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Review Article

The exploitation of human glycans by Group A *Streptococcus*

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One sentence summary: The current understanding of the glycointeractions mediated by Group A Streptococcus during infection of humans is reviewed herein, highlighting the importance of human glycans in the host–pathogen relationship.
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Abstract

Host carbohydrates, or glycans, have been implicated in the pathogenesis of many bacterial infections. Group A *Streptococcus* (GAS) is a Gram-positive bacterium that readily colonises the skin and oropharynx, and is a significant cause of mortality in humans. While the glycointeractions orchestrated by many other pathogens are increasingly well-described, the understanding of the role of human glycans in GAS disease remains incomplete. Although basic investigation into the mechanisms of GAS disease is ongoing, several glycointeractions have been identified and are examined herein. The majority of research in this context has focussed on bacterial adherence, however, glycointeractions have also been implicated in carbohydrate metabolism; evasion of host immunity; biofilm adaptations; and toxin-mediated haemolysis. The involvement of human glycans in these diverse avenues of pathogenesis highlights the clinical value of understanding glycointeractions in combatting GAS disease.

Keywords: Group A Streptococcus, glycans, glycointeractions, N-glycans, glycosidases, bacteria

Introduction

Carbohydrates, or glycans, are the most abundant macromolecules in nature (Nelson and Cox 2013). In the cellular environment, glycans are abundant at epithelial surfaces and mediate many vital biological processes (Varki 1993). While carbohydrates can exist in a number of forms, the term 'glycan' commonly refers to oligosaccharides built enzymatically from a limited array of monosaccharide substrates. Glycans can occur freely, but more commonly occur as conjugates upon other biomolecules such as lipids and proteins. It is estimated that over 50% of human proteins are post-translationally modified by glycosylation, whereby glycans are typically conjugated to proteins in the endoplasmic reticulum and processed in the Golgi (Apweiler, Hermjakob and Sharon 1999). Glycosylation influences the proteome in many ways, including cellular localisation; enzymatic function and substrate specificity; membrane receptor specificity; protein folding; and quality control (Scheiffele, Peranen and Simons 1995; Skropeta 2009; Hebert and Molinari 2012; Xu and Ng 2015; de Haas *et al.* 2020). In addition to modulating protein structure and function, glycans mediate many crucial interactions between cells (reviewed in Varki 2017). Glycans added to asparagine residues are termed N-glycans, whereas O-glycans are a structurally distinct class typically added to serine or threonine, and less frequently to tyrosine. Historically speaking, the study of carbohydrates in biology has lagged behind that of nucleic acids and proteins. Unlike genomics or proteomics, respectively, glycomics is limited by the inability to amplify material from the source, and by the low abundance of individual molecules within a sample. These difficulties are compounded by the remarkable diversity and intrinsic complexity generated from the non-linear, non-template-driven biosynthesis of glycans. Glycobiology is an evolving discipline and modern technologies have enabled researchers to partially overcome these challenges, allowing more sensitive and insightful investigations into the structures and roles of glycans. The growing appreciation of glycans is reflected in a range of biological contexts, in some cases leading to the development of therapeutic strategies (reviewed in Shriver, Raguram and Sasisekharan 2004; Dube and Bertozzi 2005; Hudak and Bertozzi 2014; Tra and Dube 2014; Taniguchi and Kizuka 2015). In the narrower context of microbiology, the importance of host glycans in pathogenesis is now well-established for a number of clinically important pathogens such as coronaviruses, Influenza A virus, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Streptococcus pneumoniae* (Schembri *et al.* 2001; Shivshankar *et al.* 2009; King 2010; Magalhaes and Reis 2010; Wilks *et al.* 2012; Kautto *et al.* 2016; Seib, Jennings and Day 2018; Semchenko *et al.* 2019; Tortorici *et al.* 2019). While further study is needed to elucidate the role of host glycans in Group A *Streptococcal* (GAS) disease, there are several GAS-mediated glycointeractions reported in the literature, which have not been reviewed.

Streptococcus pyogenes (Group A *Streptococcus*; GAS) is a Gram-positive species which is a major cause of infectious disease in humans. Superficial disease occurs primarily as pharyngitis, which

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has an estimated annual incidence of 616 million cases worldwide (Carapetis *et al.* 2005), but may also manifest as scarlet fever or impetigo. Invasive infection involves penetration of GAS into deeper tissues, causing severe disease states with poor survival rates, such as necrotising fasciitis and streptococcal toxic shock syndrome. Auto-immune complications, such as acute glomerulonephritis, acute rheumatic fever, and rheumatic heart disease, also contribute heavily to the global burden of disease. In a 2005 report, the most recent global epidemiological survey to date, GAS caused over half a million deaths and ranked in the top ten infectious causes of mortality worldwide (Carapetis *et al.* 2005). Translational research such as vaccine development is ongoing in the effort to curtail GAS disease (Rivera-Hernandez *et al.* 2016; Dale and Walker 2020), and basic research into the host–pathogen relationship remains crucial in informing potential therapeutic and prophylactic strategies. The interactions governing this relationship are complex and involve a vast array of host elements, including glycans. The exploitation of epithelial glycans is now recognised as an important facet of pathogenesis in many microorganisms (reviewed in (Poole *et al.* 2018)). Relative to other infections, this aspect of GAS disease has not been investigated to the same extent. However, there is a growing body of work implicating human glycans in GAS adherence to host tissue, particularly via the streptococcal M protein (Section 2). Other types of glycointeractions discussed herein include the identified and putative scavenging of simple and complex carbohydrates from the pharynx (Section 3); evading host immunity through deglycosylation of IgG and interrupting GAG:chemokine interactions (Section 4); the involvement of host glycans in biofilm formation (Section 5); and the haemolytic activity of the GAS toxin Streptolysin O (Section 6).

Adherence to host tissue

Adherence of bacteria to epithelial surfaces is the first step in colonisation, and is a complex, multimodal process involving the specific binding of host molecules. Strong adherence is particularly necessary in the oropharynx, in order for bacteria to withstand the mechanical stresses of mucosal and salivary flow. A number of GAS adhesins have been characterised, including lipoteichoic acid (Ofek *et al.* 1975; Tylewska, Fischetti and Gibbons 1988; Courtney *et al.* 1992), collagen-binding protein (Visai *et al.* 1995) and laminin-binding protein (Terao *et al.* 2002). Several other virulence factors not primarily considered as adhesins have been ascribed adhesin properties, such as M protein (Sanford, Davison and Ramsay 1982; Tylewska, Fischetti and Gibbons 1988; Courtney *et al.* 1992; Courtney *et al.* 1994; Wang and Stinson 1994a,b; Okada *et al.* 1995; Eyal *et al.* 2003; Smeesters, McMillan and Sriprakash 2010) and the cysteine protease SpeB (Hytönen *et al.* 2001). In addition to the primarily proteinaceous ligands of these adhesins such as fibrinogen, several glycan targets have also been identified (Table 1).

Host glycosaminoglycans are bound by the M protein

The streptococcal M protein is a highly variable surface protein that forms the basis of categorising GAS isolates in three systems of increasing specificity: *emm*-pattern, *emm*-cluster and *emm*-type (Hollingshead *et al.* 1994; Sanderson-Smith *et al.* 2014). As the M protein is highly expressed by most GAS, it initially drew interest as a potential target for GAS vaccine development. Classically, the M protein has been regarded primarily as an immune evasion molecule (reviewed in (Fischetti 1989; Smeesters, McMillan and Sriprakash 2010), though it is likely to have additional roles in GAS pathogenesis as it binds a wide range of ligands at the host cell surface and in extracellular matrix (ECM) *in vitro*. Its role in adherence to pharyngeal tissue, however, remains disputed in the literature. While there are several reports of M protein-negative mutants ($M^{(-)}$) exhibiting decreased adherence to human cells *in vitro* (Ellen and Gibbons 1972; Tylewska, Fischetti and Gibbons 1988; Wang and Stinson 1994b; Schrager *et al.* 1998; De Oliveira *et al.* 2017), other authors report no difference in adherence between $M^{(-)}$ and wild-type ($M^{(+)}$) (Caparon *et al.* 1991; Courtney *et al.* 1994). Conversely, a 2013 study demonstrated that a $M^{(-)}$ mutant demonstrated greater attachment to pharyngeal cells relative to its $M^{(+)}$ counterpart (Anderson *et al.* 2014), further obfuscating the role of M protein as an adhesin. The highly variable amino acid sequence between M proteins within the species (Smeesters, McMillan and Sriprakash 2010; McMillan *et al.* 2013) may explain these contrasting reports and underline the difficulties in achieving a consensus regarding the contribution of the M protein to adherence. This is further complicated by the inconsistency of *in vitro* models of infection employed in GAS research. Nevertheless, it is clear that the M protein is involved in adherence in some cases, and efforts to characterise and validate this role are ongoing.

Although the majority of research focusses on host proteins as ligands of the M protein, there are several descriptions of the M protein as a glycan-binding protein, or lectin. Glycosaminoglycans (GAGs) are among the non-protein ligands recognised by M protein and have been identified as targets of bacterial adhesins in several species (reviewed in (Wadstrom and Ljungh 1999; Menozzi *et al.* 2002)). They are large carbohydrate molecules composed primarily of polymerised amino and uronic sugars, expressed on eukaryotic cell surfaces and in ECM. Early research identified human GAGs, such as heparin, as targets of GAS binding, following observations that exogenous heparin and heparan sulphate inhibited attachment of GAS surface proteins to proteoglycans on basement membranes *in vitro* (Bergey and Stinson 1988). It has since been shown that the streptococcal M1 protein is responsible for the binding of various GAGs, including heparin, heparan sulphate and dermatan sulphate (Frick, Schmidtchen and Sjobring 2003) (Fig. 1). Dermatan sulphate, an abundant glycan in the skin, was bound by the majority of the 49 different M proteins tested. Furthermore, enzymatic removal of GAGs from human epithelia significantly reduced bacterial adherence, suggesting that the lectin properties of the M protein promote attachment to host tissue. However, the effect of removing these GAGs upon adherence was not investigated in M-negative mutants.

Histo-blood group antigens are involved in GAS adherence

Glycans other than GAGs are also targeted by streptococcal lectins. The M protein of throat-tropic M1 GAS specifically binds the ABO(H) histo-blood group antigens (HBGAs; Fig. 1) which are abundant in the oropharynx and saliva of most individuals (Slomiany and Slomiany 1978; Ravn and Dabelsteen 2000). HBGA binding M1 is thought to be mediated by the B repeat domains (De Oliveira *et al.* 2017). *In vitro* experiments have shown that M1 protein exhibits the highest affinity for H antigen-bearing structures (De Oliveira *et al.* 2017). In the same study, the corresponding M1 GAS isolate demonstrated the greatest association with H-expressing primary human buccal epithelial (HBE) cells. Such data implicate the specific binding of these glycans by M protein as an important process in adherence. Furthermore, M3 and M12 proteins of *emm*-types frequently isolated from throat infections have also been shown to bind HBGAs with additional experiments showing binding of members of the Lewis antigen family,

Table 1. Summary of host glycan receptors targeted by GAS lectins during adherence. GAG = glycosaminoglycan, BEC = buccal epithelial cells, PEC = pharyngeal epithelial cells, LEC = laryngeal epithelial cells, IAV = influenza A virus, MDCK = Madin-Darby canine kidney.

Lectin	*emm*-types	Glycan receptor	Experimental model	Refs.
M protein	M6	GAGs: Heparin, heparan sulphate	Protein—basement membrane	(Bergey and Stinson 1988)
	49 *emm*-types	GAGs: Heparin, heparan sulphate, dermatan sulphate	Protein—GAG Bacteria—GAG	(Frick *et al.* 2003)
	M1, M3, M12	Histo-blood group antigens (A, B, H antigen)	Protein—glycan Bacteria—BEC	(De Oliveira *et al.* 2017; De Oliveira *et al.* 2019)
		Lewis antigens (Le^A, Le^B, Le^X, Le^Y)	Protein—glycan	(De Oliveira *et al.* 2019)
		Sialic acid	Bacteria—BEC	(De Oliveira *et al.* 2019)
	M6		Protein—mucinBacteria—PEC Protein—PEC	(Ryan *et al.* 2001)
			Bacteria—IAV-infected MDCK cells	(Hafez *et al.* 2010)
	M1, M12	N-acetylgalactosamine, Type II (disialo)gangliosides, galactose	Bacteria–galactose-sepharose Bacteria–PEC	(Wadström and Tylewska 1982)
	M6	Fucose	Protein–LEC	(Wang and Stinson 1994a)
SpeB	M2	Glycoproteins: thyroglobulin, fetuin, mucin, asialofetuin, laminin	Protein–glycoprotein Bacteria–laminin	(Hytönen *et al.* 2001)
PulA	M2, M49	Glycoproteins: thyroglobulin, fetuin, mucin, asialofetuin	Protein–glycoprotein Bacteria–glycoprotein Bacteria–four oral epithelial cell lines	(Hytonen *et al.* 2003; Hytonen *et al.* 2006)
	M1	Glycogen	Protein–glycogen	(van Bueren *et al.* 2007)

which is another system of human blood group antigens (Fig. 1) (De Oliveira *et al.* 2019). The M protein-expressing GAS strains were shown to preferentially associate with HBE cells expressing H-antigen. While it is tempting to hypothesise that blood group may influence host susceptibility, it is unclear how influential HBGA binding is for adherence to the oropharynx, as epidemiological studies have not found significant correlations between GAS pharyngitis and HBGA status (Haverkorn and Goslings 1969; Cooling 2015). Interestingly however, there are reports of higher GAS carriage and tonsillectomy in non-secretors: individuals who lack oropharyngeal HBGA expression and comprise ~20% of the population (Mourant and MAD 1954; Watkins 1980; Jaff 2010). Secretors, who represent the majority, possess Lewis B or Y ($Le^{B/Y}$) glycans, whereas non-secretors instead express only Lewis A or X ($Le^{A/X}$) in oropharyngeal tissue. It has been shown *in vitro* that M1, M3 and M12 proteins bind with greater affinity to $Le^{A/X}$ trisaccharides than $Le^{B/Y}$ tetrasaccharides, which differ by the presence of an extra fucose residue (De Oliveira *et al.* 2019). It could thus be postulated that GAS has developed the capacity for multimodal recognition of host glycans as a response to the evolution of distinct HBGAs in humans. Although the role of the M protein in adherence remains unclear, these data nevertheless suggests the importance of the interaction between M proteins and host glycans. It should be noted that the M protein is highly variable, and phenotypes observed in one M protein are rarely generalisable to the entire suite of over 200 M proteins within the species. Future endeavours may localise lectin capacities to defined regions of the M protein, allowing *in silico* prediction of glycan-binding across diverse *emm*-types. Interestingly, there have also been reported interactions between host glycans and $M^{(-)}$ mutants, suggesting that lectins other than M protein are present at the bacterial surface (De Oliveira *et al.* 2017). These novel GAS lectins have not yet been characterised.

Sialic acid residues are specifically recognised by GAS lectins

The association of GAS with HBE cells has been shown to decrease significantly following the pre-treatment of cells with Sialidase A, an exoglycosidase which removes terminal sialic acids (De Oliveira *et al.* 2019). This suggests that host sialic acid residues are targeted by GAS lectins as a mechanism of adherence (Fig. 1). Sialic acid refers to a large family of nine-carbon sugar residues that exhibit marked structural and functional diversity (reviewed in Cohen and Varki 201)). The most commonly described sialic acid is *N*-acetylneuraminic acid, which occurs abundantly upon glycolipids and termini of N- and O-glycans. As described in detail later, the utilisation of sialic acid is a strategy employed by many pathogenic species (reviewed in Severi, Hood and Thomas 2007). In addition to its acquisition in the context of immune evasion and nutrient acquisition, sialic acid may also be a target of bacterial adhesins, as seen in *S. pneumoniae* (Barthelson *et al.* 1998), a phylogenetically related species which occupies the nasopharynx.

The importance of sialic acid has been exhibited by the finding that an M6 GAS strain binds to human mucins through sialic acid residues (Ryan, Pancholi and Fischetti 2001). Mucins are heavily *O*-glycosylated glycoproteins abundant in saliva and pharyngeal epithelia (Berggärd and Werner 1958; Leach 1963) and are also the predominant carrier of sialic acid and HBGAs in these tissues (Prakobphol *et al.* 1993). Mucin-binding by GAS was attributed to M6 protein, as it was observed in wild-type ($M^{(+)}$) but not the isogenic M-knockout mutant $M^{(-)}$ (Ryan, Pancholi and Fischetti 2001). Notably, pre-treatment of bacteria with sialic acid, but not other sugars, attenuated this binding. In a similar approach, adherence of M6 GAS to human pharyngeal cells was shown to be mediated by M6 protein and sialic acid interactions, particularly α2–6-linked sialic acid. Adherence was significantly reduced when GAS was pre-treated with sialic acid at a minimum inhibitory concentra-

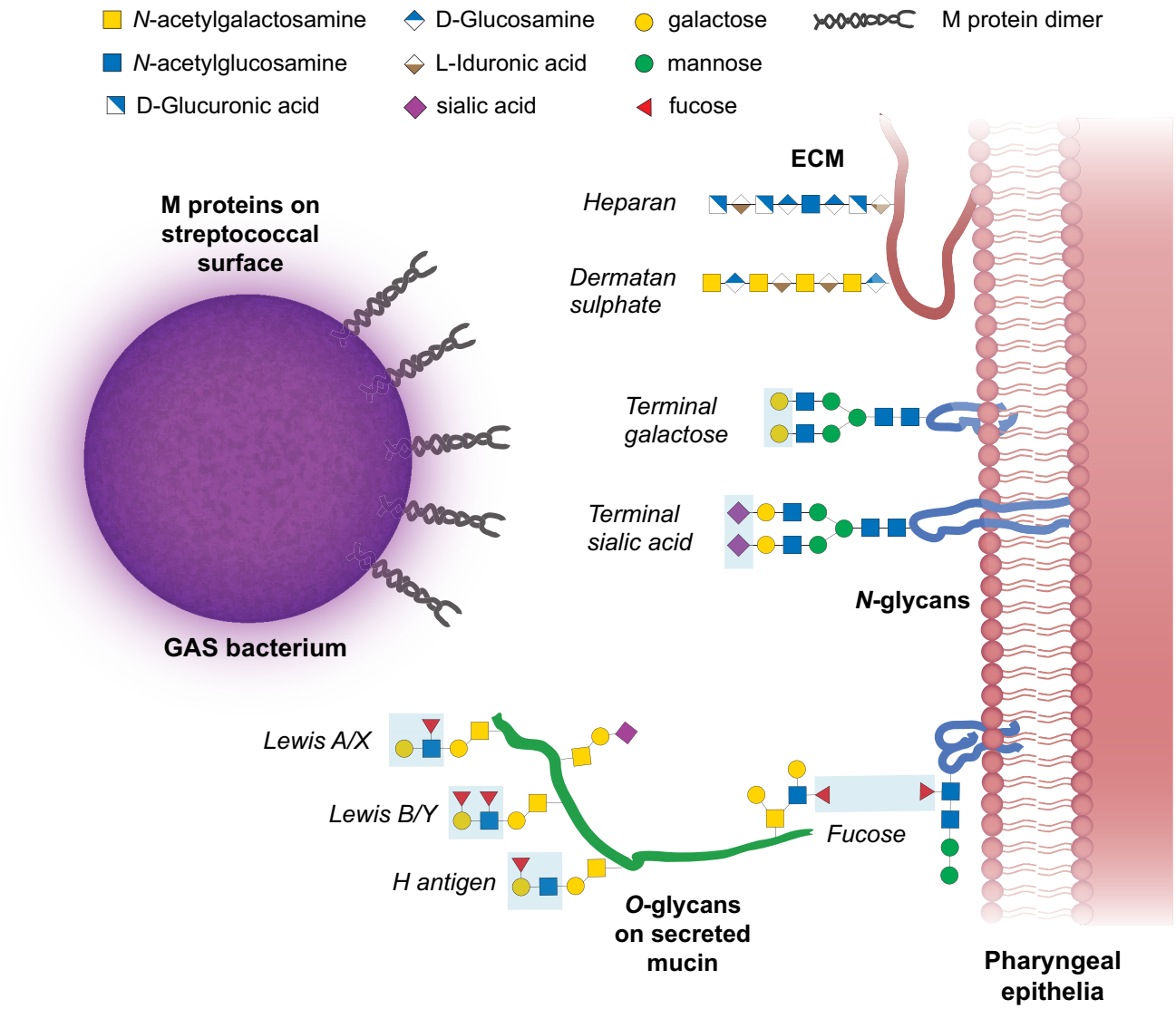

Figure 1. Streptococcal M proteins contain lectin domains with varying affinity for diverse host glycan receptors. Proteoglycan components of the ECM such as heparan and dermatan sulphate have been identified as targets of various M proteins. Smaller motifs such as the Lewis and blood group antigens are bound by many M proteins, as are monosaccharides such as galactose, sialic acid and fucose.

tion of 1 mM. This inhibitory effect was not observed in the corresponding $M^{(-)}$ mutant. Moreover, pre-treatment of wild-type GAS with several heavily sialylated glycoproteins, and pre-treatment of pharyngeal cells with neuraminidase, were respectively shown to inhibit adherence. The role of sialic acid in GAS adherence has been further suggested in experiments involving co-infection with influenza A virus (IAV), which, although performed on a non-human (MDCK) cell line, revealed several interesting results worth discussion. Firstly, $M^{(+)}$ M6 GAS exhibited markedly higher adherence to IAV-infected cells compared to control, an effect abolished in a corresponding $M^{(-)}$ strain (Hafez, Abdel-Wahab and El-Fouhil 2010). M6 GAS adherence decreased when cells were pre-treated with neuraminidase immediately prior to GAS infection. This result, coupled with the observation that pre-treatment of GAS with exogenous sialic acid also inhibited relative adherence, suggests that host sialo-glycoconjugates on IAV-infected cells are targeted by GAS lectins. Importantly, given that neither control nor IAV-infected cells supported association of a corresponding $M^{(-)}$ GAS mutant under any conditions, sialic acid-binding was attributed to the M6 protein. Adherence was also impeded by pre-treatment of cells with monensin, an inhibitor of *O*-glycosylation (Tartakoff 1983; Yanagishita and Hascall 1985) during growth and pre-treatment of GAS with exogenous MUC1, respectively (Hafez, Abdel-Wahab and El-Fouhil 2010). MUC1 is known to be heavily glycosylated with diverse glycoprofiles depending on tissue type (Shimizu and Yamauchi 1982; Patton, Gendler and Spicer 1995; Parry *et al.* 2006), but the source of MUC1 in this study is ambiguous. It is thus unclear which carbohydrate moieties are responsible for the MUC1-mediated inhibition, though these data suggest that GAS lectins, including the M6 protein, bind to sialic acid. Similarly, it is apparent that the M protein binds mucin O-glycans which most likely display a range of monosaccharide ligands.

Other monosaccharides may be involved in GAS adherence

In addition to sialic acid, other monosaccharides have been implicated in GAS adherence to host tissue (Fig. 1). Wild-type M1 and M12 GAS have been shown to bind to *D*-galactose-sepharose beads, whereas $M^{(-)}$ mutants exhibited no binding (Wadström and Tylewska 1982). The same authors reported impeded adherence

of M1 and M12 GAS to primary pharyngeal cells after pre-treating the bacteria with *D*-galactose, with the effect abrogated in corresponding $M^{(-)}$ mutants. This effect in primary pharyngeal cells was not observed in HBE cells, suggesting the presence of distinct receptors for GAS adhesins between the cell types. Earlier work had demonstrated the binding of M6 protein to fucosylated glycoproteins on human laryngeal cells (Wang and Stinson 1994a). Pre-treatment of laryngeal cells with α-*L*-fucosidase reduced binding of M protein to the cell surface by 80%, which was the largest effect relative to other glycosidase pre-treatments. In whole bacterial experiments, various glycosidase treatments of HBE cells have been shown to attenuate association, in a manner dependent on both GAS serotype and blood group antigen expression (De Oliveira *et al.* 2019). Considered together, these data support the notion that host glycan expression patterns mediate GAS colonisation in an M protein-dependent manner. However, given the remarkable diversity of M proteins in GAS, research into a broad range of M proteins is warranted in order to holistically elucidate the importance of blood group antigen recognition in GAS adherence. The importance of the lectin-glycan interactions in mediating GAS adherence is exemplified by recent evidence, whereby N-glycans released from saliva were shown to inhibit association of M1, M3 and M12 GAS to HBE cells (De Oliveira *et al.* 2019). Glycan-based inhibitors may thus represent a promising area for the development of novel therapeutics.

Other streptococcal lectins

In addition to the M protein, two other GAS proteins have been suggested as lectin-like adhesins based on their capacity to bind host glycoproteins. Many host glycoproteins, such as fibronectin and laminin, which are expressed at the epithelial surface and/or in ECM, are implicated in GAS adherence (Simpson and Beachey 1983; Switalski *et al.* 1984; Stanislawski *et al.* 1985; Terao *et al.* 2002). However, these proteins are bound specifically by GAS adhesins in a peptide- rather than glycan-dependent manner. Hytönen *et al.* however, found that an initially unidentified streptococcal surface protein could bind a range of bovine glycoproteins (thyroglobulin, fetuin, asialofetuin, submaxillary mucin), a phenomenon they described as 'strepadhesin' activity (Hytonen *et al.* 2000). This protein exhibited no binding to non-glycosylated proteins (horse myoglobin and bovine erythrocyte carbonic anhydrase), suggesting that glycoprotein-binding was mediated by glycan, rather than peptide, recognition. These authors later convincingly demonstrated that the protein responsible was SpeB (Hytönen *et al.* 2001), a well-documented streptococcal cysteine protease with activity on both streptococcal and host proteins, previously thought to exist exclusively in a secreted form (Lukomski *et al.* 1997; Lukomski *et al.* 1998; Nelson, Garbe and Collin 2011). Importantly, it was demonstrated that binding to glycoproteins could be inhibited by other glycoproteins, suggesting SpeB recognises a common structure across the four glycoproteins tested. Given the sequence diversity of the glycoproteins tested, it is likely that this common structure is a glycan epitope. However, this hypothesis is yet to be conclusively proven as preliminary experimentation by Hytönen *et al.* with glycans derived from the parent glycoproteins did not appreciably inhibit glycoprotein binding by SpeB.

Strepadhesin activity was later observed in SpeB-negative GAS mutants. Another protein, termed PulA, was identified, with glycoprotein-binding behaviour highly similar to SpeB (Hytonen, Haataja and Finne 2003). It was identified as a pullulanase, a pullulan-degrading enzyme, based on functional assays and sequence similarity to other pullulanases. Pullulan is a polysaccharide in which maltotriose units (three glucose molecules with α1–4 glycosidic linkage) are polymerised by an α1–6 glycosidic linkage, produced by some fungal species but absent in humans (Cheng, Demirci and Catchmark 2011). As the strepadhesin phenomenon was initially observed in experiments using bovine glycoproteins, Hytönen *et al.* later corroborated its relevance in the human context by examining the association of isogenic GAS mutants deficient in SpeB and PulA, respectively, with four human oropharyngeal cell lines (Hytonen, Haataja and Finne 2006). Of the two GAS strains employed in this work (M2 and M49 *emm*-types), both PulA-deficient mutants associated significantly less with human cells from all four lines compared to the respective wild-type strains. The corresponding analysis of SpeB-knockout mutants did not reach statistical significance, suggesting that when both SpeB and PulA are expresses, PulA is the major mediator of strepadhesin activity. Interestingly, PulA contains the Gram-positive cell-wall anchoring LPXTG motif, strongly suggesting that it is expressed at the bacterial surface and directed towards host substrates. PulA was shown to be active against pullulan and starch *in vitro*, but not glycogen (Hytonen, Haataja and Finne 2003) which is the closest polysaccharide analogue in humans (Calder 1991).

PulA has since been further investigated, with more recent research by Shelburne III *et al.* demonstrating increased adherence to pharyngeal epithelial cells of wild-type M1 GAS relative to *pulA* knockouts (Shelburne III *et al.* 2011). The *pulA* gene is well-conserved within GAS (Davies *et al.* 2019) and interestingly, *pulA* transcripts are elevated in saliva, and detectable in humans with pharyngitis (Shelburne III *et al.* 2011). Moreover, pullulanase-specific antibodies are present in the sera of GAS-infected patients (Reid *et al.* 2001; Hytonen, Haataja and Finne 2003). PulA is thus expressed during infection, despite the absence of pullulan substrate in the human pharynx, suggesting that it contributes significantly to adherence. This hypothesis is supported by an *in vivo* study in which colonisation of the murine oropharynx was significantly decreased for an isogenic *pulA* knockout compared to wild-type (Shelburne III *et al.* 2011). PulA is thus likely to be an important example of a lectin involved in the early stages of GAS disease. Given its α-glucan degrading properties and its upregulation in saliva, it may well have other roles in pathogenesis, such as in metabolism.

Carbohydrate metabolism

GAS is an unusually versatile pathogen with regard to its capacity to persist and cause disease in a variety of microenvironments within the human host. Given this adaptability to diverse nutritional landscapes, the metabolic activities and requirements of GAS represents an area of interest in fundamental research (reviewed in (Pancholi and Caparon 2016)). GAS is primarily reliant on the glycolytic pathway (Pancholi and Caparon 2016), however, free glucose is not readily available in the two primary sites of GAS colonisation: the oropharynx (Gough *et al.* 1996; Wood *et al.* 2004); and skin (Calderon-Santiago *et al.* 2015). Within the GAS genome, a number of pathways for the utilisation of diverse carbohydrates have been identified, with approximately 15% of the GAS genome dedicated to carbohydrate transport and metabolism (Shelburne III *et al.* 2006; Davies *et al.* 2019). These includes several phosphoenolpyruvate-dependent phosphotransferase systems, responsible for the cellular import of non-glucose mono- and disaccharides (Fig. 2). Many of the encoded products are predicted to be glycoside hydrolases: enzymes responsible for cleavage of glycosidic bonds. Glycoside hydrolases are classified into numbered families (GH) based on sequence and structural

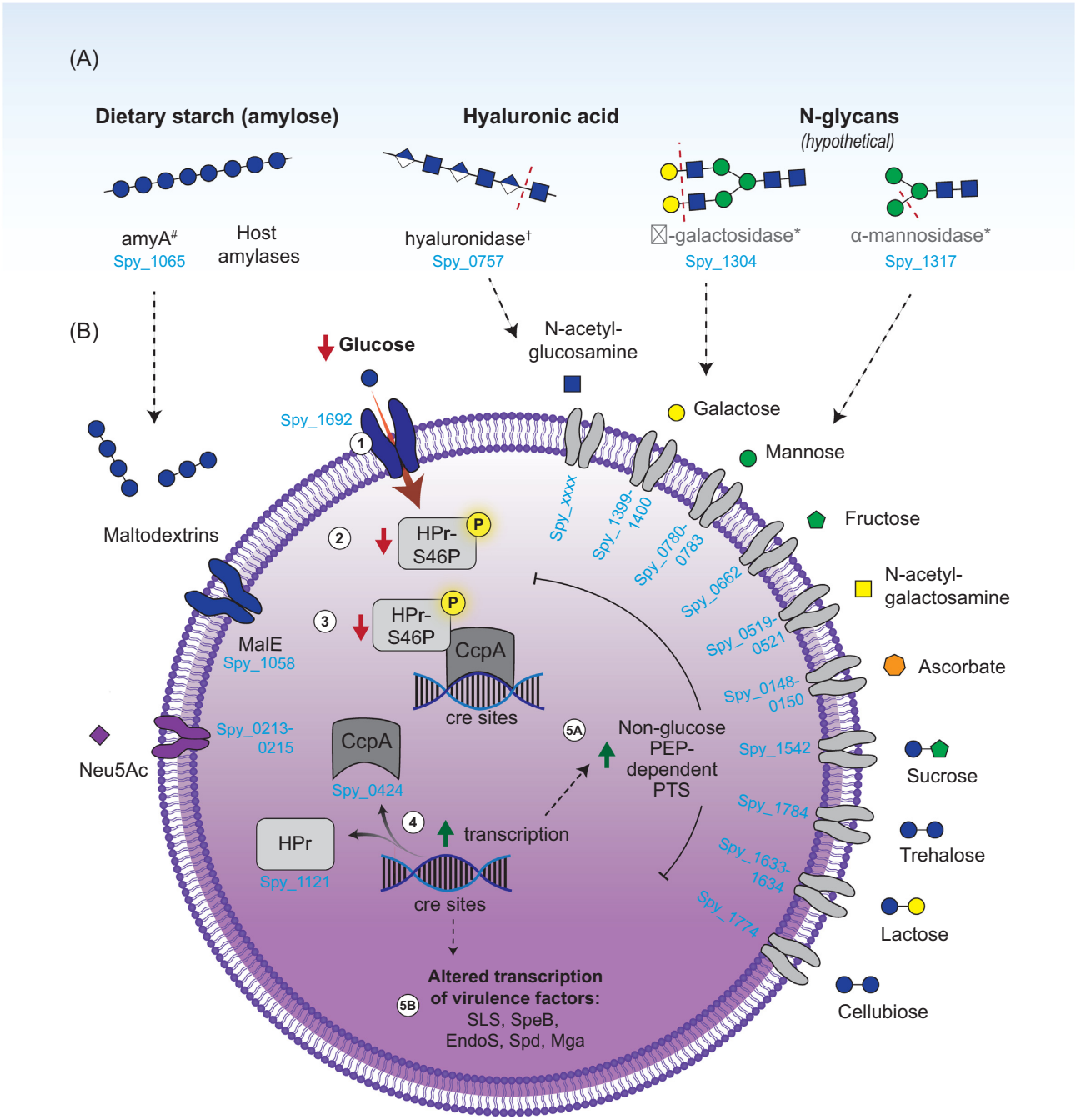

Figure 2. GAS sugar transport and major catabolite repression pathway in the oropharynx. Some mechanisms of degrading complex host substrates have been described and suggested **(A)**. Glucose and several non-glucose substrates can be imported by GAS via dedicated transporters **(B)**, many of which are phosphoenolpyruvate (PEP)-dependent phosphotransferase systems (PTS); multi-component assemblies illustrated as one unit for simplicity. In the oropharynx, low concentrations of glucose (1) result in decreased phosphorylation at the serine-46 (S46P) residue of heat-stable protein (HPr) (2). HPr-S46P is a co-repressor of carbon catabolite protein A (CcpA) which enhances binding to catabolite repression (cre) sites (3). Decreased phosphorylation of HPr results in complex disassembly (4), enabling increased transcription of sugar transport factors (5A) and altering transcription of non-metabolic virulence factors (5B). All sugars are depicted using the universal symbol nomenclature for glycans, except ascorbate which is not included in the nomenclature. Spy_xxxx assignments are based on GAS M1 strain MGAS5005, in which the N-acetylglucosamine transporter is yet to be identified. All genes are core GAS genes as per Davies *et al.* 2019 unless otherwise indicated. # Gene not conserved in all strains, † Not functional in all strains, * Gene is conserved in all strains but is yet to be functionally characterised.

similarity, which enable prediction of activity and substrate specificity (Henrissat and Davies 1997; Lombard *et al.* 2014). The identification of a putative mannosidase, for example, within a bacterial genome, suggests the utilisation of mannose as a non-glucose carbohydrate. As this system relies on *in silico* prediction and not experimental validation, further research is necessary to conclusively establish the suite of glycan substrates available for GAS metabolism and holistically elucidate the relevant biochemical pathways. The foundations for such investigation are discussed herein.

Metabolism of simple carbohydrates

Maltose, mannose, lactose and galactose have been identified as simple carbohydrate alternatives to glucose, with GAS possessing the relevant enzymes required for their import and eventual entry into the glycolytic pathway (Shelburne III *et al.* 2006; Loughman and Caparon 2007; Shelburne III *et al.* 2007a, 2008a,b). However, the mechanisms by which GAS acquires mannose and galactose from the oropharyngeal environment remain unclear. *Streptococcus pneumoniae* expresses a number of exoglycosidases that provide a fermentable source of carbon in the absence of glucose (Burnaugh, Frantz and King 2008). Though these pathways should not be inferred to occur in GAS, *S. pneumoniae* is, in this regard, a well-studied Gram-positive inhabitant of the oronasopharynx, making it an interesting point of comparison. In the absence of exogenous sugars, wild-type *S. pneumoniae* is able to grow in media supplemented with human alpha-1-acid glycoprotein, a highly glycosylated serum protein, but not in a protein-only control medium. Moreover, isogenic mutants lacking functional exoglycosidases could not grow on alpha-1-acid glycoprotein, indicating their important role in metabolism (Burnaugh, Frantz and King 2008). The same result was demonstrated using porcine mucin, a heavily O-glycosylated molecule, similar to human mucins expressed in saliva and the oropharynx (Yesilkaya *et al.* 2008).

GAS possesses both an intact metabolic pathway necessary for galactose fermentation and a highly conserved gene encoding a β-galactosidase (Davies *et al.* 2019). It is thus reasonable to postulate that GAS may have the capacity to scavenge monosaccharides from host glycoconjugates similar to *S. pneumoniae*. The β-galactosidase of *S. pneumoniae* has been identified as a bacterial surface protein (Zähner and Hakenbeck 2000), but a corresponding homologue in GAS has not been characterised. An α-mannosidase has been identified in an M1 GAS strain with activity on α1,3-mannosidic linkages (Suits *et al.* 2010). The lack of distinct signal peptide or cell-wall anchoring motif suggests that the enzyme resides in the cytosol and degrades glycans following their acquisition from the host environment, as opposed to the exoglycosidases of *S. pneumoniae* which appear to act extracellularly. In studies of the GAS mannosidase, no activity was observed on mature human glycans bearing terminal mannoses (Suits *et al.* 2010), which are typically α1,2-linked. Co-incubation with an exogenous α1,2-mannosidase, however, did yield a paucimannose product (e.g. Fig. 2; top-right) that could be degraded by the GAS mannosidase. As the enzyme is highly conserved (Davies *et al.* 2019), it is plausible that other mannosidases are expressed by GAS to act in a sequential manner, not unlike the exoglycosidases previously described in *S. pneumoniae* (Burnaugh, Frantz and King 2008). In fact, a putative α-mannosidase has been identified as part of the core genome *in silico* (Davies *et al.* 2019), which is encoded directly upstream of the aforementioned α1,3-mannosidase (Suits *et al.* 2010). It is yet to be characterised, and it is unclear if this enzyme is secreted or cytosolic.

In addition to galactose and mannose, sialic acid may be a monosaccharide substrate of GAS metabolic pathways as both a carbon and nitrogen source. The utilisation of sialic acid from the environment is a phenomenon observed in many human pathogens. While *de novo* synthesis occurs in some species such as *E. coli* and *Campylobacter jejuni*, other pathogens, such as *Vibrio cholerae*, scavenge from the host (Vimr *et al.* 2004; Thomas 2016). Interestingly, bioinformatic analyses have revealed that the distribution of genes associated with sialic acid acquisition and utilisation are limited almost entirely to mammalian colonisers (Almagro-Moreno and Boyd 2009), suggesting that sialic acid exploitation conveys a significant competitive advantage in colonisation. Although predominantly exhibited by commensals and pathogens that occupy the intestinal tract, the exploitation of host-derived sialic acid also occurs in non-intestinal pathogens such as *N. gonorrhoea* and *S. pneumoniae* (Mubaiwa *et al.* 2017). The latter in particular has been investigated in-depth for its utilisation of host sugars (reviewed in (King 2010)). In addition to metabolism, the sialic acid-degrading enzyme NanA of *S. pneumoniae* has been implicated in adherence, biofilm formation and resistance to neutrophil-mediated killing (King, Hippe and Weiser 2006; Burnaugh, Frantz and King 2008; Parker *et al.* 2009; Brittan *et al.* 2012; Wren *et al.* 2017). Given the common niche of the two organisms, it is reasonable to hypothesise that the acquisition of sialic acid, along with other monosaccharides, occurs in GAS. A sialic acid-binding protein has been identified in GAS and its expression is upregulated in saliva (Shelburne III *et al.* 2005; Virtaneva *et al.* 2005). The gene responsible is well-conserved (Davies *et al.* 2019), but it and its related genes have not been characterised. In addition to this, there are early reports of extracellular neuraminidase activity detected in GAS supernatants (Davis, Baig and Ayoub 1979; Potter *et al.* 1982), but these have not been further validated and no candidate genes have been proposed, to date. While there is thus some evidence of sialic acid utilisation by GAS, further investigation is needed. Given the importance of this capacity in many human colonisers, and the abundance of sialylated glycans in the oropharynx, an examination of sialic acid utilisation may be a promising area for novel research into GAS pathogenesis.

Metabolism of complex carbohydrates

In addition to monosaccharides, complex carbohydrates may also be the target of bacterial scavenging pathways. For example, *S. pneumoniae* can utilise human hyaluronic acid (HA) as a carbon source through the action of hyaluronate lyase (Marion *et al.* 2012). The core genome of GAS also encodes a hyaluronate lyase (*hylA*) (Davies *et al.* 2019), but the enzyme is inactive in the majority of *emm*-types due primarily to a well-conserved amino acid substitution (Hynes, Johnson and Stokes 2009; Henningham *et al.* 2014). Importantly, GAS is encapsulated with HA, which is widely regarded as an essential virulence factor in invasive disease-causing isolates (Cunningham 2000; Cole *et al.* 2011). However, Henningham and colleagues identified that M4 GAS, an emm-type frequently associated with invasive disease, is unencapsulated and possess functional *hylA* (Henningham *et al.* 2014). The authors draw further attention to the ostensible mutual exclusivity between intact HA capsule and hylA; expression of functional *hylA* in invasive, capsulated M1 5448 GAS resulted in loss of capsule. The evolutionary reasons for why M4 GAS appears to have lost the capacity to synthesise HA capsule while retaining the functional *hylA* remains unclear, though its potential role in metabolism can be speculated. Active hyaluronidase expressed by GAS can degrade murine HA *in vitro* and *in vivo*, releasing the monosaccharide product *N*-acetylglucosamine (GlcNAc) (Fig. 2A). (Starr and Engleberg 2006). It has been shown that minimal media supplemented solely with GlcNAc supports the growth of wild-type GAS and the corresponding hyaluronidase-deficient mutant (Starr and Engleberg 2006). However, only the wild-type strain could grow in media where intact HA was the exclusive carbon source. Host or bacterial HA may therefore be an example of the diverse range of carbohydrate sources that GAS may exploit in the oropharynx (Fig. 2). Further investigation into HA as a substrate involved a

Table 2. Characterised and putative exoglycosidases within the core GAS genome. #Spy number refers to the annotated sequence numbers in the genome of M1 GAS strain MGAS5005 (accession no. CP000017.2). *Gene annotations are from Tables S3 and S4 (Supporting Information) in (Davies *et al.* 2019). †GH family refers to the classification of putative or characterised glycoside hydrolases based on the presence of conserved amino acid domains (Henrissat and Davies 1997; Lombard *et al.* 2014).

Spy#	Gene*	Annotated/characterised as	GH Family†	Substrate
0476	*bglA*	β-glucosidase	GH1	Not characterised
1304	*lacZ*	β-galactosidase	GH2	Not characterised
1313	Unnamed	6-phospho-β-glucosidase	GH1	6-phospho-β-glucose (Stepper *et al.* 2013)
1314	*hyl*	N-acetylglucosaminidase	GH84	O-GlcNAc (Sheldon *et al.* 2006)
1316	Unnamed	α-mannosidase	GH125	Not characterised
1317	Unnamed	α-mannosidase	GH38	α1–3-mannosyl linkages (Suits *et al.* 2010)
1632	*lacG*	6-phospho-β-galactosidase	GH1	Not characterised

core gene (Davies *et al.* 2019) whose product had initially been annotated as a putative hyaluronidase based on the presence of a domain associated with hyaluronan degradation. Interestingly, a study of the GH84 enzyme encoded by *hyl* revealed that it had no activity upon hyaluronan, but was active upon O-GlcNAc moieties of mammalian glycoproteins (Sheldon *et al.* 2006). Notably, the authors present no examination of its activity upon N-linked glycans despite GlcNAc being a common terminus of pharyngeal N-glycans. The Gram-positive intestinal pathogen *Clostridium perfringens* possesses four genes encoding GH84 enzymes, all of which are active on N-glycans (Pluvinage *et al.* 2020). It is thus conceivable that the GH84 enzyme in GAS is active on the N- and O-glycans present in the pharyngeal epithelia. The example of *C. perfringens* also illustrates that the GH domain classification should be taken as predictive, rather than definitive, with regard to the substrate specificity of an enzyme. There are a number of putative glycosidases encoded within the GAS genome identified by the GH domain approach that are yet to be functionally validated (Table 2).

In a substantial body of work, Shelburne III *et al.* have extensively studied GAS carbohydrate metabolism. They demonstrated that growth in saliva significantly alters the transcriptome as compared to laboratory media, including increased transcription of genes responsible for complex carbohydrate acquisition and metabolism (Shelburne III *et al.* 2005). *In vivo* transcriptome analyses of GAS-infected nonhuman primates revealed similar trends (Virtaneva *et al.* 2005). The *malE* gene was selected for further investigation and was shown to be a key component of a complex carbohydrate transport system for maltodextrins (Shelburne III *et al.* 2006, 2007a), which are constituents of α-glucan polymers such as starch, pullulan and glycogen. While pullulan is not present in humans, glycogen and starch are abundant in the oropharynx. Maltotriose availability be due in part to amyA and PulA, two α-glucanases secreted by GAS (Shelburne III *et al.* 2009). Of the two, amyA is primarily responsible for starch hydrolysis (Shelburne III *et al.* 2009). However, the *amyA* gene is not well-conserved across the species and it appears dispensable for oropharyngeal colonisation in mice (Shelburne III *et al.* 2009). Instead, it is likely that host salivary amylases, active on polysaccharides from the diet, are primarily responsible for the accumulation of maltotriose in the oropharynx (Roberts and Whelan 1960; Humphrey and Williamson 2001). Streptococcal MalE was identified as the major transporter of maltotriose and longer maltodextrins and is expressed at substantially greater levels in saliva as compared to glucose- or nutrient-rich medium. Notably, *malE* knockout resulted in decreased growth in human saliva and decreased oropharyngeal colonisation in mice (Shelburne III *et al.* 2006). The scavenging of maltodextrins as a carbon source in a niche devoid of glucose is thus an important aspect of GAS pathogenesis. The transcription of genes responsible for complex carbohydrate utilisation is regulated through the involvement of factors such as catabolite control protein A, MalR and SptR/S, whose activities are linked to the production of seemingly unrelated virulence factors (Shelburne III *et al.* 2007b, 2008a,c, 2011). Thus, the differential availability of host glycans not only drives the regulation of carbohydrate-utilising pathways, but also directly influences virulence, a phenomenon observed in other streptococcal species (reviewed in Shelburne III *et al.*, 2008b).

Immune evasion

EndoS

In addition to adherence, host glycans may be exploited in the context of immune evasion. As an example, *S. pneumoniae* actively degrades host glycans to evade the immune system, via exoglycosidases whose activities confer resistance to neutrophils (Dalia, Standish and Weiser 2010). In contrast to exoglycosidases, which remove terminal monosaccharides from larger oligosaccharides, endoglycosidases cleave oligosaccharides, or remove the entire oligosaccharide from its parent glycoprotein. Human immunoglobulin G (IgG) is one such glycoprotein, bearing an N-glycan on an arginine residue (Asn-297) within the Fc region (Tao and Morrison 1989). Although all IgG molecules are modified with N-glycans in all healthy individuals, there is evidence that the identities of N-glycans at Asn-297 are subclass-specific (Bakovic´ *et al.* 2013; de Haan *et al.* 2017). In 15%–25% of individuals, a single N-glycan is also present in the Fab region (van de Bovenkamp *et al.* 2016). Many studies have collectively established the importance of Fc region N-glycosylation. In mice and in humans, IgG lacking Fc glycosylation has impaired or ablated capacities to activate complement; interact with macrophage Fc receptors; and trigger antibody mediated cytotoxicity (Koide. Nose and Muramatsu 1977; Nose and Wigzell 1983; Leatherbarrow *et al.* 1985; Tao and Morrison 1989; Walker *et al.* 1989; Radaev and Sun 2001). Therefore, the ability to deglycosylate human IgG may confer a survival advantage for bacterial pathogens.

Endo-β-*N*-acetylglucosaminidase (EndoS) is a 108 kDa secreted protein expressed in GAS and encoded by *ndoS* with endoglycosidase activity specifically against the N-glycan on the Fc region of IgG (Fig. 3). It was first described by Collin and Olsén, who demonstrated its activity on both soluble IgG and IgG bound to the bacterial surface (Collin and Olsen 2001). These authors also showed that EndoS-deficient mutants could not hydrolyse IgG glycans, suggesting that EndoS is the only IgG-specific endoglycosidase ex-

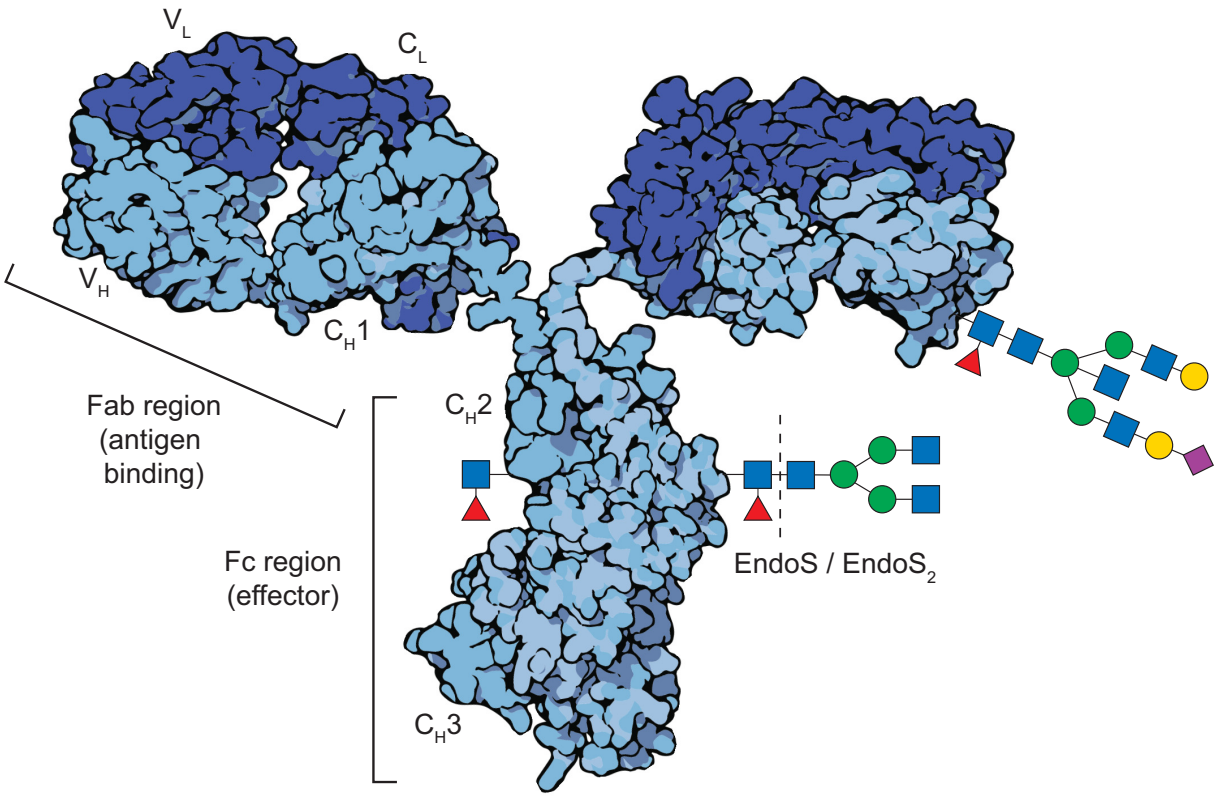

Figure 3. EndoS and EndoS$_2$ hydrolyse the Fc glycans on IgG. The Fc region of human IgG is post-translationally modified with an N-glycan at Asn-297, which is targeted by GAS EndoS and EndoS2. In some individuals, the Fab region is also glycosylated. The structures on the right are examples of commonly encountered IgG N-glycans. The truncated structure on the left represents the remnant glycan following EndoS-mediated cleavage. C = conserved; V = variable; $_H$ = heavy; $_L$ = light. Adapted from van de Bovenkamp *et al.* 2016. Space-filled illustration of immunoglobulin reproduced from 123rf.com with permission.

pressed by GAS. It has been shown that functional EndoS significantly enhances survival of GAS in human blood *ex vivo*, an effect attributed in part to the observation that EndoS-treated human IgG exhibits impaired binding to Fc receptors and is unable to activate the classical complement pathway *in vitro* (Collin *et al.* 2002). EndoS has not been comprehensively studied *in vivo*, with only two animal studies reported, to date. In interpreting these studies, it should be noted that the mouse IgG N-glycome is more variable than in humans and glycosylation profiles are highly strain-specific (Krištić *et al.* 2018). The repertoire of glycans however, does not differ markedly between humans and mice, and when comparing certain subclasses there are notable similarities in glycopatterns (Zaytseva *et al.* 2018).

The more recent and more comprehensive of the two mouse studies performed to date suggest EndoS provides an advantage in the context of adaptive immunity. Naegeli and colleagues immunised mice with M1 protein prior to subcutaneous infection with an M1 strain (AP1), reporting significantly greater survival, decreased weight loss and decreased bacterial load in the *ndoS* mutant, relative to wild-type (Naegeli *et al.* 2019). Notably, this effect was not apparent in naïve mice, where the only difference observed between the two strains was a modest increase in splenic bacterial load following wild-type infection; weight loss, skin bacterial load and survival were similar between both groups (Naegeli *et al.* 2019). The ostensible irrelevance of *ndoS* in M1 infection of naïve mice is in accord with an earlier study (Sjogren *et al.* 2011), which employed wild-type M1 5448, a globally prevalent serotype frequently isolated from severe, invasive GAS disease (Aziz and Kotb 2008). The authors observed no significant difference in 10-day lethality following intraperitoneal infection between wild-type and *ndoS* mutant strains (Sjogren *et al.* 2011). In the same study, the mouse experiment was repeated with a less virulent, naturally EndoS-deficient strain (NZ131; M49 (McShan *et al.* 2008)) transformed with *ndoS*. The *ndoS*-expressing strain exhibited significantly greater 10-day lethality compared to the untransformed control. The authors speculate that this strain-biased effect of EndoS may be due to the overexpression of *ndoS* in the transformed strain, relative to 5448 wild-type. Interestingly, it has since been shown that this M49 strain expresses a similar endoglycosidase, EndoS$_2$ that has not been described for any other *emm*-type (Sjogren *et al.* 2013). EndoS$_2$ acts on human α-1-acid glycoprotein in addition to IgG and has distinct activity and glycoform selectivity (Sjogren *et al.* 2015). Ostensibly only expressed in a minority of GAS strains, EndoS$_2$ nevertheless represents a further example of an immunomodulatory interaction of host glycans in GAS infection. *ndoS* is highly conserved among GAS (Davies *et al.* 2019), suggesting an important role in bacterial fitness or pathogenicity. Notably, it has been shown that EndoS is not involved in nutrient acquisition (Collin and Olsen 2001), confirming its primary role as an immunomodulatory factor.

The importance of EndoS and EndoS$_2$ in infection is unclear and warrants further scrutiny, particular in the context of human infection. In a study involving throat swab samples from GAS pharyngitis patients and blood samples from sepsis patients, the authors observed significantly higher levels of glycan-hydrolysed IgG relative to healthy controls in both kinds of samples (Naegeli

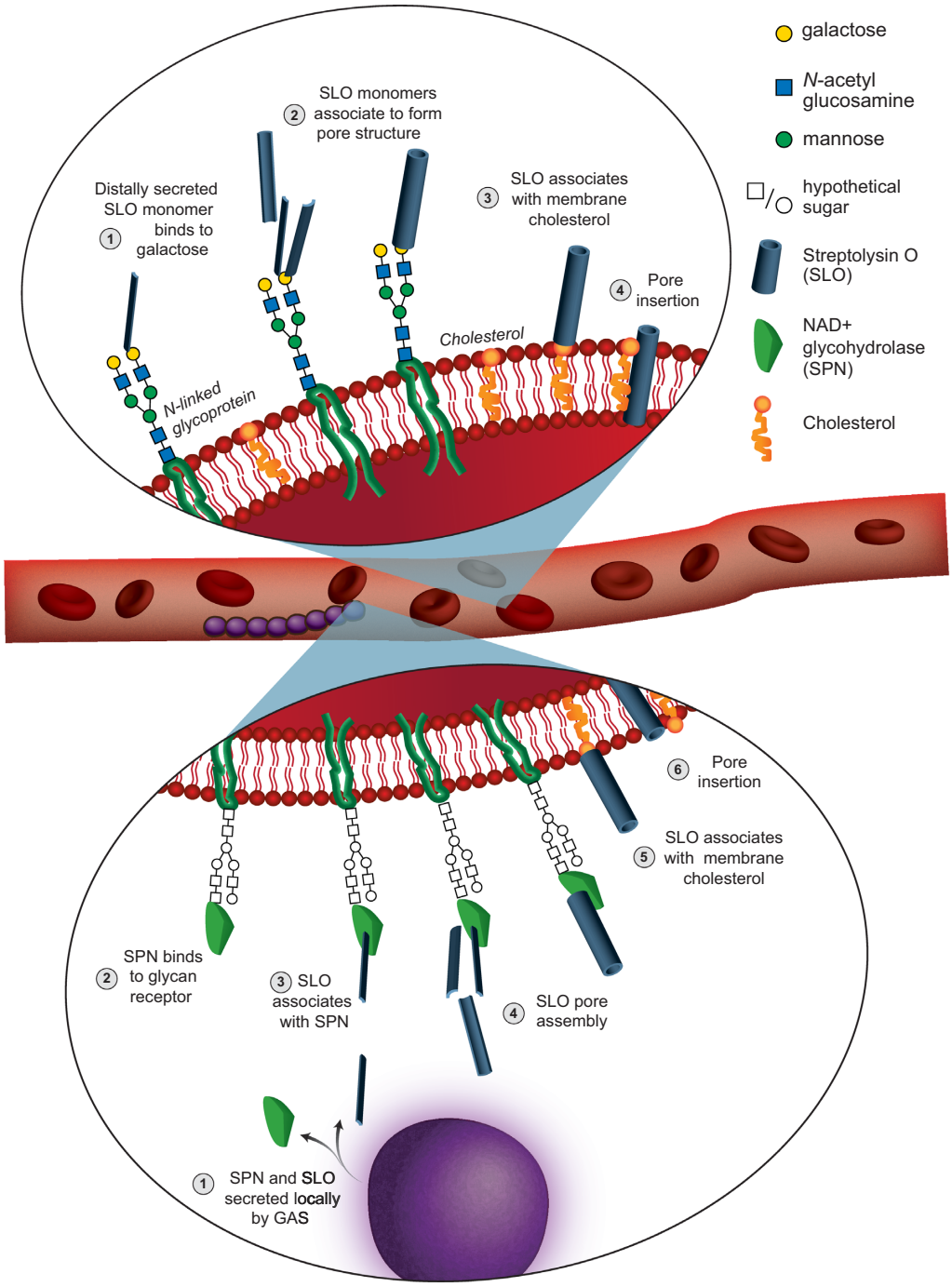

Figure 4. Proposed model for the recognition of glycans in erythrocyte membranes by the pore-forming toxin SLO **(A)** and its co-toxin SPN **(B)**. In the absence of streptococcal NAD^+ glycohydrolase (SPN)**,** secreted streptolysin O (SLO) polymers assemble at local or distal erythrocyte membranes by first binding to a galactose residue (A). Locally secreted SPN binds to a hypothetical glycan receptor and serves as the site for SLO polymer assembly in the absence of galactose (B). In both cases, the assembled SLO polymers interact with membrane cholesterol prior to pore insertion.

et al. 2019). The authors also reported decreased virulence of EndoS knockout mutant GAS (AP1, M1 serotype) in a mouse model. The inherent limitations of mouse models for GAS disease necessitates the examination of human samples, as conducted here. However, analyses were limited by patient availability and confounded by variation in the GAS strains responsible for infection. Further investigation is thus necessary to provide clarity about the expression and role of EndoS during infection. Human infection models, such as the Controlled Human Infection for Vaccination Against Streptococcus (CHIVAS) trial (Osowicki *et al.* 2019) exemplify a platform conducive for the rigorous exploration of these kinds of host-pathogen interactions without the limitations inherent in hospital samples. However, CHIVAS primarily facilitates the study of the host-pathogen relationship in the oropharynx, where EndoS is perhaps less relevant as compared to more severe infection. Thus, alternative approaches such as non-human primate studies may be necessary to elucidate the importance of EndoS in GAS pathogenesis.

SpyCEP

Streptococcus pyogenes cell envelope protease (SpyCEP) is a GAS virulence factor implicated in the pathogenesis of severe GAS disease. It is upregulated in invasive isolates and has been identified as a major cause of the hallmark diminished neutrophil recruitment at sites of necrotising infection (Edwards *et al.* 2005; Turner *et al.* 2009). SpyCEP cleaves the human chemokine Interleukin-8 (CXCL8) at the C-terminus, impeding neutrophil chemotaxis and recruitment. Although SpyCEP does not directly interact with host glycans, its capacity to confer protection from innate immunity relies heavily on the involvement of host GAGs in neutrophil functions. It has been established that the C-terminal portion of CXCL8 is responsible for binding host GAGs and that the N-terminal region interacts with its cognate receptors, CXCR1 and CXCR2 (Gayle 3rd *et al.* 1993; Ahuja, Lee and Murphy 1996). The mechanisms for how SpyCEP-mediated C-terminal cleavage impede chemokine:receptor interactions, however, have only recently been explored. It has been proposed by Goldblatt *et al.* that surface-bound GAGs sequester intact CXCL8 in close proximity to its receptors, enabling more efficient chemotaxis (Goldblatt *et al.* 2019). The authors evidence the necessity of the CXCL8:GAG interaction by demonstrating aberrant, impeded neutrophil migration following the enzymatic removal of host GAGs, or SpyCEP-mediated CXCL8 cleavage, respectively. SpyCEP thus exploits the dependence of neutrophils upon host glycans in the process of chemokine binding, effectively attenuating this crucial component of the innate immune response. SpyCEP and EndoS/EndoS2 are examples of mechanisms that interrupt host glycosylation to defend GAS against the human immune system. These mechanisms are protective in nature, as opposed to pathways that directly elicit host cell damage such as those mediated by bacterial toxins.

The Streptolysin O toxin

Many bacterial toxins interact with host glycan structures, including the cholera, Shiga, tetanus, and pneumolysin toxins (reviewed in (Schiavo and van der Goot 2001; Kulkarni, Weiss and Iyer 2010; Poole *et al.* 2018)). Streptolysin O (SLO) is a cholesterol-dependent, pore-forming cytolysin secreted by GAS with lytic activity against a range of mammalian cells (Sierig *et al.* 2003; Ahmad, Lutfullah and Ali 2011). Toxicity is exerted by SLO-mediated translocation of streptococcal NAD^+ glycohydrolase (SPN) into the target cell (Madden, Ruiz and Caparon 2001; Bricker *et al.* 2002). While its recognition of membrane cholesterol has long-since been established, SLO has more recently been identified as a lectin, with reported binding of a number of physiologically relevant human glycans. It was demonstrated that exogenous lacto-*N*-neotetraose (LNnT) inhibited SLO binding to erythrocyte membranes (Shewell *et al.* 2014). LNnT terminates with a galactose residue and is present as a ceramide on the surface of human neutrophils (Schwarting and Marcus 1977). Interestingly, it has been demonstrated that SLO-induced haemolysis can be inhibited by a galactose-binding lectin isolated from the marine invertebrate *Aplysia kurodai* (AKL) (Hasan *et al.* 2014). This inhibition was dose-dependent and was suppressed with exogenous galactose, suggesting that AKL and SLO compete for terminal galactose in the erythrocyte membrane. Further research showed that mutation of SLO residues at the predicted glycan-binding sites impaired or abolished haemolytic activity on erythrocytes, despite retaining affinity to target cell membranes (Mozola and Caparon 2015). Furthermore, SLO was unable to form pores in cells lacking galactose-containing glycoproteins or glycolipids. Taken together, these results strongly implicate the involvement of host galactose in SLO pore formation and haemolysis. Co-expression of SPN rescued pore-forming ability in the absence of galactose, indicating that galactose is not necessary for SLO binding in the presence of SPN (Fig. 4B). However, SPN is also predicted to contain a glycan-binding domain (Kelley and Sternberg 2009; Smith *et al.* 2011) and notably, point mutation within this putative domain abolished pore-forming ability. Considered together, these studies suggest that the haemolytic activity exerted by SLO and SPN relies on recognition of host membrane glycans.

Later research employing glycan microarray analysis, a technique increasingly utilised in GAS-glycome research (Indraratna *et al.* 2020), revealed that SLO binds a range of host glycans, with especially high affinity for B antigen type IV pentasaccharide (Shewell *et al.* 2020). Although B type IV pentasaccharide does not occur on erythrocytes, it is a galactose-bearing oligosaccharide similar to type I or II antigen abundant on erythrocyte membranes in individuals of B blood type. Notably, pre-incubation of SLO with this pentaose completely abolished haemolytic activity (Shewell *et al.* 2020). Further research may inform the therapeutic plausibility of harnessing this glycan-mediated negation of SLO, given its contribution to invasive GAS disease.

Biofilms

The formation of biofilms in both commensal and pathogenic contexts is an important aspect of the host-bacteria relationship. Biofilms exhibit markedly different characteristics to their planktonic counterparts, including phenotypic heterogeneity; resistance to host immunity; presence of persister cells and antibiotic tolerance, all of which contribute to increased virulence (reviewed in Dufour, Leung and Lévesque 2010). Although there is notable paucity of research in the roles of and processes concerning GAS biofilms, there is considerable evidence of biofilm formation and consequent antibiotic tolerance in clinical GAS infections (reviewed in Vyas *et al.* 2019). The involvement of host and bacterial glycans with respect to biofilm formation and phenotype is a niche that is well-explored in only a few pathogenic species, such as *P. aeruginosa* (Tielker *et al.* 2005; Wheeler *et al.* 2019) and *S. pneumoniae* (Trappetti *et al.* 2009; King 2010). With regard to the former, several glyco-based molecules have shown biofilm-modulating properties in vitro, including a C-fucosyl derivative, rhamnose-binding protein and mannose-conjugated polymers (Consoli *et al.* 2011; Fu *et al.* 2019; Limqueco *et al.* 2020). There has been far less investigation into the role of host glycans in GAS biofilm formation, despite the abundance of glycans in the oropharynx. One 2020 study however, demonstrated a change in GAS biofilm phenotype following modification of the host surface glycome *in vitro* (Vyas *et al.* 2020). Indiscriminate removal of N-glycans from the surface of human pharyngeal monolayers resulted in increases in biofilm biomass. Further experiments demonstrated that enzymatic removal of terminal mannose and sialic acid residues, respectively, also resulted in increases in biofilm biomass across a diverse selection of *emm*-types. Moreover, M12 biofilms grown on these exoglycosidase pre-treated surfaces resulted in a two-fold increase in penicillin tolerance relative to control biofilms. The mechanisms by which host glycans influence antibiotic susceptibility are yet to be uncovered but represent a promising avenue for future innovative research.

Concluding remarks

The involvement of the host glycome in GAS infection remains only partially uncovered. However, it is clear that many streptococcal factors exploit host glycans in achieving a range of

pathogenic outcomes, including adherence to host tissue; carbohydrate acquisition within the oropharyngeal niche; degradation of host IgG; biofilm formation and SLO-mediated haemolysis. The examination of the many glycointeractions involved in these processes also reveals the substantial paucities in the understanding of the role of human carbohydrates in GAS disease. Many of these gaps represent promising opportunities for novel research into GAS pathogenesis and more generally, may advance our broader understanding of the host–pathogen relationship.

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