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Deciphering the bacterial glycode: recent advances in bacterial glycoproteomics

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Abstract

Bacterial glycoproteins represent an attractive target for new antibacterial treatments, as they are frequently linked to pathogenesis and contain distinctive glycans that are absent in humans. Despite their potential therapeutic importance, many bacterial glycoproteins remain uncharacterized. This review focuses on recent advances in deciphering the bacterial glycode, including metabolic glycan labeling to discover and characterize bacterial glycoproteins, lectin-based microarrays to monitor bacterial glycoprotein dynamics, crosslinking sugars to assess the roles of bacterial glycoproteins, and harnessing bacterial glycosylation systems for the efficient production of industrially important glycoproteins.

Introduction

Pathogenic bacterial strains that are resistant to existing antibiotics pose a widespread health risk. Thus, there is an urgent need to identify new pathogen-associated molecules that can be targeted for the selective eradication of bacterial infections. Bacterial glycoproteins – proteins that are post-translationally modified by the covalent addition of one or more monosaccharides – are an attractive class of untapped targets.

It was long believed that bacteria could not synthesize glycosylated proteins due to the simplicity of their cellular structure and concomitant lack of sub-cellular organelles. Despite this long-standing dogma, the synthesis of glycoproteins in select bacterial strains has been firmly established by a number of leaders in the field[1–3]. Two important themes have surfaced since the discovery of bacterial glycoproteins. First, bacterial glycoproteins are frequently linked to pathogenesis (see review in [4]). For example, in some pathogenic bacteria, protein glycosylation is essential for locomotion, and without the appropriate flagellar glycans these bacteria cannot colonize their hosts[5]. In other bacterial pathogens, the glycans on proteins mediate interactions with host cells, such as adhesion to host cells[6] or evasion of the host's immune system[7]. In several cases, bacterial strains with altered protein glycosylation display decreased fitness within the host[3,5]. The link between bacterial glycoproteins and pathogenesis indicates that this class of biomolecules should be exploited for therapeutic intervention.

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The second theme that has emerged is that bacterial glycoproteins contain unique glycans that are absent from humans[4,8]. Although bacteria are capable of synthesizing the two major classes of glycoproteins that are found in eukaryotes – those that have asparagine-linked (N-linked) glycans and those that have serine/threonine (O-linked) glycans – their structures often contain sugars that are distinctly bacterial. For example, the amino- and deoxy-monosaccharides pseudaminic acid[5], bacillosamine[9], 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH)[10], and *N*-acetyl fucosamine[11] are found in bacterial glycans but are absent from human glycans (Figure 1). Thus, in addition to their links to pathogenesis, bacterial glycoproteins distinguish bacterial cells from human cells. Together, these attributes make bacterial glycoproteins attractive targets for new antibacterial treatments.

Despite their potential therapeutic importance, many bacterial glycoproteins remain understudied. This relative paucity of information is due, in part, to the structural complexity of the bacterial glycoproteome[8,12]. Bacterial glycoproteins contain heterogeneous glycans composed of myriad monosaccharides that can be linked in a branched or linear manner; moreover, unlike in DNA and protein synthesis, no template directs glycan synthesis. This structural complexity poses analytical challenges, yet this very complexity encodes the biological information that provides the impetus for studying bacterial glycoproteins. In this review, we focus on the latest methods developed to decipher the bacterial glycode and efforts to harness this information for biotechnological applications.

Discovering bacterial glycoproteins

The discovery of bacterial glycoproteins is typically a three-step process that includes the detection, isolation, and characterization of these biomolecules. In this section, we discuss recent advances in chemistry that facilitate these steps.

Detection of bacterial glycoproteins

The first step in discovering bacterial glycoproteins is to broadly detect their presence. Eukaryotes are expected to synthesize glycoproteins, but not all bacteria do; thus, the synthesis of glycoproteins by a given bacterial strain is not known in advance. Traditional biological methods to detect bacterial glycoproteins include using glycan-binding proteins such as lectins or antibodies that recognize and bind to particular glycan structures[8] (Figure 2). Conventional chemical approaches, such as periodic acid/hydrazide labeling or boronic diester modification of *cis* diols, employ reagents that covalently modify carbohydrate functionalities[8] (Figure 2). Both of these approaches have enabled the detection of glycoproteins in numerous species[3,9,13] yet suffer from limitations. As an example of a limitation, biological approaches require *a priori* knowledge of glycan structure and the existence of a biological reagent that binds to bacterial glycans. In contrast, traditional chemical methods detect a wide array of glycan structures but their promiscuity leads to undesired interactions[14], resulting in low signal to noise and false positives.

To address these limitations, metabolic oligosaccharide engineering, a chemical method that was pioneered by Bertozzi, Reutter, and coworkers in eukaryotic systems[15–17], has recently been expanded for use in bacterial systems. In this approach, cells are supplemented with a monosaccharide that has been chemically modified with a bio-orthogonal functional group – one that is normally absent from biological systems, well tolerated by cells, and non-reactive with endogenous biological functionalities[18]. Permissive carbohydrate biosynthetic pathways process the unnatural monosaccharide and incorporate it into cellular glycoproteins. The bio-orthogonal functional group then acts as a chemical handle and is covalently tagged with complementary reactive partners (Figure 2). For example, once incorporated into cellular glycoproteins, unnatural sugars containing azides or alkynes are

elaborated with probes *via* Staudinger ligation[19] or 1,3-dipolar cycloaddition[20,21] (“click” chemistry) to enable detection (Figure 2). The specificity of the bio-orthogonal chemistries makes metabolic glycan labeling a robust approach for glycoprotein discovery. The method is also general, as the types of glycans (e.g. N-linked, O-linked) that are labeled can be tailored by the choice of unnatural sugar and its subsequent fate in the cell[16].

Metabolic glycan labeling has had success in a range of bacteria, including the gastric pathogens *Helicobacter pylori*[22] and *Campylobacter jejuni*[23], and the intestinal *Bacteroidales* species[24]. In each case, this strategy has revealed fresh insights. For example, Koenigs *et al.* employed an azide-labeled *N*-acetylglucosamine (GlcNAc) analog to profile glycoproteins present in *H. pylori*[22]. These experiments detected a large number of uncharacterized glycoproteins and produced the first evidence that *H. pylori* synthesizes N-linked and O-linked glycoproteins. In another example, Wu and coworkers employed an alkyne-modified fucose derivative to visualize fucose-containing glycoproteins in *Bacteroides* and *Parabacteroides* species[24]. Finally, Liu *et al.* demonstrated that an azide-containing, dedicated metabolic precursor of pseudaminic acid is only incorporated into two flagellar glycoproteins in *C. jejuni* [23]. These seminal studies indicated that metabolic glycan labeling is robust, general, complementary to existing approaches, and gives strong signals. Metabolic labeling thus offers promise for deciphering the bacterial glycome.

Isolation of bacterial glycoproteins

Identification of glycosylated proteins requires that they be isolated or enriched from non-glycosylated cellular proteins. Traditionally, glycoproteins are isolated via column chromatography, where immobilized lectins, antibodies, or chemical functionalities (e.g. hydrazides, boronic diesters) are employed to selectively enrich for glycan-modified proteins[25]. As with many detection methods, these isolation methods are valuable but suffer from either a lack of generality or non-specific binding, depending on the capture reagent. Newly introduced metabolic labeling approaches have the potential to greatly accelerate the isolation of bacterial glycoproteins by overcoming these challenges. In particular, the choice of metabolic substrate engenders generality, while the specificity of bio-orthogonal reactions circumvents unintended binding. Indeed, azide- and alkyne-labeled glycoproteins have been enriched and identified in large-scale studies in eukaryotic systems with a high degree of confidence[16,26,27]. Briefly, previous studies have employed either the Staudinger ligation or “click” chemistry to attach affinity tags (e.g. biotin, hexahistidine) to labeled glycoproteins for subsequent biochemical purification. Recently described covalent capture methods to isolate azide-labeled proteins further simplify enrichment by decreasing the number of purification steps[28]. Ongoing studies in our lab have utilized an azidosugar/Staudinger ligation-based strategy to successfully enrich and identify more than one hundred glycosylated proteins from *H. pylori* (S. A. Longwell *et al.*, unpublished). Large-scale studies of this nature will therefore facilitate the study of the bacterial glycome.

In addition to physically isolating glycoproteins, there are approaches to isolate glycoproteins spectrometrically. Bertozzi and colleagues have recently developed a technique in which a 1:2:1 mixture of three GlcNAc isotopologs (termed an IsoMix) is used to metabolically label N-glycan-modified proteins prior to mass spectrometry analysis[29]. The unique isotopic distribution of the IsoMix selectively perturbs the mass envelope of labeled glycopeptides. Despite the presence of significant noise from abundant unlabeled biomolecules, these characteristic mass envelopes can then be distinguished algorithmically to generate an inclusion list of mass peaks for targeted tandem mass spectrometry (MS²). This approach identified 133 N-glycosites in yeast and doubled the list of experimentally observed N-glycosites in the yeast proteome. Although IsoMix has yet to be applied to bacterial systems, it could be extremely useful for computationally distinguishing glycopeptides for the identification of bacterial glycoproteins and glycosylation sites.

Characterization of bacterial glycoproteins

Once glycosylated proteins and peptides are separated from other biomolecules by either physical or spectrometrical means, they can be characterized to establish their identities, sites of glycosylation, and attached glycan structures[12]. The bottom-up mass spectrometry approach to broadly inventory the glycosylated proteins of a bacterial species focuses on characterizing glycoproteins present in a stringently isolated sample. Putative glycoproteins are identified based on unique peptide sequences and are then validated by MS² to detect characteristic glycan signatures. Additionally, sites of glycosylation can be determined through glycan removal and isotopic tagging[12]. As representative examples, Szymanski[9], Comstock[3], Koomey[13] and Cordwell[30] have utilized this type of bottom-up approach to broadly characterize glycoproteins from *C. jejuni*, *Bacteroides* sp., and *Neisseria* sp.

An alternative to the above-described bottom-up approach to identifying glycoproteins is a top-down analysis, in which intact proteins are analyzed by electrospray-ionization mass spectrometry (ESI-MS) to ascertain the glycoproteins' molecular weights[12]. Any significant mass excess relative to the molecular weight predicted by the primary sequence alone is suggestive of glycan modification, which can be validated through fragmentation analyses[12]. Logan and coworkers successfully applied this approach to identify unique glycans on the flagellar proteins of *H. pylori*, *C. jejuni*, *Listeria monocytogenes*, and *Aeromonas caviae*[31]. More detailed structural analysis of glycans is possible with sequential MS (MSⁿ)[32] and nuclear magnetic resonance (NMR) spectroscopy[33]. Ultimately, information from the characterization of a bacterial glycome could inform the development of therapeutics by identifying new targets and elucidating the structures of glycoproteins implicated in host-pathogen interactions.

Monitoring bacterial glycan dynamics: lectin microarrays

While discovering bacterial glycoproteins represents an important first step in deciphering the glycome, deeper layers of information can be revealed by monitoring bacterial glycan dynamics. For example, changes in glycoprotein expression levels upon exposure of bacterial cells to host cells may identify glycoproteins involved in host-pathogen interactions. Furthermore, comparison of glycomes from bacterial strains with a range of pathogenicity could identify factors that make certain strains particularly virulent. Finally, time-dependent glycomic analyses have the potential to yield a dynamic picture of glycosylation across various stages of growth. Established techniques to monitor glycan dynamics include quantitative mass spectrometry[34], metabolic glycan labeling[26,35], and flow cytometry[36].

An alternative method that is particularly well suited to monitoring bacterial glycan dynamics employs lectin-based microarrays. In this approach, originally developed by Pilobello and Mahal[37], a variety of lectins are immobilized separately in an array. A particular glycoprotein or cell population is then labeled for detection (e.g. with fluorescence) before or after application to the array (Figure 3). The resulting "glycopatterns" of multiple samples can be easily compared to determine glycosylation states associated with different strains or experimental conditions (Figure 3). In their landmark work, Mahal and Hsu employed lectin arrays to distinguish strains of *Escherichia coli* and to observe temporal changes in glycosylation states across various growth phases[38,39]. More recently, lectin arrays have been used by Gao *et al.* to study the effect of growth media on the glycosylation profile of *E. coli*[40], while Semchenko *et al.* used lectin-based arrays to elucidate structural aspects of lipooligosaccharide in *C. jejuni*[41].

Although lectin arrays offer an approach to study changes in glycosylation, they currently suffer from shortcomings that limit their utility for studying bacterial glycan dynamics[42]. One shortcoming is that unique bacterial glycans do not always have a known lectin binder. Conversely, some lectins display poor affinity for their glycan ligands, rendering the extraction of structural information about bacterial glycoproteins from lectin array results challenging. To address these issues, work is in progress to identify and characterize additional carbohydrate-binding proteins using glycan arrays[43,44]. Known lectins could also be modified through rational design and/or combinatorial approaches in order to alter lectin specificity and expand the diversity of lectins available for use[45]. With continued improvement, lectin arrays have the potential to become even more valuable tools for rapidly characterizing the dynamics of bacterial glycans.

Assessing the role of bacterial glycoproteins

Assessing the functions of bacterial glycoproteins provides the next level of depth in deciphering the bacterial glycome. There are instances in which the roles of bacterial glycans are clearly defined; however, there is an abundance of cases in which they remain unknown. Traditional approaches to assess the function of bacterial glycoproteins include the generation and characterization of glycosylation mutants (Figure 4a). Szymanski[46], Logan[5], Comstock[3], Koomey[13] and others[47] have conducted seminal studies in which they identified bacterial glycosylation systems, genetically interrupted these systems, and studied the effects on bacterial fitness and protein biology. These conventional genetic approaches have yielded the majority of what we currently know about bacterial glycoprotein function – e.g. its importance in survival within the host, binding to host cells, and stabilizing protein structure – and will continue to shed light in this area.

While genetic studies have the potential to reveal broad roles of glycans by inducing phenotypic changes, they do not provide a molecular level of detail about the function of bacterial glycans. Metabolically incorporated crosslinking sugars[48,49] offer one approach to yield insight into the molecular roles of bacterial glycoproteins. In particular, this method enables the delineation of glycan-protein interactions, which are crucial for mediating cellular events such as bacterial adhesion. In this technique, unnatural sugars that contain photoactivatable cross-linkers (e.g. aryl azides or diazirines) are metabolically incorporated into cellular glycoproteins[48–51]. After exposing cells to light, glycoproteins of interest are purified with their binding partners covalently attached. Mass spectrometry and immunochemical analyses are then conducted to reveal the identities of candidate ligands. Paulson, Kohler, and coworkers have utilized this approach to study glycan-binding proteins in mammalian cells[48–51]. Similar to precedents in eukaryotic systems, the use of metabolically incorporated cross-linkers will enable the unambiguous identification of bacterial glycan binding partners (Figure 4b). For example, bacterial monosaccharides such as pseudaminic acid or bacillosamine could be modified with cross-linkers, metabolically incorporated into *C. jejuni*'s glycoproteins, and used to assess the role of these monosaccharides in mediating adhesion of *C. jejuni* to host proteins. Such studies have the potential to elucidate the function of bacterial glycoproteins in host-pathogen interactions.

Harnessing bacterial glycosylation systems

Glycoproteins are very important commercially, as they form the basis of a number of therapeutics (e.g. erythropoietin, Herceptin) and carbohydrate-based conjugate vaccines (e.g. Prevnar). Commercial glycoproteins are typically accessed via expression in eukaryotic cells or by semi-synthesis, both of which are costly and time-consuming processes. The finding that bacteria synthesize glycoproteins is particularly exciting because it indicates that bacteria, and their glycosylation machinery, might be harnessed for the cheap and efficient

production of industrially important glycoproteins[52]. Indeed, Aebi, Feldman, and others have demonstrated that transfer of general glycosylation loci from other bacteria into *E. coli* directs the synthesis of recombinant glycoproteins in *E. coli*, a bacteria that lacks endogenous glycoproteins[53,54]. Both N-linked[54] and O-linked[55,56] glycoprotein assembly occurs in *E. coli* if the proper oligosaccharyltransferase is introduced. Concurrent expression of glycan modifying enzymes tailors glycan structures, enabling even human-like glycoproteins to be manufactured in *E. coli*[57,58]. Thus, the discovery of bacterial glycoproteins has propelled glycoengineering forward and made the facile production of therapeutic glycoproteins in *E. coli* a reality.

Conclusion

Bacterial glycoproteins represent a new frontier in glycomics. This recently discovered class of biomolecules contains unusual structures that are frequently linked to virulence and thus offers promise for the development of novel classes of anti-bacterials. Despite their potential therapeutic importance, our current knowledge of bacterial glycoproteins is far behind our knowledge of eukaryotic glycoproteins. Novel applications of metabolic glycan labeling and mass spectrometry complement traditional glycomics techniques to identify bacterial glycoproteins. Moreover, lectin-based microarrays enable the study of bacterial glycan dynamics. Studies that disrupt the synthesis of bacterial glycoproteins and unveil their binding partners yield phenotypic and molecular-level insights into their functions. Finally, bacterial glycosylation systems are being harnessed for glycoengineering purposes to produce commercially important glycoproteins with defined structures. Therefore, new approaches are deciphering the bacterial glycode and driving this field forward.

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Highlights

- Bacterial glycoproteins are potential drug targets and are worthy of study.
- Metabolic glycan labeling enables discovery and characterization of glycoproteins.
- Lectin arrays permit monitoring of bacterial glycoprotein dynamics.
- Crosslinking sugars reveal binding partners of glycoproteins to elucidate function.
- Bacterial glycosylation systems facilitate efficient glycoprotein production.

Figure 1.

Examples of bacterial glycan structures. Deoxy- and amino-monosaccharides, such as bacillosamine, pseudaminic acid, 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH), and *N*-acetyl fucosamine, are found in bacterial glycans but are absent from human glycans. These glycan structures can be elaborated with additional monosaccharides at the indicated positions (-OR).

Figure 2.

Methods to detect and isolate bacterial glycoproteins. Bacterial glycoproteins are tagged via (1) binding to glycan-binding reagents, such as lectins or antibodies (top arrow), (2) periodic acid-assisted oxidation followed by hydrazide attack (middle arrow), or (3) metabolic labeling with an unnatural sugar, such as an azide-containing sugar (shown on left), followed by bio-orthogonal chemistry (e.g. Staudinger ligation, bottom arrow). Once glycoproteins are tagged, they can be detected by Western blot or isolated via affinity chromatography from other cellular proteins.

Figure 3.

Representative workflow for a lectin-based microarray experiment to characterize bacterial glycan dynamics. In this technique, bacteria cultured in two conditions, A and B, are differentially labeled (e.g. with green and red fluorophores, respectively). The pool of labeled cells is then mixed and hybridized to a lectin microarray. Glycopatterns reveal differential binding of cells from conditions A and B and thus reflect alterations in surface glycan structures.

Figure 4.

Methods to decipher glycoprotein function. (a) In a traditional genetic approach, bacteria with wild type glycosylation (wt) are compared to glycosylation mutants (ΔGT) to reveal phenotypic changes, such as a change in the pathogenicity of the strain. (b) Crosslinking sugars can reveal molecular details of glycoprotein function, in particular the identity of glycan binding partners. In this technique, bacteria are metabolically labeled with a crosslinking sugar, and then exposed to host cells or proteins (shown). Non-covalent complexes are trapped by irradiating cells with light. Subsequent biochemical purification of complexes and analytical characterization reveals the identity of glycan binders.