Current Topics in Biochemical Research
Vol. 14, *2*, 2012

Review

The bitter-sweet relationships of the host-pathogen glycome

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ABSTRACT

Virulence and the extent to which a microbe will cause disease are determined by dynamic host-pathogen interactions. It is now recognized that many of the processes involved depend upon a complement of carbohydrate structures associated with both the host and the invading microorganism, collectively termed the host-pathogen glycome. As glycomics tools and technologies develop, we are gaining a broader appreciation for the exquisite diversity of bacterial glycans and their role in pathogenesis and host immunity. Though deciphering the interactions between the host and the bacterial glycome is challenging and understudied, it promises to unveil avenues for novel biomedical applications in the fields of diagnostics, therapeutics, and vaccines that will reduce the burden of infectious diseases. This review will focus on the impact of bacterial glycoconjugates in host-pathogen interactions using specific examples and highlight the biomedical applications that stem from advances in our understanding.

KEYWORDS: host-pathogen glycome, host-pathogen interactions, bacterial glycome, microbial glycome, glycobiology, carbohydrate, glycan, vaccine, biomarker, lipopolysaccharide, peptidoglycan, glycoprotein

ABBREVIATIONS

PG: peptidoglycan, LPS: lipopolysaccharide, LOS: lipooligosaccharide, S-LPS: smooth LPS, R-LPS: rough LPS, *O*-PS: *O*-antigen polysaccharide, LLO: lipid-linked oligosaccharide, CPS: capsular polysaccharide, EPS: Extracellular/Exo-polysaccharide, CWG: cell wall glycopolymer, P-CWG: peptidoglycan anchored CWG, M-CWG: membrane anchored CWG, TA: teichoic acid, LTA: lipoteichoic acid, LAM: lipoarabinomannan, OS: oligosaccharide, PS: polysaccharide, OPG: osmoregulated periplasmic glucan, MDO: membrane derived oligosaccharide, fOS: free oligosaccharide, MurNAc: *N*-acetylmuramic acid, GlcNAc: *N*-acetylglucosamine, Glc: glucosamine, Glu: glucose, Gal: galactose, Man: mannose, Fuc: fucose, Xyl: xylose, SA: sialic acid, GlcA: glucuronic acid, IdoA: iduronic acid, Kdo: 3-deoxy-D-manno-octulosonic acid, Ko: D-glycero-D-talo- oct-2-ulosonic acid, HexNAc: *N*-acetylhexosamine, OTase: oligosaccharyltransferase, GT: glycosyltransferase, UDP: uridine diphosphate, TLR: toll-like receptor, Nod: nucleotide oligomerization domain, PAMP: pathogen associated molecular pattern, PRR: pathogen recognition receptor, PGRP: peptidoglycan recognition protein, LBP: LPS binding protein, PGCT: protein glycan coupling technology, MS: mass spectrometry, MS/MS: tandem MS, nLC: nano-flow liquid chromatography, HPLC: high pressure liquid chromatography, RPLC: reversed phase liquid chromatography, IP-NPLC: ion pairing normal phase liquid chromatography, NMR: nuclear magnetic resonance, PAS: periodic acid-Schiff

1. INTRODUCTION

While perhaps as many as a thousand microbial species exist within a human host in a symbiotic or commensal state [1-4], those that lead to disease or death are termed pathogenic [5]. Though only a

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small minority of all bacteria are pathogenic, together they are one of the leading causes of human mortality, responsible for an estimated 22% of deaths worldwide in 2008 [6]. A disease state is both pathogen and host specific [7, 8] and the severity of disease is the result of a dynamic interplay between host defences and pathogen virulence factors. Processes such as host invasion, colonization, sporulation, germination, immune stimulation, and host immune evasion play a direct role in determining the outcome of infection. It is now recognized that many of these interactions depend on a complement of carbohydrates on both sides of the relationship [9]. In fact, carbohydrate expression of the host is often influenced by the presence of a pathogen and vice versa [1, 7, 10, 11].

Carbohydrates are ubiquitous, complex, and functionally diverse biomolecules important in all domains of life [12]. In addition to serving as a major energy source for cells, they are also frequently cell surface associated and involved in cell-cell interactions as glycoconjugates. Mono-, di-, poly-, and oligo- saccharides may exist as free glycans or may be coupled with proteins, lipids, or nucleotides to form glycoconjugate macromolecules in linear or branched structures. Glycomics, an analogous field to genomics and proteomics, is the comprehensive study of all glycoconjugates in a biological system. It has proven to be more challenging than its predecessor 'omics' fields due to the non-template driven biosynthesis of glycoconjugates [7, 13, 14] and complex glycan biosynthetic pathways. Eukaryotic glycomes are comprised of essentially ten well characterized monosaccharides, assembled in varying numbers and conformations: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), mannose (Man), fucose (Fuc), xylose (Xyl), sialic acid (SA), glucuronic acid (GlcA), and iduronic acid (IdoA) [14-16]. Across the domain of Bacteria, there is an as yet unknown number of different monosaccharides [17-19], with numerous sugars such as pseudaminic acid [20] and legionaminic acid [21] being unique to prokaryotes [18, 22]. The diversity is increased by the potential for these microbial glycans to be additionally modified by functional groups [23] or amino acids [24-26]. As a result of different analytical challenges, the analysis and bioinformatics of microbial glycomes have lagged behind those of eukaryotes.

Despite the differences in glycome composition, the tools used to study prokaryotic and eukaryotic glycomes are often quite similar. These techniques include liquid chromatographic separation/ enrichment [27-29], advanced mass spectrometry (MS) [27, 28, 30-36], nuclear magnetic resonance (NMR) [37-42], electron microscopy [43], periodic acid-Schiff (PAS) staining [44, 45], lectin chromatography [46, 47], chemical labeling [48-55], and physical [56], chemical [57, 58] or enzymatic [59, 60] glycan removal. Detailed reviews of the analytical techniques can be found elsewhere [18, 61, 62]. Through the study of both prokaryotic and eukaryotic glycomes has come the realization that carbohydrates play an essential role in host-pathogen interactions. The host-pathogen glycome would therefore encompass all the sugar molecules involved in these interactions during a microbial infection.

In bacteria, the complement of glycans and glycoconjugates includes lipopolysaccharides (LPS), lipooligosaccharides (LOS), peptidoglycan (PG), extracellular (or exo-) polysaccharides (EPS), capsular polysaccharide (CPS), cell wall glycopolymers (CWG), nucleotide-activated sugars, lipid-linked oligosaccharides (LLO), *N*- and *O*- linked glycoproteins [18], and free glycans [63, 64]. Many of these sugar related structures are surface associated, as seen in Figure 1, and are consequently important for interactions with the external environment. In the case of pathogenic bacteria, the external environment is frequently a host organism. Our current knowledge of the many diverse roles of bacterial carbohydrates involved in host-pathogen interactions allow them to be loosely grouped into the following categories: structure and physical protection; adherence and colonization; antigenicity and immunogenicity; pathogenicity and virulence; and host evasion. With an increasing understanding of these mechanisms in human pathogens comes the opportunity for targeted medical applications. For example, the identification of novel sugars as pathogen biomarkers can lead to improved diagnostics, as has been shown in *B. anthracis* [65-67]. As has been demonstrated by work in *Yersinia pseudotuberculosis*, inhibiting bacterial motility through the disruption of carbohydrate synthesis pathways holds promise for the development of novel antimicrobials [68-70].

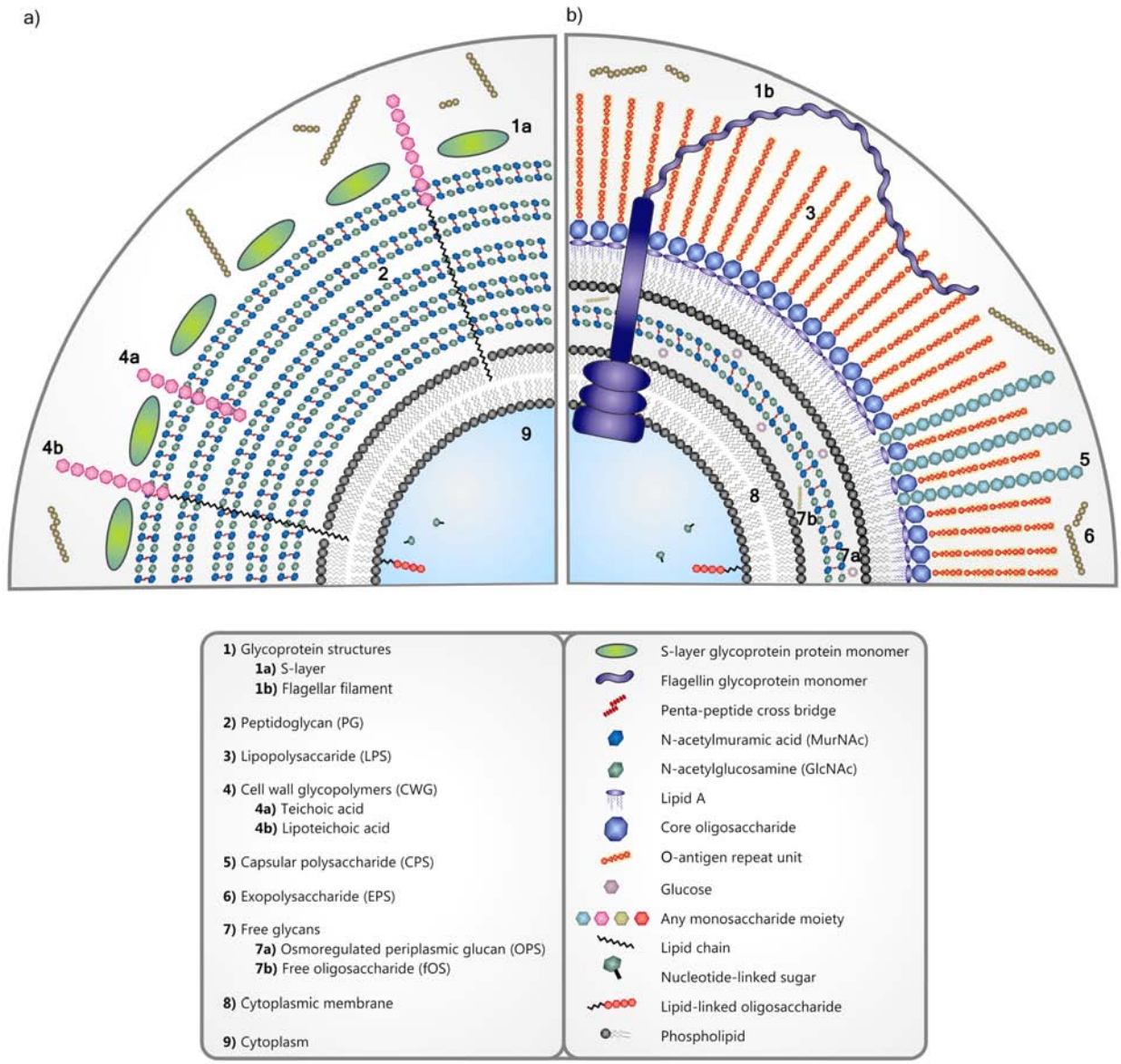

Figure 1. Schematic representation of the pathogen glycome. a) Gram positive bacteria uniquely express cell wall glycopolymers such as teichoic acid and lipoteichoic acid embedded in or through a thick multi-layered peptidoglycan. **b)** Gram negative species possess a single layer of peptidoglycan, but also express a complex lipopolysaccharide structure (lipid A-core-*O*-antigen) as part of an outer membrane and free periplasmic glycans that are not present in Gram positive species. Although only depicted in one or the other in the figure, many species of both Gram types additionally produce capsular polysaccharide and exopolysaccharide structures, and glycosylate proteins such as flagellin and S-layer. Nucleotide-linked sugar precursors and lipid-linked oligosaccharide intermediates involved in the biosynthesis of many carbohydrate structures are located in the cytoplasm of both Gram types. Whether direct or indirect, all of these glycans and glycoconjugates have important roles in pathogenesis and host-pathogen interactions.

Understanding the role of sugars in antigenic immune stimulation, through both innate and adaptive responses [71], will improve vaccine safety and efficacy.

This review will focus on the role of the bacterial glycome in host-pathogen interactions and the medical applications that have come from advances in our understanding of its impact.

2. THE BACTERIAL GLYCOME

The bacterial glycome is complex and comprises several categories of carbohydrate associated biomolecules, the structure and function of which can vary substantially between Gram positive and Gram negative species (see Figure 1), between species of the same Gram type, and even between strains of the same species. Production of this complex glycome is metabolically costly and involves substantial enzymatic machinery, suggesting that carbohydrates have important roles in prokaryotic life cycles. Furthermore, many carbohydrate structures are surface associated where they are likely to interact with host cells and tissues. Bacterial carbohydrates offer many advantages for pathogenesis, but their unique structures often provide targets against which the host can mount a more specific and targeted immune response. Below we describe the general characteristics of various microbial glycans and glycoconjugates, and provide evidence for their role in host-pathogen interactions citing a pathogen specific example for each.

2.1. Glycoproteins

Glycoproteins, proteins that have been co- or post-translationally modified by carbohydrate, fulfill many structural and functional roles in all domains of life. The carbohydrate modifications allow for further diversification of the proteome and subsequently increases the scope and complexity of protein activity [10]. The first glycoprotein identified and characterized was hen egg albumin in 1938 [72]. For many years, though additional eukaryotic glycoproteins were discovered, there was no evidence for prokaryotic glycosylation. It was not until the mid 1970s that the surface layer (S-layer) of *Halobacterium salinarium* (an archaean) [73-75], and the S-layers of *Clostridium thermosaccharolyticum* and *Clostridium thermo-hydrosulfuricum* (bacteria) [76, 77] were reported to be glycoproteins. This began the path to dispelling the belief that eukaryotes were unique in their ability to modify protein with sugar moieties. Since that time, an increasing number of bacterial species have been reported to express glycoproteins [17, 78, 79], including numerous human pathogens. These include, but are not limited to: *Clostridium botulinum* [80], *Clostridium difficile* [24], *Campylobacter coli* [81-83], *Campylobacter jejuni* [20, 84], *Helicobacter pylori* [85], *Neisseria gonorrhoeae* [86, 87], *Neisseria meningitidis* [88, 89], *Aeromonas hydrophila* [90], *Aeromonas caviae* [91], *Francisella tularensis* [92-94], *Pseudomonas aeruginosa* [95-103], *Listeria monocytogenes* [104, 105], *Escherichia coli* [106], *Acinetobacter baumannii* [107], *Burkholderia thailandensis* [108], *Burkholderia pseudomallei* [108], *Borrelia burgdorferi* [109], *Streptococcus pyogenes* [110], *Aggregatibacter actinomycetemcomitans* [111], *Haemophilus influenzae* [112-115] and *Bacillus anthracis* [116-118]. Many bacterial glycoproteins are components of surface structures and appendages, such as S-layer, flagella, pili, fimbriae, and exosporium layers, suggesting relevance for intercellular interactions and pathogenesis.

The carbohydrate portion of a glycoprotein, known as a glycan, can be a mono-, di-, poly-, or oligo-saccharide chain, typically linked together by glycosidic bonds in either a linear or a branched conformation. Glycans can be classified as either *N*-linked or *O*-linked depending on their mechanism of biosynthesis and attachment to the protein or polypeptide. Traditional *N*-glycosylation (also refered to as general glycosylation) involves the assembly of a series of monosaccharides from nucleotide activated sugars to a phosphorylated isoprenoid lipid carrier in the cytosol, resulting in a lipid-linked oligosaccharide (LLO). The glycan is then covalently bound *en bloc* by an oligo-saccharyltransferase (OTase) to the amide nitrogen of asparagine (Asn, or N) side chains, where the Asn residue resides within a consensus sequence [Reviewed extensively in [26]]. In archaea, as in eukaryotes, the glycosylation consensus sequence is Asn-X-Ser/Thr. In bacteria, the consensus sequence has been expanded in some cases to Asp/Glu-X_1-Asn-X_2-Ser/Thr (X, X_1, and X_2 represent any amino acid other than proline (Pro, or P)) [119]. *O*-linked glycans are synthesized by the sequential addition of monosaccharides directly on the hydroxyl oxygen of threonine (Thr, or T), serine (Ser, or S), and less frequently tyrosine (Tyr, Y) side chains by various glycosyltransferases (GTs) [120]. Although some species specific motifs have been proposed [87, 121-124], no absolute *O*-linked glycan consensus sequence has been identified. It has also recently been suggested

that a three dimensional structural epitope (a right-handed β-helix) rather than a strict amino acid sequence may represent a glycosylation motif for at least one family of *O*-glycosyltransferases [125, 126]. *C. jejuni* possesses both of the aforementioned glycosylation systems. Flagellin is modified through the *O*-linked pathway while more than 60 additional proteins, many of which are periplasmic or membrane associated [28], are confirmed modified via a highly conserved *N*-linked pathway [64, 84, 127-130]. Recently, a novel 'general' *O*-glycosylation system has been described in some bacteria including *Neisseria* sp. [87, 131-133], *Pseudomonas* sp. [131], and *Acinetobacter* sp. [107] whereby the transfer of glycan to the *O*-linked site occurs *en bloc* through a lipid carrier, similar to the *N*-glycosylation mechanism [Reviewed in [130]].

Carbohydrate modifications have the potential to alter the physical, chemical, and biological properties of a protein. However, deciphering the biological role of protein glycosylation in bacteria is challenging. Although a substantial number of bacterial glycoproteins have now been identified, only a subset has a characterized function in host-pathogen interactions (see Table 1). To emphasize the significance of glycoproteins in host-pathogen interactions, we will describe in more detail recent insights with respect to the biological impact of flagellar glycosylation in *C. difficile*.

The Gram positive anaerobe, *C. difficile*, is a nosocomial enteric pathogen that primarily affects immunocompromised individuals or patients who have been treated with antibiotics. However, in recent years, an increase in community acquired *C. difficile* has been observed [134]. Increasing antibiotic resistance, particularly for the emerging hypervirulent strains of the 027 ribotype, means that *C. difficile* is a continually growing problem [135]. Due to a dormant and recalcitrant spore form, *C. difficile* infection recurs after treatment and apparent cessation of symptoms in 20-40% of cases [134]. Secretion of two toxins, TcdA and TcdB, upon colonization of the intestinal lining leads to *Clostridium difficile* associated diseases (CDAD), including diarrhoea, toxic megacolon, pseudomembranous colitis [136], and even death.

Many strains of *C. difficile* are motile, and as in many other bacteria, the flagellin monomer is a glycoprotein. The first indication of flagellar glycosylation in *C. difficile* in several strains was an apparent increased molecular mass as compared to the predicted molecular mass when electrophoretically separated by SDS-PAGE [137, 138]. Later it was shown that *C. difficile* strain 630 harbours a unique 398 Da glycan, composed of a *N*-acetylhexosamine (HexNAc) linked to a methylated aspartic acid through a phosphate bridge [24]. Twine *et al.* [24] have putatively reported that this carbohydrate modification is required for proper flagellar assembly, and consequently motility. In this study, an insertionally inactivated mutant strain was generated by the ClosTron method [139] targeting *CD0240*, a gene encoding a conserved putative glycosyltransferase. This glycosyltransferase is located immediately downstream of *fliC*, the gene encoding the structural protein monomer. Through SDS-PAGE of purified flagellin and nLC-MS/MS plus *de novo* sequencing analysis of flagellin peptides, it was determined that disruption of the 0240 glycosyltransferase eliminated flagellin glycosylation. While the FliC protein was expressed, albeit at reduced levels compared to wild type, electron microscopy showed that the flagellar filament was not properly assembled in the mutant, resulting in truncated and fragile flagellar filaments. Consequently this mutant strain was immotile in stab agar tests [24]. Loss or reduction in motility due to absence of flagellin glycosylation is not unique to *C. difficile*. The same has been observed for a number of other bacteria, including *A. caviae* [140, 141], *A. hydrophila* [142], *B. pseudomallei* [108], *C. coli* [143], *C. jejuni* [20, 82, 143, 144], and *H. pylori* [85, 145]. Motility represents an important virulence factor for bacteria, as without it most gastrointestinal pathogens are hindered in their ability, if not entirely unable to reach their target colonization sites [69]. It is also interesting to note that flagellin glycosylation has been linked to virulence through regulation of toxin secretion, although the mechanism is not clear. Aubry *et al.* [146] demonstrated by cell rounding assay that the 0240 mutant strain secreted slightly more toxin than wild type bacteria at 24 hours growth. The increase, however, was not as significant as loss of FliC entirely [146]. Taken together, these studies demonstrate that loss of carbohydrate modification can simultaneously impact multiple

Table 1. Highlighted roles for glycoproteins in host-pathogen interactions. Characterized biological functions pertaining to human pathogens are summarized. (*proposed function*) indicates a role that has been proposed but has not yet been unambiguously characterized. *denotes a role for protein glycosylation that has not been assigned to a particular protein. It is important to note that some or all of the pleiotropic effects associated with the general *N*-linked protein glycosylation system may be impacted by the fOS that was recently discovered in *C. jejuni*, which is synthesized through the same pathway [357].

Gram +/-	Species	Protein	Linkage	Involvement in host-pathogen interaction and pathogenesis
+	*Bacillus anthracis*	BclA/BclB (exosporium glycoproteins)	O	Unique antigenic carbohydrate (anthrose) on spore surface [377, 426-428]
	Clostridium difficile	Flagellin	O	Glycosylation required for flagellar filament assembly and motility [24] Slight impact on toxin regulation [146]
	Clostridium botulinum	Flagellin	O	Host mimicry to diminish immune response (*proposed function*) [80, 429]
	Mycobacterium	Apa (45/47 kDa)	O	Immunodominant antigens: Delayed-Type Hypersensitivity (DTH) reaction and T lymphocyte stimulation [430, 431] Adhesion [432]
	Streptococcus sanguis	SrpA	Unknown	Adhesion to platelets via GPIb [433]
-	*Acinetobacter baumannii*	OmpA/MotB (A1S_1193) Hypothetical proteins: (A1S_3626, A1S_3744, A1S_0556, A1S_2371, A1S_3580, A1S_3658)	General O	Biofilm formation and virulence* [107]
	Aeromonas caviae	Flagellin	O	Flagellar filament assembly and motility (*O*-antigen also affected) [140, 141]
	Aeromonas hydrophila	Polar flagellin	O	Flagellar filament assembly, motility, adhesion, biofilm formation [142]
	Aggregatibacter actinomycetemcomitans	EmaA	O	Collagen adhesion [111]
	Burkholderia pseudomallei	Flagellin	O	Motility (but not flagellar assembly) [108]
	Campylobacter coli	Flagellin	O	Motility [143] Serospecificity: Terminal sialic acid of flagellin glycan involved in LAH antibody strain specific epitopes [81, 434] Unique carbohydrate PseAm involved in flagellar filament serospecificity [82]

Table 1 continued..

	Campylobacter jejuni	Flagellin	O	Flagellar filament assembly and motility [20, 82, 143, 144] Autoagglutination [434-436] Cell charge [435] Biofilm formation [435] Colonization of chicks [435, 437] Adherence to host cells [434, 437] Virulence (ferret diarrhoeal model) [434] Serospecificity via LAH2 antibody [82]
		Cjp3/VirB10	N	Natural transformation [438]
		Pleiotropic effects of general *N*-linked glycosylation (>60 proteins modified through this mechanism) [84, 129, 417]	N	Adherence* to Caco-2 cells [437] and INT407 intestinal cells [439] Invasion* of Caco-2 cells [437] and INT407 cells [439] Colonization* of Leghorn chicks [437] and Hsd:ICR mice [439] Host immune stimulation* [440]
	Chlamydia trachomatis	OMP	N	Adherence to and infectivity of HeLa cells [441]
	Escherichia coli	TibA	Unknown	AIDA mediated adherence and invasion of host epithelial cells [106, 442, 443]
	Haemophilus influenzae	HMW1	N	Adherence to Chang epithelial cells [112-114]
	Helicobacter pylori	Flagellin	O	Motility, CagA-phosphorylation, NF- kB activation [85, 145]
	Neisseria gonorrhoeae	PilE (Type IV pilin subunit)	General O	Complement recptor 3 activation [444] Growth rate [445]
	Neisseria meningitides	PilE (Type IV pilin subunit)	General O	Impacts soluble pilin production [446, 447] Piliation [446]
	Porphyromonas gingivalis	Gingipains	Unknown	Protease activation [448]
		OMP85	Unknown	Biofilm formation (*proposed function*) [449]
		Mfa1	Unknown	DC-SIGN-targeting or cross-linking (*proposed function*) [450]
	Pseudomonas aeruginosa	Pilin	O	Virulence (*proposed function*) [451]
		Flagellin type b	O	Activation of IL-8 release from A549 cells via TLR5 [452]
		LecB	N	Transport to the surface where it is involved in adhesion to host cells (*proposed function*) [453]
	Tannerella forsythia	S-layer	O	Biofilm formation [454] T-helper (Th)17 response suppression [455]

virulence factors and disrupt host-pathogen interactions.

2.2. Peptidoglycan (PG)

With only a few exceptions (including mycoplasmas and Planctomyces), all bacteria possess peptidoglycan (PG), also known as murein, as part of their cell wall. In Gram negative species, the peptidoglycan exists as a thin and usually singly layered sacculus (ranging from approximately 2.5-7 nm in thickness) [147-149] between the cytoplasmic and outer membranes within the periplasmic space. In Gram positive species, however, which do not have an outer membrane or lipopolysaccharide (LPS), the peptidoglycan is layered and significantly thicker (estimated to be up to 50 nm) [150]. It is this thick peptidoglycan that distinguishes Gram positive species from Gram negative species [151, 152]. Other glycan associated molecules such as glycoproteins and cell wall glycopolymers (CWG) are often anchored to or through the Gram positive peptidoglycan layer. The carbohydrate portion of this proteoglycan is a series of alternating β-(1,4) linked *N*-acetyl-glucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) moieties, which are cross linked through short amino acid sequences, typically penta-peptides. The resultant matrix is both strong and flexible [153, 154], contributing greatly to cell shape [155] and acting as a physical protective barrier.

Peptidoglycan synthesis is a complex but conserved process, beginning in the cytoplasm with the assembly of nucleotide (uridine diphosphate (UDP))-sugar precursors: UDP-GlcNAc and UDP-MurNAc. UDP-MurNAc is formed from UDP-GlcNAc by the action of the Mur ligases MurA and MurB. Mur C-F catalyze the non-ribosomal addition of five amino acids to UDP-MurNAc before MraY couples the entire structure to the membrane bound undecaprenyl phosphate lipid carrier. This lipid linked intermediate structure, Lipid I, is then converted to Lipid II by the addition of GlcNAc from UDP-GlcNAc by MurG [156]. Lipid II is subsequently translocated through the membrane by the action of a flippase enzyme [157], however this step is not as well characterized as the rest of the peptidoglycan biosynthesis. Penicillin binding proteins (PBPs) are then responsible for the glycosyltransferase and transpeptidase activity that polymerizes the Lipid II monomers into the full mesh-like peptidoglycan sacculus. There are also a series of specific hydrolases and lytic transglycosylases responsible for targeted cleavage permitting the removal or insertion of new lipid II monomers, thus allowing for cell growth, division, and flexibility [158].

Production of peptidoglycan is a dynamic process, with highly spatially and temporally regulated synthesis and hydrolysis. For example, excessive or uncontrolled hydrolase activity would result in the deterioration of peptidoglycan and eventual cell lysis. As a defence against infection, many hosts have evolved enzymes that function in much the same way as the bacterial hydrolases to aid in destruction of invading pathogens. Lysozyme, for example, is present on most human mucosal surfaces and breaks the β-(1,4) glycosidic bond between GlcNAc and MurNAc [159-162], making the sugar component of this glycoconjugate a direct target of the host innate immune response. Once cells are lysed, peptidoglycan fragments are released. These fragments (muropeptides) are recognized as conserved pathogen associated molecular patterns (PAMPs) by a family of host pattern recognition receptors (PRRs) in the cytoplasm designated nucleotide oligomerization domain (Nod) proteins [163-166]. Recognition of specific peptidoglycan fragments by Nod1 stimulates a proinflammatory immune response and macrophage recruitment, which is amplified by induction of Nod2 [167, 168]. Peptidoglycan is also recognized by host type I transmembrane receptor protein, toll-like receptor 2 (TLR2), and peptidoglycan recognition proteins (PGRPs). Recognition by TLR2 in TLR2:TLR1 or TLR2:TLR6 heterodimer complexes initiates expression of cytokines and chemokines, whereas the effect of PGRP recognition is less clear in humans. Due to co-evolution of humans and pathogens, some bacteria have consequently developed lysozyme resistance by masking their PAMP signature [159] through secondary modification of the carbohydrate backbone, for example by *O*-acetylation [158, 169-173] or *N*-deacetylation [169, 174-178]. Gram positive bacteria also possess numerous cell wall glycopolymers (CWG) within the peptidoglycan layers that can contribute to protection of peptidoglycan by blocking lysozyme binding sites [179].

One of the most virulent food borne pathogens, *Listeria monocytogenes*, is responsible for a spectrum of diseases, collectively referred to as listeriosis. Characterized by conditions ranging from febrile gastroenteritis, to meningoencephalitis, to septicemia [180], it has a mean fatality rate of 20-30% [181]. This virulence is due in large part to its natural lysozyme resistance [182]. As a result, the peptidoglycan of this Gram positive, intracellular pathogen has been the focus of numerous studies. The mechanism by which *L. monocytogenes* achieves this resistance provides an excellent example to further elaborate on the role of peptidoglycan in host-pathogen interactions.

Though the peptidoglycan is an essential component of bacterial architecture, it is clear that its PAMP signature is detrimental to bacterial survival as it stimulates a prompt innate immune response by the host. *L. monocytogenes*, like several other bacteria, has evolved mechanisms of evading host detection by secondarily modifying the carbohydrate backbone of the cell wall structure. *N*-deacetylation of GlcNAc residues within the peptidoglycan structure confers lysozyme resistance to *L. monocytogenes*, contributing to its ability to escape host immunity and cause serious food borne illness. In 1982, Kamisango *et al.* [183] discovered through dinitrophenylation treatment that up to 30% of the glucosamine residues of the lysozyme resistant *L. monocytogenes'* peptidoglycan are not *N*-acetylated as is normally the case for most Gram positive bacteria. Furthermore, *N*-acetylation of purified *L. monocytogenes* peptidoglycan rendered it susceptible to lysozyme degradation [183]. Boneca *et al.* [175] later generated a knockout mutant of the *pgdA* gene (previously designated *lmo0415*) to evaluate its proposed *N*-deacetylase activity and the impact of this *N*-deacetylation in lysozyme resistance. Using reversed phase high pressure liquid chromatography (RP-HPLC) and mass spectrometry (MS) to compare purified peptidoglycan of the mutant strain with wild type, it was determined that closer to 50% of the wild type peptidoglycan GlcNAc residues were secondarily modified by *N*-deacetylation. However, in the mutant strain, only GlcNAc was observed, confirming the proposed enzymatic activity of PgdA. The mutant strain also showed increased lysozyme sensitivity as compared to wild type by cell rounding assay [175]. To further evaluate the biological impact of GlcNAc *N*-deacetylation for *L. monocytogenes*, Rae *et al.* [169] designed an *in vitro* intracellular growth model using murine bone marrow-derived macrophage cell lines that were capable (BBM) or incapable (BBM Lys-) of producing lysozyme. In this study, they demonstrated that while a lysozyme sensitive mutant (Δ*pgd*) grows as well as wild type within BBM Lys- cells, growth of the mutant strain in the presence of lysozyme was hindered. In addition, the cytokines expressed by BBMs infected with wild type *L. monocytogenes* showed increased expression following infection with the lysozyme sensitive strain: IL-1β and IL-12 intracellularly, and IFN-β. This cytokine signalling led to increased bacteriolysis and pyroptosis in the cytosol. These increases in cytokine production and bacterial cell death were not observed in BBM Lys- cells. Finally, an *in vivo* murine model of infection supported the *in vitro* experiments. The *pgd* mutant, but not wild type, showed growth defects in B6 mice, while both strains grew normally in Lys- mice [169]. This demonstrates the conflicting roles of peptidoglycan that are both advantageous and detrimental to bacterial pathogenesis.

2.3. Lipopolysaccharide (LPS)/Lipooligosaccharide (LOS)

As the name suggests, glycolipids are amphipathic molecules consisting of hydrophilic carbohydrate covalently linked to hydrophobic lipid. The distinct physiochemical properties of the two constituents result in multifaceted roles for glycolipids, including roles in pathogenesis. Lipopolysaccharide (LPS) is a prominent glycolipid component of most Gram negative bacterial cell walls, while it is entirely absent in Gram positive species. LPS forms the outer monolayer of the outer membrane, and is comprised of three distinct regions: a lipid A, a central core oligosaccharide (OS), and an *O*-antigen polysaccharide (*O*-antigen or *O*-PS) chain. In some cases, most commonly associated with musocal Gram negative species, LPS lacks the *O*-antigen chain resulting in a lipooligosaccharide (LOS). Based on colony morphology, normal LPS has also been classified as "smooth" LPS (S-LPS), while LOS is categorized as "rough" LPS (R-LPS) [184-186]. The biosynthesis of LPS proceeds through one of three pathways: the Wyz

polymerization system, the ATP-binding cassette (ABC) transporter-dependent system, or the synthase-dependent system. These mechanisms are complex and a detailed explanation is beyond the scope of this review. Extensive overviews of LPS biosynthesis are provided in [187-189].

Lipid A is the most cellularly proximal and most conserved domain, generally consisting of a phosphorylated disaccharide (most commonly di-glucosamine) linked to fatty acid chains. The number of acyl chains, as well as their length and saturation, provide some variability to an otherwise very conserved structure [187]. Structurally, lipid A serves to anchor LPS into the outer membrane, exposing the carbohydrate portion on the cell surface. Release of lipid A following cell death or cell lysis by the host immune system produces a range of toxic effects. In fact, LPS is otherwise known as endotoxin for this reason. The lipid A structure is extensively reviewed in [186].

The core OS, which bridges lipid A and *O*-antigen, is usually limited to 1-15 monosaccharide moieties [190]. The inner core, although made up of more unusual sugars, is far more conserved than the outer core. In fact, all inner core structures characterized to date contain at least one (but usually more than one) 3-deoxy-D-manno-octulosonic acid (Kdo) residue that links the core to the lipid A [191]. In only a few species, including *Burkholderia cepacis* [192, 193], *Yersinia pestis* [194], *Acinetobacter* [195, 196], and *Serratia marcescens* [197, 198], the closely related sugar D-glycero-D-talo- oct-2-ulosonic acid (Ko) replaces one or more of the Kdo residues [186, 190]. Although heptose is also considered a conserved monosaccharide component of the inner core structure, there are some pathogenic species that are entirely lacking heptose, including *Francisella tularensis* [199], *Legionella pneumophila* [21, 200], *Chlamydia trachomatis* [201], *Moraxella catarrhalis* [202, 203], and most *Acinetobacter* species. Additional micro-heterogeneity of the inner core can be achieved through substitution with charged groups such as phosphate, phosphorylethanolamine, and pyrophosphate, as well as acetyl groups or amino acids. The outer core, by contrast, is far more variable as it incorporates a more diverse range of carbohydrates, predominently hexoses, in varying numbers. Recently, however, the first pathogen to lack an outer core was identified. In this case, the *O*-antigen of *Halomonas stevensii* is attached directly to the inner core [204].

A series of oligosaccharide repeating units form the *O*-antigen polysaccharide structure, the most distal region of smooth LPS. The monosaccharide composition, the number of sugars in each repeating unit, and the number of repeating units is highly variable between species and can also be host dependent [205]. Usually, the repeating unit consists of 2-8 monosaccharides [206]. These variables, combined with the potential for non-carbohydrate based additional modification of the sugars, results in a staggering diversity of *O*-antigen structures. Owing to this capacity for diversity, *O*-antigen is frequently responsible for serospecificity. *O*-antigen structure and biosynthesis have recently been reviewed in more detail [206].

Like peptidoglycan, LPS is a conserved pathogen associated molecular pattern (PAMP) [207] that is effectively recognized by pattern recognition receptors (PRRs) [208, 209], which trigger the complement system as well as a toll-like receptor mediated innate immune response. The TLR mediated response is initially triggered by binding of LPS (or more specifically lipid A) to LPS binding protein (LBP), a host serum glycoprotein which acts as a lipid transport [210, 211]. Since lipid A is well conserved across Gram negative species, LBPs are broadly reactive with many pathogens [210, 212, 213]. LBP-lipid A associates with membrane-bound CD14 glycoprotein [214] on the cell surface of innate immune cells, particularly phagocytes such as macrophages, monocytes, and dendritic cells (DCs), which subsequently associates with the type I transmembrane receptor protein, toll-like receptor 4 (TLR4) [189, 215-218] and the accessory myeloid differentiation 2 (MD2) glycoprotein [219-222]. This complex initiates a cascade of intracellular signalling, beginning with the activation of proinflammatory cytokines, IFN-β, and NF-κB [216, 223-226]. These innate responses contribute to positive feedback that further increases the innate immune response and also recruits the adaptive immune system through elaborate cross talk [207, 227-229]. In severe cases, excessive exposure to LPS can result in systemic inflammation, sepsis, endotoxic shock, and death. While the endotoxic activity is believed to be caused by the lipid A portion, in at least

some Gram negative species lipid A alone is incapable of attaining the same biological activity as lipid A conjugated to one or more sugar(s) of the core OS [206]. The sugar moiety, most often the Kdo residue of the core OS, modifies the lipid A structure such that it is recognized and bound by the LBPs and other receptor molecules [189, 220, 230]. This highlights a role for bacterial carbohydrates in host immune stimulation and virulence. The impact of lipid A associated carbohydrates has thus far been demonstrated in *Salmonella* [231, 232], *Bordetella pertussis* [233, 234], *Neisseria meningitides* [235-238], and *Capnocytophaga canimorsus* [239]. It is also interesting to note the involvement of multiple glycoproteins in the host recognition system and that this glycosylation has been shown to be crucial for recognition of LPS via TLR4 [221].

Francisella tularensis is a Gram negative, intracellular pathogen and the etiological agent of tularemia, a potentially fatal respiratory condition. This CDC category A pathogen has been heavily studied due to its potential use in bioterrorism [240-243]. *F. tularensis* LPS is relatively non-stimulatory as compared to that of most other Gram negative bacteria. It also produces natural colony variants with altered *O*-antigen structure [244]. Taken together, *F. tularensis* offers an interesting example to illustrate the importance of LPS, particularly the *O*-antigen polysaccharide, in host-pathogen interactions.

In *F. tularensis* subsp. holartica live vaccine strain (LVS) [245], spontaneous colony variants with reduced virulence and immunogenicity, along with an as yet undefined mechanism for inducing protection [246], have hindered licensing as a vaccine by the Food and Drug Administration (FDA) [247]. Two prominent variants have been designated blue phase (LVS-B) and gray phase (LVS-G) based on the appearance of the colonies under oblique light [248]. LVS-G has been shown to be less virulent and less immunogenic than the normal LVS-B. There have been conflicting reports of the *O*-antigen structure associated with gray variants. Some have reported gray variants to have significantly altered *O*-antigen structure [249], reduced *O*-antigen [250], or to have no *O*-antigen at all [251]. It is possible that all three of these *O*-antigen altered gray variants exist. Hartley *et al.* [251] showed by matrix assisted laser desorption ionization (MALDI) mass spectrometry that in their hands LVS-G is devoid of *O*-antigen polysaccharide. Unlike proteinase K digests of LVS-B, equivalent digests LVS-G were not reactive with monoclonal antibodies generated by LVS LPS by western blot. Also in contrast to LVS-B, the LVS-G variant failed to elicit a protective adaptive immune response in mice and showed a diminished ability to survive within murine J774 macrophages [251]. Interestingly, macrophage uptake of the two variants was shown to be similar *in vitro*. However, addition of complement in normal mouse serum stimulated increased LVS-G uptake, suggesting that the lack of *O*-antigen revealed the PAMP signature of the lipid A moiety. While addition of complement did not affect the uptake of LVS-B, serum from LVS immunized mice did increase its uptake, further demonstrating the role of LVS *O*-antigen in stimulating an adaptive immune response. Interestingly, the *O*-antigens of *F. tularensis* subsp. *tularensis* and subsp. *novicida* have also been shown to be protective antigens [252]. Clay *et al.* [247] further showed that LVS-B is resistant to complement mediated lysis, while LVS-G is susceptible. The same serum sensitivity was observed for an *F. tularensis* LVS genetic knockout mutant (Δ*wbtA*) lacking *O*-antigen [253]. In other bacteria, it has also been shown that LPS lacking *O*-antigen can more easily stimulate the innate immune response by bypassing LBPs and CD14 [254], resulting in a different cytokine expression profile. Although these experiments suggest that *O*-antigen is responsible for the stimulation of the adaptive immune response, Conlan *et al.* have determined that neither lipid A nor core-*O*-antigen complex alone can elicit protection from future challenge [255]. In summary, it would seem that while the *O*-antigen portion of the LPS generates an adaptive (and in this case protective) immune response, it also masks and protects the PAMP signature of the endotoxic lipid A portion from the host's innate immune response and complicates the recognition process. Furthermore, it has been suggested that the gray phase variant may serve to take advantage of the host immune response by eliciting a nitric oxide response that inhibits T-cell proliferation, allowing the blue phase variant to spread [249]. This is just

one example demonstrating multiple roles for the *O*-antigen polysaccharide in host-pathogen interactions during infection.

2.4. Cell wall glycopolymers

Throughout their peptidoglycan, Gram positive organisms contain various cell wall glycopolymers (CWGs) *in lieu* of the LPS containing outer membrane. In the case of peptidoglycan anchored CWG (P-CWG), these polymers are attached to MurNAc residues through a phosphodiester linkage [256]. Membrane anchored CWG (M-CWG), have a glycolipid portion that extends below the peptidoglycan and attaches to the cytoplasmic membrane [257, 258]. Typically, the CWGs are comprised of repeating units of sugar-phosphate residues (poly(glycerol phosphate) or poly(ribitol phosphate)). These polymers are frequently additionally substituted with other sugar moieties, and non-carbohydrate molecules such as pyruvate or *D*-alanine, for example. The specific composition of CWGs, particularly in the P-CWG category, is extremely diverse, and tends to be species or strain specific [259]. Though their structures and physiochemical properties are highly variable, they typically function in conjunction with peptidoglycan to produce an anionic matrix on the surface of the cell [256, 260].

P-CWGs include teichoic acid (TA; WTA), teichuronic acid, uronic acid, and the more recently discovered teichulosonic acid [261]. M-CWGs include lipoteichoic acid (LTA), lipoglycans, lipomannan, and lipoarabinomannan [262, 263]. The best characterized of these polymers are the TAs [256, 264-266], and LTAs [267, 268]. Many Gram positive bacteria possess both TA and LTA. Species lacking TA and LTA tend to substitute them with functionally similar polymers such as teichuronic acid and lipomannan [258], respectively.

CWG have been shown to be involved in a vast variety of biological functions. Since Gram positive bacteria lack the protective outer membrane (including LPS), the CWG can contribute to protecting the cell within a host by blocking lysozyme binding sites [179] or by filling spaces in the peptidoglycan, thereby preventing entry of defensins [269], antibiotics [270], and other host defences [271-273]. They are also capable of tethering other surface associated molecules (i.e. S-layer proteins) to the cell wall [274, 275], and provide additional strength and rigidity for the cell [260]. It has also been suggested that these polymers direct cell division [276], help regulate autolysin activity [270, 277, 278], participate in ion-exchange and homeostasis [277-279], and have involvement in adhesion to host cells [280-282]. Furthermore, CWGs are antigenic, stimulating both an innate and adaptive immune response [283-286], but also show a role in host immune evasion [287]. For a more complete description of the many diverse biological roles of CWG, the reader is refered to the following reviews [256, 259, 288].

One of the most well studied CWGs is the TA of the Gram positive *Staphylococcus aureus*, which was first described in 1961 [289-291]. *S. aureus* is both a nosocomial and community acquired opportunistic pathogen [292] that can lead to skin and soft tissue infections (SSTIs) [292-294], pneumonia [292, 295, 296], bacteremia/septisemia [280, 292, 297, 298], and endovascular infection [299]. Though it is a persistent commensal organism in 20% of the human population, approximately 60% of people are carriers intermittently [300]. Colonization of epithelial and endothelial cells, particularly in the nasopharynx, is a risk factor that can lead to serious future infection through an incidental open wound or following surgery [301]. The influx of methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains throughout the world is of even greater concern [292, 300], prompting increased study of the mechanisms of adherence and colonization involving the CWGs [297] [Reviewed in [302]].

Though adherence of *S. aureus* to host cells is a multifactorial process [Reviewed in [301]], its TA was first implicated in adherence in the 1980s [303, 304]. This important CWG is composed of approximately 40 GlcNAc and *D*-alanine modified ribitol phosphate units [305]. Weidenmaier *et al.* (2004) [280] generated a viable mutant strain of *S. aureus* lacking the UDP-GlcNAc transferase *tagO*, which is involved in the first step of TA biosynthesis. Lack of this enzyme resulted in an absence of TA without significant impact on growth, generation time, or survival rates of the bacteria. The pattern of proteins anchored to the cell wall of the mutant strain also appeared to be unaffected. The *ΔtagO* mutant did, however, show reduced adherence

in vitro to human nasal epithelial cells (HNECs) [280], human airway epithelial cells [280], and human umbilical vein endothelial cells (HUVECs) [282] as compared to the wild type strain. Evidence of direct interaction between the TA and these epithelial and endothelial cells was demonstrated by the dose dependent adherence of beads coated with TA to the same human cells lines [280, 282]. In later studies using a cotton rat model (*Sigmodon hispidus*), the nares of which are representative of humans', the effect of TA on *S. aureus* colonization was evaluated. As compared to the 100% colonization rate of wild type bacteria, the teichoic acid lacking *ΔtagO* mutant failed to colonize even a single rat [280]. Additionally, *S. aureus* strains that had colonized cotton rat nares showed increased transcript levels of genes known to be involved in TA biosynthesis, including *tagO* [306]. The *ΔtagO* mutant was also less virulent and impaired in its ability to colonize cardiac, kidney, and liver cells in a rabbit model of *S. aureus* endocarditis [282].

2.5. Capsular polysaccharide (CPS), Extracellular polysaccharide (EPS), and Biofilms

In addition to the *O*-antigen of LPS, most bacteria produce other polysaccharides that are important to host-pathogen interactions, including capsular polysaccharide (CPS or *K*-antigen), and extracellular (or exo-) polysaccharide (EPS). These carbohydrate polymers often share precursor substrates and biosynthetic pathways such as the Wyz polymerization system, the ATP-binding cassette (ABC) transporter-dependent system, and the synthase-dependent system noted above in the context of *O*-antigen [Reviewed in [307]]. As a result, the distinction between these polysaccharides is subtle and the topic of some debate.

Some general definitions, however, do exist and are based more on localization and function rather than primary structure. CPS is attached directly to the phospholipid of the outer membrane of some Gram negative bacteria [308], while it attaches either to the peptidoglycan [309-312] or cytoplasmic membrane [313] of some Gram positive organisms. CPS is generally an anionic carbohydrate polymer that forms a hydrated capsule around the cell. This capsule offers protection against harsh environmental pressure, such as dessication [314, 315]. For pathogenic species, the CPS can shield the cell from host immune responses [307, 314] including complement mediated killing and phagocytosis [316, 317]. In some cases, the CPS even mimics host carbohydrates to avoid detection [318].

EPS, on the other hand, is secreted and only loosely associated with the cell. Planktonic cells increase EPS production to initiate biofilm formation when exposed to various environmental signals: antimicrobials, host signals and defences, nutrient deficiencies, mechanical stimuli, and osmolarity [319-321]. This biofilm "slime" (or glycocalyx) is a community of bacteria, held together in an EPS based matrix that also contains some protein, lipid, and nucleic acid components [322]. By encompassing a group of cells, it creates a more hospitable environment protected from external stresses, allowing nutrient sharing, increased horizontal gene transfer [323], and cooperative survival under otherwise harsh conditions [322]. Of clinical interest, biofilms can protect a community of bacteria from various host immune responses, suggesting a clear role for these structures in pathogenesis [324]. Additionally, antibiotic resistance of many pathogens has been associated directly or indirectly with biofilm formation [325, 326]. Some of the latter mechanisms are activated or expressed as a consequence of biofilm formation and include slower growth rates, poor membrane permeability, efflux pumps, DNA based chelation of microbial cations, and enzymes that degrade antibiotic compounds (β-lactamases) [327-330]. They have been shown to confer antibiotic resistance [331-333] up to a thousand fold more than planktonic cells [334], leading to persistent and recalcitrant infections, as well as contaminated medical implants and devices in hospitals [335].

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen that is responsible for an exceptionally wide range of human diseases as a result of its ability to colonize and persist within numerous biological (and non-biological) niches. The resultant pathologies include, but are not limited to dermatitis, pneumonia, meningitis, endocarditis, otitis externa, bactermia, and sepsis [327]. As a result of aginate-based EPS biofilm [336], *P. aeruginosa* frequently causes persistent infections, despite antibiotic treatment and combination therapy [337-339], particularly in the

lungs of cystic fibrosis patients. Tobramycin, an aminoglycoside, is one of the few effective antibiotics used to combat *P. aeruginosa* infection.

The capacity for *P. aeruginosa* to form biofilms was first demonstrated in 2000 by electron microscopy and measurement of quorum-sensing signals from clinical isolates of the pathogen [340]. Biofilm formation has been shown to be initiated by its own EPS (aginate) production [320] following exposure to certain environmental stressors or signals, such as antibiotics, nutrient deficiencies, cell density, and salt concentrations [311]. Once initiated, the complex process is genetically regulated [341]. However, it is important to note that a biofilm is a heterogenous substance with a gradient of microenvironments [342, 343]. When it comes to antibiotic resistance, colonies within the biofilm may have need for different defence mechanisms depending on their particular location. *P. aeruginosa* is armed with a number of these mechanisms, and does appear to display a spectrum of reversible biofilm specific phenotypes that act in concert to produce a matrix that is as a whole resistant [322, 335, 344]. For example, Drenkard *et al.* demonstrated a reversible phenotypic shift to a rough-small colony variant (RSCV) with significantly increased antibiotic resistance compared to the normal planktonic cells of the same clinical isolate [311].

The polysaccharide matrix offers direct protection to *P. aeruginosa* cells embedded within by providing a barrier that reduces diffusion and penetration of antibiotics, antimicrobials, and biocides [345-349]. Further emphasizing the importance of the carbohydrate polymer in protection, Hentzer *et al.* reported that an aginate overproducing strain was more resistant to tobramycin as compared to a normal aginate producing strain. This was demonstrated by a distinct decrease in biofilm biomass following exposure to the antibiotic [350].

Other biofilm specific mechanisms of resistance are expressed by individual colonies as needed depending on the environmental signals they encounter. For example, *P. aeruginosa* colonies at the perimeter of a biofilm are less protected from diffusion of antibiotics than those at the centre and therefore must activate their own individual defences, such as increased β-lactamase production [351]. One such mechanism in *P. aeruginosa* that is of particular relevence to the topic of this review involves another polysaccharide: cyclic glucans. As described by Mah *et al.*, a mutation to the glucosyltransferase *ndvB* resulted in increased susceptibility of *P. aeruginosa* to several antibiotics, including tobramycin, gentamicin, ciprofloxacin, and ofloxacin when grown in biofilms as compared to free floating planktonic cells. In the same study, they also showed an absence of glucose in the periplasmic space of the mutant as compared to wild type. Finally, they demonstrated direct interaction and binding of the glucans to tobramycin via hydrophobic interaction chromatography. These glucans seem to sequester antibiotic compounds in the periplasm, preventing them from penetrating further into the cell and reaching their target sites [334]. Although other non-carbohydrate based defences exist within *P. aeruginosa* [328, 330, 352-354], this example highlights the vital role of carbohydrate in more than one antibiotic resistance mechanism. When the physical barrier of the polysaccharide matrix is insufficient, another carbohydrate polymer is upregulated to produce an alternate mode of defence.

2.6. Free glycans

In addition to the previously described glycoconjugate molecules, many Gram negative Proteobacteria also produce non-conjugated free glycans [63, 334, 355-359]. These soluble poly- or oligo- saccharides are released into and accumulate within the periplasmic space between the cytoplasmic and outer membranes, and have been associated with several critical biological functions.

One such class of free glycans consists of osmoregulated periplasmic glucans (OPGs), which were previously called membrane derived oligosaccharides (MDO) after their discovery in *E. coli* [360]. These polysaccharides, comprised of glucose chains, are divided into four families based on their structures. In general, Family I OPGs are linear β-1,2 linked glucose polymers, with β-1,6 branching. Family II OPGs are cyclic structures with β-1,2 linkages. Family III is also made up of cyclic glucose polymers, but they contain β-1,6 or β-1,3 linkages (and less frequently β-1,4 linkages). Family IV OPGs are cyclic β-1,2 linked glucose polymers that each contain a single

α-1,6 linkage [Reviewed in [63, 355]]. Within these families, further diversification of the structures is achieved through varying degrees of polymerization and additional non-carbohydrate modifications of the glucose residues such as succinate, acetate, phosphoglycerol, phosphocholine, and phosphoethanolamine groups [356, 361, 362]. With the exception of *Brucella* spp. OPGs [363-365], these structures are osmotically regulated, with low environmental osmolarity leading to increased OPG production. It has been speculated that increased OPG in the periplasm serves to maintain a higher solute concentration, preventing efflux of metabolites and possible cellular rupturing [64, 366-368].

The effects of OPGs, however, seem to be more pleiotropic. Several studies have even implicated biosynthesis of OPGs with virulence factors of human pathogens. However the direct involvement is often unclear. In *P. aeruginosa*, both cyclic and linear glucans have been identified. As described above, these cyclic glucans contribute to antibiotic resistance of biofilms [334]. The linear glucans are not involved in this antibiotic resistance directly, but are required for complete and normal biofilm formation in low osmotic conditions [356]. Optimal growth of *Shigella flexneri* in hypoosmotic conditions requires proper OPG synthesis [358]. The same is true for *Salmonella enterica* serovar Typhimurium. In this bacterium, however, motility, virulence, and biofilm formation are additionally influenced by OPG production [359, 369, 370]. It has also been shown that the cyclic glucans of *Brucella abortis* stimulate the human and murine immune system through TLR4 mediated activation of dendritic cells [371] and is also required for intracellular survival [363].

Free oligosaccharides (fOS) encompass a more recently discovered class of free glycans. Although fOS have now been detected in nearly all *Campylobacter* species [129], they were first detected in *C. jejuni,* one of the leading causes of gastroenteritis. In *C. jejuni,* the free oligosaccharide is structurally identical to the *N*-linked heptasaccharide that modifies over 60 proteins [64]. While fOS and OPG are structurally very different, they are similarly regulated by osmolarity. Therefore, fOS has been suggested to play a role in homeostasis. Further evaluation of the impact of these fOS is difficult, however, since fOS and *N*-glycans are synthesized through the same Pgl pathway. Therefore phenotypes arising from disruption or deletion of genes within this pathway may be the result of either a loss of protein *N*-glycosylation or a loss of periplasmic fOS [129, 357].

3. BIOMEDICAL IMPACT AND APPLICATIONS

In order to effectively recognize and combat a microbial infection, the host immune system must be able to differentiate between “self” and “non-self.” Medical intervention must also rely on these distinctions. Diagnostics require the detection of foreign substances, while therapeutics and vaccines must target or elicit protection against the invading organism with minimal harm to host cells and tissues. The unique sugars found preferentially or exclusively within the bacterial glycome offer an excellent means of distinguishing pathogen from host. Exploiting these differences is at the heart of many medical intervention strategies currently under development (see Figure 2). Carbohydrates and their associated structures that are highly conserved across bacterial species can be employed in broad spectrum therapies, while unique carbohydrates make excellent biomarkers for pathogen detection as well as targets for specific medical treatments.

3.1. Carbohydrate biomarkers

Symptomology of bacterial infections and disease is often insufficient for precise diagnosis, which often necessitates objective and measurable diagnostic tests. A marker of infection can be a measure of a host response to a foreign organism or a unique and distinguishable feature of the bacterium itself. These markers are most effective when present in easily accessible biological fluids suchs as blood, urine, saliva, or sputum. Distinguishing between elements of the host and the invading organism is necessary, but it is also important to distinguish between different genera, species, and strains of bacteria in order to provide the most appropriate treatment. The unique sugars that comprise the bacterial glycome have the potential to provide the differentiation required for accurate diagnosis [80, 372]. In addition, using

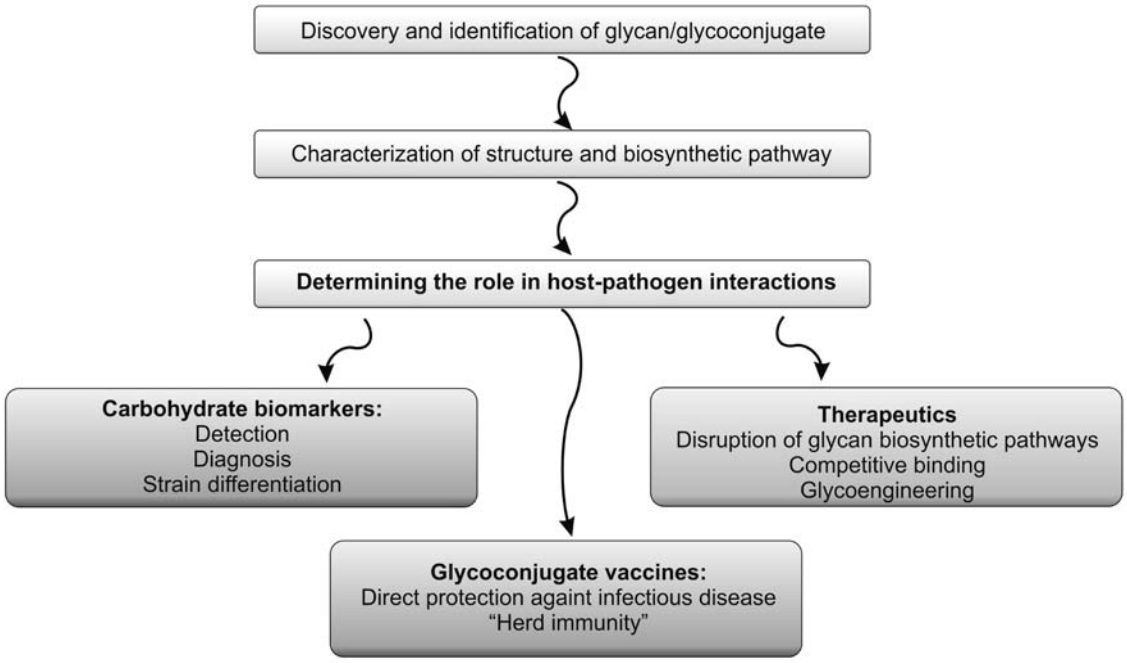

Figure 2. Biomedical applications derived from understanding the host-pathogen glycome. Development of such applications involves a lengthy workflow, starting with the initial discovery of the bacterial glycan or glycoconjugate, followed by characterization and determination of its biological role in pathogenesis. In some cases, the biological significance can be exploited for preventative, diagnostic, or therapeutic medicines.

glycan-related bacterial biomarkers for detection of pathogenic species in food and water, and on abiotic surfaces (particularly nosocomial pathogens in the hospital environment) can offer a means of preventing the spread of infections.

There are numerous examples of assays and diagnostics at various stages of development that take advantage of unique carbohydrate structures in bacteria. For example, the cell wall glycopolymer lipoarabinomannan (LAM) found in *Mycobacteria* is exploited as a urine biomarker in the 'Clearview TB ELISA' (Inverness Medical Innovations, now Alere Inc.) for the detection of *Mycobacterium tuberculosis* [373, 374]. Currently under development is a more rapid, inexpensive, and clinician friendly point-of-care diagnostic strip test, 'Determine TB-LAM' (Alere Inc.) based on the same principles of the LAM-ELISA [317, 374-376]. Using a photogenerated glycan array [377] and a bead-based assay [378], *B. anthracis* spores can also be detected by a unique carbohydrate, termed 'anthrose', which decorates the exosporium BclA and BclB proteins [66, 67]. A multiplexed bead-based assay shows promise for detecting and differentiating 13 strains of *Steptococcus pneumonia* based on sero-specific polysaccharides [379], while a microarray chip for the detection of specific *O*-antigen structures is being developed under the Consortium for Functional Glycomics (CFG) for the detection of several *Salmonella* strains [380]. Recently, a lateral flow immunochromatography test was developed for the monoclonal antibody based recognition of *Staphylococcus aureus* peptidoglycan [381]. Another carbohydrate microarray was designed for simultaneous screening to detect three potential bioterrorism agents: *F. tularensis*, *B. anthracis*, and *B. pseudomallei* [382]. Point-of-care diagnostics for infectious diseases will benefit from increased knowledge of the unique carbohydrates that decorate bacterial pathogens.

3.2. Glycoconjugate vaccines

Beginning with the near eradication of diseases such as smallpox and polio, vaccines have revolutionized the way we manage disease. Prophylactic vaccines aim to generate a host immune response which protects against future

infection, thereby preventing the spread of disease. Vaccines have the potential to reduce morbidity and mortality directly through immunizations, but also indirectly through "herd immunity" [383-387]. There are several types of vaccines, including live vaccine strains (LVS), subunit vaccines, toxoid vaccines, and conjugate vaccines, each with advantages and disadvantages. The specificity of an immune response generated towards a unique bacterial sugar would suggest that carbohydrates are good candidates for safe, targeted, and efficacious vaccine development. Some carbohydrates interact directly with human major histocompatibility complex (MHC) molecules [388], however, most do not [389] and consequently cannot generate a T-cell dependent immune response [390]. Many carbohydrates are therefore inherently poor at stimulating immunological memory that will offer protection in the future. Through glycoengineering, carbohydrates can be conjugated to other molecules, such as protein carriers, that can help induce a stronger adaptive immune response [69, 391]. In this way, glycoconjugates are capable of stimulating both a T-cell dependent and independent host immune response, making them highly effective vaccines [390, 392-394].

There are currently 15 successfully approved and licensed glycoconjugate vaccines for bacterial pathogens on the market. ActHib®, Hiberix®, HibTiter®, PedvaxHib®, and Synthetic Quimi-Hib® are all licensed *Haemophilus influenzae* type b vaccines. *Neisseria meningitides* has six licensed vaccines for various serogroups: MenAfrica, Menjugate®, Meningitec®, NeisVac-C®, Menactra®, and Menveo®. Prevnar-7®, Prevnar-13®, and Synflorix® are licensed for vaccination against *Streptococcus pneumonia*, while Peda Typh® and Vi-rEPA are licensed *Salmonella enterica* serovar Typhi vaccines [389]. Additionally, there are several glycoconjugate vaccines currently in late stage clinical trials for *S. auereus*, *Shigella flexneri* and s*onnei*, and Group B *Streptococcus* [389].

Beyond the licensed and late clinical trial vaccines noted above, there are numerous other glycoconjugate vaccines at various stages of development for a number of pathogens. *Vibrio cholera* [395], *Burkholderia pseudomallei* and *mallei* [396, 397], *Francisella tularensis* [398], *Helicobacter pylori* [399, 400], *Bacillus anthracis* [401, 402], and *Moraxella catarrhalis* [403] are just a few of the pathogens for which glycoconjugate vaccines are being investigated. For more extensive information about glycoconjugate vaccines, the reader is referred to [389, 394, 404].

3.3. Therapeutic compounds and targets

As our understanding of the bacterial glycome, its biosynthetic pathways, and its important role in host-pathogen interactions increases, so does the potential for pharmaceutical advancements to combat infectious diseases. Glycans and glycoconjugates that are unique to bacteria, while necessary for bacterial survival and/or pathogenesis are promising targets for novel therapeutic compounds. Those carbohydrate structures that are conserved across numerous bacterial species offer novel targets for broad spectrum treatment [187, 405-409], while carbohydrates unique to a particular species or strain may allow for more targeted intervention. Inhibitor compounds may also be directed at the biosynthetic pathways responsible for production of various carbohydrate associated structures [69, 372, 410]. While antibiotics apply a strong selective pressure that favours resistant colonies (leading to antibiotic resistance), inhibitory compounds are less likely to promote this sort of resistance. These 'antimicrobials' therefore offer an exciting alternative to traditional antibiotic therapies.

In contrast to offering drug targets, knowledge of the bacterial glycome can be exploited for designing carbohydrate based therapeutics to combat infectious diseases, for example bioactive glycans that retain their biological function [69, 411]. Another approach involves the metabolic labelling of key surface associated carbohydrates with chemical reporters allowing for targeted elimination of invading microorganisms via toxin or antibody mediated mechanisms [49]. Carbohydrate 'mimics' and 'decoys' have also been investigated as anti-adhesion drugs, as they can block binding of key bacterial surface glycans to host lectins and receptors [412, 413].

Beyond antibiotic and antimicrobial applications, in-depth understanding of bacterial glycosylation systems has provided an opportunity to improve production of eukaryotic derived therapeutic

proteins for non-pathogen associated diseases, including several forms of cancer and autoimmune disease [414]. Since 70% of therapeutic proteins are glycosylated [415], it has been necessary to recombinantly express these proteins in eukaryotic hosts rather than the preferred *E. coli* system which does not have the appropriate glycosylation machinery. As an alternative, chemical conjugation of glycan to protein is also possible, but both of these approaches are time consuming and require multiple undesireable purification steps. With the discovery of a bacterial *N*-linked glycosylation system in *C. jejuni* [84], and the functional transfer of the entire gene locus into *E. coli* [393, 394, 416, 417], came the opportunity to begin producing eukaryotic glycoproteins in an easily manipulated bacterial host [418, 419]. Protein glycan coupling technology (PGCT) exploits bacterial glycosyl-transferases and their relaxed substrate specificity. This "living glycoprotein factory" has the potential to reduce costs while increasing yields of important therapeutic glycoproteins [415], including antibodies and cytokines [414]. There are, however, still some challenges facing this technology and further "humanizing" of these living factories will be required [415]. Glycoengineering also holds promise for improving efficacy and half-life of other current pharmaceutical products [420-425].

4. FUTURE DIRECTIONS

Bacterial pathogens represent a significant threat to human health and have been the target of significant study. Since the discovery of penicillin in 1928 by Alexander Flemming (a finding that earned him the Nobel Prize in 1945), we have relied heavily on antibiotic therapy as a primary means of combatting microbial infection. However, with declining antibiotic discoveries and increasing antibiotic resistance in pathogenic bacteria, there is a growing need for novel medical intervention strategies.

Successful development of antimicrobial medicines, whether preventative or therapeutic, requires an understanding of how pathogens interact with their hosts. More and more it is being acknowledged that microbial carbohydrates play a significant role in these interactions, participating in processes from colonization to host immune activation and evasion. Full characterization of the bacterial glycome is a daunting task, given its diversity and complexity, but offers an avenue for novel treatment options.

Though progress has been made, there is still a need to adapt methods and bioinformatic tools traditionally used in the study of eukaryotic glycomes for the study of bacterial glycomes. New strategies designed to study the more unusual bacterial sugars are also required. However, studying pathogen glycomes in isolation is insufficient. To truly appreciate the role of carbohydrates in host-pathogen interactions, a shift from *in vitro* based experiments to *in vivo* studies will be extremely important, particularly for evaluating the evolving impact of these structures during various stages of pathogenesis while subjected to host stresses. Advancement in this field will also benefit from a collaborative, multidisciplinary approach that brings together information not only from glycomics, but also proteomics, transcriptomics, genomics, metabolomics, interactomics, and bioinformatics. A more complete understanding of the host-pathogen glyco-interactome in context should provide opportunities for the development of novel and improved therapeutic options to reduce the burden of infectious diseases.

ACKNOWLEDGEMENTS

We would like to thank Dr. Felicity Stark for critical reading of this review. We would also like to apologize to those authors whose work could not be included in this manuscript due to space limitations.

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