Chemical biology tools to probe bacterial glycans

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Abstract. Bacterial cells are covered by a complex carbohydrate coat of armor that allows bacteria to thrive in a range of environments. As a testament to the importance of bacterial glycans, effective and heavily utilized antibiotics including penicillin and vancomycin target and disrupt the bacterial glycocalyx. Despite their importance, the study of bacterial glycans lags far behind their eukaryotic counterparts. Bacterial cells use a large palette of monosaccharides to craft glycans, leading to molecules that are significantly more complex than eukaryotic glycans and that are refractory to study. Fortunately, chemical tools designed to probe bacterial glycans have yielded insights into these molecules, their structures, their biosynthesis, and their functions.

Introduction

Bacteria adorn their cell envelopes with a dense glycocalyx that provides structural support and rigidity, offers protection from the environment, and mediates interactions with surrounding cells. Proper construction of bacterial glycans is critical for bacterial fitness and survival[1]. Therefore, disrupting bacterial glycans has been a potent and effective antibiotic strategy[2-4]. From a structural perspective, bacteria utilize more than 700 monosaccharide building blocks to construct their glycans[5], and most of these monosaccharides are exclusively bacterial sugars. Therefore, the classes of glycans crafted by bacterial cells, including capsular polysaccharide (CPS), lipopolysaccharide (LPS), and peptidoglycan (PG) (Fig. 1A), have no counterpart in eukaryotes. Further, each bacterial species uses only a subset of monosaccharides, leading to dramatic structural variability across glycans on different bacteria and consequently a plethora of bacteria-selective targets[6,7]. Taken together, the important roles of glycans in bacterial physiology coupled to their distinctive structures make bacterial glycans prime candidates for novel and selective antibiotics and vaccines.

The potential of bacterial glycans remains relatively untapped due to the challenges associated with studying them. Genetic methods cannot be used to probe glycans or assess glycan composition due to the lack of a direct linkage between the genetic code and the complex, branched, and heterogenous glycans produced by cells. Further, mass spectrometry approaches struggle to differentiate between a variety of isomers with identical molecular mass[8]. The study of bacterial glycans is further compounded by their structural diversity; the presence of exclusively bacterial monosaccharides renders the use of methods developed to study eukaryotic glycans not directly transferable for the study of bacterial glycans[9]. As a result, there is an urgent need to develop tools that are designed to meet the challenges associated with studying bacterial glycans.

In response to this call to action, our laboratory and others have harnessed the power of chemistry to expedite the study of bacterial glycans. Chemical tools tailored for these studies, including bacterial monosaccharide-based reporters and inhibitors, have made important inroads toward understanding and altering bacterial glycans[10-16]. Probes bearing bioorthogonal or fluorescent moieties have facilitated the discovery of bacterial glycans and the genes involved in their biosynthesis, and have enabled the tracking of glycans on live bacteria in complex settings. These studies have inspired the development of novel glycan-binding and glycan-disrupting agents, and they set the stage for the creation of glycoconjugate vaccines. This review highlights recent advances in the creation and application of enabling tools that have utility for chemical biologists and glycoscientists pursuing basic and applied research in a range of bacterial systems.

Development of metabolic probes to label bacterial glycans

A widely adopted strategy to probe bacterial glycans is termed metabolic glycan labeling (MGL) and entails coopting biosynthetic machinery to metabolically install unnatural carbohydrates into newly synthesized glycans. Typically, the probes utilized resemble very closely molecules that bacteria incorporate natively, but also include functional groups that enable detection. The resulting labeled bacterial glycans can then be measured through covalent addition of a secondary reporter molecule for indirect MGL (Fig. 1B), or by direct MGL if the unnatural probe comprises a built-in reporter (Fig. 1C).

MGL was first pioneered as an indirect, two-step labelling process by Bertozzi and colleagues[17-19] in eukaryotic systems that built on the work of Reutter and coworkers[20,21]. MGL has since been expanded to study PG[22-24], bacterial glycoproteins,[25-27] LPS,[28,29] CPS,[30] arabinogalactan[31], and trehalose-containing glycolipids[32] (Table 1). In essence, this method harnesses permissive carbohydrate biosynthesis enzymes to process substrates with minor structural perturbations, including the presence of a small bioorthogonal functional group (e.g., azide, alkyne), into cellular glycans. *In vitro* or with live cells, glycans bearing bioorthogonal

functional groups can be conjugated to fluorophores or other detectable moieties in a second step using exquisitely selective bioorthogonal reactions[33] (e.g., strain-promoted azide-alkyne cycloaddition[34]) that were recognized by the 2022 Nobel Prize in Chemistry[35] (Fig. 1B). A suite of novel bioorthogonal probes for studying exclusively bacterial sugars has expanded the breadth of studies possible (Table 1). New azide-containing analogs developed in the past few years have facilitated detection of glycan epitopes on a range of bacteria that there was no way to probe previously[24,31,36-40]. Novel probes enable tracking of bacterial glycans containing monosaccharides from arabinose (Table 1H), inositol (Table 1I) and muramic acid (Table 1A) to rare L-sugars (Table 1K), pseudaminic acid (Table 1E), legionaminic acid (Table 1F) and Kdo (Table 1C).

Alternatively, direct metabolic glycan labeling obviates the need for a bioorthogonal ligation step by using carbohydrate-fluorophore conjugates that are biosynthetically installed by permissive enzymes (Fig. 1C)[41]. Direct labeling also offers the possibility of designing probes that are natively in their “off”-state and turn “on” to produce a signal when very specific conditions are met, such as arrival at a predetermined cellular destination, or following interaction with a specific molecular partner such as a bacterial enzyme, or upon a user-induced stimulus like photo-activation. For example, Banahene *et al.* took advantage of the congested lipophilic interior of the mycomembrane to install molecular-rotor fluorogenic trehalose probes that only fluoresce upon reaching the mycomembrane (Table 1J) [42]. The development of “smart” probes that are activated *in situ* allows for no-wash labeling in live cells and offers enhanced sensitivity, attributes that are well suited to probing glycans in real time and in complex settings. Unfortunately, not all glycosylation enzymes are tolerant of substrates bearing sterically large fluorophores.

MGL is possible even when native bacterial pathways have stringent substrate specificity or are absent in an organism altogether. In such cases, permissive versions of the enzyme or transporter from another organism can be genetically introduced or exogenously delivered to allow incorporation of modified substrates. Though *E. coli* lacks the requisite bypass enzymes to

metabolically incorporate *N*-azidoacetyl glucosamine (GlcNAz) into peptidoglycan, Xu *et al.* were able to circumvent this barrier by expressing the glycosyltransferase OleD in *E. coli* and treating the transformed bacteria with the activated substrate 2-chloro-4-nitrophenyl-GlcNAz (Table 1B) [43]. In a similar vein, de Jong *et al.* recently described the use of exogenously delivered enzymes to install azido-sialic acids onto the LPS of *Neisseria gonorrhea* (Table 1 D) [44]. Thus, enzyme introduction can expand the suite of probes available to tag and track bacterial glycans in a range of systems.

Discovery of bacterial glycan selective carbohydrate-binding proteins

To complement metabolism-based approaches, carbohydrate-binding proteins, termed lectins, can be used to bind to and detect bacterial glycans (Fig. 1D). Although plant-based lectins with broad-binding specificities have been used in the past to bind and detect eukaryotic as well as bacterial glycans, not until recently have lectins with selectivity for bacterial glycans been discovered [45,46]. Building on years of research exploring the human gut lectin *Intelectin*, Ghosh *et al.* recently described the human oral lectin ZG16B as a cell envelope polysaccharide probe that binds selectively to oral commensal bacteria and regulates their growth [47]. Further, Wu *et al.* discovered that the innate immune lectin, Galectin-7, specifically targets microbes that express blood group-like antigens in their glycocalyx [48]. These works serve as models for the discovery of other human lectins that bind to microbial glycans, setting the stage to understand how host lectins mediate interactions with commensal bacteria and how host cells use these interactions to tailor the composition of the microbiome.

Moving beyond lectins, some efforts have focused on identifying or creating proteins that bind bacterial carbohydrate motifs[49]. Toward this end, Eddenden *et al.* recently reported a catalytically-dead glycoside hydrolase probe that binds to the prominent biofilm polysaccharide poly-β-1,6-*N*-acetylglucosmine (PNAG)[50]. This probe allowed for monitoring of PNAG

production during biofilm formation. Newly developed screens for anti-glycan antibodies[49] offer promise for identifying antibodies that bind distinctive bacterial glycan epitopes. Increasing the suite of bacterial glycan-binding proteins will provide an expanded toolkit for probing bacterial glycans and their functions *in situ*.

Probing bacterial glycans in physiologically relevant settings

Recently, glycan probes have been applied in physiologically relevant settings to gather insights into bacteria-host interactions and to report on glycan epitopes present on bacteria in mixed microbial communities (Fig. 2). Focused studies have assigned the physiological roles of bacterial glycans in model systems. Further, large-scale studies have probed these epitopes in more complicated settings. The combination of these two approaches has furthered our understanding of bacterial glycans and demonstrated the value of chemical tools at small-scale and large-scale levels.

Chemical probes offer an approach to study and tailor immune-mediated anti-bacterial responses (Fig. 2A). Using azido bacterial PG precursors and subsequent click-chemistry, Wodzanowski *et al.* visualized live bacteria within macrophages in a three-dimensional hydrogel matrix and measured concomitant cytokine production[51]. Dzigba *et al.* utilized MGL to decorate *Mycobacteria* with trehalose conjugated to antibody recruiting molecules, resulting in increased antibody recognition and phagocytosis by macrophages[52]. Using interleukin-10 deficient mice and a two-step MGL approach, Weiss *et al.* tracked the immune response against intestinal commensal and pathogenic bacteria, showing a nuanced bacteria-specific interleukin response and highlighting the importance of tracking species-level glycan epitope expression[53]. Ghosh *et al.* reported that the PG-binding oral lectin ZG16B reduced bacterial cell proliferation without triggering cell death and allowed commensal bacteria to form clusters at specific sites within the oral cavity[47]. Thus, bacterial glycans can serve as arbiters of immune recognition that can be

tailored to enhance or mute the immune response, as well as to select for propagation of commensal bacteria in a host-directed manner.

Novel approaches in high-throughput screening of bacterial glycans and identification of novel glycan-binding molecules have set the stage to propel the field. Following an MGL- and genome sequencing-based approach, Han *et al.* experimentally isolated numerous bacterial strains bearing MGL-tagged glycans from complex mouse and human intestinal microbiomes[54] (Fig. 2B). Using lectins rather than MGL, McPherson *et al*. employed Lectin-Seq to profile lectin-microbe interactions in native gut microbial communities [55] (Fig. 2B). Utilizing a glycan microarray with hundreds of distinct glycans and a Python algorithm, Ho *et al.* developed a screening method capable of characterizing binding dynamics of macromolecules to glycans, and then experimentally validated this approach with human Galectin 7 and an array of bacteria[56]. Finally, an *in silico* prediction method developed by Bonnardel *et al.* identified and predicted upwards of 100,000 bacterial lectins capable of binding to eukaryotic glycans within a specific microbiome[57]. These novel methods for high-throughput prediction and testing will accelerate the pace at which we gain understanding into bacterial glycans and their roles in bacteria-host interactions.

Insights into bacterial glycan biosynthesis, recycling, and degradation

The bacterial glycan life cycle involves the choreography of myriad events, from monosaccharide activation and glycan construction to glycan tailoring, recycling, and degradation (Fig. 3A). Much of our current understanding of bacterial glycans and their functions was developed by studying and interfering with enzymes required for glycan biosynthesis and turnover. However, studies to probe glycan-active enzymes, including glycosyltransferases that synthesize bacterial glycans and glycosyl hydrolases that tailor and degrade them, face substantial technical barriers. Chiefly, these enzymes require substrates, including nucleotide

sugar donors and elaborated glycans bearing exclusively bacterial monosaccharides, that are not readily available. Onerous chemical synthesis or biological purification is needed to access substrates for *in vitro* studies of these enzymes.

With the aid of chemical tools, some of the limitations with studying glycosyltransferases have been addressed. For example, Zheng *et al.* characterized and modulated substrate selectivity of bacterial nucleotidyltransferases involved in activating monosaccharides for subsequent transfer to growing glycans [58,59]. By identifying constraints and enhancing the activity of these enzymes, these studies have eased access to activated nucleotide sugar donors, setting the stage for directed biosynthesis of nucleoside-diphosphate-sugars and downstream glycoconjugates (Fig. 3B). Moreover, Moulton *et al.* developed an MGL-based screen to identify genes required for glycan biosynthesis in the absence of glycan structural information [60,61] (Fig. 3C). By relying on azide-labeled glycan production as a readout of intact glycan biosynthesis, the authors were able to perform a cell-based assay to screen for glycan biosynthesis defects in putative glycosyltransferase mutants. This strategy could be applied more broadly to reveal glycosylation genes in uncharacterized pathways.

New chemical tools have led to the development of robust and tractable assays to study glycosyl hydrolases. Toward this end, Luijkx *et al.* designed activity-based profiling probes to detect fucosidase activity in bacterial samples, including on the surface of live bacteria [62,63]. Design criteria of these fucose-based probes include a cross-linking site for covalent trapping of fucosidases and an azide tag for detection of trapped glycosidases with bioorthogonal chemistry. Using a similar activity-based profiling strategy, Killinger *et al.* employed a glucose-iodoacetamide probe bearing an azide at the 6-position [64] to crosslink and detect *Bifidobacterium* enzymes involved in mucin degradation [65]. High-throughput fluorogenic probes developed by Wang *et al.* to detect glucosidase activity [66] and poly-*N*-acetylglucosamine (PNAG)-degradation [67] further expanded the available toolkit (Fig. 3D). Access to these probes, coupled to rigorous downstream biochemical characterization of reconstituted enzymes [68,69], opens the door to identifying

bacterial glycan-active enzymes that modulate a variety of biological processes, from host immune detection to biofilm degradation.

Strides have been made integrating glycosyltransferases and glycosidases into pathway-level pictures. MGL with azido-D-ala probes in *M. smegmatis* allowed tracking of enzymes, substrates and products of peptidoglycan biosynthesis and revealed how cell wall biosynthesis occurs directionally via horizontal compartmentalization of precursors in the membrane [70] (Fig. 3E). In a series of biochemical and structural studies, a view of the structural dynamics regulating cell wall synthesis[71] and degradation[68] machinery has emerged. Finally, through *in vitro* reconstitution and mutation experiments, a mechanism has emerged to explain how bacteria acylate cell envelope polymers [72]. Together, these results deepen our understanding of the glycan life cycle, from building blocks to assembly, recycling, and degradation.

Perturbing and harnessing bacterial glycan biosynthesis

Insights into metabolic substrates and biosynthetic pathways have opened the door to the development of small molecules that inhibit glycan biosynthesis. For example, Morrison *et al.* synthetized chain terminating 6-fluoro and 6-deoxy GlcNAc analogs that truncate polymerization of PNAG and reduce biofilm in *E. coli* at concentrations as low as 10 μM [73]. Coopting an alternative inhibition mechanism, Quintana *et al.* developed thioglycoside substrate decoys based on rare bacterial monosaccharides that altered glycan biosynthesis and fitness in pathogenic bacteria, yet had no notable effect on glycosylation or growth in beneficial bacteria or mammalian cells [74]. Biosynthetic pathway mapping and access to glycosylation mutants offer an alternative approach to identifying glycan inhibitors. In particular, Muscato *et al.* demonstrated the extraordinary efficiency of using a synthetic lethal screen to discover compounds that disrupt a specified glycan biosynthesis pathway [75]. Their synthetic lethal screen of *S. aureus* against a library of 230,000 compounds led to the identification of two molecules that disrupt lipoteichoic

acid biosynthesis by inhibiting UgtP, a lipoteichoic acid glycosyltransferase. Treatment of methicillin-resistant *S. aureus* (MRSA) with the UgtP inhibitors re-sensitized MRSA cells to oxacillin [75]. Finally, using an activity-based profiling strategy targeting serine hydrolases, Li *et al.* identified a series of compounds with inhibitory concentrations as low as 0.1 μM that inhibited growth of replicating and non-replicating *Mycobacterium tuberculosis* and caused morphological changes typically associated with cell wall disruption [76]. These studies offer different tactics to disrupt bacterial glycan biosynthesis and have the potential to be applied across other classes of glycans and species of bacteria.

While perturbing glycan biosynthesis has the potential to underpin treatment of bacterial disease, glycoconjugate vaccines offer a means to prevent bacterial disease[77]. Although there are well established approaches to make glycoconjugate vaccines, substantial hurdles remain with the transport and storage of these vaccines once they are synthesized. Remarkable advances in cell-free protein expression technology have enabled the production of self-assembling, freeze-dried glycoconjugate vaccines that bypass the need for cold-chain transfer and storage. Jewett and colleagues demonstrated proof of concept for a bioconjugate vaccine production system containing lyophilized bacterial glycan biosynthesis machinery. Essentially, shelf-stable freeze-dried glycosylation machinery were rehydrated and used to produce polysaccharide and glycoprotein antigens *in situ* that subsequently produced a robust immune response in animal models [78,79]. Kowarik *et al.* demonstrated the feasibility of producing an *E. coli* O25B bioconjugate vaccine by using glycosylation machinery in *E. coli* to produce the O25B polysaccharide, then enzymatically transferring the purified O25B antigen onto the exotoxin A carrier protein (EPA) of *Pseudomonas aeruginosa* in a cell-free manner [80]. The modularity and flexibility of coupling cell-based production to enzymatic synthesis offers significant potential for crafting other glycoconjugate vaccines. These enzymatic methods can be complemented by synthetic methodologies that have been used to chemically produce a wide range of bacterial glycoconjugate vaccines[77,81,82].

Conclusions and Perspectives

The last few years led to an expansion of the suite of probes available to metabolically label bacterial glycans and the suite of accessible glycan-binding proteins. These probes laid the foundation for studying glycan epitopes in physiologically relevant settings, including in the context of the human gut microbiome, and teasing out the role of specific glycan epitopes in triggering a host immune response. Novel applications of existing probes were extended to identify genes required for glycosylation events and to track membrane compartmentalization of glycan biosynthesis. To meet unmet needs, new fluorogenic probes were developed to detect and identify glycosyl hydrolases, shedding light on the role of these enzymes in breaking down dietary sugars and dismantling bacterial biofilms. Finally, advancements in understanding bacterial glycosylation set the stage for developing small molecule inhibitors of bacterial glycan biosynthesis and harnessing glycan production machinery for vaccine development.

Despite these advances, gaps remain in our ability to survey and harness bacterial glycans. Although bacteria utilize over 700 monosaccharides in glycan biosynthesis[5,83,84], only a small percentage of these sugars have been chemically modified into metabolic probes[10]. Thus, the knowledge learned from existing probes does not adequately capture the enormous amount of glycan epitope variability across the bacterial domain. Advances in expedient synthesis of exclusively bacterial sugars promise to address this gap by easing access to otherwise inaccessible reagents[85,86]. Once a bacterial sugar becomes synthetically accessible, it can be converted into a bioorthogonal probe, an activity-based probe, a fluorogenic glycosyl hydrolase substrate, and a metabolic inhibitor, and it can be incorporated into glycoconjugate vaccines (Fig. 4). The design principles found in the papers highlighted in this review promise to serve as a blueprint for future advances.

While the metabolic probes and inhibitors described above allow structure-function studies that were heretofore impossible, most of these molecules were bioactive at concentrations higher

than feasible for use in living subjects. For use in ever-more complex and physiologically relevant settings, including animal infection models and, potentially, the clinic, these molecules would need to be optimized for use at much lower concentrations. Fortunately, existing probes provide clues about suitability of potential targets, and they offer a starting point for accessing mechanistic and structural information to serve as a basis for refinement. Chemical tools have had a significant mark in this subfield and will pave the way for the development of new antibiotics and vaccines that harness the tremendous untapped potential of the bacterial glycocalyx.

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• Dugan AE, Jain S, Neznanova L, *et al*.: **Human oral lectin ZG16B acts as a cell wall polysaccharide probe to decode host-microbe interactions with oral commensals**. *Proc Natl Acad Sci U S A* 2023, **120**:e2216304120.

This article describes the discovery and characterization of a human oral lectin that binds to glycans on commensal bacteria and directs growth of beneficial bacteria in discrete locations within the oral cavity. The authors found that both the location and extent of bacterial growth is regulated by this lectin-carbohydrate interaction.

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This report describes the development and implementation of a high-throughput flow-cytometry-based screen to identify interactions between human lectins and glycans presented on the surface of bacteria within complex gut microbial communities. Mannose-binding lectin and human intellectin exhibited vastly different extents and patterns of binding to glycans on commensal bacteria within the gut microbiome.

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Application of an activity-based probe to detect and characterize glycosylhydrolases in complex microbial communities. The authors observed that the microbiome of mice responded to a high fiber diet by increasing the activity of glycosyl hydrolases, polysaccharide lyases, and sugar transport proteins.

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This compelling work focuses on the development of chain-terminating *N*-acetylglucosamine analogs that are incorporated into glycan polymers and lead to truncated glycan biosynthesis. In particular, 6-fluoro and 6-deoxy analogs were potent inhibitors of biofilm formation.

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Describes a highly efficient genetic and chemical screen to identify small molecules that target bacterial glycan biosynthesis pathways. The authors screened ~230,000 compounds and identified lipoteichoic acid inhibitors that resensitized Methicillin-resistant *Staphylococcus aureus* to oxacilin.

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This paper describes a major advance in the production of glycoconjugate vaccines from freeze-dried components. Briefly, the authors demonstrated that freeze-dried lysates containing vaccine production machinery can be rehydrated and drive the synthesis of glycoconjugate vaccines that have efficacy in murine infection models.

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Figure Legends

Figure 1. Overview of bacterial cell envelope glycans and major approaches to study them discussed in this review. A) The Gram-negative bacterial cell envelope contains a variety of exclusively bacterial glycans including peptidoglycan, lipopolysaccharide (LPS), capsular polysaccharide (CPS), and, for some bacteria, glycosylated proteins (glycoproteins). B) Bacterial glycans can be studied using an indirect two-step metabolic labeling approach in which they are first metabolically labeled with an unnatural sugar bearing a bioorthogonal functional group (e.g., azide) and then detected in a second step via bioorthogonal chemistry (e.g., strain-promoted azide-alkyne cycloaddition). C) Direct metabolic labeling with a sugar bearing a detectable probe such as a fluorophore presents an alternative to indirect labeling. D) Proteins that bind to carbohydrates (e.g., lectins, antibodies) can be used to detect and study bacterial glycans.

Figure 2. Approaches to probe bacterial glycans in physiologically relevant settings. A) Bacteria-host interactions can be probed in cell culture models to understand the role of glycans in eliciting immune recognition and cytokine production from host cells (left). Direct metabolic labeling with sugars bearing antibody-recruiting molecules (e.g., trehalose-dinitrophenyl conjugates) can be used to induce antibody binding to and immune recognition of bacterial targets (right). B) Glycans on the surface of gut microbiota isolated from human stool samples can be probed using a two-step metabolic labeling approach in which they are first metabolically labeled with an azidosugar and then detected in a second step via strain-promoted azide-alkyne cycloaddition (top). Alternatively, lectin-based approaches can be used to bind glycan epitopes on microbiome constituents and then enrich and identify species presenting those epitopes (bottom).

Figure 3. Insights into bacterial glycan biosynthesis, recycling, and degradation. A) The bacterial glycan life cycle begins with monosaccharide activation to produce sugar-nucleotide

donors, followed by glycan construction by a series of glycosyltransferases (GTs) that catalyze monosaccharide addition to growing glycans in a directional manner, and *en bloc* glycan transfer to yield fully elaborated glycoconjugates. Cell envelope and cell wall glycans can be tailored by modifying enzymes (e.g., glycosidases, acetyltransferases) and ultimately degraded and recycled by glycosyl hydrolases. Recent advances have yielded (B) nucleotidyltransferases with enhanced substrate flexibility to facilitate access to sugar-nucleotide donors and ease glycan production *in vitro*, (C) metabolic labeling-based screens to identify genes encoding glycosyltransferases that play a role in glycan biosynthesis, (D) probes to detect and identify glycosyl hydrolase activity, and (E) metabolic labeling-based methods to track glycan biosynthetic intermediates within membrane domains to yield insight into directional glycan biosynthesis.

Figure 4. Expedient synthesis of rare bacterial sugar scaffolds opens the door to create novel chemical biology tools by adopting precedented design principles.

Table Legend

Table 1. Chemical probes highlighted in this review, including classes of glycans they label, probe structures, and metabolically labeled cellular structures.

Figures

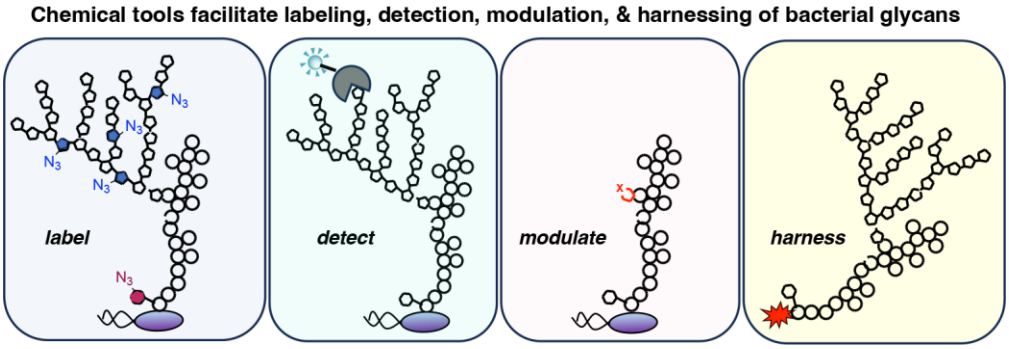


Table of contents entry

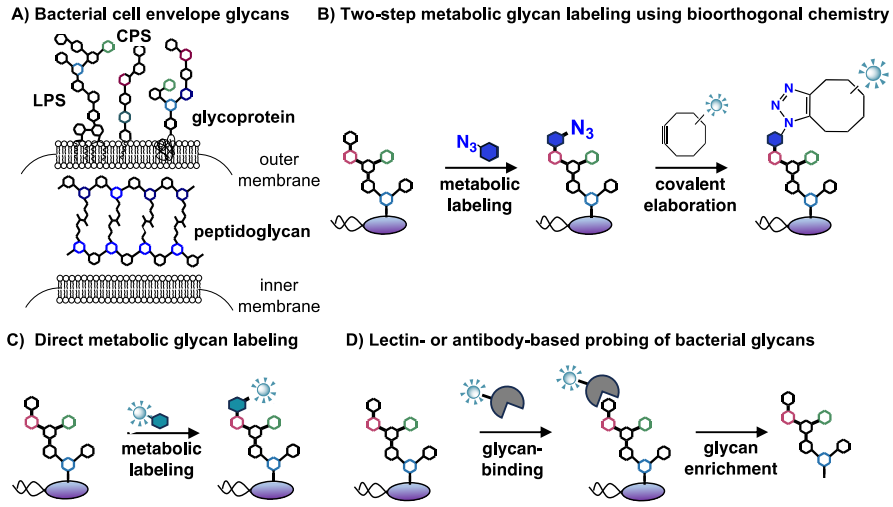

A) Bacterial cell envelope glycans
CPS
LPS
glycoprotein
outer membrane
peptidoglycan
inner membrane
B) Two-step metabolic glycan labeling using bioorthogonal chemistry
N3
metabolic labeling
N3
covalent elaboration
C) Direct metabolic glycan labeling
metabolic labeling
D) Lectin- or antibody-based probing of bacterial glycans
glycan-binding
glycan enrichment

Figure 1

Table 1. Chemical probes highlighted in this review, including classes of glycans they label, probe structures, and metabolically labeled cellular structures.

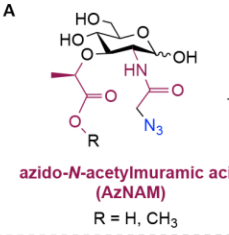
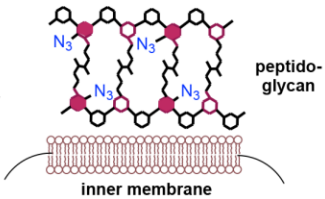
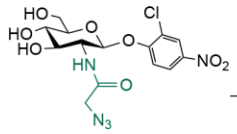
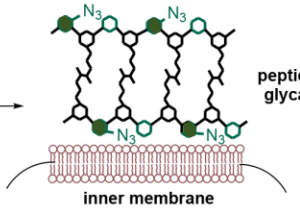
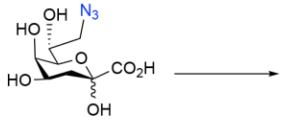
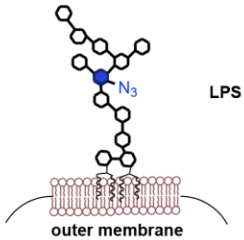
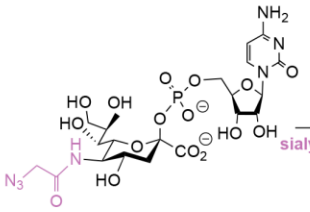
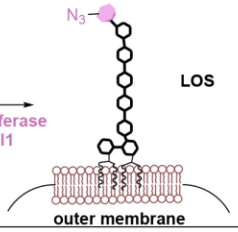
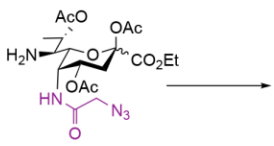
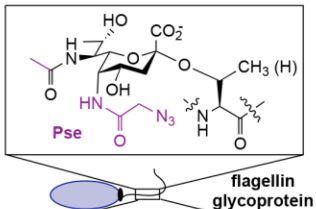
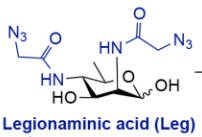
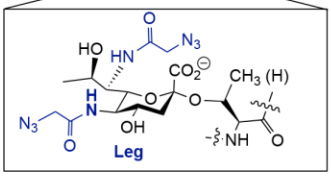
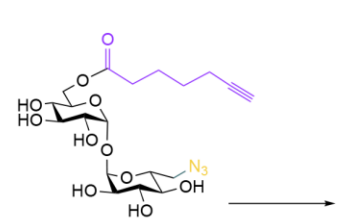
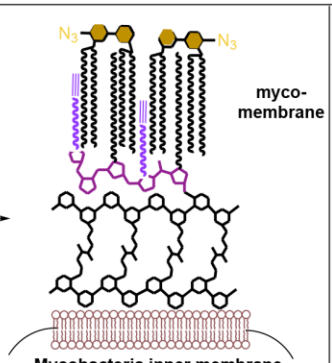
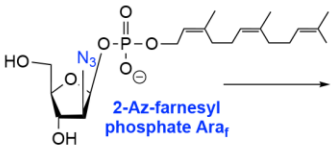
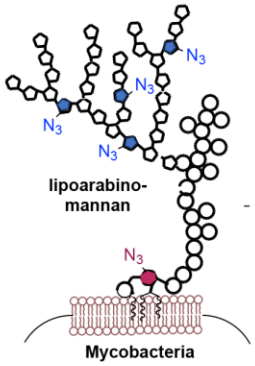
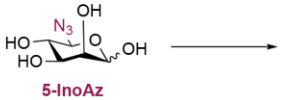
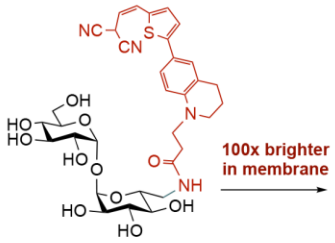
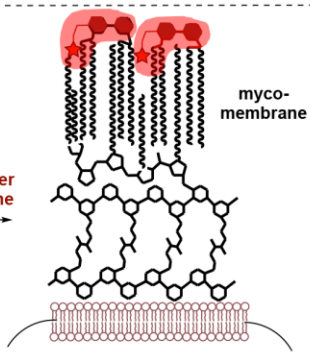
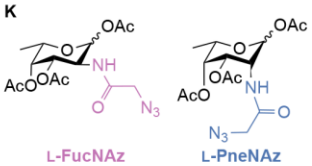
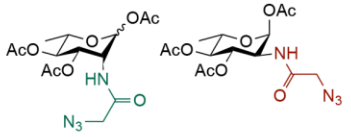
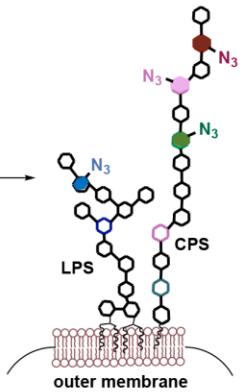
Probes for peptidoglycan		
Probe Structure	**Cellular Structure**	**Notes**
A  **azido-*N*-acetylmuramic acid (AzNAM)** R = H, CH_3	 **peptido-glycan** **inner membrane**	**Class of probe:** indirect MGL **Organisms tested on:** Gram-negative bacteria including *E. coli, H. pylori, Tannerella forsythia* **Effective concentration:** 60 μM - 6 mM **Reference:** Brown *et al.*, 2021[24]
B  **2-chloro-4-nitrophenol *N*-azidoacetylglucosamine (GlcNAz-CNP)**	**OleD**  **peptido-glycan** **inner membrane**	**Class of probe:** indirect MGL with expressed enzyme **Organisms tested on:** *Escherichia coli* expresing OleD **Effective concentration:** 1.2 mM **Reference:** Xu *et al.*, 2022[43]
Probes for lipopolysaccharide		
Probe Structure	**Cellular Structure**	**Notes**
C  **8-N_3-Kdo**	 **LPS** **outer membrane**	**Class of probe:** indirect MGL **Organism tested on:** *Myxococcus xanthus* **Effective concentration:** 2.5 mM **Reference:** Saidi *et al.*, 2022[29]
D  **CMP-*N*-azidoacetyl sialic acid**	**sialyltransferase ST6Gal1**  **LOS** **outer membrane**	**Class of probe:** indirect MGL with exogenous enzyme **Organism tested on:** *Neisseria gonorrhea* **Effective concentration:** 50 μM **Reference:** de Jong *et al.*, 2022[44]
Probes for glycoproteins		
Probe Structure	**Cellular Structure**	**Notes**
E  **protected pseudaminic acid (Pse)**	 **flagellin glycoprotein**	**Class of probe:** indirect MGL **Organism tested on:** *Campylobacter jejuni, Bacillus thuringiensis* **Effective concentration:** 2.5 mM **Reference:** Vibhute *et al.*, 2021[37]
F  **Legionaminic acid (Leg) hexose precursor**		**Class of probe:** indirect MGL **Organism tested on:** *Campylobacter jejuni* **Effective concentration:** 1 mM **Reference:** Meng *et al.*, 2021[38]

Table 1 *(continued)*

Probes for glycolipids		
Probe Structure	**Cellular Structure**	**Notes**
G  **O-AzAlk trehalose monomycolate** **bifunctional chemical reporter of trehalose and mycomembrane metabolism**	 **myco-membrane** **Mycobacteria inner membrane**	**Class of probe:** indirect MGL **Organisms tested on:** *Mycobacterium smegmatis* **Effective concentration:** 50 μM **Reference:** Pohane *et al.*, 2022[40]
H  **2-Az-farnesyl phosphate Ara$_f$**	 **lipoarabino-mannan** **Mycobacteria**	**Class of probe:** indirect MGL **Organisms tested on:** *Mycobacterium smegmatis, Cornebacterium glutanicum* **Effective concentration:** 250 μM **Reference:** Marando *et al.*, 2021[31]
I  **5-InoAz** **azido inositol probe to label inositol-containing glycans**		**Class of probe:** indirect MGL **Organisms tested on:** *Mycobacterium smegmatis* **Effective concentration:** 1 mM **Reference:** Hodges *et al.*, 2023[39]
J  **100x brighter in membrane** **far-red molecular rotor-based trehalose**	 **myco-membrane**	**Class of probe:** direct MGL, "turn on" **Organisms tested on:** *Mycobacterium smegmatis* *Mycobacterium tuberculosis* **Effective concentration:** 100 μM **Reference:** Banahene *et al.*, 2022[42]
Probes acting on multiple glycan biosynthesis pathways		
Probe Structure	**Cellular Structure**	**Notes**
K  **L-FucNAz** **L-PneNAz**  **L-RhaNAz** **L-QuiNAz** **L-sugar analogs to metabolically label LPS and CPS**	 **LPS** **CPS** **outer membrane**	**Class of probe:** indirect MGL **Organisms tested on:** Gram-negative bacteria including *Pleisiomas shigelloides, Vibrio vulnificus, Helicobacter pylori* **Effective concentration:** 1 mM **Reference:** Luong *et al.*, 2022[36]

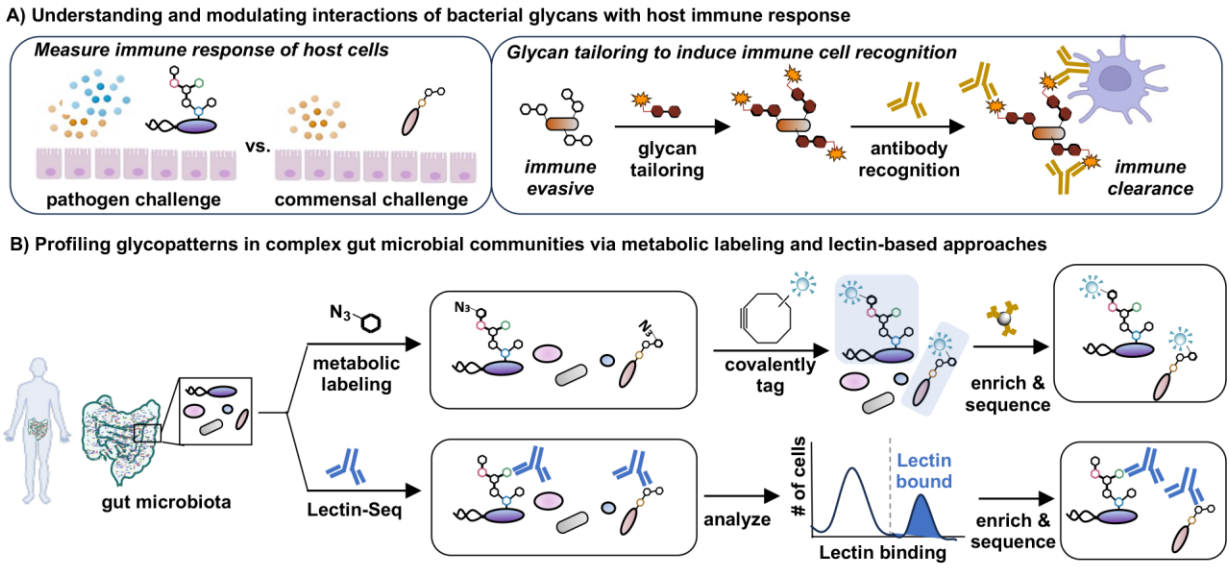

A) Understanding and modulating interactions of bacterial glycans with host immune response
Measure immune response of host cells
vs.
pathogen challenge
commensal challenge
Glycan tailoring to induce immune cell recognition
immune evasive
glycan tailoring
antibody recognition
immune clearance
B) Profiling glycopatterns in complex gut microbial communities via metabolic labeling and lectin-based approaches
gut microbiota
metabolic labeling
Lectin-Seq
covalently tag
enrich & sequence
analyze
# of cells
Lectin bound
Lectin binding
enrich & sequence

Figure 2

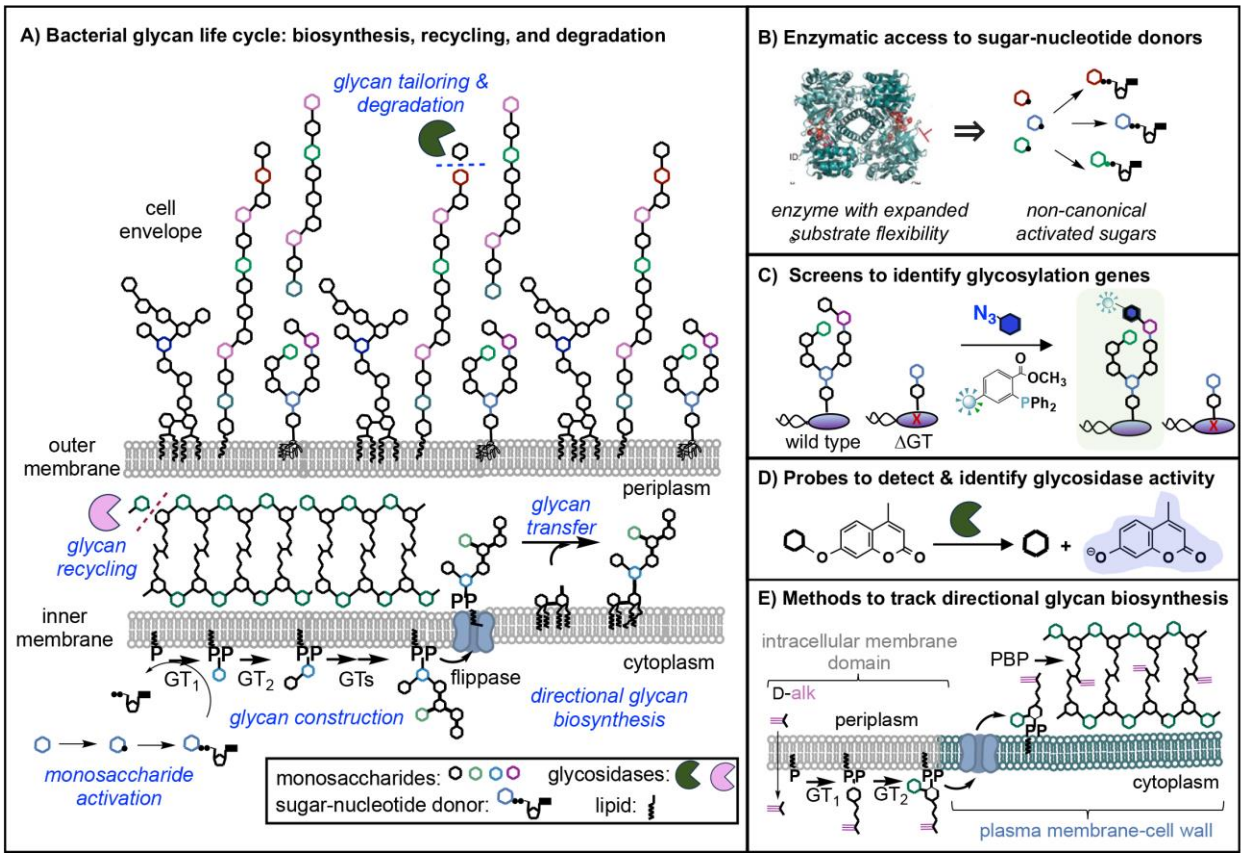

A) Bacterial glycan life cycle: biosynthesis, recycling, and degradation
glycan tailoring & degradation
cell envelope
outer membrane
periplasm
glycan recycling
glycan transfer
inner membrane
cytoplasm
GT1
GT2
GTs
flippase
glycan construction
directional glycan biosynthesis
monosaccharide activation
monosaccharides:
glycosidases:
sugar-nucleotide donor:
lipid:
B) Enzymatic access to sugar-nucleotide donors
enzyme with expanded substrate flexibility
non-canonical activated sugars
C) Screens to identify glycosylation genes
wild type
ΔGT
D) Probes to detect & identify glycosidase activity
E) Methods to track directional glycan biosynthesis
intracellular membrane domain
D-alk
periplasm
PBP
cytoplasm
plasma membrane-cell wall

Figure 3

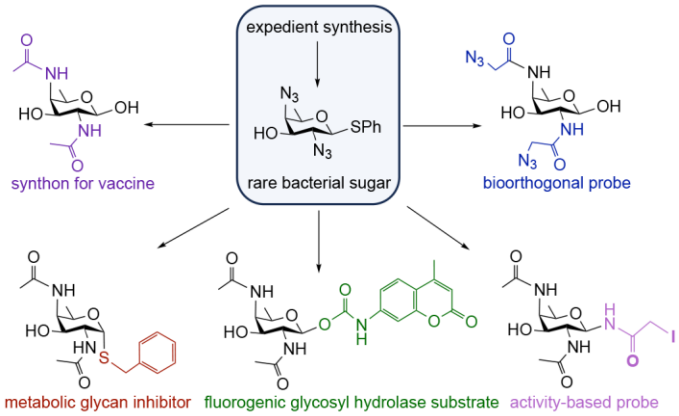

expedient synthesis
rare bacterial sugar
synthon for vaccine
bioorthogonal probe
metabolic glycan inhibitor
fluorogenic glycosyl hydrolase substrate
activity-based probe

Figure 4