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Probing Monotopic Phosphoglycosyl Transferases from Complex Cellular Milieu

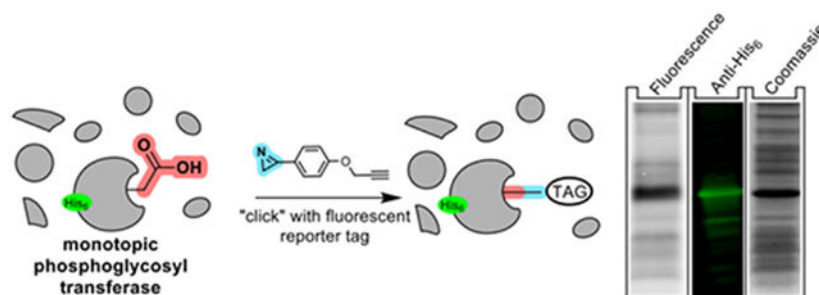
Alyssa J. Anderson^{a,†}, Leah M. Seebald^{a,†,§}, Christine A. Arbour^a, Barbara Imperiali^{a,*}

^aDepartment of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

Abstract

Monotopic phosphoglycosyl transferase enzymes (MonoPGTs) initiate the assembly of prokaryotic glycoconjugates essential for bacterial survival and proliferation. MonoPGTs belong to an expansive superfamily with a diverse and richly annotated sequence space; however, the biochemical roles of most monoPGTs in glycoconjugate biosynthesis pathways remain elusive. To better understand these critical enzymes, we have implemented activity-based protein profiling (ABPP) probes as protein-centric, membrane-protein compatible tools that lay the groundwork for understanding the activity and regulation of the monoPGT superfamily from a cellular proteome. With straightforward gel-based read outs we demonstrate robust, covalent labeling at the active site of various representative monoPGTs from unfractionated cell membrane fractions using 3-phenyl-2H-azirine probes.

Graphical Abstract



*Corresponding Author Barbara Imperiali - Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge 02139, United States, imper@mit.edu.

†Equal contributions

Alyssa J. Anderson – Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge 02139, United States

Leah M. Seebald - Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge 02139, United States

Christine A. Arbour - Department of Biology and Department of Chemistry, Massachusetts Institute of Technology, Cambridge 02139, United States

§Present Addresses

Haverford College, Haverford PA

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website <http://pubs.acs.org>. Supporting experimental methods include protein expression and CEF preparation, protocols for gels and Western blots, detailed synthetic procedures and full structural characterization of new compounds.

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INTRODUCTION

Bacterial glycoconjugate biosynthesis is initiated at the cytoplasmic face of the cell by phosphoglycosyl transferases (PGTs), which catalyze the transfer of a phospho-sugar from a soluble nucleoside diphosphate (NDP)-activated donor to a polyprenol phosphate (Pren-P) acceptor (Figure 1).¹ The membrane-bound PGT product then undergoes sequential glycosylations by the action of glycosyltransferases (GTs), and is ultimately flipped to the periplasm to afford the substrate for mature glycoconjugate biosynthesis. These glycoconjugates contribute to the structural integrity of cells and feature macromolecular landscapes that interact with human hosts, and include the O-antigen of lipopolysaccharide (LPS),^{2, 3} capsular polysaccharide (CPS),⁴ and peptidoglycan (PG).⁵

PGTs are represented by two broad enzyme superfamilies that are distinct in both structure and mechanism of action.⁶ The polytopic PGTs (polyPGTs) are exemplified by the mammalian GPT and bacterial WecA and have been shown to invoke a mechanism involving a ternary complex utilizing a conserved Asp-Asp-X-X-Asp motif.⁷⁻⁹ In contrast the monotopic PGTs (monoPGTs), exemplified by the *Campylobacter* PglC, invoke covalent catalysis exploiting an active site aspartic acid carboxyl group. This nucleophilic aspartic acid activity is unique and a hallmark of the mechanism of monoPGTs, which supports probe development with specificity towards the monoPGT superfamily.¹⁰

The monoPGTs are named for the distinct membrane topology of the minimal functional unit in which a re-entrant membrane helix penetrates a single leaflet of the bilayer and anchors the enzyme to the cytoplasmic face of the membrane. The monoPGT superfamily is exclusively prokaryotic and members share a signature functional core that can be elaborated at the N- or C- terminus with various modalities.¹¹ A recent sequence similarity network (SSN) analysis has classified over 38,000 non-redundant sequences in the monoPGT superfamily; the SSN has illuminated a wide range of functional domains, including sugar-modifying enzymes, glycosyl transferases or regulatory domains that may be appended to the monoPGT functional core.¹² The diversification of a common basic scaffold across the superfamily could allow for differential regulation of pathways with shared membrane-embedded substrates. In particular, one cluster in the SSN contained PGTs fused to regulatory domains, suggesting that PGTs may play a central role in the regulation of glycoconjugate biosynthesis pathways. Additional recent work showed that Cap5M, a monoPGT in the pathway for CPS production in *Staphylococcus aureus*, is activated upon Tyr phosphorylation by the kinase CapB,¹³ further corroborating the idea that regulation of bacterial glycosylation may occur at the PGT step.

To understand these critical regulatory processes, the gap in knowledge between gene sequence and protein activity must be addressed. Implementation of protein-forward techniques and analyses can serve to complement the invaluable genetic information uncovered by the SSN. However, protein-forward analyses typically rely on purified proteins, and membrane protein isolation and handling is rife with technical challenges: membrane proteins, such as the monoPGTs, are prone to low expression levels and solubilization from lipidic environments typically relies on detergents that can perturb structure and function, which can further promote aggregation and precipitation from

solution.¹⁴ As such, the expression and activity of monoPGTs is best studied in the context of the native complex cellular milieu.

Activity-based protein profiling (ABPP) is a powerful proteomics technique used to selectively interrogate the functional proteome by covalently targeting the active sites of classes of proteins with shared mechanistic signatures.^{15, 16} ABPP is uniquely suited for probing complex proteomes, and is suited to membrane protein analysis in lysates or in cell envelope fractions (CEFs). By extension, ABPP is potentially ideal as a proteomics approach for probing the expansive monoPGT superfamily. Biochemical studies and sequence analysis have identified a highly-conserved active site in the monoPGT superfamily, including an Asp-Glu dyad catalytic motif (Figure 2A). This motif contributes to monoPGT catalysis, through a Bi-Bi ping pong mechanism.¹⁰ In this mechanism, the first reaction step involves formation of a covalent phosphoglycosyl derivative of the carboxyl group of the active site aspartic acid (Figure 2B). Within glycoconjugate biosynthesis pathways, this mechanism is exclusive to monoPGTs and affords the potential for selective intervention as the nucleophilic Asp is primed for probing with active-site targeted electrophilic agents. In contrast, the polyPGTs include a conserved active site Asp-Asp-Xaa-Xaa-Asp motif that plays a role in substrate orientation and catalysis, but *not* in nucleophilic catalysis.

Carboxylic acid-modifying ABPP probes in general have shown notoriously poor amino acid specificity; amino acids that are more nucleophilic, such as cysteine or serine, can out-compete carboxylic acids in reactions with electrophiles.¹⁷ Typical moieties used for carboxylic acid labeling include carbodiimides, epoxides, diazo compounds, phenylsulfonate esters, α -chloroacetamides, and α - β unsaturated ketones.¹⁷⁻¹⁹ We explored many of these classical labeling options for monoPGTs. Compounds containing carbodiimides and phenylsulfonate esters resulted in nonspecific labeling, and compounds comprising epoxides and α - β unsaturated ketones led to no observable monoPGT labeling. Alternatively, specificity can be controlled employing a warhead approach by synthesizing known protein ligands strategically equipped with electrophiles. However, the warhead approach requires prior knowledge of either protein structure or protein ligands and many important enzyme classes do not yet have this information available. Notably, amino acids with carboxylic acid side chains have been shown to be efficiently labeled in glycosidases exhibiting the Koshland double displacement mechanism.²⁰ These probes employed deoxyfluorosugar substrate mimics²¹ as well as aziridine-based cyclophellitol inhibitor scaffolds.²²

Recently, Ma *et al.* have described a 2H-azirine (2H-Az) electrophilic moiety for carboxylic acid labeling,²³ and the 3-phenyl-2H-Az compounds were shown to be ideal for ABPP; the reactive group is small, stable and shows carboxylate-selective reactivity. Utility was demonstrated in live cells, and derivatives can be prepared using straightforward chemistry, enabling integration of reactive handles such as azides or alkynes for protein labeling and detection as well as mass spectrometry (MS)-based proteomic analysis. Herein, we present ABPP of monoPGTs with 3-phenyl-2H-Az probes that target the highly-conserved nucleophilic Asp of the Asp-Glu dyad. This work lays the foundation for the identification of glycoconjugate biosynthesis pathways in diverse prokaryotes and provides

tools for studying regulatory mechanisms in the multistep processes. We also provide a straightforward synthetic route that will allow for tunable 3-phenyl-2H-Az derivatives.

METHODS

Preparation of Cell Envelope Fractions (CEFs)

The CEF preparation was carried out at 4 °C. Cell pellets from 500 mL culture were re-suspended in 50 mL buffer A (50 mM PBS, pH 7.5) containing lysozyme (50 mg) (Research Products International, cat L38100), protease inhibitor cocktail (50 µL) (Roche, cat 11836170001) and DNase I (100 µL) (New England BioLabs cat M0303S). Cells were sonicated (50 % amplitude, 1 sec ON/2 sec OFF, 2 x 2 min). Cells were kept on ice during sonication. The resultant lysate was centrifuged at 9,000 g for 45 min at 4 °C. The cloudy supernatant was collected in a new centrifuge tube and further centrifuged at 140,000 g for 65 min at 4 °C. A membrane pellet (cell envelope fraction, CEF) was produced. The CEF was re-suspended using a Dounce homogenizer in 10 mL buffer A,

Probe labeling and click conditions

CEF was diluted in Buffer A to a total protein concentration of 10 mg/mL, and was subsequently treated with DMSO-dissolved probe at the appropriate concentrations. The probe-treated CEF was allowed to react with the probe for either 18 h at 4 °C, or 2 h at RT. Click with the azide-conjugate (N₃-biotin or N₃-fluorophore) was carried out following previous literature reports.⁴ Azide-conjugate was added to the eppendorf containing the probe reaction to a final concentration of 1 mM. This was followed by the addition of 100 µM TBTA, 1 mM sodium ascorbate, and 100 µM CuSO₄. The reaction was agitated for 2 h at RT, then quenched with loading dye comprising BME.

Western blot analysis

Labeled CEF samples were separated by gel electrophoresis on Biorad 4-20% gradient gels. 7 µL of sample was loaded per lane for Western blot and SDS-PAGE analysis. Western blot analyses were run at 100 V for 1 h at 4 °C. For the detection of PGTs, membranes were incubated with a 1:5000 dilution of mouse anti-His antibody (LifeTein) in TBS-T 3% BSA followed by a 1:5000 dilution of IRDye 800CW secondary goat anti-mouse antibody in Odyssey blocking buffer (LI-COR Biosciences). The blots were imaged using a LICOR imager. For subsequent detection of labeled protein, the Western blots were stripped by incubation at 37 °C in stripping buffer (62.5 mM Tris, pH 6.8, 1% SDS, 0.35% β-mercaptoethanol (v/v)) for 1 hour. After thorough washing in TBS-T, the membrane was blocked in Odyssey blocking buffer for 1 hour at RT, and incubated with 0.1 mg/mL Streptavidin-647 in TBS-T 3% BSA overnight at 4 °C. The blots were washed three times in TBS prior to imaging. Labeled proteins were visualized by scanning fluorescence on a Typhoon FLA 9000 (GE Healthcare).

RESULTS AND DISCUSSION

Representative enzymes from the monoPGT superfamily were selected for activity profiling: PglC from *Campylobacter jejuni* (*Cj*), PglC from *Helicobacter pullorum* (*Hp*), and PglB

from *Clostridium botulinum* (*Cb*). *C. jejuni* is a human food borne enteropathogen that is a significant cause of gastroenteritis worldwide.²⁴ *H. pullorum* is a zoonotic pathogen known to colonize the gastrointestinal tract of poultry. *C. botulinum* is a food borne pathogen that can produce botulinum toxin. PglC from *C. jejuni* and *H. pullorum* catalyze the first membrane-committed step in the biosynthesis of *N*-linked glycoproteins, and PglC (*Hp*) displays 68% sequence identity to PglC (*Cj*). *C. botulinum* PglB catalyzes this same reaction in an unknown glycoconjugate biosynthesis pathway and shares 36% sequence identity with PglC (*Cj*).

Following the established protocol, we first resynthesized the principal 3-phenyl-2H-Az probe described by Ma, *et al.* The final product matched the literature characterization including NMR analysis and mass spectrometry. For a broader range of ABPP applications we established a revised route that would enable diversification of the probe structures without impacting the carefully balanced electrophilic reactivity. With an activated C=N double bond and a lone pair of electrons on the nitrogen, the 2H-Az is a key precursor for a wide variety of transformations due to the strong reactivity towards a wide range of electrophiles and nucleophiles.¹⁷ This makes developing synthetic routes for ABPP challenging because many standard reaction conditions to incorporate biologically useful pharmacophores or functional groups used in copper catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry may not be orthogonal to both the synthesis and the preservation of the 2H-Az moiety. To avoid introducing multiple synthetic routes that are only orthogonal to specific moieties, we instead employed a late-stage diversification strategy that uses modular attachments to bring together the *p*-phenyl-2H-azirine and select building blocks at the final synthetic step. An alkyl halide sulfonyl chloride linker is key to this strategy; it has two distinct electrophilic groups capable of selective step-wise nucleophilic addition. We exemplified this route by first chlorinating sodium sulfonate (**1**) with oxalyl-chloride, then selectively reacting the sulfonyl chloride with dipropargyl amine to introduce the essential terminal alkyne for click chemistry (Figure 3A). The resulting compound (**2**) is then used to alkylate the base-activated *p*-phenol-2H-azirine (**3**), yielding **Az-2**.

Probes **Az-1** and **Az-2** (100 μ M) were first applied to the cell envelope fraction (CEF) of *Escherichia coli* BL21 overexpressing PglC-His₆ (*Cj*).²⁵ Although we are heterologously overexpressing PglC, expression yields are modest using this method; membrane protein overexpression is considerably lower compared to that of soluble proteins. Each probe was incubated with CEF for 18 h at 4 °C and subsequently conjugated by CuAAC click chemistry with N₃-biotin. As a negative control, these probes were also tested against BL21 CEF not overexpressing a monoPGT. Labeling was analyzed by Western blot with Alexa Fluor™ 647 streptavidin. The presence of PglC-His₆ was confirmed by anti-His antibody detection and total protein was visualized by Coomassie staining (Figure 3B). Analysis of the resulting labeled proteome using fluorescence imaging revealed robust covalent labeling of PglC (*Cj*) by **Az-1** and **Az-2**, with the most prominent band occurring at the expected molecular weight (23 kDa). Additional fluorescence labeling of background proteins was minimal when directly compared to the prominence of the PglC (*Cj*) band. Notably, **Az-2** maintains reactivity towards activated protein carboxylic acids, demonstrating that the balanced reactivity of the 2H-azirine is not impacted by the additional chemical moieties.

This provides a viable strategy for introducing features such as known protein ligands to boost probe affinity and selectivity. The fluorescent labeling for both **Az-1** and **Az-2** occurred with an intensity that is dependent on the final concentration of the probe (Figure S1). This concentration dependent experiment allowed us to select the probe concentration for our experiments to afford the best visual for our gel-based read outs.

Active site specificity was confirmed by probe labeling Asp-Glu active site mutants. These experiments were carried out using PglC from *C. concisus* (*Cc*), because active site mutations are not well tolerated in PglC (*Cj*). Furthermore, we used a SUMO construct, His₆-SUMO-PglC, to express active site variants; the N-terminal SUMO tag moderately mitigates the low expression yields and poor stability these mutants display. Asp93, which has been biochemically determined to form the covalent intermediate,¹⁰ was mutated to Asn (D93N). Separately, Glu94, which is proposed to coordinate with the Mg²⁺ cofactor, was mutated to Gln (E94Q). The same labeling methodology was applied to the variants. Labeling was analyzed by Western blot with Alexa Fluor™ 647 streptavidin, and the presence of PglC was confirmed with anti-His₆ antibody detection (Figure 3C). By fluorescence, each active site mutant displays attenuated labeling relative to WT protein. Labeling is eliminated with the D93N mutant, capturing the essentiality of the catalytic Asp93 residue. In contrast, the E94Q variant also shows reduced labeling, but not to the extent of the catalytically essential D93N. We propose that the reactivity of the Asp is perturbed by this local mutation change. Interestingly, the Glu94 appears to impact the nucleophilicity of the Asp93, but it is clearly not the main contributor. The observed labeling corroborates the two-step ping-pong mechanism of the monoPGTs, involving a catalytic Asp-Glu (DE) dyad, wherein the Asp carboxyl group attacks at the β-phosphate of the NDP-sugar substrate forming a covalent phospho-sugar intermediate.¹⁰

To further test the specificity of **Az-1** for catalytic residues, we carried out a concentration-dependent gel-shift assay on CEF containing PglC (*Cj*). Labeling was carried out in the same manner as previously described, and the probe was clicked with monodisperse N₃-PEG₃₆. A direct fluorescence read-out visualized by anti-His antibody detection correlates the number of new bands to the number of probe additions to a monoPGT, where each new band represents a mass shift of approximately 1.5 kDa. Probe concentrations ranged from 100 μM to 10 mM (Figure 3D). Importantly, only a single mass shift was visualized with each concentration, even with treatment up to the 10 mM probe; this clearly demonstrates site-specific probe modification to PglC (*Cj*). The efficiency of labeling is concentration dependent. This single labeling is presumed to occur predominantly at the Asp93 residue, based on the previously demonstrated enhanced chemical nucleophilicity and supporting biochemical analysis.¹⁰

To validate the specificity of labeling for active site residues, we measured changes in activity of *Cj*PglC upon labeling with **Az-1**. CEF containing overexpressed *Cj*PglC was treated with 1 mM and 10 mM **Az-1** for 18 h at 4 °C. We selected these probe concentrations based on densitometry from the previous gel-shift assay. At these concentrations, labeling is observed at an efficiency that would sufficiently attenuate activity if active site residues are in fact targeted by **Az-1**. We applied a radioactivity-based assay which directly measures Und-PP-sugar product by monitoring the transfer of a radiolabeled-phosphosugar from the

UDP-[³H]-diNAcBac to the unlabeled Und-P acceptor; substrate conversion is quantified by scintillation counting after extraction.²⁶ A time course of activity was carried out under linear initial rate conditions, and the sample treated with 10 mM **Az-1** had activity diminished to 53% compared to an unlabeled CEF sample (WT unlabeled CEF maintains complete activity under these conditions). The sample treated with 1 mM **Az-1** had activity that was reduced to 84% compared to unlabeled CEF (Figure S2). This concentration dependent decrease in activity is related to active-site labeling, inhibiting catalysis and product formation.

After characterizing probe labeling of PglC (*Cj*), we employed the activity probes to test labeling of additional superfamily members, including PglC (*Hp*) and PglB (*Cb*) (Figure 4). These select monoPGTs comprise the minimal functional catalytic core. Following the same labeling methodology, probes **Az-1** and **Az-2** showed very robust and targeted labeling of monoPGTs from the complex protein-rich CEF mixture. Strong fluorescent bands are seen at the molecular weight corresponding to each monoPGT, indicating high levels of labeling, and importantly, visualization also showed very low levels of non-specific protein labeling. These labeling results exemplify how effective **Az-1** and **Az-2** are at targeting the highly-conserved monoPGT catalytic dyad, and how these probes label other members of this expansive superfamily. Based on several biological replicates, we did not observe a significant difference in reactivity of **Az-1** and **Az-2** towards monoPGTs. Native expression levels of monoPGTs, such as PglC, are expected to be too low to visualize labeling by gels and Western Blot. Labeling of CEF from *Campylobacter jejuni* NCTC 11168 was carried out, however no labeled PglC was observed, likely due to low endogenous expression levels. Labeling of CEF from *Campylobacter jejuni* NCTC 11168 was carried out, however no labeled PglC was observed, likely due to low endogenous expression levels (data not shown). We assessed the sensitivity of the current probe and have determined that monoPGTs at concentrations down to 300 nM can be observed by fluorescence in gels of CEF by **Az-1** labeling (Figure S3).

In summary, we have demonstrated that we can covalently target the active site Asp in the catalytic Asp-Glu by employing 2H-Az probes. We have established active site-specific covalent labeling of monoPGTs from complex cellular fractions based on simple efficient gel-based read outs, and carried this out with representative monoPGTs, including PglC from *C. concisus*, PglC from *C. jejuni*, PglC from *H. pullorum*, and PglB from *C. botulinum*.

This work provides a strong foundation for employing activity-based protein profiling (ABPP) as a tool for understanding the activity and regulation of the monoPGT superfamily. Future work will focus on increasing the specificity of the probes to support ABPP of monoPGTs at the low native expression levels. To achieve this goal, we will use the presented synthetic route to tailor a small library of probes to monoPGTs. Specifically, we will utilize previously described monoPGTs inhibitors²⁷ as templates and will incorporate small structural components of the best inhibitors via addition to the sulfonyl chloride linker. We will then identify the best structural features that enhance binding affinity to carry out more targeted biological profiling. With targeted ABPP probes in hand, characterization of unknown glycoconjugate biosynthesis pathways in pathogenic bacteria can begin, with the

ultimate goal of identification of new mono PGTs as targets for mechanistic analysis and screening for inhibitors in cells and cell membrane fractions.

ABPP has been invaluable to proteomics providing the ultimate springboard for enzyme discovery, and has greatly contributed to our understanding of myriad of diverse enzyme classes including metalloproteins,²⁸ oxidoreductases,²⁹ and glutathione *S*-transferases.³⁰ With these comprehensive ABPP studies we are now poised to accelerate monoPGT research in an analogous path.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ABPP	activity based protein profiling
PGT	phosphoglycosyl transferase
mono	monotopic
NDP	nucleoside diphosphate
GT	glycosyltransferase
LPS	lipopolysaccharide
CPS	capsular polysaccharide
PG	peptidoglycan
CEF	cell envelope fraction
SSN	sequence similarity network
UDP	uridine diphosphate
UMP	uridine monophosphate
Az	azirine
Cj	Campylobacter jejuni
Cc	Campylobacter concisus
Hp	Helicobacter pullorum

Cb	Clostridium botulinum
Pren-P	polyprenol phosphate
diNAcBac	di- <i>N</i> -acetylbacillosamine
Pgl	protein glycosylation
SUMO	Small Ubiquitin-like Modifier

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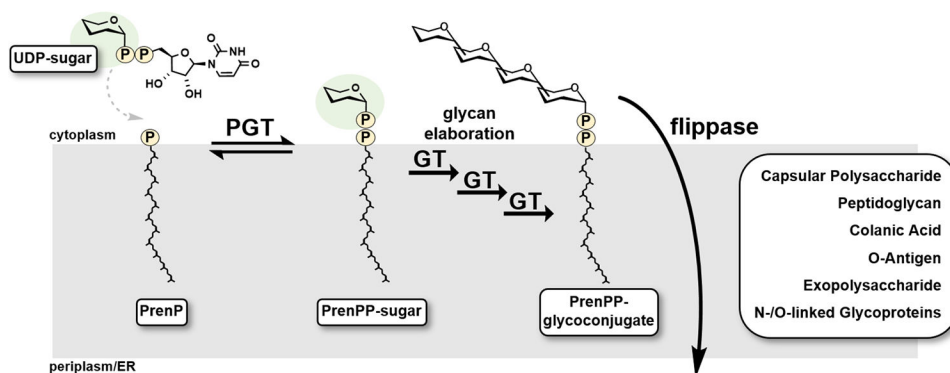
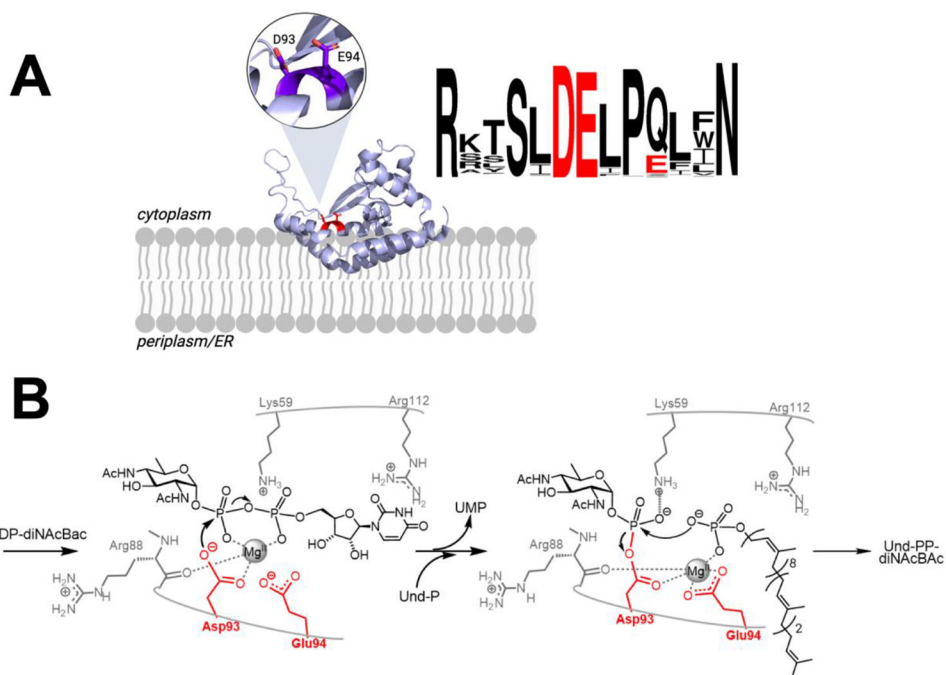
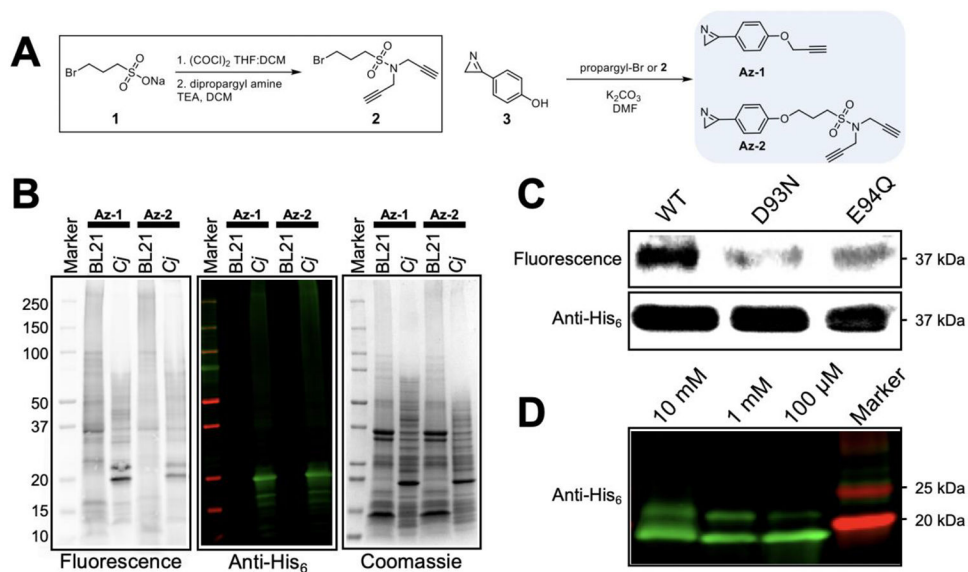


Figure 1. Phosphoglycosyl transferases (PGTs) catalyze the initial membrane-committed step in the *en bloc* mechanism of glycoconjugate biosynthesis. The PrenPP-sugar product of the PGT reaction is further elaborated via sequential modification by glycosyltransferases (GTs) to afford PrenPP-glycoconjugates, that are translocated to the periplasmic space by a flippase. These membrane-bound glycoconjugates then continue on support essential functions related to bacterial survival and proliferation.

**Figure 2.**

A) Ribbon diagram of monoPGT PglC (PDB 5W7L), inset and red coloring shows close-up view of active site highlighting the PglC Asp-Glu catalytic dyad. Sequence logo showing conservation in the catalytic residues among PglC orthologs. Logo generated using <https://weblogo.berkeley.edu/logo.cgi>. **B)** Bi-Bi ping-pong mechanism between a UDP-*N,N*-diacetylbaucillosamine (UDP-diNacBac) as the UDP-sugar donor, and undecaprenol phosphate (Und-P) as the Pren-P acceptor. Proposed roles for binding are shown and catalytic residues are highlighted in red (numbering from *Campylobacter concisus* PglC).

**Figure 3.**

A) Synthesis the sulfonamide linker, and synthesis of probes **Az-1** and **Az-2**. **B)** Western blot analysis of CEF containing overexpressed PglC-His₆ (*Cj*) in BL21, compared to CEF without any overexpressed proteins. The left most Western blot shows the fluorescence read out from the streptavidin fluorophore conjugate, and the middle shows the same Western blot imaged with Anti-His₆ fluorophore conjugate. The right most is a gel with the total protein stained by Coomassie. **C)** Probe labeling of His₆-SUMO-PglC (*Cc*) active site variants with **Az-1** (100 μM). **D)** Probe labeling with **Az-1** in a concentration dependent manner, followed by a click reaction with monodispersed N₃-PEG₃₆, demonstrating a single covalent modification visualized with a single mass shift.

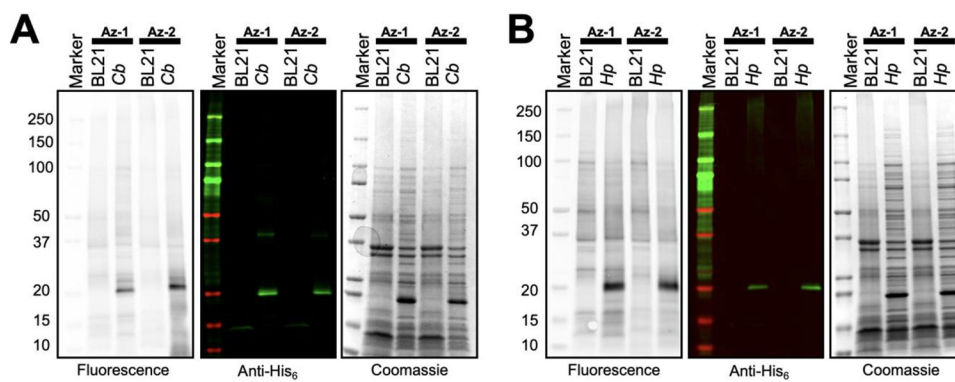


Figure 4. Western blot analysis of CEF containing overexpressed PglC-His₆ in BL21, compared to CEF without any overexpressed proteins. The left most Western blot shows the fluorescence read out from the streptavidin fluorophore conjugate, and the middle shows the same Western blot imaged with Anti-His₆ fluorophore conjugate. The right most is a gel with the total protein stained by Coomassie. Analysis with **A)** *Cb* and **B)** *Hp*.