Never take candy from a stranger: the role of the bacterial glycome in host–pathogen interactions

Christopher W Reid, Kelly M Fulton & Susan M Twine†

†Author for correspondence: National Research Council – Institute for Biological Science, Ottawa, ON, K1A 0R6, Canada. ■ Tel.: +1 613 949 7545 ■ Fax: +1 613 952 9092 ■ Susan.Twine@nrc-cnrc.gc.ca

With the comprehensive study and complete sequencing of the *Haemophilus influenzae* genome in 1995 came the term ‘genomics’ and the beginning of the ‘omics’ era. Since this time, several analogous fields, such as transcriptomics and proteomics, have emerged. While growth and advancement in these fields have increased understanding of microbial virulence, the study of bacterial glycomes is still in its infancy and little is known concerning their role in host–pathogen interactions. Bacterial glycomics is challenging owing to the diversity of glyco-conjugate molecules, vast array of unusual sugars and limited number of analytical approaches available. However, recent advances in glycomics technologies offer the potential for exploration and characterization of both the structures and functions of components of bacterial glycomes in a systematic manner. Such characterization is a prerequisite for discerning the role of bacterial glycans in the interaction between host defences and bacterial virulence factors.

Carbohydrates constitute the most structurally diverse class of natural products and can serve many functions in cells and organisms [1]. In addition to their role in energy metabolism, carbohydrates are also found attached to proteins and lipids, or as loosely associated polysaccharides on the cell surface. Glycobiology encompasses the determination of the structure and function of complex carbohydrates (glycans). More recently, there has been a shift within glycobiology towards large-scale systematic studies of the entire complement of glycan structures within a given cell or organism, referred to as the ‘glycome’. Glycomics, therefore, is the study of the significance of glyco-conjugate assembly and expression in biological systems. Since glycosylation involves a series of metabolic events, some of the concepts in lipidomics [2] and metabolomics [3] are common to glycomics. As a field, glycomics has lagged behind genomics and proteomics, owing to the inherent difficulties in the isolation of glycans, the subsequent analysis of their structure and function and the limited number of different analytical techniques available for use in their study.

Surface polysaccharides represent the predominant structures on all bacterial cell surfaces, and they are often important players in the interactions between pathogens, their hosts and the environment. These structures can be involved in the maintenance of surface charge, phase variation and serum resistance. In bacteria, the role of these glycan moieties in symbiosis, pathogenesis, biofilm formation, cell–cell interactions and evasion of the immune response [4–7] is just

beginning to be understood. In Gram-negative bacteria, the vast majority of carbohydrate moieties are found in lipopolysaccharides (LPS)/ lipooligosaccharides (LOS) or capsular polysaccharide (CPS). There are an increasing number of reports describing the modification of bacterial proteins with *N*- or *O*-linked glycan moieties (FIGURE 1) (reviewed in [8,9]).

One of the main challenges in the study of glycomes is the complexity of carbohydrate structures. In mammals, carbohydrates are assembled from a group of approximately ten common monosaccharides. By contrast, the absolute number of potential monosaccharide building blocks in bacteria is unknown. Bacteria can synthesize pentose and heptose sugars not commonly found in mammals, and this diverse array of glycans is proving to be an analytical challenge. In addition, carbohydrate complexity increases as monosaccharides are assembled into linear and branched polymers based on the regiochemistry and stereochemistry (α or β) of the anomeric carbon in the glycosidic bond. For example, it is estimated that, for a hexasaccharide, all possible oligosaccharide isomers would yield approximately 10^{12} structures [10]. In addition to the monosaccharide and linkage diversity, a number of modifications to the monosaccharide subunits can occur, including methylation, sulfation, phosphorylation and, in some bacteria, the addition of amino acids [11,12]. Finally, the method of display of the glycan, by attachment to lipids or via *N*- and *O*-linkage to proteins, adds further complexity to the glycome. This complexity is compounded by the

Keywords

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nontemplate-driven nature of glycan biosynthesis. Taking all these factors into consideration, the analytical challenge to dissect these structures, and assign biological function, is substantial.

In this review we provide an overview of bacterial glycomes and the analytical techniques that are employed in their study. Owing to the

diversity in this field, it is not feasible to discuss all the contributions made to bacterial glycomics; therefore, this review will focus on surveying the recent literature and describing select techniques that can be applied to the analysis of the bacterial glycome. We then focus upon how this work has specifically contributed to

our understanding of the role of the bacterial glycome in the host–pathogen interactions of *Campylobacter jejuni*.

Deciphering the bacterial glycome using mass spectrometry

Mass spectrometry in bacterial glycoprotein discovery

For many years, glycoproteins were thought to be expressed solely by eukaryotes and archaea, but it is now evident that glycoproteins are found in all domains of life. The first noneukaryotic glycoprotein was the surface layer protein (SLP) from the archaean *Halobacterium salinarum* [13,14]. More recently, research has demonstrated that several medically relevant human pathogens also modify their proteins with glycan moieties [15].

In the last decade, protein *N*-glycosylation (via asparagine residues) has been identified in the intestinal pathogen *C. jejuni* [16], and *O*-linked protein glycosylation (via serine or threonine residues) has been described in pathogens such as *Helicobacter pylori* [17], *Pseudomonas aeruginosa* [18,19], *Neisseria* spp. [20,21], *Escherichia coli* [22,23] and *Clostridium difficile* [24]. Protein glycosylation has been increasingly reported in both Gram-positive and Gram-negative bacteria, commonly including flagellins [9], pilins [25–27], fimbriae [28], cell surface proteins (e.g., adhesins [29–31]) and structural proteins (e.g., SLPs of Gram-positive bacteria [32]). Although the function of the glycan moiety of these proteins has yet to be determined, there are a number of reports describing a role in virulence, colonization and macromolecular assembly [33–35].

Two different mechanisms for protein glycosylation have been observed and are based on the mechanism through which the glycans are transferred to proteins. In the first mechanism, the transfer of carbohydrates occurs directly from nucleotide-activated sugars to acceptor proteins. This mechanism is typical of *O*-glycosylation in eukaryotes, and flagellar glycosylation in several bacterial species [9]. Alternatively, an oligosaccharide can be preassembled on a polyisoprene carrier before being transferred *en bloc* to protein acceptors by an oligosaccharyltransferase. This type of mechanism is found in the *N*-glycosylation pathways in eukaryotes, bacteria [36] and archaea, and *O*-linked glycosylation of pili in *P. aeruginosa* [27,37,38] and *Neisseria* species [21]. Of particular note, the *N*-linked glycosylation pathway of *C. jejuni* produces a conserved heptasaccharide with the structure GalNAc- α 1,4-GalNAc- α 1,4-(Glc- β 1,3)-GalNAc- α 1,4-GalNAc- α 1,4-GalNAc- α 1,3-Bac2,4diNAc- β 1,

where Bac2,4diNAc is 2,4-diacetamido-2,4,6-trideoxyglucopyranose [39] and is assembled by proteins encoded in the protein glycosylation (*pgl*) locus [16]. This glycan is synthesized on a polyisoprenyl carrier at the cytoplasmic face of the inner membrane through the action of a phosphoglycosyltransferase (PglC) and several glycosyltransferases (PglA, PglH, PglI and PglJ). The assembled oligosaccharide is then flipped to the periplasm by the action of the membrane-associated flippase (PglK). Once in the periplasm, the oligosaccharide is transferred to proteins that possess the extended sequon Asp/Glu-X-Asn-Y-Ser/Thr (where X and Y are any amino acid except Pro) by the oligosaccharyltransferase (PglB) [40,41]. The similarities between the Pgl pathway in *Campylobacter* and the eukaryotic *N*-glycosylation machinery have resulted in an explosion of research on this pathway, as the pathway serves as a simplified model for studying the eukaryotic system. Additionally, it can be functionally transferred to *E. coli* [42], where it shows potential for applications in glycoengineering [43].

Detection of bacterial glycoproteins is often a challenging task owing to the diverse array of monosaccharide building blocks available to bacteria. Nevertheless, detecting novel bacterial glycoproteins and predicting the structure of the attached glycans is virtually impossible, and must be deduced using a wide range of bioanalytical techniques. These include high-performance liquid chromatography (HPLC) [44], mass spectrometry (MS) [45–48], nuclear magnetic resonance (NMR) [49], polyacrylamide gel electrophoresis (PAGE) [50] and capillary electrophoresis (CE) [51–53]. Typically studies commence with glycoprotein detection by 1D or 2D gel electrophoresis, where aberrant protein migration is often the first clue. Commercially available lectin-binding kits or carbohydrate-reactive dyes are frequently used. Putative glycoproteins are then typically isolated and their initial characterization generally relies heavily upon MS, exploiting so-called ‘top-down’ or ‘bottom-up’ techniques. An outline of these approaches, as applied to bacterial glycoprotein discovery and analysis, is presented in FIGURE 2. The higher throughput study of the entire complement of glycoproteins within a bacterial cell is an emerging field known as ‘glycoproteomics’, a description of which is beyond the scope of this article. Progress in bacterial glycoproteomics has recently been reviewed [54].

‘Bottom-up’ MS analyses of putative bacterial glycoproteins can be carried out on purified glycoprotein(s) or protein(s) separated by gel electrophoresis. The protein is digested with

Figure 2. Mass spectrometry workflow for the identification and characterization of individual bacterial glycoproteins. ‘Bottom-up’ analyses of glycoproteins at the peptide level: the bottom-up method involves the tandem mass spectrometry (MS/MS) analysis of enzymatically digested protein/glycoprotein. **(A)** The diverse and unusual bacterial glycans make the interpretation of bacterial glycopeptide MS/MS data challenging. *De novo* peptide sequencing is often required for identification of bacterial glycopeptides. **(B)** Mass spectrometers equipped with electron transfer dissociation (ETD) or electron capture dissociation (ECD) can be used to determine individual sites of glycan linkages in glyopeptides. Both ETD and ECD afford an alternative method of peptide fragmentation to more widely used collision-induced dissociation, preserving delicate glycan modifications; **(B)** shows a representative ETD spectrum of *Clostridium botulinum* glycopeptide. A combination of these complementary techniques allows a comprehensive characterization of a putative glycoprotein sequence. **(C)** Typical peptide sequence coverage obtained for FlaA protein from MS/MS and ETD data. These data showed that glycan was attached through O-linkage, with seven sites of modification (Ser126, Ser139, Ser142, Ser165, Ser171, Ser176 and Ser182). ‘Top-down’ MS analyses of purified intact glycoproteins: the top-down approach uses mass spectrometry techniques to analyze the intact protein. **(D)** An observed mass in excess of the mass expected based on the translated genome sequence is often indicative of post-translational modification, such as glycosylation. The MS-profile of an intact protein usually results in a broad envelope of multiply charged protein ions in the MS spectrum, from which the reconstructed molecular mass profile can be calculated. The data shown in **(D)** shows the observed masses of FlaA from *C. botulinum* Alberta, with three distinct masses of 32297, 31879 and 31462 Da. Of note, each of the major intact mass peaks was separated by a mass of 417 Da, which corresponds to the mass of the flagellin glycan. MS/MS analysis of a single, multiply charged protein ion, can often yield information regarding the masses of labile, protein-associated, glycan ions. Analysis of *C. botulinum* Alberta flagellins using this method revealed at least two distinct marker ions of mass-to-charge ratio 512.3 and 418.2. By adjusting the collision energy at the front end of the mass spectrometer, these glycan associated ions can also be observed (known as front-end collision-induced dissociation) **(E)**. Subsequently, MS/MS of the observed glycan fragment ions can yield information about the glycan composition. For the FlaA protein of *C. botulinum*, the spectrum of the glycan oxonium ion at mass-to-charge ratio 418 gave an MS/MS spectrum with predominant peaks at 274, 240 and 181 Da. The fragmentation pattern of this glycan moiety closely resembled that previously observed for pseudaminic acid or legionaminic acid, with an additional unknown mass of 143 Da. Overall, these data showed the FlaA protein to be modified with a pseudaminic acid-like sugar of 417 Da, at seven sites via O-linkage via serine residues. The structure of this sugar was determined by nuclear magnetic resonance of a purified glycopeptide, and shown to be a novel legionaminic acid derivative, 7-acetamido-5-(N-methyl-glutam-4-yl)-amino-3,5,7,9-tetra-deoxy-d-glycero- α -D-galacto-nonulosonic acid [13]. Data represented uses *Clostridium botulinum* strain Alberta flagellin, FlaA, as a representative glycoprotein [13].

a proteolytic enzyme, most commonly trypsin, and analyzed by tandem MS (MS/MS). Consequently, strategies to rapidly identify and evaluate glycopeptides are required. The use of software tools for assignment of peptide fragmentation data are widely used as a matter of routine (e.g., MASCOT™; Matrix Science, London, UK). By contrast, the development and use of bioinformatics tools and databases for glycobiology and glycomics research has been slow. Software-assisted data analysis of bacterial glycopeptide tandem mass spectra is often hampered by a lack of knowledge of the glycan produced by a particular bacterium. Therefore, *de novo* glycopeptide analyses are necessary, despite being time consuming and often a bottleneck in such studies. Additional information obtained from ‘bottom-up’ studies includes linkage site information and glycan sequence information [55]. Observation of glycan MS/MS fragmentation patterns can potentially provide clues as to the glycan structure.

‘Top-down’ MS permits the study of the intact protein, rather than the peptides generated from proteolytic digestion of the protein. Recently, a top-down MS approach was applied to expedite the identification of unusual bacterial glycoprotein-associated ions (so-called ‘oxonium’ ions) [56]. The selectivity and specificity of this approach was exploited for the identification of the unusual glycan modification of flagellin from protein *H. pylori* [56]. Studies of *Clostridium botulinum* strains also used this approach as a higher-throughput method of characterizing flagellin from a wide range of *C. botulinum* isolates, and showed potential for flagellin glycan-associated marker ions in strain differentiation [12,57].

Newer strategies are currently being developed to specifically enrich glycoproteins or peptides from bacteria. Some of these approaches are being adapted from methods developed for the study of eukaryotic glycans [58]. For example, hydrazide capture of *N*-linked glycoproteins, lectin affinity purification and ion-pairing normal phase liquid chromatography (IP-NPLC) are being applied to prokaryotic glycoprotein discovery, and are increasing the study of glycoproteomics [59,60].

Of particular interest, a recent study used IP-NPLC to resolve glycosylated peptides from a tryptic digest of *C. jejuni* 11168 periplasmic proteins. The method used acids as ion-pairing reagents in normal-phase chromatography to increase the hydrophobicity differences between nonglycopeptides and glycopeptides. This permitted the reproducible isolation of bacterial *N*-linked glycopeptides, modified with nonsialylated

heptasaccharides, and expression comparisons of glycopeptides between *C. jejuni* 11168 and its *pglD* mutant [59]. This study reported the first observation of heptasaccharides containing monoacetylated bacillosamine-modifying *C. jejuni* glycoproteins [59].

Identification of glycoproteins is the first challenge; the second is gathering structural information regarding the attached sugar. In many cases, this requires release of the glycan from the protein, to allow detailed structural analyses of the glycan using MS or NMR. Release of bacterial glycans from proteins can be challenging – many existing methods were developed for release and study of eukaryotic glycans. These techniques may not be applicable to bacterial proteins because the carbohydrate–peptide linkages are different and the enzymes capable of releasing the carbohydrate moieties are rarely available. However, a universal glycomics method was used to address the challenges of glycan structural investigations. This method, based on the combination of nonspecific proteolytic digestion and permethylation, was applied as a complementary approach to study the diversity of *C. jejuni* 11168 *N*-linked sugars [61]. *N*-glycome analysis of *C. jejuni* 11168 allowed, for the first time, detection of free heptasaccharide that were unexpectedly produced by the *N*-glycan pathway within the periplasm. The authors were able to clearly distinguish between free glycan and asparagine-linked glycan [61].

Unambiguous structural assignment is most readily obtained from NMR studies of purified sugars or short glycopeptides. In addition to challenges in releasing glycans from bacterial proteins, obtaining sufficient glycan material from purified proteins is difficult. In attempts to overcome this obstacle, focused metabolomics studies have targeted intracellular nucleotide-activated glycan precursors in order to characterize glycan biosynthetic pathways, using both MS and NMR. Such studies have been pivotal in the structural analysis of *C. jejuni* protein-associated glycans, and in functional studies of the associated biosynthetic pathways [17,62–67]. Such work is often a prerequisite for further functional studies of the role of the glycoprotein in virulence.

MS & related technologies in bacterial nonprotein glycan discovery

Detection of bacterial glycolipids by MS

Technological advancements in genomics, transcriptomics and proteomics have allowed scientists to probe biological systems with increased

precision and sensitivity. While the analysis of lipids from microorganisms has been around since the 1950s, the tools for the examination of the lipidome and, in particular, the glycolipidome are still emerging [68–70]. The role of glycolipids in host–pathogen interactions is an emerging field, with the bulk of the scientific literature revolving around the analysis of lipid A [71,72]. Traditional methods for the analysis of lipid-linked oligosaccharides (LLOs), a subclass of biologically relevant glycolipids, usually involves chemical or radioactive labeling of the oligosaccharide component followed by chromatographic or electrophoretic separation [73]. In the realm of bacterial glycolipidomics, there is an increasing body of work on the assembly of bacterial polysaccharides and the polyisoprenyl carriers upon which they are assembled. MS has been the tool of choice to investigate the levels of undecaprenyl phosphate in bacterial membranes [74], levels of lipid II, an intermediate in peptidoglycan biosynthesis [75], and levels of polyisoprene intermediates in LPS biosynthesis in *Salmonella* spp., *E. coli* and *Francisella novicida* [76–78]. While glycolipidomics is still an emerging field, the *C. jejuni* Pgl pathway (described in detail previously) will be used as an example of the information that can be obtained by analyzing lipid-mediated steps in oligosaccharide biosynthesis *in vivo*.

Lipooligosaccharide profiling of the Pgl pathway using the newly developed affinity-capture CE-MS has provided unique insight into the synthesis of the *N*-glycan oligosaccharide [79]. This method has allowed for the rapid enrichment of LLOs, utilizing an immobilized lectin (soybean agglutinin) with specificity towards the GalNAc residues in the *C. jejuni* LLO of interest. In order to characterize the membrane-associated steps in the Pgl pathway, both wild-type *C. jejuni* and isogenic mutants of the pathway were used to investigate the assembly of LLO intermediates. Analyses of isogenic mutants in the oligosaccharyltransferase (*pglB*) and membrane flippase (*pglK*) revealed an accumulation of undecaprenyl-pyrophosphate (Und-PP) heptasaccharide, with a significantly greater amount found in the flippase mutant. The lower levels of Und-PP-heptasaccharide in the *pglB* mutant is most likely due to the presence of enzymes in the periplasm responsible for the recycling of Und-PP. The accumulation of LLO intermediates in these mutants confirmed that the *C. jejuni* *N*-glycan is assembled on undecaprenyl phosphate. Analysis of isogenic mutants in steps upstream of the flippase PglK revealed

an absence of accumulating truncated LLO intermediates, suggesting feedback regulation of the pathway. This conclusion was supported by the observation of the accumulation of UDP-diacetamido-trideoxy-Hex in the galactosyltransferase mutants, *pglJ* and *pglH*, and the observed allosteric regulation in the analogous yeast oligosaccharyltransferase complex [80]. Since undecaprenol is in limited abundance in the bacterial cell and is involved in a plethora of cellular activities, some of which are vital for cell survival, it is not surprising that a feedback regulation of the Pgl pathway would exist to prevent the accumulation of LLOs and sequestering of undecaprenol from other biological processes.

The application of affinity capture-MS to the analysis of membrane-associated processes in carbohydrate biosynthesis pathways provides a simple and rapid method to gather structural information for the oligosaccharides under study. Unlike conventional techniques, radioisotope labeling and chromatographic separation are not required for analysis [81]. In addition, by varying the lectin used for affinity capture, this method can be applied to a wide range of systems. Analysis of complex bacterial carbohydrate biosynthetic pathways, such as those for pilin *O*-glycosylation and peptidoglycan assembly, may provide new insights into the synthesis and control of these glycans. Targeted glycolipidomic profiling is a powerful tool for bacterial glycobiology. It provides a high-throughput method for assessing the role of genes in a biosynthetic pathway and allows for the identification of potential regulatory mechanisms, by detecting the accumulation or absence of intermediates in the pathway. With the recent advances in glycoengineering in bacteria, affinity-capture CE-MS also allows for a more comprehensive assessment of the efficiency and potential bottlenecks associated with producing potential protein glycoconjugates in bacteria. Current work on the analysis of bacterial LLOs is focused on the development of more general and nonselective enrichment procedures.

LPS profiling by CE-MS

Capillary electrophoresis is a high-resolution technique for the separation of complex biological mixtures [82]. When coupled with MS, CE provides a powerful approach for the identification and structural characterization of analytes. This technique has been used for the characterization of bacterial glycolipids and glycoconjugates from bacteria, including *H. influenzae* [83–87], *Neisseria meningitidis* [85,88], *Pasteurella*

multocida [89–91], *H. pylori* [92,93], *C. jejuni* [94–96], *Vibrio anguillarum* [97] and *Aeromonas salmonicida* [98]. CE–MS has been widely used for characterizing differences in LPS glycoform and phosphorylated populations [84,88,99]. This is achieved by exploiting differences in the molecular conformations and charge distributions of closely related LPS molecules during electrophoretic separations.

A recent study employed immunoaffinity capture CE–MS to investigate LPS changes in the fish pathogen *A. salmonicida* under co-culture conditions [100]. When co-cultured with trout macrophages in intraperitoneal chambers, significant differences were observed in the mass spectral fragmentation patterns of the LPS *O*-chain polysaccharide isolated *in vivo*. The *O*-antigen repeating unit was previously reported to contain a rhamnose residue substituted with side chain glucose and *O*-acetyl group [101]. Compared with LPS isolated from cells grown *in vitro*, glycoforms containing multiple glucose residues and *O*-acetyl groups were observed in the *in vivo* samples.

Lipopolysaccharide is implicated as a major virulence factor of *H. influenzae*. Nontypeable *H. influenzae* (NTHi) is a significant cause of otitis media in children, and is the most common pathogen recovered from the middle ear of children with recurrent episodes [102]. Although immunity against NTHi appears to develop after acute otitis media, protection is strain-specific, permitting recurrent episodes owing to antigenically distinct isolates. Extensive structural studies of LPS from *H. influenzae* have led to the identification of a conserved glucose-substituted triheptosyl inner-core linked to lipid A [103–109]. The LPS of *H. influenzae* mimics host glycolipids and possesses several phase-variable modifications, which allow the bacterium to evade the host immune system. Recent work has shown that NTHi LPS oligosaccharides replaced by terminal sialic acid (*N*-acetyl neuraminic acid) residues are critical virulence factors in the pathogenesis of otitis media in chinchillas [110] and gerbils. In the chinchilla, sialylation of LPS modifies the interaction with complement [111]. This altered recognition could be attributed to sialylation masking the LPS epitopes recognized by complement [111] or molecular mimicry of host-like structures [110]. It has also been proposed that the expression of sialylated LPS glycoforms may play a role in pathogenesis by promoting biofilm formation [112–114]. One study, using a CE electrospray ionization MS (CE–ESIMS) technique on NTHi isolated from chinchilla, suggested that the

virulence afforded by LPS sialylation depends on the ability of NTHi to scavenge sialic acid from the host [110]. Further work on NTHi LPS glycoforms followed changes in LPS up to 7 days postchallenge in the chinchilla and constituted the first detailed description of LPS glycan patterns during the course of a bacterial infection [115]. Over the course of the study, a trend toward less-complex glycoforms was observed as the infection progressed. It was suggested that the organisms responsible for maintaining active infection may undergo major changes in their metabolic profile as a consequence of available nutrients and their response to the stresses mediated by the host's plethora of innate and acquired immune responses [112]. The biosynthesis of shortened LPS glycoforms may represent a metabolic consequence or an adaptive response to limited energy resources available within the host environment [115]. The role of the *in vivo* LPS *O*-antigen in pathogenesis is not clear, but it is interesting that the LPS carbohydrate structure is influenced by the host environment. This underlines the importance of further studies of LPS and other glycans from bacteria grown within the host environment.

One drawback to LPS analysis by CE–MS is the limitations posed by the buffer used in CE separation, and the deleterious effects it can have on detection limits. Gas-phase ion mobility separations have the potential to separate glycan structural isomers, based upon their 3D shape [116,117]. High-field asymmetric waveform ion mobility spectrometry (FAIMS) is a gas-phase ion separation technique based on compound-dependent differences in ion mobility at high field [118,119]. The FAIMS system acts as an ion filter which continuously transmits a selected type of ion, independent of mass-to-charge ratio. This allows users to selectively isolate target ions based upon physical properties. The approach also reduces chemical background and other interferences in MS spectra. FAIMS is increasingly being combined with front end LC or CE separations. The coupling of a FAIMS to a CE–MS system was successful in separation of trace levels of LPS oligosaccharide glycoforms from NTHi [83]. It was found that the signal of interest was increased as many as 7.5-fold when the FAIMS device was used [83].

Bioinformatics for glycomics

The development and use of informatics tools and databases for glycobiology and glycomics research has increased considerably in recent years. However, the general development in

this field can still be considered as being in its infancy, when compared with the bioinformatics tools available to the genomics and proteomics communities. For example, the automated identification of peptides in proteomics studies is becoming almost routine, with the use of tools such as Mascot [301]. The major factor hindering the development of equivalent tools for glycoproteomics and glycomics is the lack of automated tools that can retrieve structural information from MS data. This challenge is compounded by the structural diversity of glycans and the variety of methods required to characterize both glycopeptides and the modifying glycan. The development of library-based glycan sequencing tools are slowed by the lack of well-curated collections of glycan sequences.

Presently, MS-based glycan and glycopeptide analysis is generally restricted to specialist laboratories that possess the expertise required for manual interpretation of glycan-containing spectra. Even for trained mass spectrometrists, analysis of multiple glycan MS or MS/MS spectra frequently becomes the bottleneck in data analysis. In many cases, manual annotation can be resource limiting or even prohibitive for a high-throughput glycomics screen. Tools are being developed to address issues in glycan profiling, particularly the recent development of an algorithm called Cartoonist, which performs automated annotation of MALDI-TOF spectra [120]. This works with single MS spectra to determine the composition of a particular glycan structure. Cartoonist assigns plausible glycan structures and provides a confidence level assignment to aid further analysis. The graphical forms consist of the spectra annotated with either cartoons of the structures in symbol form, or with formulas of each monosaccharide.

Other algorithms and tools for either glycan structure prediction or glycan annotation also exist. For example, the GLYCH method was developed for the interpretation of MS data, to list all possible fragment types of glycans [121]. Other online tools for annotating glycan structures from mass peaks include GlycoPep ID [122], Glyco-Mod [123] and GlycoPeakFinder [124].

In addition to interpretation tools, there are currently three major databases that house glycan structure data: KEGG Glycan [125], Glycosciences.de [126] and a relational database hosted by the Consortium for Functional Glycomics (CFG) [127], an international initiative funded by the National Institute of General Medical Sciences (MD, USA). The CFG's database is a web portal connected to

the integrated interfaces of diverse datasets in the CFG's relational databases, which contain information regarding glycan-binding proteins, glycan structures and associated glycosyltransferases. The Kyoto Encyclopedia of Genes and Genomes database (KEGG) have produced an extension, KEGG Glycan, which is managed and developed by the Kyoto University Bioinformatics Center (Kyoto, Japan). Glycosciences.de is maintained by the German Cancer Research Center (Heidelberg, Germany) and provides researchers with MS and glycan structure data, as well as applications for glycan analysis. Although the three current databases share the same initial collection of glycan structure, they use different file formats, which, in proteomics and glycoproteomics alike, is a huge informatics stumbling block. GlycoVault is a web-based informatics gateway that contains databases, ontologies and other glycan structure-related data. This application also contains the Glycomics Browser, a web-based visualization and analysis tool for glycan data. The use of these databases in the analysis of bacterial glycomes will rely upon the continued accumulation of bacterial glycan structural information. For further information, readers are directed to an excellent recent tutorial article [128].

Application of NMR to bacterial glycomics

Nuclear magnetic resonance, in particular liquid state NMR, has been employed to study microbial metabolism for more than 30 years [129], and has played a critical role in the determination of the structure of a myriad of microbial glycoconjugates. NMR has been fundamental for the structural analysis of CPS and LPS from numerous microbial pathogens [130–138]. While NMR has provided detailed information regarding the biomimicry of these molecules, this review will focus on the application of whole-cell and solid-state NMR to the elucidation of the pathogen glycome.

HR-MAS whole-cell NMR

High-resolution magic angle spinning NMR (HR-MAS NMR) was initially developed in the late 1990s and has been used to characterize a variety of biological samples [139,140]. In HR-MAS NMR, the sample is spun at the magic angle of 54.7° in order to reduce spectral line broadening caused by chemical shift anisotropy and contributions from magnetic susceptibility [141,142], making this technique amenable to the acquisition of high-resolution spectra from various biological matrices, including intact bacterial cells. The majority of the

literature on HR-MAS whole-cell NMR has focused on the CPS of Gram-negative pathogens, such as *C. jejuni* [96] and *N. meningitidis* [143]. In recent years, HR-MAS whole-cell NMR has been expanded to cell wall components of mycobacteria, such as arabinogalactan [144].

High-resolution magic angle spinning NMR has been used extensively to study the CPS and the *N*-linked Pgl pathway (described previously) in the intestinal pathogen *C. jejuni* [40,145,146]. The CPSs produced by *C. jejuni* are important virulence factors involved in colonization and invasion and are the major antigenic component of Penner's serotyping system [147]. To date, there have been several *C. jejuni* CPS structures reported that identify a number of phase-variable structural variations, such as the incorporation of methyl, ethanolamine and aminoglycerol groups on the CPS sugars [148,149]. One of the more unusual modifications reported is the *O*-methyl phosphoramidate $\text{CH}_3\text{OP}(\text{O})(\text{NH}_2)$ (OR) (MeOPN) group – a labile, phase-variable phosphorylated structure. HR-MAS NMR has been employed as a rapid, high-throughput means to directly examine animal and human isolates from diverse clinical presentations and geographical locations for the presentation of the MeOPN CPS modification [150]. An interesting aspect of this study by McNally *et al.* was the application of HR-MAS whole-cell NMR to examine MeOPN expression within an avian host [150], providing evidence that this phase-variable modification is present during colonization of the GI tract (FIGURE 3). In addition, this technique was used to demonstrate the commonality of this phase-variable modification in 61 of 111 strains tested. Through the combination of bioinformatic analysis of the *C. jejuni* genome, mutagenesis and whole-cell NMR, the authors were able to identify the genes responsible for the biosynthesis of this unique modification. The applicability of HR-MAS whole-cell NMR as a high-throughput screen for identifying genes involved in the biosynthesis of surface polysaccharides is further illustrated by the identification of the genes responsible for D-glycero-L-glucose heptose biosynthesis in *C. jejuni* 11168 CPS [145].

The applicability of HR-MAS NMR is not restricted solely to the analysis of surface polysaccharides. It has also served to elucidate the *N*-linked Pgl pathway in *C. jejuni* 11168 [96]. Through the use of a CPS-negative strain, whose signals interfere with the detection of *N*-glycan signals, the anomeric signals of the *N*-glycan heptasaccharide were observed *in vivo*. This

Figure 3. High-resolution magic angle spinning nuclear magnetic resonance analysis of the cecal contents of a representative *Campylobacter jejuni* colonized chick. (A) High-resolution magic angle spinning (HR-MAS) ¹H nuclear magnetic resonance (NMR) spectrum of plate-grown 11168H cells (256 scans). The inset shows the spectrum for the same sample analyzed with a ¹H-³¹P heteronuclear single quantum coherence HR-MAS NMR experiment (1024 scans, ³¹P decoupled, ¹J_{HP} = 12 Hz). (B) The HR-MAS ¹H NMR spectrum of the cecal contents of a chick that has been colonized with *C. jejuni* 11168H at 48 h post-inoculation (256 scans). The inset shows the spectrum for the same cecal sample examined using the ¹H-³¹P heteronuclear single quantum coherence HR-MAS NMR experiment (1024 scans). OMe is the –OCH₃ group located at C-6 of residue (F) and HOD is the deuterated water resonance. All spectra were acquired at 500 MHz (¹H). The labeling of resonances (C–E) correspond to the anomeric signals of the monosaccharides in the 11168H capsular polysaccharide structure presented at the bottom. Reproduced with modifications from [150]. MeOPN: *O*-methyl phosphoramidate.

provided the capacity to quantify the levels of *N*-glycan heptasaccharide in isogenic mutants of the Pgl pathway, and assess the ability of the oligosaccharyl transferase PglB to transfer truncated glycans. It should be noted that the ability to observe the *N*-linked glycan by HR-MAS NMR is most likely due to the production of protein-free heptasaccharide in the periplasm, resulting from competition between the transfer of the lipid-linked heptasaccharide to protein, and hydrolysis by water [61].

While the vast majority of applications of HR-MAS NMR to glycomics have utilized *C. jejuni*, there have been several applications of this technique to the study of other microbial glycomes, particularly in the structural characterization of the cell wall of living mycobacterial cells [144,151]. In these studies, *Mycobacterium smegmatis* was used as a model organism for the pathogen *M. tuberculosis* in an attempt to elucidate the major carbohydrate components of the mycobacterial cell wall. The major challenges in studying the mycobacterial cell wall revolve around deciphering the cell wall biosynthetic pathways and its interaction with antimycobacterial drugs and host tissues. From a 'systems biology' viewpoint, identifying how the various cell wall components interact with each other could facilitate the development of new anti-TB drugs and treatment strategies. Lee and coworkers have successfully applied multidimensional HR-MAS NMR to the analysis of whole-cell mycobacteria [144,151]. The use of selective labeling techniques, such as heavy nitrogen (^{15}N) and carbon (^{13}C) sources has allowed for the resolution of signals from different mycobacterial macromolecular structures, including mycolyl arabinogalactan, lipoarabinomannan and glycolipids. Through the use of selective labeling, the authors were able to measure the levels of arabinogalactan and lipoarabinomannan in a semi-quantitative manner, which is a useful feature when investigating the biosynthetic pathways of these molecules. An interesting feature of using multidimensional HR-MAS NMR is the ability to utilize a whole-systems approach to study the effects of antibiotics on the mycobacterial cell wall. In the case of *M. smegmatis*, the effects of ethambutol (EMB), a first-line treatment for TB known to reduce the arabinan content of the cell wall through inhibition of the arabinosyltransferases EmBA–C, were investigated [152]. HR-MAS NMR indicated a significant decrease in most arabinose signals in the mycobacterial cell wall upon treatment with EMB, with the exception of the 5'-linked Araf in

the arabinogalactan core, suggesting that EMB inhibits multiple enzymes in the arabinan biosynthetic pathway, but not the arabinosyltransferase, which builds the 5'-linked core [144]. This approach allows for the observation of the effects of drug treatment and gene mutation on the cell wall, which could help in defining mechanisms of action for other cell wall inhibitors, and the screening of new cell wall-acting drugs. It will be interesting to see if this methodology can be applied to the elucidation of the role of the mycobacterial cell wall in host cell interactions.

Solid-state NMR of peptidoglycan

Another component of the bacterial glycome that can sometimes be overlooked owing to its inherent heterogeneity and insolubility is peptidoglycan. With peptidoglycan serving as a major structural component of the bacterial cell, studying the biosynthesis, metabolism and dynamics of this critical structure by traditional techniques can be difficult. While advances have been made in the characterization of peptidoglycan-acting enzymes with their peptidoglycan substrates [153,154], these are *in vitro* techniques that cannot begin to dissect the complex interactions that occur with such a heterogeneous substrate. In addition, peptidoglycan biosynthesis and metabolism has traditionally been a well-spring of antimicrobial targets, and still holds vast potential for the development of new antimicrobials. Recent applications of solid-state NMR to the study of the dynamics and structure of the Gram-positive cell wall demonstrate the potential of this technique for studying peptidoglycan dynamics *in vivo* [155], as well as for dissecting the mode of action and binding specificity of cell wall-acting antibiotics [156–161].

To date, solid-state NMR of Gram-positive cell walls has predominantly focused on the analysis of the stem peptide and pentapeptide bridges linking adjacent glycan strands in *Staphylococcus aureus* [155]. Through the use of ^{13}C - and ^{15}N -labeled amino acids typically found in the peptidoglycan stem peptide, a quantitative measurement of the degree of cross-linking could be obtained in intact *S. aureus* cells. Traditional methods for analysis of peptidoglycan cross-linking usually involve enzymatic digestion and analysis of mucopeptides by HPLC or MS [162]. The use of solid-state NMR and labeling experiments provided information regarding the tertiary structure of the peptidoglycan sacculus [163]. Spin diffusion experiments can be used as a molecular ruler to measure the distance between the glycan backbone and stem peptide,

which provided information on the orientation of peptidoglycan strands in the Gram-positive cell wall. The results obtained from these experiments bring into question the traditional view of Gram-positive cell wall architecture. The commonly held view is that the glycan backbone adopts a helical twist so that the stem peptides within a plane perpendicular to the glycan main chain are antiparallel [162,164,165]. The use of solid-state NMR and spin diffusion studies seems to suggest that the stem peptides can be antiparallel as well [166]. This *in vivo* analysis of peptidoglycan provides relevant data on the organization of this essential bacterial polysaccharide under physiologically relevant conditions. Solid-state NMR provides a powerful tool to assess the interaction of potential antimicrobial candidates developed to target the bacterial cell wall [158,159,166–168] and provides direction in the refinement of activity or target specificity for medicinal chemists.

Probing the bacterial glycome using chemical & physical methods

Chemical labeling: bioengineering of bacterial glycans

Sialic acids have been shown to be involved in many host–pathogen recognition events. As a result, there has been much activity in the search for methods to enrich for and identify sialation in complex tissues [169–172]. Studies have demonstrated that analogues of glycan precursors, for example azido analogues of sugar precursors, can successfully be incorporated in living cells [171–184]. The resulting azido sugars, expressed on the cell surface, can then be labeled via reactions such as the Staudinger ligation (commonly referred to as ‘click chemistry’), functioning as a bio-orthogonal reporter group [175]. This type of approach is being exploited for the study of both eukaryotic and bacterial cells [172]. For example, the pseudaminic acid (Pse) pathway of *C. jejuni* has been targeted using this type of approach. Pse is a nine-carbon α -keto acid that is structurally related to the sialic acids. The sugar is *O*-linked to flagellin proteins in pathogenic bacteria, such as *H. pylori* and *C. jejuni*. Glycosylation is required for assembly of the flagellar filament, and has been implicated in the virulence of these bacteria [176].

In a recent report, Liu and coworkers synthesized a neutral azido sugar precursor that was taken up by *C. jejuni* cells and converted into an azido-labeled Pse derivative *in vivo* [177]. The third intermediate in the Pse pathway, 6-deoxy-Alt α NAc, was targeted for the introduction of

the labeled analogue, 6-deoxy-Alt-NAc α NAz, based on synthetic considerations. The analogue was incorporated into the growth medium, where it competed readily with the natural substrate and was tolerated by downstream steps in the Pse pathway. The bacteria harboring the azido-labeled Pse were motile and the azido functionality was maintained after *in vivo* incorporation into flagella. While the use of these tags in glycomic imaging experiments has been reported for eukaryotic systems [178,179], applications for these modified glycans in bacteria have yet to be demonstrated.

Lectin microarrays: the potential for dynamic studies of the bacterial glycome

A lack of high-throughput analytical tools for the characterization of bacterial glycans has been an obstacle in the examination of the dynamics of bacterial carbohydrate variation over the course of infection. Recently, lectin-based microarrays have been developed as a glycoprofiling tool. This technology exploits many features of more traditional microarray tools, but with a series of lectins immobilized on a single chip, allowing high-throughput screening of the entire cellular glycome [180–182]. Lectins are naturally occurring glycan-binding proteins [183]. A wide variety of lectins has been described, each with a unique glycan-binding profile. Some lectins bind to a very specific glycan structural feature, while others show a wider or more complex binding profile [184]. The commercial availability of purified lectins has led to their wide use as a means of detecting glycoproteins by western blotting, and isolating glycoproteins and glycopeptides by affinity chromatography. Lectin arrays have the advantage of requiring small amounts of samples, from picogram [182] to microgram quantities [180], under mild conditions that potentially allow the detection of secondary modifications that might be lost under more traditional sample preparation methods. In addition, this approach does not require glycan release or purification prior to analysis, nor does it rely upon costly and complex MS equipment for analysis.

A recent application of lectin microarrays to the study of *E. coli* surface glycoconjugates demonstrated that the array was able to differentiate between 21 closely related strains [185,186]. This opens the door to rapid carbohydrate-based bacterial fingerprinting [180,182,185,186]. Perhaps more importantly, the platform allowed dynamic alterations in the *E. coli* cell surface glycome to be followed and enables the examination of changes in the glycome in response to the host over time.

While this study highlights the promise of the technology, there are some limitations in its use for bacterial studies. Since whole bacteria were screened, only accessible carbohydrate motifs on the cell surface were visualized, rather than the whole bacterial glycome. However, as the authors acknowledge, it is probable that the surface-accessible carbohydrates are those most likely to be involved in immune recognition and cell adhesion [185,186]. Perhaps more importantly, the lack of lectins that recognize sugars unique to bacteria is a significant limitation to this technique. The above study used a group of lectins with well-characterized binding to mammalian carbohydrate epitopes, which are also found on bacterial pathogens known to express mammalian-like glycans [184,185,187–190]. Lectin microarrays have the advantage of parallel analyses by many lectins simultaneously, but suffer the disadvantage that they are limited by a panel of approximately 60 existing commercially available lectins. These lectins suffer unexpected cross reactivities and often their interaction with bacterial glycans cannot be predicted [186,191]. This is in addition to potential problems, such as linking chemistry and the impact of lectin orientation upon binding. In this regard, alternatives to the use of lectins are being sought, including nonprotein carbohydrate binding moieties or short peptides that might function as lectin analogues [192]. As the repertoire of well-characterized bacterial glycan-binding proteins increases, the applications of lectin binding will likely be expanded and applied to screen the surface glycomes of other bacteria.

Role of the *C. jejuni* glycome in host–pathogen interactions

Campylobacter jejuni, a Gram-negative intestinal food-borne pathogen, is a major cause of human gastroenteritis and can be associated with the postinfectious neuropathy Guillain–Barré syndrome [193]. Transmission of *C. jejuni* predominantly occurs through consumption of contaminated meat products, in particular poultry. Genome sequencing provided evidence of an unexpected capacity for this organism to produce a wide range of glycoconjugates, including LOS, CPS and proteins modified with *N*- or *O*-linked sugars [194]. Glycobiologists have focused their efforts on *C. jejuni* as a toolbox for understanding bacterial glycan biosynthetic pathways. A wealth of information on the role of these glycoconjugates in pathogenesis exists in the literature, which makes *C. jejuni* a model organism for the discussion of the role of the glycome in host–pathogen interactions.

The outermost glycoconjugate expressed on the cell surface of *C. jejuni* is the CPS, the major serodeterminant of the Penner serotyping scheme [147]. Genomic analysis of *C. jejuni* isolates indicates a high degree of genetic diversity in the CPS gene clusters, resulting in a large array of CPS structures and modifications being expressed. Bacterial capsules have long been thought to play a protective role against adverse conditions that bacteria might encounter, either within the host or the environment [195]. The *C. jejuni* CPS is involved in bacterial adherence and colonization of host tissues, protection of the bacterium from desiccation and impairment of phagocytosis or complement mediated killing [146]. CPS structures from several different *C. jejuni* isolates were determined using solution NMR and, as mentioned earlier, HR-MAS NMR. These studies have illustrated the diversity of this cell surface polysaccharide [148–150,196]. Overall, there is still much to be learned about the role of the CPS in *C. jejuni*, with regards to host interactions.

The outer membrane of *C. jejuni* is composed of a phase-variable LOS, which mimics several human ganglioside structures [197]. This molecular mimicry in some patients is thought to lead to the development of the autoimmune neuropathies Guillain–Barré syndrome and/or Miller Fisher syndrome, caused by the host immune response to LOS ganglioside mimics that have cross-reactivity with host glycolipids [198]. *C. jejuni* can vary LOS biosynthesis genes through homopolymeric tract variation, which occurs with a relatively high frequency. LOS diversity can vary owing to environmental pressures, allowing the pathogen to adapt and survive in a variety of environments inside and outside the host. Early work on this glycoconjugate supports a role for LOS in cellular adherence and invasion [199]. The application of analytical methods with increased sensitivity has recently allowed a more detailed analysis of the structural diversity of *C. jejuni* LOS. Sialic acid moieties within LOS have been demonstrated to be *O*-acetylated, while heptoses have been observed to harbor *O*-linked glycine modifications [200,201]. The biological role of this additional level of LOS glycan diversity remains to be elucidated.

Whole-genome comparisons of 111 strains of *C. jejuni*, using comparative phylogenomics, demonstrated that isolates from a variety of hosts fell into two clades, defined as livestock and non-livestock [202]. Of the genes identified as significantly prevalent in either clade, six genes within the *O*-linked flagellin glycosylation biosynthetic

locus were of particular interest. The strain NCTC11168, found in the livestock clade, has a greater number of putative flagellin-associated biosynthetic genes (50) compared with a sequenced human isolate, 81–176 (26 genes) [33]. This would suggest that livestock-associated strains have the potential to elaborate more complex flagellin modifications in comparison with strains within the nonlivestock clade. However, the structure of the flagellin glycan of livestock strains is not known. The structure of the flagellin glycan from *C. jejuni* 81–176 is known and is perhaps one of the most well-studied flagellin glycosylation systems. Flagellin structural proteins, FlaA and FlaB, are decorated with the nine-carbon Pse sugar (5,7-diacetamido-3,5,7,9-tetradecoxy-L-glycero- α -L-manno-nonulosonic acid [Pse5Ac7Ac], as well as an acetamidino form 5-acetamido-7-acetamidino-3,5,7,9-tetradecoxy-L-glycero- α -L-manno-nonulosonic acid [Pse5Ac7Am]). Derivatives of these sugars have also been identified in strains of *C. jejuni* and contribute to the structural variability of flagellin glycosylation [65,66,203,204]. In *C. jejuni* 81–176, glycosylation of flagellin is required for flagellar filament biogenesis and bacterial motility and, therefore, has biological significance [33]. The sites of glycan modification of flagellin are predicted to be surface exposed in the fully folded protein, pointing to a role in surface interactions, either with the host cells or other bacterial cells. Autoagglutination (AAG), a known marker of virulence in some bacterial pathogens, including *C. jejuni*, has been shown to be dependent upon flagellin glycosylation [33]. Studies of defined mutants, deficient in various glycan-associated biosynthetic genes, pointed to a role for both Pse and PseAM in AAG. A further study of a *C. jejuni* 81–176 PseA mutant, which only modifies flagellin with a single sugar, Pse5Ac7Ac, demonstrated altered AAG compared with wild-type strains. Interestingly, the mutant exhibited alterations in its ability to adhere to a human intestinal epithelial cell line (INT407 cells). During shorter incubation times, only minor defects were observed but, as incubation times were extended, wild-type cells formed microcolonies apparently interacting via flagellar filaments. By contrast, the PseA mutant adhered as single cells, with no visible microcolony formation [33,205]. In an *in vivo* study using the ferret diarrheal model of disease, the *pseA* mutant was attenuated relative to the wild-type *C. jejuni*. Although microcolony formation is the first step in the formation of bacterial biofilms, and *C. jejuni* has been shown to form biofilms *in vitro* [206], there is no evidence

that the pathogen forms biofilms *in vivo*. In this regard, a recent study investigated the role of the *O*-linked glycosylation locus in chicken colonization. In particular, the gene *Cj1324* was shown to be involved in the biosynthesis of novel legionaminic acid derivatives on flagellin of *C. jejuni* 11168H. These modifications altered AAG, cell charge and the ability of the mutant strain to colonize chickens [62]. Studies in these animal models point to a role of flagellin, and flagellin glycosylation, in colonization and disease. As with many studies of bacterial glycomes, the details of pathogen glycan interactions with cells of the host have yet to be explained.

As discussed previously, *Campylobacter* possesses an *N*-linked protein glycosylation system, found within a 16 kB region of the genome [39,42]. Addition of the *N*-linked glycan occurs within the periplasm, with an estimated 150 periplasmic proteins harboring the target sequence for glycosylation (so-called *N*-linked sequon). Several glycoproteins were identified in early work – Cj1594c, PEB3 and *Campylobacter* glycoprotein A (CgpA). Glycoproteomic analyses by 2D gels showed the glycosylation of both PEB3 and CgpA to be dependent upon the presence of a functional PglB protein [207]. Since then, an increasing number of *N*-linked glycoproteins have been identified, contributing to the total *C. jejuni* glycome. These include VirB10, a component of the type IV secretion system, and an inner membrane lipoprotein, CjA [208].

Discerning the role the *N*-linked heptasaccharide plays in pathogenesis is challenging. The variety of proteins that are modified would suggest that any mutation that disrupts the *pgl* pathway will have multiple pleiotropic effects. Indeed, disruption of the *pgl* biosynthetic pathway results in bacteria that have a diminished capacity to adhere to and invade human intestinal cells (INT407) *in vitro* and a reduced ability to colonize chickens [205,209–211]. Given the number of glycosylated proteins, attributing a direct role for *N*-linked protein glycosylation in colonization and invasion is tenuous at best. Its role may be more general, and may be related to aspects such as protein stability, protection against proteolytic degradation and promoting specific protein–protein interactions [212,213]. This view is supported by the observation that the *N*-glycosylated VirB10 is less stable when not glycosylated [214]. Loss of *N*-glycosylation appears to affect other bacterial processes, such as iron acquisition [215], amino acid transport and biosynthesis and *O*-linked flagellin glycosylation [214]. As mentioned above, these observations

could be due to secondary effects. Beyond the ambiguous role of the Pgl glycosylation system in bacterial virulence, studies have opened the door towards the use of this pathway in glycoengineering. This is made possible due to the observation that the entire *pgl* locus can be functionally transferred into *E. coli* [39] and the fact that the oligosaccharyltransferase PglB to possess relaxed specificity for the donor glycan [43].

Future perspective

Despite the growing awareness of the biological significance of bacterial glycoconjugate structures, the study of bacterial glycomics is still in its infancy. Neither the role of bacterial glycans in virulence nor the impact of the bacterial glycome in host–pathogen interactions is clear. Many studies have instead focused upon the eukaryotic host carbohydrates to which many bacterial pathogens bind. As such, there is a pressing need for the adaptation of methods and bioinformatic tools designed for eukaryotic glycomic studies to the study of bacterial glycans. For example, eukaryotic glycan libraries are already being created for use in large-scale studies of carbohydrates. In addition to the traditional, albeit complicated, chemical methods, there are now chemoenzymatic approaches being employed that take advantage of known glycosyltransferases [216]. This sort of technology needs to be adapted for the production of bacterial glycans on a large scale. However, this progress is, and will likely continue to be, hindered by the fact that microbes tend to produce such diverse, unique and unusual sugar modifications.

Although *in vitro* studies are important for the dissection and study of individual glycans, there will need to be a shift towards increased *in vivo* work, as the field of bacterial glycomics continues to grow. It is only through the integration of both *in vitro* and *in vivo* studies that we will begin to fully understand the biological role and significance of bacterial glycans on a molecular level, particularly with respect to the interplay between pathogens and their hosts. The dynamic nature of the bacterial glycome should also be investigated by future *in vivo* research, directed at determining how the glycome changes over the course of infection and is impacted by conditions within a host. There is already preliminary evidence that LPS glycoforms of NTHi become shorter and less complex over the course of infection, possibly owing to environmental stresses such as the host's immune response or as a result of depleted nutrients [112]. This may be the most challenging task faced by

bacterial glycomics, as it is in proteomics as well, owing to the inherent difficulties associated with studying bacteria grown within a host.

A full understanding of the complex relationship between a pathogen and its host does not lie in a single field of study. Despite its significance, glycomics alone will not provide a complete picture. The potential exists for future systems-level analyses through the incorporation of continued advancements in other fields, such as genomics, proteomics, transcriptomics, lipidomics and metabolomics with the ever-growing field of glycomics. In particular, advancements in genome sequencing technologies will certainly improve understanding of the genetic basis of glycosylation. Furthermore, metabolomic-based studies of lipid-linked intermediates of glycoconjugate molecules will help in the elucidation of their biosynthetic pathways. Only through such multidisciplinary collaboration will a complete understanding of host–pathogen interactions be possible.

As the field of pathogen glycomics continues to grow, there will be significant potential for diagnostic and therapeutic applications. Glycoengineering, in particular, offers a number of medically relevant opportunities based on bacterial glycomic research. This technology has the capacity to produce humanized glycoprotein therapeutics and to alter protein-associated carbohydrates to improve both the activity and half-life of these therapeutic proteins [217]. With advances in glycoengineering, the large-scale production of recombinant glycoconjugate antigens for use in the diagnosis of infection and vaccination against known pathogens will also be possible [218,219]. In addition to glycoengineering, inhibitors designed to disrupt the biosynthetic pathways of glycan moieties shown to be involved in pathogen virulence may offer alternative treatment options to antibiotics. This is particularly important given the ever-growing issue of antibiotic resistance.

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Executive summary

Mass spectrometry of the bacterial glycome

- Mass spectrometry is a pivotal technology in glycomics studies.

Analysis of bacterial glycoproteins

- There are increasing numbers of reports of bacterial glycosylation in pathogenic bacteria.
- Discovery of bacterial glycoproteins can be challenging due to the diversity of glycans bacteria use to modify proteins.
- To date, well-characterized bacterial glycoproteins include pilins, flagellin and other surface-associated proteins of both Gram-negative and Gram-positive bacteria.
- Single proteins, isolated by gel electrophoresis or purification, can be analyzed at the protein or peptide level.
- At the protein level, so-called ‘top-down’ mass spectrometry studies of intact protein can rapidly characterize bacterial glycan ions.
- At the peptide level, interpretation of individual bacterial glycopeptide tandem mass spectra can be challenging, owing to the diverse range of bacterial glycans produced.
- A combination of mass spectrometry and nuclear magnetic resonance (NMR) over many years has been used to increase our knowledge of the structures of glycans that form the bacterial glycoproteome.
- Reports of methods to specifically isolate bacterial glycopeptides are advancing knowledge of bacterial glycoproteomes.
- Metabolomics studies offer an alternative approach to study *N*- or *O*-linked protein glycosylation biosynthetic pathways.

Application of NMR to bacterial glycomics

- High-resolution magic angle spinning (HR-MAS) NMR is a powerful tool for the screening of bacterial capsular polysaccharides, and a high-throughput method for identifying genes that assemble or modify bacterial carbohydrates.
- The application of HR-MAS NMR to the analysis of the mycobacterial cell wall provides a valuable tool for studying the biosynthesis of the various carbohydrate components of this highly complex structure.
- Solid-state NMR is one of the only methods to study peptidoglycan tertiary structure, as this insoluble, heterogeneous bacterial polymer is not amenable to classical structural analytical techniques, such as x-ray crystallography. The ability to study cell wall drug interactions using this technique will provide valuable information towards the development of new or modified antimicrobials.

Probing the bacterial glycome using chemical & physical methods

- Lectins (carbohydrate-binding proteins) have been used as tools for glycoprofiling, most recently in microarray formats. This is a high-throughput approach that has the potential to differentiate between bacterial strains, based upon surface carbohydrate profiles.
- Lectin microarrays have also been used to characterize the dynamics of the bacterial surface glycome during the course of infection.
- The use of lectins in bacterial studies is hindered by a lack of predictable lectin binding to bacterial sugars. Lectins also suffer from sometimes unpredictable cross reactivities.
- Incorporation of sugar analogues into eukaryotic glycans has been achieved and exploited for imaging the glycome. Metabolic labeling of *Campylobacter jejuni* flagellin glycan was carried out using an azido-labeled pseudaminic acid precursor. This could be exploited in the future for labeling for imaging and other purposes.

Role of the *C. jejuni* glycome in host–pathogen interactions

- Possibly the best characterized bacterial glycome is that of *Campylobacter*. This bacterium devotes a considerable proportion of its genome to carbohydrate biosynthesis.
- Studies typically generate deletions of individual glycan-associated biosynthetic genes, and monitor the impact upon glycan structure and bacterial virulence in model systems.
- Components of the *C. jejuni* glycome have a key role in the interaction with the host, including adherence and biofilm formation.
- The molecular targets on host cells with which bacterial glycan interact are largely unknown.
- Rapid progress is leading to an increasing number of studies to determine the role of individual bacterial glycans in interactions with the host.

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www.matrixscience.com

Affiliations

- Christopher W Reid
National Research Council – Institute for Biological Science, Ottawa, Ontario, K1A 0R6, Canada
Tel.: +1 613 991 2141
Fax: +1 613 952 9092
chris.reid@nrc-cnrc.gc.ca
- Kelly M Fulton
National Research Council – Institute for Biological Science, Ottawa, Ontario, K1A 0R6, Canada
Tel.: +1 613 991 5466
Fax: +1 613 952 9092
kelly.fulton@nrc-cnrc.gc.ca
- Susan M Twine
National Research Council – Institute for Biological Science, Ottawa, Ontario, K1A 0R6, Canada
Tel.: +1 613 949 7545
Fax: +1 613 952 9092
susan.twine@nrc-cnrc.gc.ca