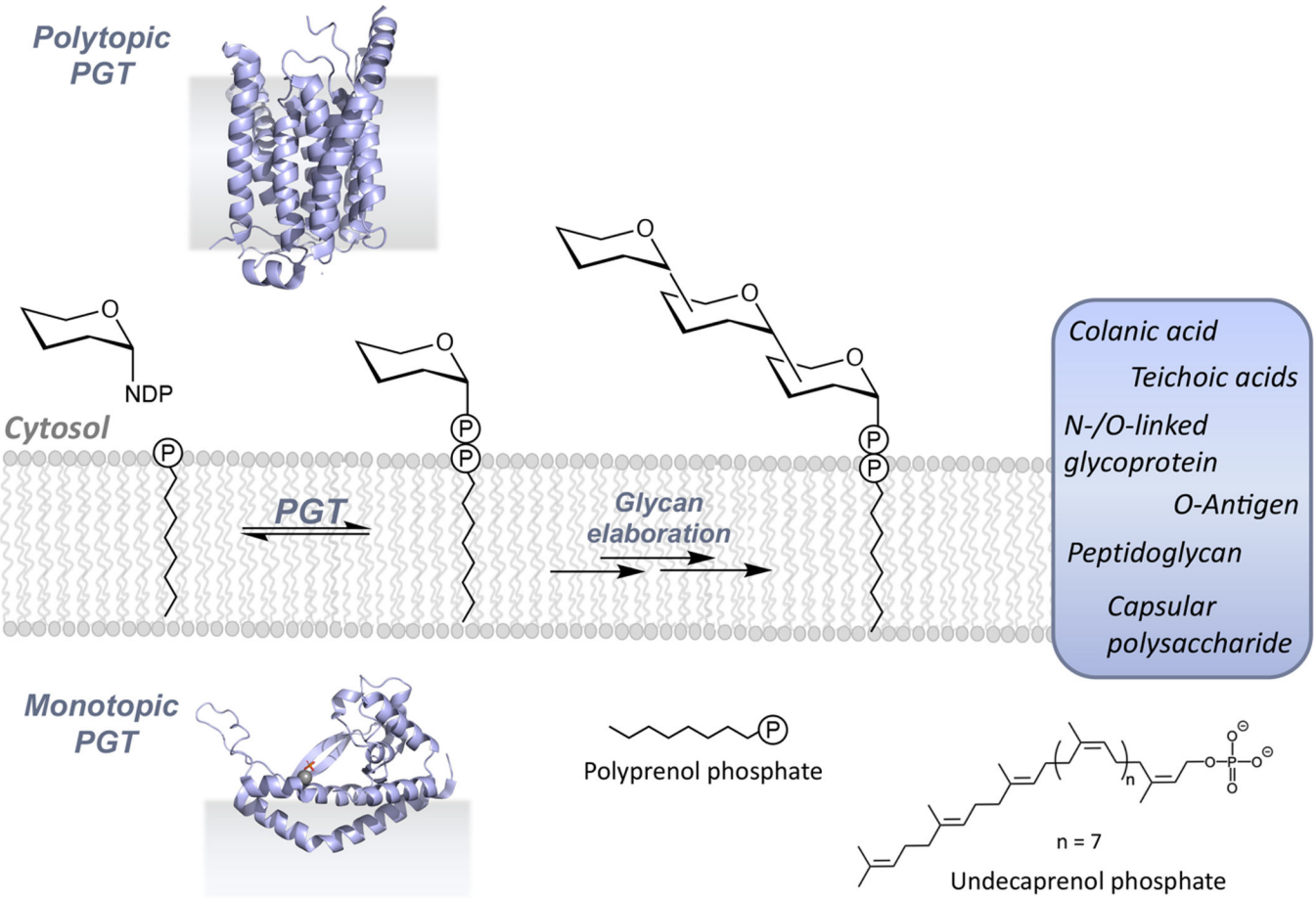

Figure 1. Enzymes and stepwise pathways in glycoconjugate biosynthesis

Polytopic and monotopic phosphoglycosyl transferases (PGTs) catalyze the initial, membrane-committed step in the *en bloc* mechanism of glycoconjugate biosynthesis. Inserts show ribbon diagrams of superfamily members PglC from *Campylobacter concisus* (PDB 5W7L) and MraY from *Aquifex aeolicus* (PDB 3CKR). Glycoconjugates are assembled via sequential modification of the PrenPP-linked product of the PGT reaction and ultimately polymerized and/or conjugated with lipids or proteins. PrenPs such as the bacterial undecaprenol phosphate are important lipid carriers in glycoconjugate assembly.

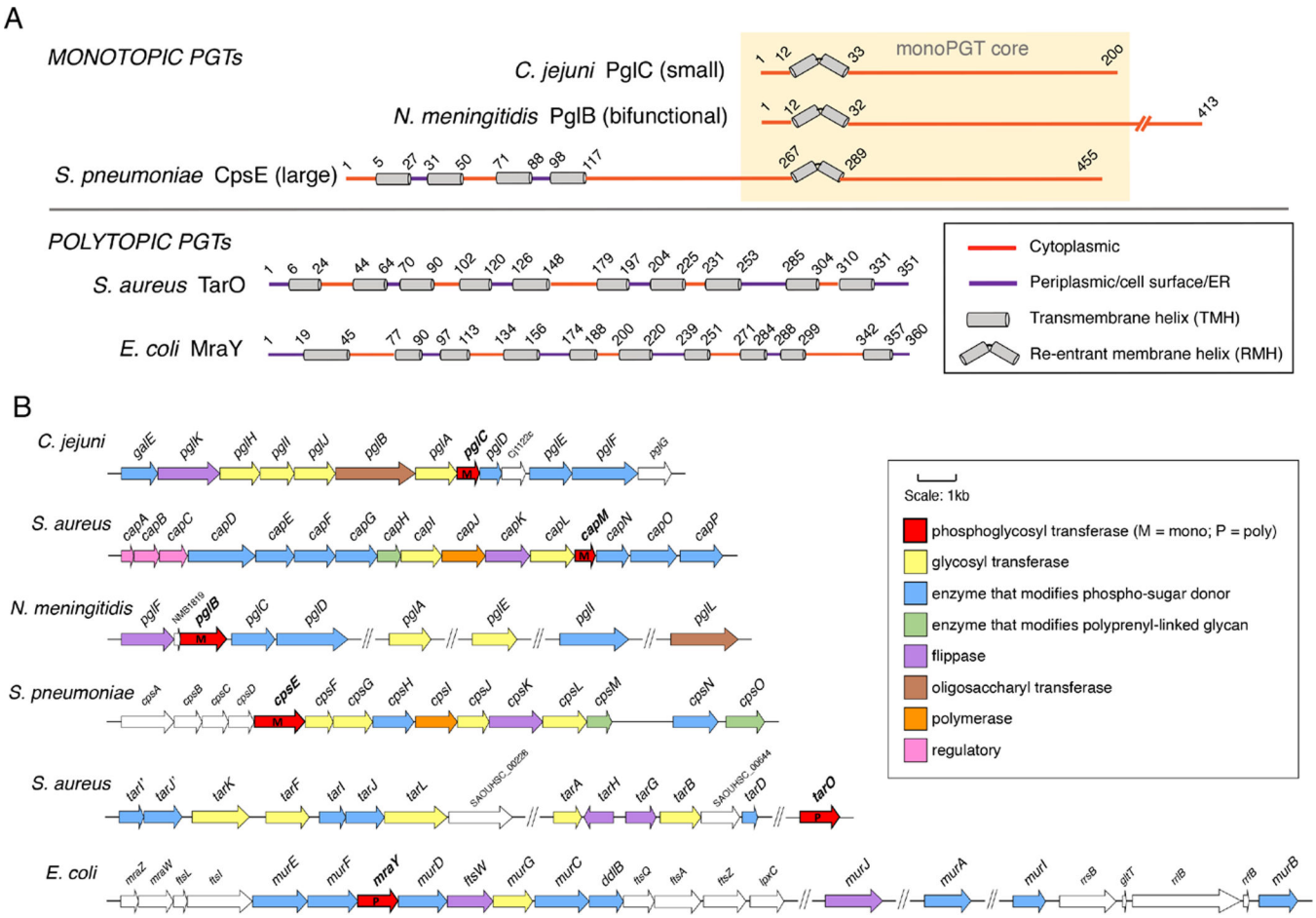

Figure 2. PGT topologies and genes associated with bacterial PGT-dependent pathways

A. Topology diagrams showing the disposition of TMH and RMH motifs for poly- and monoPGTs. The topology of PglC (*Campylobacter jejuni*) is determined *via* biochemical analysis and by analogy with the structure of the *Campylobacter concisus* PglC (PDB 5W7L). The topology of the mono-PGT domain in PglB (*Neisseria meningitidis*) is inferred by homology, and the topology of CpsE (*Streptococcus pneumoniae*) is deduced by analogy with the experimentally determined structure of WcaJ (*Escherichia coli*). The topologies of the TarO (*Staphylococcus aureus*) and MraY (*E. coli*) are based on the experimental structures of homologous polyPGTs, (6FWZ and 3CKR respectively). B. Bacterial glycoconjugate assembly genes and pathways featuring mono- and polyPGTs including pathways from: *C. jejun*i (NCTC 11168), *N*-linked protein glycosylation; *Staphylococcus aureus* (NCTC 8325) - capsular polysaccharide; *N. meningitidis* (MC58) - *O*-linked protein glycosylation; *S. pneumoniae* (Serotype 9V) - capsular polysaccharide; *S. aureus* (NCTC 8325) – wall teichoic acid; *E. coli* (K-12 W3110) - peptidoglycan. Functionally-annotated genes related to the PGT-containing pathways are color coded by function (see insert).

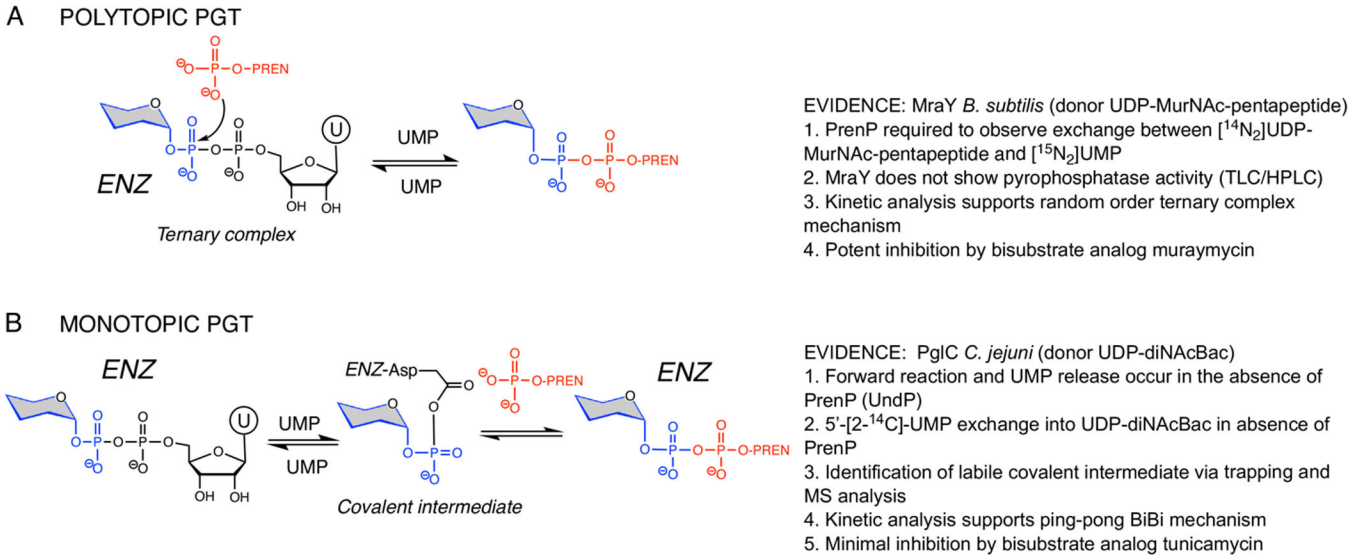

Figure 3. Summary of mechanistic studies on poly- and monoPGT enzymes

A. Experiments leading to mechanistic model and assignment of ternary complex (random binding) mechanism for the polyPGT MraY from *Bacillus subtilis* (31, 32). B. Experiments leading to mechanistic model and assignment of Bi-Bi ping-pong mechanism for PglC from *Campylobacter jejuni* (33).

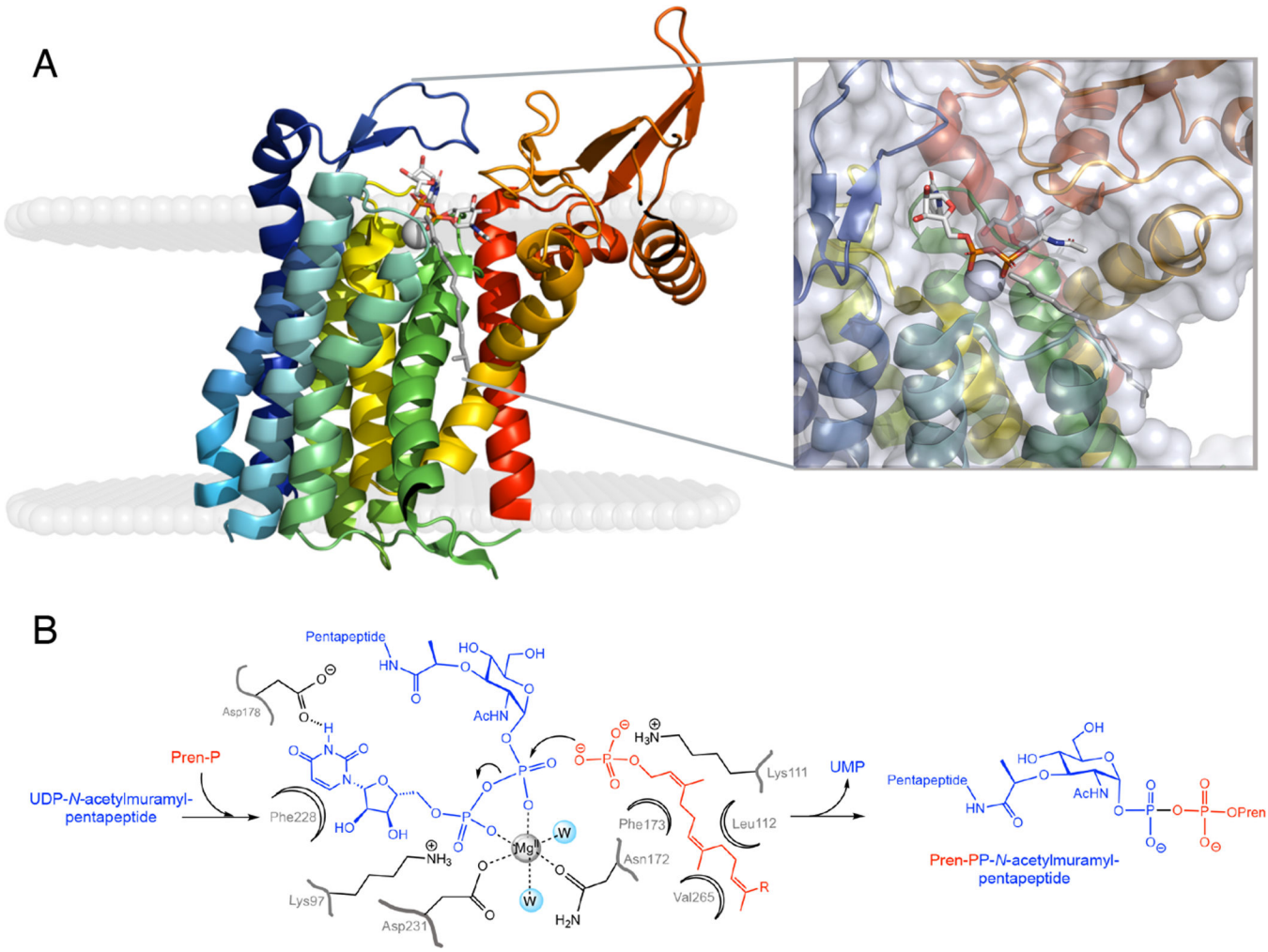

Figure 4. Structure and mechanism of the polyPGTs

A. Ribbon diagram of polyPGT DPAGT1 (PDB 6FWZ) color ramped from N- to C-terminus (blue to red) shown in membrane as calculated using the OPM server (43) with UDP-GlcNAc (white stick) and Mg^{2+} ion (grey sphere). Also shown is polyprenyl moiety (grey stick) from superposition of tunicamycin bound complex (PDB 6BW6). Inset shows close-up view of active site with protein transparent surface. B. Ternary complex mechanism of polyPGTs with proposed roles for binding and catalytic residues (numbering from *Clostridium bolteae* MraY).

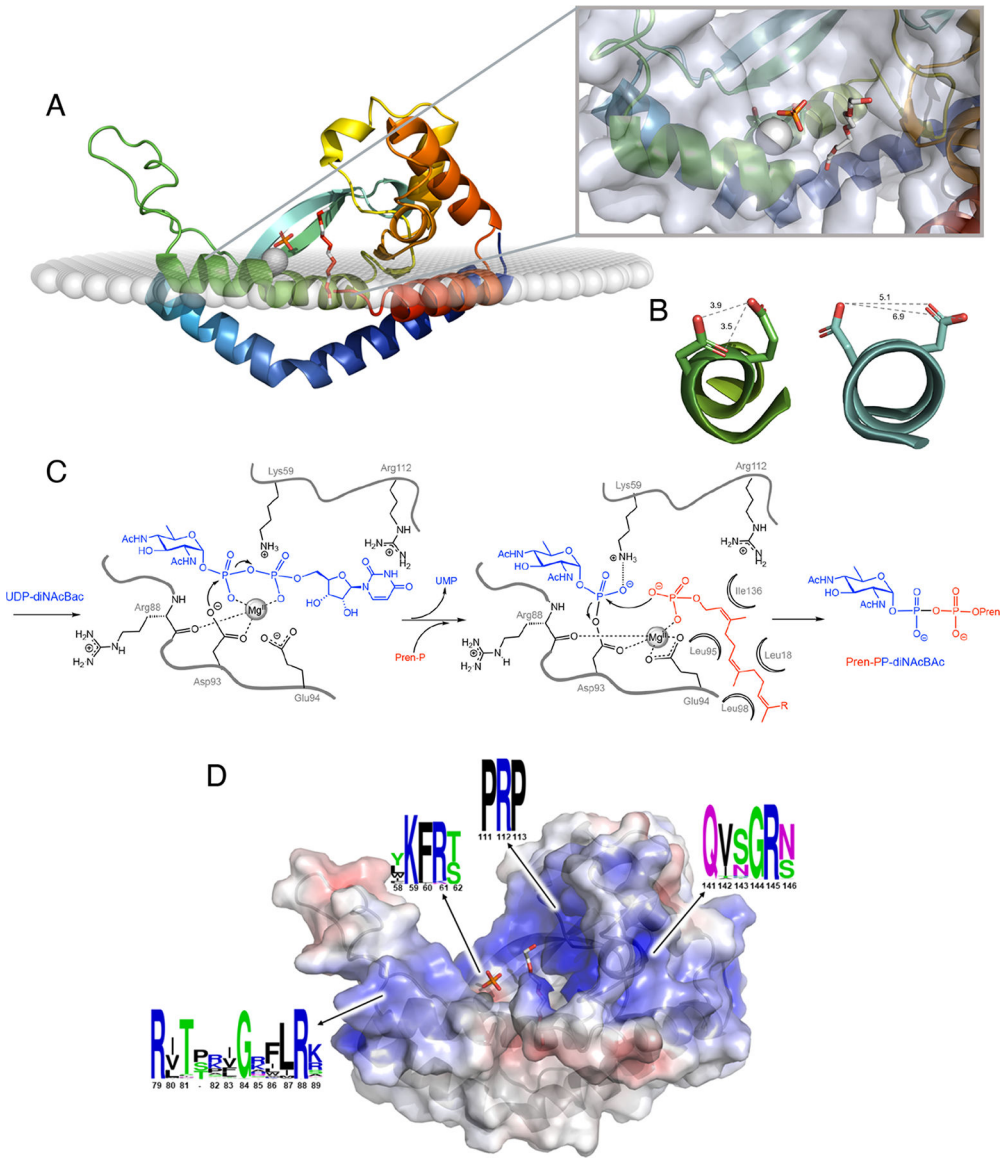

Figure 5. Structure and mechanism of the monoPGTs

A. Ribbon diagram of monoPGT PglC (PDB 5W7L) color ramped from N- to C- terminus (blue to red shown in membrane as calculated using the OPM server (43) with Mg^{2+} ion (grey sphere), phosphate and PEG (white stick; PEG molecule shows proposed position for UndP binding). Inset shows close-up view of active site with protein transparent surface. B. Active-site helix 3_{10}-geometry allows for co-facial positioning of Asp-Glu catalytic dyad. MonoPGT PglC residues 91–99 shown in ribbon representations colored as in panel A with side chains of the catalytic dyad Asp93-Glu94 shown as stick. PolyPGT DPAGT1 (PDB 6FWZ) residues 100–118 shown in ribbon representation colored as in Figure 4A with residues Asp115 and Asp116 from the conserved aspartyl motif shown as stick. In polyPGTs these residues do not play an analogous role in catalysis (see text). Shortest distances between oxygen atoms on proximal residues (gray dashed lines). C. Bi-Bi ping-pong mechanism of monoPGTs with proposed roles for binding and catalytic residues (numbering

from *Campylobacter concisus* PglC). D. Electrostatic mapping of PglC revealing positive funnel for substrate binding. Ribbon diagram of PglC with transparent surface colored by electrostatic potential ramped from −5 kT/e (red) to 5 kT/e (blue). Ligands shown as in panel A. The sequence conservation of positive pockets was calculated using sequences of all three classes of monoPGT (small, large, and bifunctional), visualized using WebLogo (*C. concisus* PglC numbering).

Table 1.

Regulation of PGT-initiated pathways

Regulatory Protein/Receiver Domain	PGT	Organism	Target protein	Regulated step	Effect of Regulation	Pathway
Polytopic Pathways						
PknB (PASTA-eSTKs)	MraY	*Mycobacterium tuberculosis*	GlmU	UDP-GlcNAc synthesis	reduced activity	PG
			MviN	glycan translocation	inhibited by FhaA	PG
			PonA1	transglycosylation/ transpeptidation	activation	PG
			CwlM	lipid II export via MurJ	cellular localization, activation of MurA	PG
PrkA (PASTA-eSTKs)	MraY	*Listeria monocytgenes*	YvcK	cell-wall homeostasis	unknown	unknown
			GpsB	transglycosylation/ transpeptidation	ClpCP-dependent degradation of MurA	PG
Wnt/β-catenin	DPAGT1	*Homo sapiens*	---	gene expression	increased N-glycosylation of E-cadherin	N-glycosylation
Monotopic Pathways						
RcsB/RcsC	WcaJ	*Escherichia coli*	RcsA	gene expression	RcsA regulates gene expression	colanic acid
CapB/CapA	CapM	*Staphylococcus aureus*	CapO	UDP-ManNAc synthesis	activation	CPS
			CapM	phosphoglycosyl transfer	increases activity	CPS
			CapE	UDP-FucNAc synthesis	increases activity	CPS
Wzc	WcaJ	*Escherichia coli*	Ugd	UDP-glucuronic acid synthesis	increases activity	colanic acid
CpsD/CpsC	CpsE	*Streptococcus pneumoniae*	unknown	unknown	regulates CPS length	CPS
EpsD/EpsC	EpsE	*Streptococcus thermophilus*	EpsE	phosphoglycosyl transfer	activation	EPS