Bowdoin College
Bowdoin Digital Commons

Chemistry Faculty Publications | Faculty Scholarship and Creative Work

2-1-2013

Deciphering the bacterial glycocode: Recent advances in bacterial glycoproteomics

Scott A. Longwell
Bowdoin College

Danielle H. Dube
Bowdoin College

Follow this and additional works at: https://digitalcommons.bowdoin.edu/chemistry-faculty-publications

Recommended Citation

Longwell, Scott A. and Dube, Danielle H., "Deciphering the bacterial glycocode: Recent advances in bacterial glycoproteomics" (2013). *Chemistry Faculty Publications*. 10.
https://digitalcommons.bowdoin.edu/chemistry-faculty-publications/10

This Article is brought to you for free and open access by the Faculty Scholarship and Creative Work at Bowdoin Digital Commons. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of Bowdoin Digital Commons. For more information, please contact mdoyle@bowdoin.edu, a.sauer@bowdoin.edu.

ELSEVIER

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Chemical Biology

Deciphering the bacterial glycocode: recent advances in bacterial glycoproteomics

Scott A Longwell and Danielle H Dube

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 glycocode, 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.

Address
Department of Chemistry and Biochemistry, Bowdoin College, 6600 College Station, Brunswick, ME 04011, USA

Corresponding author: Dube, Danielle H (ddube@bowdoin.edu)

Current Opinion in Chemical Biology 2013, **17**:41–48

This review comes from a themed issue on **Omics**

Edited by **Matthew Bogyo** and **Pauline M Rudd**

For a complete overview see the Issue and the Editorial

Available online 29th December 2012

1367-5931/$ – see front matter, © 2012 Elsevier Ltd. All rights reserved.

http://dx.doi.org/10.1016/j.cbpa.2012.12.006

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 subcellular 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 [1,2,3•]. 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.

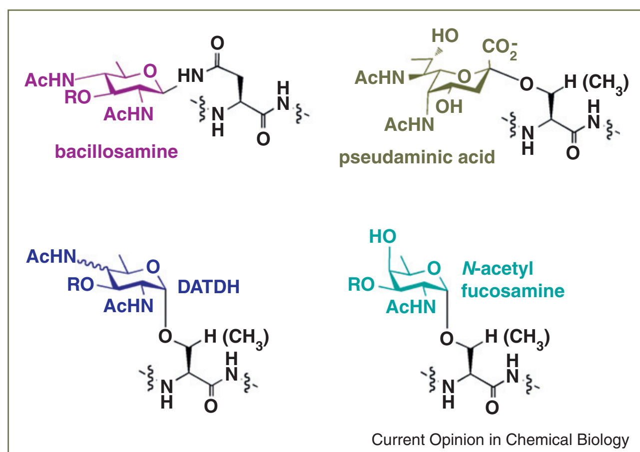
The second theme that has emerged is that bacterial glycoproteins contain unique glycans that are absent from humans [4•,8]. Although bacteria are capable of synthesizing the two major classes of glycoproteins that are found in eukaryotes – those that have asparagine-linked (N-linked) glycans and those 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, partly, 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 glycocode 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.

Figure 1



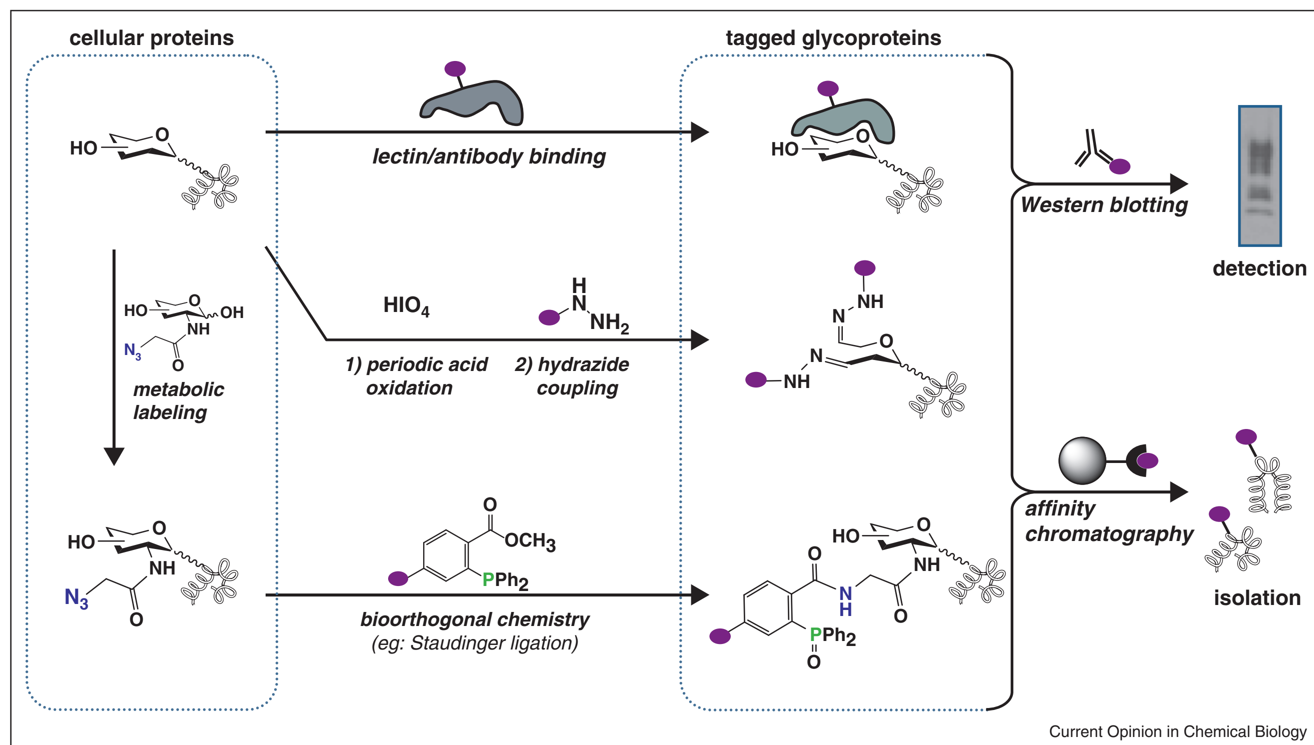
Examples of bacterial glycan structures. Deoxy- and amino-monosaccharides, such as bacillosamine, pseudaminic acid, 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH), and *N*-acetyl fucosamine, are found in bacterial glycans but are absent from human glycans. These glycan structures can be elaborated with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

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. By 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

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.

Bertozzi, Reutter, and co-workers 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 co-workers 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* (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 co-workers 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 before 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 (MS^2). 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 MS^2 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 co-workers 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 (MS^n)[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.

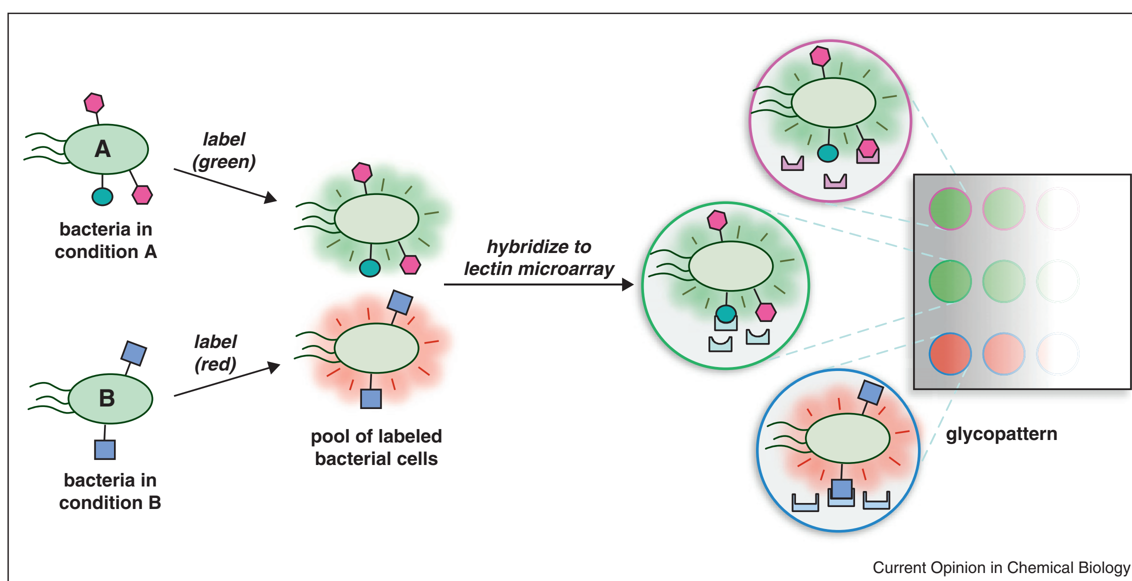
Monitoring bacterial glycan dynamics: lectin microarrays

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

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.

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

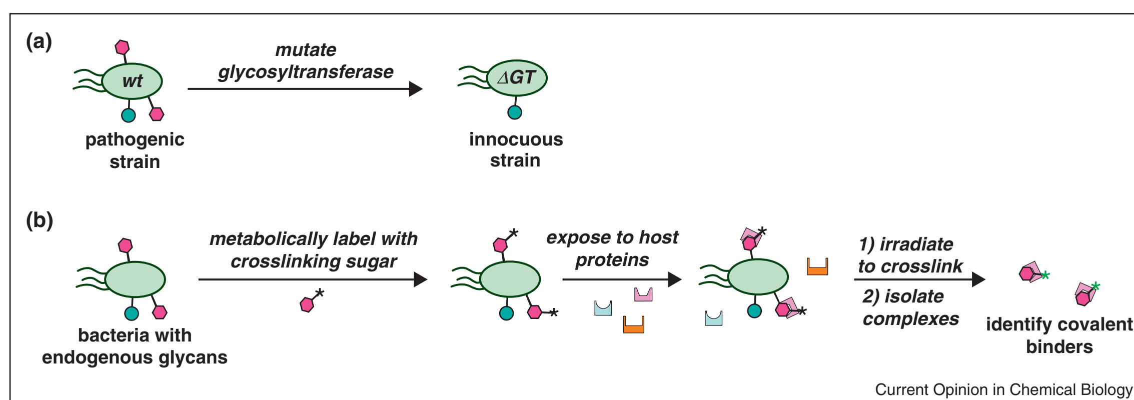
Assessing the functions of bacterial glycoproteins provides the next level of depth in deciphering the bacterial glycome. There are instances in which the roles of bacterial glycans are clearly defined; however, there is an abundance of cases in which they remain unknown. Traditional approaches to assess 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 – for example 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 [48,49•,50,51•]. 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 [48,49•,50,51•]. 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

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.

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 glycocode and driving this field forward.

Acknowledgements

We thank Margot Paulick, Ben Gorske, Aimee Eldridge, Van Tra, and Andrea Koenigsberg for providing helpful comments on the manuscript. SL thanks the Arnold and Mabel Beckman Foundation for an undergraduate research fellowship and the Idea Network for Biological Research Excellence for a post baccalaureate research fellowship. We gratefully acknowledge the National Institutes of Health (award R15GM093867) for supporting this work. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

References and recommended reading

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

- • of special interest
- •• of outstanding interest

1. Abu-Qarn M, Eichler J, Sharon N: **Not just for Eukarya anymore: protein glycosylation in Bacteria and Archaea**. *Curr Opin Struct Biol* 2008, **18**:544-550.

2. Benz I, Schmidt MA: **Never say never again: protein glycosylation in pathogenic bacteria**. *Mol Microbiol* 2002, **45**:267-276.

3. • Fletcher CM, Coyne MJ, Villa OF, Chatzidaki-Livanis M, Comstock LE: **A general O-glycosylation system important to the physiology of a major human intestinal symbiont**. *Cell* 2009, **137**:321-331.
This seminal paper identifies an O-glycosylation system in *Bacteroides fragilis* that glycosylates a number of proteins. The authors utilized a fucose-binding lectin to purify and identify glycosylated proteins. Further, they demonstrated that protein glycosylation is necessary for *B. fragilis* to competitively colonize the mammalian intestine.

4. • Dube DH, Champasa K, Wang B: **Chemical tools to discover and target bacterial glycoproteins**. *Chem Commun* 2011, **47**:87-101.
This recent review article summarizes our current understanding of bacterial glycoproteins, including their distinctive structures, links to pathogenesis, and challenges associated with their study. Moreover, the review describes how chemistry can be harnessed to study and target these biomolecules

5. Schirm M, Soo EC, Aubry AJ, Austin J, Thibault P, Logan SM: **Structural, genetic and functional characterization of the flagellin glycosylation process in *Helicobacter pylori***. *Mol Microbiol* 2003, **48**:1579-1592.

6. Gross J, Grass S, Davis AE, Gilmore-Erdmann P, Townsend RR, Geme JWS: **The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification**. *J Biol Chem* 2008, **283**:26010-26015.

7. Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schaffer C: **Potential of the *Tannerella forsythia* S-layer to delay the immune response**. *J Dent Res* 2011, **90**:109-114.

8. Balonova L, Hernychova L, Bilkova Z: **Bioanalytical tools for the discovery of eukaryotic glycoproteins applied to the analysis of bacterial glycoproteins**. *Expert Rev Proteomics* 2009, **6**:75-85.

9. Young NM, Brisson J-R, Kelly J, Watson DC, Tessier L, Lanthier PH, Jarrell HC, Cadotte N, St Michael F, Aberg E *et al.*: **Structure of the N-Linked glycan present on multiple glycoproteins in the Gram-negative bacterium, *Campylobacter jejuni***. *J Biol Chem* 2002, **277**:42530-42539.

10. Stimson E, Virji M, Makepeace K, Dell A, Morris HR, Payne G, Saunder JR, Jennings MP, Barker S, Panico M: **Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose**. *Mol Microbiol* 1995, **17**:1201-1214.

11. Castric P, Cassels FJ, Carlson RW: **Structural characterization of the *Pseudomonas aeruginosa* 1244 pilin glycan**. *J Biol Chem* 2001, **276**:26479-26485.

12. Hitchen PG, Dell A: **Bacterial glycoproteomics**. *Microbiology* 2006, **152**:1575-1580.

13. Vik A, Aas FE, Anonsen JH, Bilsborough S, Schneider A, Egge-Jacobsen W, Koomey M: **Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae***. *Proc Natl Acad Sci USA* 2009, **106**:4447-4452.

14. Hopf PS, Ford RS, Zebian N, Merkx-Jacques A, Vijayakumar S, Ratnayake D, Hayworth J, Creuzenet C: **Protein glycosylation in *Helicobacter pylori*: Beyond the flagellins?** *PLoS ONE* 2011, **6**:e25722.

15. Dube DH, Bertozzi CR: **Metabolic oligosaccharide engineering as a tool for glycobiology**. *Curr Opin Chem Biol* 2003, **7**:616-625.

16. Laughlin ST, Bertozzi CR: **Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation**. *Nat Prot* 2007, **2**:2930-2944.

17. Keppler OT, Horstkorte R, Pawlita M, Schmidts C, Reutter W: **Biochemical engineering of the N-acyl side chain of sialic acid: biological implications**. *Glycobiology* 2001, **11**:11R-18R.

18. Prescher JA, Bertozzi CR: **Chemistry in living systems**. *Nat Chem Biol* 2005, **1**:13-21.

19. Saxon E, Bertozzi CR: **Cell surface engineering by a modified Staudinger reaction**. *Science* 2000, **287**:2007-2010.

20. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB: **A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes**. *Angew Chem Int Ed* 2002, **41**:2596-2599.

21. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG: **Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition**. *J Am Chem Soc* 2003, **125**:3192-3193.

22. • Koenigs MB, Richardson EA, Dube DH: **Metabolic profiling of *Helicobacter pylori* glycosylation**. *Mol Biosyst* 2009, **5**:909-912.
The study applied metabolic glycan labeling to detect myriad glycoproteins in *Helicobacter pylori*. Glycan cleaving enzymes suggest that the labeled species observed are both N-linked and O-linked glycoproteins.

23. • Liu F, Aubry AJ, Schoenhofen IC, Logan SM, Tanner ME: **The engineering of bacteria bearing azido-pseudaminic acid-modified flagella**. *Chembiochem* 2009, **10**:1317-1320.
The authors selectively labeled pseudaminic acid-modified glycoproteins in *Campylobacter jejuni* with azides. Their data reveal that only flagellin proteins are modified with pseudaminic acid and set the stage for selectively targeting azide-covered *C. jejuni* with therapeutic phosphines.

24. • Besanceney-Webler C, Jiang H, Wang W, Baughn AD, Wu P: **Metabolic labeling of fucosylated glycoproteins in *Bacteroidales* species**. *Bioorg Med Chem Lett* 2011, **21**:4989-4992.
An example of how metabolic glycan labeling can complement lectin-based approaches to detect bacterial glycoproteins. These authors utilized an alkyne-modified fucose derivative to detect fucose-containing *Bacteroides* and *Parabacteroides* glycoproteins.

25. Bond MR, Kohler JJ: **Chemical methods for glycoprotein discovery**. *Curr Opin Chem Biol* 2007, **11**:52-58.

26. Laughlin ST, Baskin JM, Amacher SL, Bertozzi CR: ***In vivo* imaging of membrane-associated glycans in developing zebrafish**. *Science* 2008, **320**:664-667.

27. Zaro BW, Yang Y-Y, Hang HC, Pratt MR: **Chemical reporters for fluorescent detection and identification of O-GlcNAc-modified proteins reveal glycosylation of the ubiquitin ligase NEDD4-1**. *Proc Natl Acad Sci USA* 2011, **108**:8146-8151.

28. Nessen MA, Kramer G, Back J, Baskin JM, Smeenk LEJ, de Koning LJ, an Maarseveen JH, de Jong L, Bertozzi CR, Hiemstra H *et al.*: **Selective enrichment of azide-containing peptides from complex mixtures**. *J Proteome Res* 2009, **8**:3702-3711.

29. • Breidenbach MA, Palaniappan KK, Pitcher AA, Bertozzi CR: **Mapping yeast N-glycosites with isotopically recoded glycans**. *Mol Cell Proteom* 2012.
Developed a metabolic labeling approach that confers a distinctive isotopic tag to N-glycans, enabling glycopeptides to be distinguished from their non-glycosylated counterparts during LC–MS/MS analysis.

30. Scott NE, Parker BL, Connolly AM, Paulech J, Edwards AVG, Crossett B, Falconer L, Kolarich D, Djordjevic SP, Hojrup P *et al.*: **Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS Applied to the N-linked glycoproteome of *Campylobacter jejuni***. *Mol Cell Proteom* 2011, **10** M000031–M201.

31. Schirm M, Schoenhofen IC, Logan SM, Waldron KC, Thibault P: **Identification of unusual bacterial glycosylation by tandem mass spectrometry analyses of intact proteins**. *Anal Chem* 2005, **77**:7774-7782.

32. Ashline D, Singh S, Hanneman A, Reinhold V: **Congruent strategies for carbohydrate sequencing. 1. Mining structural details by MSn**. *Anal Chem* 2005, **77**:6250-6262.

33. Brisson JR, Vinogradov E, McNally DJ, Khieu NH, Schoenhofen IC, Logan SM, Jarrell H: **The application of NMR spectroscopy to functional glycomics**. *Methods Mol Biol* 2010, **600**:155-173.

34. Zhang Y, Yin H, Lu H: **Recent progress in quantitative glycoproteomics**. *Glycoconj J* 2012, **29**:249-258.

35. Laughlin ST, Bertozzi CR: **Imaging the glycome**. *Proc Natl Acad Sci USA* 2009, **106**:12-17.

36. Leipold MD, Ornatsky O, Baranov V, Whitfield C, Nitz M: **Development of mass cytometry methods for bacterial discrimination**. *Anal Biochem* 2011, **419**:1-8.

37. Pilobello KT, Krishnamoorthy L, Slawek D, Mahal LK: **Development of a lectin microarray for the rapid analysis of protein glycopatterns**. *Chembiochem* 2005, **6**:985-989.

38. Hsu K-L, Mahal LK: **A lectin microarray approach for the rapid analysis of bacterial glycans**. *Nat Prot* 2006, **1**:543-549.

39. Hsu K-L, Pilobello KT, Mahal LK: **Analyzing the dynamic bacterial glycome with a lectin microarray approach**. *Nat Chem Biol* 2006, **2**:153-157.

40. • Gao J, Liu D, Wang Z: **Screening lectin-binding specificity of bacterium by lectin microarray with gold nanoparticle probes**. *Anal Chem* 2010, **82**:9240-9247.
Developed a lectin-microarray-based assay to screen glycoconjugates on microbial surfaces. The authors were able to detect dynamic changes in surface glycans on microbial strains in response to exposure to various growth media.

41. • Semchenko EA, Day CJ, Moutin M, Wilson JC, Tiralongo J, Korolik V: **Structural heterogeneity of terminal glycans in *Campylobacter jejuni* lipooligosaccharides**. *PLoS ONE* 2012, **7**:e40920.
This study employed lectin arrays to analyze terminal lipooligosaccharides (LOS) glycans on *C. jejuni*. The authors found that LOS structure cannot always be predicted from the genetic composition of the biosynthetic cluster.

42. Hsu K-L, Mahal LK: **Sweet tasting chips: microarray-based analysis of glycans**. *Curr Opin Chem Biol* 2009, **13**:427-432.

43. Cholleti SR, Agravat S, Morris T, Saltz JH, Song X, Cummings RD, Smith DF: **Automated motif discovery from glycan array data**. *OMICS: J Integ Biol* 2012, **9**:2012 Online Ahead of Print: August.

44. Haab BB: **Using lectins in biomarker research: addressing the limitations of sensitivity and availability**. *Proteomics Clin Appl* 2012, **6**:346-350.

45. Propheter DC, Hsu K-L, Mahal LK: **Recombinant lectin microarrays for glycomic analysis**. *Meth Mol Biol* 2011, **723**:67-77.

46. Larsen JC, Szymanski C, Guerry P: **N-Linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81-176**. *J Bacteriol* 2004, **186**:6508-6514.

47. •• Howard SL, Jagannathan A, Soo EC, Hui JPM, Aubry AJ, Ahmed I, Karlyshev A, Kelly JF, Jones MA, Stevens MP *et al.*: ***Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens**. *Infect Immun* 2009, **77**:2544-2556.
A beautiful study that demonstrates links between glycosylation of *C. jejuni* flagella with legionaminic acid, autoagglutination of cells, biofilm formation, and the ability of *C. jejuni* strains to colonize chickens. The authors employed a genetic strategy to create *C. jejuni* glycosylation mutants and complemented strains.

48. Yu S-H, Bond MR, Whitman CM, Kohler JJ: **Metabolic labeling of glycoconjugates with photocrosslinking sugars**. *Methods Enzymol* 2010, **478**:541-562.

49. • Yu S-H, Boyce M, Wands AM, Bond MR, Bertozzi CR, Kohler JJ: **Metabolic labeling enables selective photocrosslinking of O-GlcNAc-modified proteins to their binding partners**. *Proc Natl Acad Sci USA* 2012, **109**:4834-4839.
This paper is a very recent example of how photocrosslinking sugars can be employed in cells to reveal the functional roles of glycans. In this study, a diazirine-modified GlcNAc-1-phosphate analog was incorporated into O-GlcNAc modified proteins and utilized to identify glycan binders.

50. Bond MR, Zhang HC, Vu PD, Kohler JJ: **Photocrosslinking of glycoconjugates using metabolically incorporated diazirine-containing sugars**. *Nat Prot* 2009, **4**:1044-1063.

51. • Ramya TNC, Weerapana E, Liao L, Zeng Y, Tateno H, Liao L, Yates JR, Cravatt BF, Paulson JC: **In situ trans ligands of CD22 identified by glycan-protein photocross-linking-enabled proteomics**. *Mol Cell Prot* 2010, **9**:1339-1351.
An example of how photocrosslinking sugars can be employed in cells to reveal molecular binding partners. The authors incorporated an aryl-azide-modified sialic acid analog into B-cells to search for trans ligands of CD22. The authors were able to identify the B-cell receptor IgM as a validated binder of CD22.

52. Terra VS, Mills DC, Yates LE, Abouelhadid S, Cuccui J, Wren BW:
•• **Recent developments in bacterial protein glycan coupling technology and glycoconjugate vaccine design**. *J Med Microbiol* 2012, **61**:919-926.
A very recent review article that describes the functional expression of bacterial glycosylation enzymes in *E. coli* to produce tailor-made glycoproteins.

53. Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW *et al.*: **N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli***. *Science* 2002, **298**:1790-1793.

54. Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, Morris HR, Dell A, Valvano MA, Aebi M: **Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli***. *Proc Natl Acad Sci USA* 2005, **102**:3016-3021.

55. Gebhart C, Ielmini MV, Reiz B, Price NL, Aas FE, Koomey M,
• Feldman MF: **Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in *Vibrio cholerae* and *Burkholderia thailandensis***. *Glycobiology* 2012, **22**:962-974.
The authors identified oligosacchyltransferases involved in O-glycosylation in *V. cholerae* and *B. thailandesis* and functionally transferred them into *E. coli.* These enzymes display relaxed glycan and protein substrate specificity and have the potential to be used in glycoengineering purposes.

56. Egge-Jacobsen W, Salomonsson ENs, Aas FE, Forslund A-L, Winther-Larsen HC, Maier J, Macellaro A, Kuoppa K, Oyston PCF, Titball RW *et al.*: **O-Linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-yargeting oligosaccharyltransferase and characterization of the native oligosaccharide**. *J Bacteriol* 2011, **193**:5487-5497.

57. Schwarz F, Huang W, Li C, Schulz BL, Lizak C, Palumbo A, Numao S, Neri D, Aebi M, Wang L-X: **A combined method for producing homogeneous glycoproteins with eukaryotic N-glycosylation**. *Nat Chem Biol* 2010, **6**:264-266.

58. Valderrama-Rincon JD, Fisher AC, Merritt JH, Fan Y-Y,
•• Reading CA, Chhiba K, Heiss C, Azadi P, Aebi M, DeLisa MP: **An engineered eukaryotic protein glycosylation pathway in *Escherichia coli***. *Nat Chem Biol* 2012, **8**:434-436.
A beautiful example of harnessing bacterial glycosylation enzymes for the synthesis of human-like glycoproteins in *E. coli*. The authors engineered an *E. coli* strain to express *C. jejuni*'s oligosaccharyltransferase pglB, as well as four eukaryotic glycosyltransferases, for the production of human-like N-linked glycans on eukaryotic proteins.