***Streptococcus pyogenes* adhesion and colonization**

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Abstract

Streptococcus pyogenes (group A *Streptococcus*, GAS) is a human-adapted pathogen responsible for a wide spectrum of disease. GAS can cause relatively mild illnesses such as strep throat or impetigo, and less frequent but severe life-threatening diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome. GAS is an important public health problem causing significant morbidity and mortality worldwide. The main route of GAS transmission between humans is through close or direct physical contact, and particularly via respiratory droplets. The upper respiratory tract and skin are major reservoirs for GAS infections. The ability of GAS to establish an infection in the new host at these anatomical sites primarily results from two distinct physiological processes, namely bacterial adhesion and colonization. These fundamental aspects of pathogenesis rely upon a variety of GAS virulence factors, which are usually under strict transcriptional regulation. Considerable progress has been made in better understanding these initial infection steps. This review summarizes our current knowledge of the molecular mechanisms of GAS adhesion and colonization.

Keywords: *Streptococcus pyogenes, adherence, colonization, biofilm, pathogenesis, transcriptional regulation*

Introduction

Streptococcus pyogenes, otherwise known as Group A *Streptococcus* (GAS), is a clinically important bacterial pathogen that is exclusively adapted to the human host. GAS commonly causes respiratory tract infections with mild to modest disease symptoms, such as tonsillitis and pharyngitis (strep throat). GAS may also colonize the skin, causing superficial infections such as pyoderma. Patients with such infections usually respond well to antibiotic treatment. However, under certain conditions GAS may invade deeper tissues and cause severe infections (septicaemia, necrotizing fasciitis or streptococcal toxic shock like syndrome). Repeated GAS infections may also trigger immune sequelae such as rheumatic fever, rheumatic heart disease and acute post-streptococcal glomerulonephritis. The estimated global burden of severe GAS disease places it among the most important bacterial pathogens with approximately 18 million cases per year and 517,000 deaths [1]. Exacerbating public health concerns has been recent reports of scarlet fever outbreaks [2-4], the emergence of invasive infections [5], and rising rates of antibiotic resistance [4,6]. Although all GAS strains remain exquisitely sensitive to penicillin.

Dissemination of GAS may occur by person-to-person contact or via contaminated airborne droplets. Skin infection may lead to pyoderma, with approximately 111 million cases occurring each year [1], and respiratory tract infection by GAS may result in pharyngitis, with approximately 600 million cases worldwide [1]. The epithelial lining of the upper respiratory tract represents a particularly favorable aerobic and nutrient-rich environment for the growth of many bacteria. Thus, GAS has to find a way to compete in the dynamic airway microbiota. The pathogenesis of GAS is highly complex, and has been extensively reviewed elsewhere [7,8]. Undoubtedly, one of the very first and fundamental stages of GAS pathogenesis is adhesion to and colonization of the epithelium [9-13] (Figure 1). The ability of GAS to adhere to host epithelia is serotype-specific and depends on the repertoire of adhesins and systems for sensing changes to environmental conditions. In principle, however, all adhesins fulfil the same

function by bringing the bacterial cell into close contact with the host cell by specific adhesin-receptor interactions.

After initial attachment, GAS has been observed to form microcolonies. These macroscopic structures have been implicated in streptococcal skin infection and acute bacterial tonsillopharyngitis [14,15]. When bacterial cells proliferate, such microcolonies may form complex groups that constitute organized three-dimensional structures, a sessile lifestyle commonly referred to as a biofilm. It is now broadly accepted that GAS is capable of microcolony and biofilm formation and that this sessile lifestyle plays an important role in GAS pathogenesis [16]. As there is a fluent transition between reversible and irreversible attachment during biofilm formation, most of the adhesins of GAS are also required for this multicellular surface-attached community state. Finely tuned expression of adhesins and other virulence factors is therefore critical for efficient colonization and infection.

Regulation of adherence occurs at multiple levels and has been extensively discussed in previous reviews [17-19]. These complex regulatory networks include two component regulatory systems (e.g. CovRS/CsrRS, FasBCAX) and 'stand-alone' transcriptional regulators. One example is the Mga regulator whose regulon comprises ~ 10% of the GAS genome that includes numerous genes important for adhesion and the metabolic homeostasis of GAS [20]. Other stand-alone regulators include RofA-like proteins (RALPs) and the regulator RopB (also known as Rgg) [21]. All of these systems respond to growth-related or environmental signals to control expression of genes involved in colonization. In recent years, it has become increasingly apparent that small non-coding RNAs represent a further level of regulation in GAS [19,22].

Although we do not as yet clearly understand the precise mechanisms by which GAS facilitates the initial interaction with host epithelial cells, the past few years have witnessed substantial advances in our understanding of this stage of *S. pyogenes* infection. This review aims to provide an overview of our current knowledge on the initial host-pathogen interaction during GAS colonization.

Adherence of GAS

The GAS cell surface displays a variety of proteins and other macromolecules that facilitates the colonization of host tissues (reviewed in [10]). The initial attachment process has long been hypothesized to be a two-step process, with weak and/or long-range interactions followed by more specific, high affinity binding [23]. Weak, hydrophobic interactions mediated by lipoteichoic acids may contribute to initial adherence to host surfaces [23]. This weak interaction, in turn, may permit longer distance first attachment events mediated through extending surface appendages such as pili, followed by multiple, higher affinity binding events such as protein-protein or lectin-carbohydrate interactions. The GAS cell surface incorporates numerous protein adhesins that allow GAS to colonize distinct tissue sites (Table 1). Many of these adhesive proteins are covalently attached to the cell wall peptidoglycan by sortase enzymes [24-26].

Table 1: GAS cell-wall anchored adhesins and target host receptors.

Adhesin	Receptor(s) and/or function(s)	Reference(s)
<i>FCT region</i>		
Pili	Salivary agglutinin gp-340, Bind to epithelial cells	[27-29]
Sfb1/PrtF1	Fibronectin, Fibrinogen	[30-36]
PrtF2/FbaB/PFBP	Fibronectin, Fibrinogen	[33,36-40]
Cpa/AP1	Collagen type I	[41-43]
<i>Other fibronectin-binding proteins</i>		
SOF/Sfb2	Fibronectin, Fibrinogen, Fibulin-1	[44-49]
FbaA	Fibronectin, Factor H	[50-52]
SfbX	Fibronectin	[48]
Scl1/2	Fibronectin, Laminin, $\alpha_2\beta_1$ integrin, $\alpha_{11}\beta_1$ integrin, Factor H, Lipoproteins, CFHR1	[53-62]
<i>M or M-like proteins</i>		
M proteins	Fibronectin, Plasmin(ogen), Fibrinogen, Factor H, Sialic acid, CD46, Glycosaminoglycans, Beta 2-Microglobulin, Albumin, Immunoglobulins, Collagen type I and IV	[63-76]
Mrp	Immunoglobulins, Fibrinogen	[77]
Arp	Immunoglobulins	[77-79]
Sir	Immunoglobulins	[77,80]
<i>Other laminin-binding proteins</i>		

Lsp	Laminin	[81]
Lbp	Laminin	[82]
<i>Other plasmin(ogen)-binding proteins</i>		
PAM	Plasmin(ogen)	[83]
Prp	Plasmin(ogen)	[84]
Epf	Plasmin(ogen)	[85]
<i>Other saliva-binding proteins</i>		
AspA	Salivary agglutinin gp-340	[86-88]
GrpE	Salivary proline-rich proteins	[89]
<i>Other adhesins</i>		
Protein H	Immunoglobulins	[90,91]
PulA	Glycoproteins	[92]
R28	Unknown, Binds to epithelial cells	[93]
Slr	Collagen type I	[76]
SpyAD	Human keratin 1, Collagen type VI	[94,95]

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123

124 *Sortases*

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126 A hallmark of the majority of GAS adhesins is their covalent linkage to the GAS
 127 peptidoglycan via sortase enzymes. GAS sortases are broadly classified into two
 128 types: the “housekeeping sortase” SrtA, which anchors the majority of cell
 129 surface proteins via their LPXTG motif to the cell wall peptidoglycan [25], and
 130 the pilus-associated sortases SrtB, SrtC1 and SrtC2 [25,96], which mediate the
 131 polymerization of pilin subunits into long, fibrillar surface structures [97].
 132 Sortase enzymes, and their mechanism of action, have been characterized for
 133 numerous Gram-positive organisms (for review, see [26,98-100]).

134

135 *GAS pili*

136

137 GAS pili are long, flexible rods protruding up to 3 μ m from the cell surface [97].
 138 They consist of a major backbone pilin subunit (BP), and one or more minor
 139 ancilliary protein subunits (AP1/Cpa and AP2), which are polymerized into the
 140 mature pilus structures by a series of transpeptidation reactions catalyzed by the
 141 pilus-associated sortases [96,97,101-104]. The assembled pilus structure is then
 142 ultimately anchored to the cell wall by SrtA [105]. The sortase-catalyzed
 143 assembly mechanism of Gram-positive pili has been reviewed extensively
 144 elsewhere [98,106-108].

145

146 The genes encoding pili, along with genes required for their assembly and
 147 regulation, are localized to a single genomic island. Collectively, this region is
 148 referred to as the FCT region, as this island often encodes adhesins that mediate
 149 binding to fibronectin and collagen in addition to the pili, which comprise the T-
 150 serotyping system [97,109]. At least 9 different FCT regions occur in GAS, and
 151 these have been termed FCT-1 to FCT-9 [101,110]. With few exceptions, GAS
 152 isolates belonging to the same *emm* type belong to the same FCT type. The major
 153 M types responsible for human disease in developed countries belong to the
 154 following FCT types: FCT-1 (*emm6*), FCT-2 (*emm1*, *emm28*), FCT-3 (*emm3*,
 155 *emm12*), FCT-4 (*emm12*, *emm28*, *emm89*), and FCT-5 (*emm4*) [111].

156

157 GAS pili mediate attachment to a variety of cell lines and primary human tissues,
 158 and adhesive properties are likely conveyed by the accessory, minor pilus
 159 subunits. In FCT-2 pili, deletion of the tip adhesin Cpa reduced binding to
 160 primary tonsil explants and primary skin keratinocytes, without affecting pilus
 161 production [29,112]. Surprisingly, this adhesion was not inhibited by pre-
 162 incubation with any of the published ligands for Cpa [29], suggesting that the
 163 true epithelial receptor for FCT-2 pili remains to be identified. In addition to
 164 primary cells and tissues, FCT-1 and FCT-2 pili have also been shown to mediate
 165 adhesion to a variety of epithelial cell lines [28,29,104,112]. Similarly, the FCT-1
 166 ancilliary protein AP1 was found to mediate binding to A549 epithelial cells, and
 167 *Lactococcus lactis* engineered to express FCT-1 pili were able to adhere to A549
 168 cells in an AP1-dependent manner [42]. FCT-1 and FCT-2 pili have also been
 169 shown to mediate bacterial autoaggregation, which may increase colonization
 170 through the formation of microcolonies and/or biofilms [28,42,113]. In the case
 171 of FCT-2 pili, this aggregation was mediated by host glycoprotein gp340 binding
 172 [27].

173

174 In contrast to the FCT-1 and FCT-2 pili, relatively little is known about the
 175 contribution of pili from other FCT types to GAS adhesion to host cells. FCT-3 pili
 176 from both M49 and M53 GAS strains have been found to be indispensable for
 177 adhesion to HEp-2 epithelial cells and human skin [114,115]. However, Cpa was

found to mediate adhesion to human skin [115], a finding that could be attributed to the apparent localization of Cpa at the FCT-3 pilus base rather than at the tip of the FCT-3 pili [103,104].

Fibronectin-binding proteins

A common theme in bacterial adhesion to host tissues is the involvement of proteins that bind extracellular matrix (ECM) components such as fibronectin, collagen and laminin. Collectively, these bacterial adhesins have been termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [116]. GAS produces multiple MSCRAMMs that interact with fibronectin, collagen and laminin. By far the most abundant class of these are those that bind fibronectin; a total of 10 fibronectin binding proteins have been characterized in GAS with the majority of these proteins anchored to the cell wall through an LPXTG motif (Table 1-3). Fibronectin binding proteins broadly belong to two types, depending on whether or not they contain fibronectin-binding repeats (reviewed in [117]).

The FCT region of most GAS strains encodes one or both of the major fibronectin-binding proteins PrtF1/Sfb1 [30], and PrtF2 [37,39,109]. PrtF2, in turn, exists as two separate alleles as a result of deletion mutations in the *prtF2* sequence; PFBP lacks 105 amino acids at the N-terminus in comparison to FbaB [39]. PrtF1 and PrtF2 proteins exhibit a high affinity to human fibronectin (Kd ~5 nM for PrtF1 and ~2 - 8 nM for PrtF2; [36]), and deletion of the encoding genes usually reduces fibronectin binding considerably [30,33,37,118]. In strains that do encode them, heterologous expression of PrtF1 and PrtF2 has been shown to mediate adhesion to a variety of epithelial cell lines [32,38,40,118,119]. PrtF2 was also shown to be required for adhesion of M53 GAS to human skin [115]. Thus, the FCT-encoded PrtF1 and PrtF2 surface proteins are likely to be important GAS adhesins through interaction with host fibronectin. Although considered primarily as fibronectin-binding proteins, PrtF1 and PrtF2 were also shown to bind fibrinogen [35,36]. Walden and colleagues recently demonstrated that PrtF1 covalently binds fibrinogen via an unusual thioester bond, possibly

helping the bacterial cell to irreversibly attach itself to the host's tissues [34]. The authors suggest that this novel role of bacterial adhesins as 'chemical harpoons' may in fact be a common yet unrecognized mechanism of covalent attachment of bacterial proteins to fibrinogen [34].

It is worth noting, however, that FCT-2 strains, which include the major pathogenic serotype M1T1, encode neither PrtF1 nor PrtF2. However, the M1 protein has been shown to bind fibronectin with high affinity (~48 nM) [36], indicating that FCT-2 strains bind fibronectin in an FCT-independent manner.

In addition to the FCT region, a second genomic region present in approximately half of GAS strains also contributes to fibronectin binding. This genomic region encodes two fibronectin proteins that are co-transcribed: serum opacity factor (SOF) and SfbX [48]. Both SOF and SfbX are predicted to be cell wall anchored via SrtA. While purified SOF does mediate binding to HEp-2 cells [49], an isogenic M49 *sof* mutant was not altered in its ability to bind HEp2 cells. However, SOF was required for GAS to invade these cells [120]. This may reflect the presence of other high affinity fibronectin binding proteins in M49 GAS strains, such as PrtF2. Surprisingly, while purified SOF bound HEp-2 cells, this binding was found to be independent of the Fn binding domain [47].

In addition to the FCT and SOF genomic regions described above, multiple other fibronectin-binding domain proteins have been characterized in GAS (Table 1; for review see [117]). These proteins are sporadically distributed in different GAS strains, with the exception of Fbp54, which is encoded in every strain that has been examined [39]. Many of these have also been shown to mediate adhesion to human cells with different host-cell tropism. For example, Fbp54 has been shown to mediate adherence to buccal epithelial cells, but not HEp-2 epithelial cells [121]. Likewise, there appear to be serotype-specific differences in adhesive properties of fibronectin-binding proteins. For example, the streptococcal hemoprotein receptor (Shr, see below) from M49 GAS [122], but not M1T1 GAS [123], was shown to mediate binding to HEp-2 cells. Thus, it is

important to interpret the function of GAS fibronectin binding proteins in the context of both strain serotype and host cell specificity.

There is much redundancy in the ability of GAS to bind fibronectin. This may reflect the expression of different fibronectin-binding proteins under different environmental conditions or niches, or the preferential binding of particular proteins to certain fibronectin isotypes. It is worth noting that in addition to cell and tissue adherence, several other phenotypes have been attributed to fibronectin binding proteins. These include invasion of host epithelial and endothelial cells [120,124-127], binding other host ligands such as fibrinogen [34-36,128,129], collagen [41,130], plasminogen [131,132], fibulin-1 [44] and heme [122], and evasion of host immunity [133]. Clearly, much work remains to be done in this area in order to fully understand the complex interplay of GAS fibronectin binding proteins with host tissues.

In addition to the fibronectin-binding proteins discussed above, GAS has been shown to bind other ECM proteins, including type I and IV collagen and laminin. Binding of these ECM proteins can be mediated directly, or indirectly via fibronectin acting as a bridging molecule; such GAS adhesins may additionally bind other host molecules. For example, in addition to binding fibronectin, the Sfb1 protein discussed above can recruit type IV collagen via surface-bound fibronectin, which results in the formation of large bacterial aggregates [130]. The M1 and M18 proteins (discussed in detail below), have been shown to bind type IV collagen [72,134], while the Cpa pilus protein discussed above binds type I collagen [41]. A GAS surface protein (Lbp) that mediates binding to laminin has also been identified, and shown to bind HEP2 epithelial cells [82]. Surprisingly, this protein is structurally related to bacterial zinc receptors, and as such has been suggested to mediate laminin binding via laminin-bound zinc [135].

Collagen-like proteins

In addition to the MSCRAMM proteins discussed above, GAS also possesses one or more collagen-like proteins, Scl1 and Scl2, which bind to host receptors by

mimicking human collagen. The collagen-like domain consists of varying numbers of Gly-X-X motifs, and exhibits a triple-helical elongated protein structure similar to human collagen [136]. As such, the GAS Scl proteins can bind to the host collagen receptors, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins [56-58], as well as a variety of other host ligands, including high- and low-density lipoproteins, fibronectin, laminin and the complement receptors CFHR1 and factor H [54,59-62]. Scl1, which is encoded in the genome of all GAS strains [137], has also been shown to mediate adherence and internalization into HEp2 epithelial cells [54,55,58].

Role of M proteins in adherence

The M protein, encoded by the *emm* gene, is a major surface protein and virulence factor, and forms the basis of the major typing scheme for GAS. M protein exists as an alpha helical coiled-coil dimer extending ~60nm from the bacterial surface [138], with each monomer anchored to the cell wall peptidoglycan by SrtA [25,139]. M protein is comprised of four repeat regions (A-D), which vary in size and amino acid composition. The surface exposed N-terminus exhibits considerable antigenic diversity, and sequencing of the hypervariable A-repeat region has identified >220 variants. As such, a large number of functions, and interactions with host molecules, have been ascribed to different M protein variants [140-143]. For the purpose of this review, we will focus on the role of M protein as an epithelial colonization factor.

Numerous studies have provided evidence that M protein contributes to the adherence of GAS to host cells. M proteins from multiple serotypes (M1, M3, M5, M6, M18 and M24) have all been shown to contribute to GAS adherence to immortalized cell lines such as HEp2 [29,144-148], and Detroit 562 [70,74]. In addition, several host cell molecules, including sialic acid [70], CD46 [66,71], dermatin sulfate and heparin sulfate [74], beta2-microglobulin [67], and collagen type I and IV [72,76], have been reported to bind to certain M proteins (Table 1). However, there are serotype-specific differences in the interactions with these receptors. For example, M6 protein has been reported to bind CD46 [66,71],

while M18 does not [149]. Likewise, M3 protein binds collagen type IV, while M6 and M18 proteins do not bind this ligand [72]. Given the extraordinary diversity of the surface-exposed N-terminus of M proteins, these serotype-specific differences in binding to different host cell ligands are not surprising.

Despite numerous investigations, the direct interaction of M protein with primary human cells is somewhat more controversial. M proteins have been shown in several studies not to confer significant binding to primary buccal cells [145,150], primary keratinocytes [29], immortalized primary human pharyngeal cells [75], or the HaCaT cell line [29,75]. In fact, an isogenic M1 protein negative mutant has been shown to adhere better than the wild type strain in some studies [29,75]. In research using primary human tonsil explants, M1 protein was shown to provide a minor contribution to GAS adhesion [29], and M6 protein was shown not to contribute to GAS adhesion [150]. In both of these studies, the authors noted a reduced ability of the M protein mutant strains to clump together and form microcolonies on the epithelial surface. It is therefore possible, that the role of M protein in mediating adherence to host tissues is in its ability to promote the formation of bacterial microcolonies on the host cell surface, rather than direct interaction with host cell receptors.

In addition to these roles in mediating adherence, M1 protein has also been shown to promote invasion of immortalized cell lines, via cooperative binding to CD46 and fibronectin [126].

Secreted adhesins

In addition to cell wall-anchored adhesins, GAS also secretes proteins to make physical contact with the host. These secreted adhesins lack the classical LPXTG anchoring domain, but possess a signal peptide for bacterial secretion. The well-studied adhesins in this category are the streptococcal pyrogenic exotoxin B (SpeB) and the prophage-encoded secreted phospholipase A2 (SlaA). These multifunctional proteins are both important for host cell adhesion and GAS

virulence. Further to this, we include the streptococcal hemoprotein receptor (Shr) as a secreted adhesion since it is not only expressed on the cell surface of GAS [122], but Shr is also a secreted protein [122,151].

Table 2: Host receptors of secreted GAS adhesins.

Adhesin	Receptors(s) and/or function(s)	Reference(s)
SpeB	Laminin, $\alpha_5\beta_3$ integrin, $\alpha_{2b}\beta_3$ integrin, Thyroglobulin, Glycoproteins	[152-154]
SlaA	Unknown	[155,156]
Shr	Laminin, Fibronectin, Hemoprotein	[122,151,157]

Streptococcal pyrogenic exotoxin B (SpeB)

SpeB does not contain an LPXTG anchoring motif but does incorporate an amino-terminal leader peptide [158]. SpeB is a secreted cysteine protease with the ability to degrade a wide range of host molecules in addition to streptococcal proteins (for review see [159]). Analysis of the *speB* gene sequence from 200 GAS isolates led to the classification of SpeB into three different mature SpeB variants (mSpeB1, mSpeB2 and mSpeB3) [153]. The mSpeB2 variant contains an RGD (Arg-Gly-Asp) sequence, a signature recognition sequence for integrin binding [160]. Initial experiments examining the binding properties of mSpeB2 showed that this variant was indeed able to bind to $\alpha_5\beta_3$ and $\alpha_{2b}\beta_3$ integrins on the surface of transfected cell lines, and such binding could be inhibited by an integrin-specific mAb [153]. Integrins have been previously described to be important targets for pathogen entry into host cells [161,162]. Therefore, it has been hypothesized that the mSpeB2-integrin interaction could favor GAS virulence. Although a direct correlation has not been established, the mSpeB2 variant is expressed in various GAS serotypes such as M1, which is responsible for a significant number of severe invasive infections globally [163]. The role of SpeB in apoptosis has been investigated. Interaction of recombinant SpeB containing the RGD domain caused apoptosis of A549 cells via $\alpha_5\beta_3$ integrin binding [154]. In addition, the Fas-mediated cell death pathway was also activated by the interaction of SpeB with Fas following a RGD independent mechanism [154]. Apart from integrin and Fas, SpeB also binds thyroglobulin,

laminin and other glycoproteins [152]. This binding was detected in a GAS strain that expresses a SpeB variant that lacks the RGD motif involved in integrin binding, suggesting laminin and other glycoprotein binding is mediated by a different mechanism [152]. Taken together, these observations suggest SpeB may play a role in mediating GAS adhesion and invasion into host cells, however these effects seem to be strain specific and complex. A study using GAS M1T1 and M49 strains showed that expression of SpeB protease did not significantly affect the ability of GAS to adhere to A549 epithelial cells, while it increased the ability of both GAS strains to invade [164]. Other studies have shown that deletion of the *speB* gene from M2 and M3 GAS strains increased the ability of the mutant strains to internalize into human umbilical vein endothelial cells, A549 human lung fibroblasts [165] and HEp-2 cells [166].

In addition to the adhesin functions attributed to SpeB, this protease has also been reported to influence invasion of CHO-K1 cells by inhibiting fibronectin-dependent internalization [167]. Although the mechanism is not fully elucidated, it is thought that SpeB proteolytic activity modifies the bacterial surface thus abrogating fibronectin-mediated binding [167].

Secreted phospholipase A2 (SlaA)

SlaA contains a secretion signal however lacks the LPXTG anchoring domain [168]. It possesses phospholipase activity against several phospholipid substrates [169]. Expression of SlaA is increased when GAS is co-cultured with epithelial cells and saliva [170], suggesting involvement in the initial steps of colonization. Deletion of *slaA* resulted in a M3 mutant strain with decreased ability to bind to D562 epithelial cells compared to wild type. Furthermore, addition of recombinant SlaA improved the ability of the mutant strain to adhere to D562 cells [155]. A non-human primate model (NHP) of pharyngitis in cynomolgus macaques was used to show that the SlaA mutant was unable to colonize the pharynx of NHPs and establish an infection, compared to the M3 wild type strain [155]. Further research is required to confirm which specific

host components SlaA targets and how this enhances the ability of GAS to colonize the upper respiratory tract.

Streptococcal hemoprotein receptor (Shr)

Shr is a streptococcal protein that lacks an LPXTG anchoring motif but contains an N-terminal signal peptide, and is also found associated with the membrane via a putative C-terminal transmembrane domain [122]. It is part of the heme acquisition system of GAS, where its function is to bind host heme-containing proteins [151]. Shr is able to sequester heme from methemoglobin [157] which is then transferred to Shp, another constituent of the GAS heme acquisition system [171]. In addition to its iron acquisition and transfer functions, Shr is able to bind fibronectin and laminin *in vitro*. Deletion of the *shr* gene in a M49 GAS strain resulted in a decrease in the ability of the mutant strain to adhere to HEp-2 cells and attenuated virulence in a zebra fish model compared to the wild type isogenic strain [122]. Studies using other GAS strains have shown differing outcomes. A GAS M1T1 isogenic mutant strain for *shr* showed a reduction in ability to bind laminin compared to the wild type strain, however the isogenic mutant bound fibronectin as well as wild type [123]. The laminin and fibronectin binding experiments in this study were undertaken using the GAS M1T1 strain, while in the previous study [122] binding to these proteins was determined using recombinant Shr. This might in part explain the differences regarding the ability of Shr to bind fibronectin. Binding to HEp-2 and HaCaT cells was not affected by the deletion of *shr* in the GAS M1T1 mutant strain, however this mutant strain caused smaller skin lesions compared to the wild type in a subcutaneous murine infection model [123].

Anchorless adhesins

Anchorless adhesins are a group of streptococcal surface proteins that lack the classic LPXTG motif usually present in surface anchored proteins signal peptides for secretion [10]. The mechanism by which anchorless adhesins are exported

from the cytosol to the surface is not well understood. The most important anchorless adhesins in GAS are housekeeping enzymes that not only perform important virulence roles on the surface but also carry out essential catalytic functions within the cytoplasm [172]. Here we review some of the GAS anchorless adhesins that have shown evidence for a role in GAS adhesion and colonization (Table 3).

Table 3: Host receptors of anchorless GAS adhesins.

Adhesin	Receptors(s) and/or function(s)	Reference(s)
GAPDH/SDH	Fibronectin, Lysozyme, Myosin, Actin, Plasmin(ogen), uPAR/CD87	[131,132,173-176]
α -enolase (SEN)	Plasmin(ogen)	[177-179]
Fbp54	Fibronectin	[121,145]

Streptococcal surface dehydrogenase (GAPDH/SDH)

GAPDH/SDH, also known as the plasmin receptor protein (Plr) [180], is a glyceraldehyde-3-phosphate dehydrogenase that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate during glycolysis, and is therefore an essential GAS enzyme [131]. GAPDH/SDH is also found on the GAS surface where it mediates important roles in virulence. GAPDH/SDH is able to bind to important host molecules such as fibronectin, lysozyme, myosin, actin [131] and plasmin(ogen) [132,176]. Substitution of the C-terminal lysine with a leucine in purified recombinant GAPDH/SDH showed decreased plasmin binding compared to the wild type protein [176]. However, live GAS carrying the same amino acid substitution did not show a commensurate reduction in binding, indicating that GAPDH/SDH is not the only plasmin receptor present on GAS [176].

Purified GAPDH/SDH or GAPDH/SDH present in live GAS was able to activate protein phosphorylation in pharyngeal cells [181]. In particular, the presence of GAPDH/SDH triggered phosphorylation of histone 3 protein (H3) by tyrosine kinase [181]. Although phosphorylation did not influence the ability of GAS to adhere to pharyngeal cells, it was advantageous for GAS invasion. The authors of

this study hypothesized that activation of protein tyrosine kinase may activate the influx of Ca^{2+} , increasing inositol phosphate and/or diacylglycerol facilitating GAS invasion via the actin polymerization pathway [181]. However, in order to confirm this hypothesis, further studies need to be undertaken.

GAPDH/SDH may play a further role in pharyngeal colonization via the ability to bind uPAR/CD87 (N-terminal D1 domain) on pharyngeal cells, which has been shown to be upregulated upon infection [173]. Modification of the C-terminal lysine of GAPDH/SDH abrogated binding to uPAR, demonstrating the importance of this particular residue. GAS mutants expressing a GAPDH/SDH with a substituted or deleted C-terminal lysine were less capable of adhering to Detroit 562 pharyngeal cells, suggesting a key role for this anchorless adhesin in GAS adherence and potentially colonization [173]. Introduction of a hydrophobic tail at the C-terminal end of GAPDH/SDH decreased export of the protein from the cytoplasm to the surface.

Streptococcal surface enolase (SEN)

SEN is the glycolytic enzyme α -enolase, an octameric metalloenzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate [177,179,182]. SEN is found in the cytosol and on the surface of GAS, and possesses high binding affinity for plasmin(ogen). Plasmin bound to SEN retains proteolytic activity, suggesting an important role in GAS virulence and tissue dissemination [177]. C-terminal lysine residues are in part responsible for SEN high affinity for plasminogen. Substituting the last 2 lysine residues of SEN for leucine residues (K434-435L) in a M6 GAS strain resulted in a significant reduction in the ability to bind plasminogen and penetrate extracellular matrix in an *in vitro* assay [178]. Further studies using recombinant SEN mutants in native conformation revealed that in addition to K434-435, lysine residues 252 and 255 also contribute to plasminogen affinity [179]. Substitution of all 4 lysine residues abrogates SEN's ability to bind plasminogen [183]. The interaction between SEN and plasminogen allows GAS to access tricellular tight junctions (tTJs), where plasminogen functions as a bridge between SEN and the major

component of tTJs, tricellulin. The ability of GAS to establish the SEN-plasminogen-tricellulin molecular interaction potentially facilitates bacterial translocation via a paracellular route [183], hence improving GAS ability to adhere and colonize the host.

FBP54

FBP54 is an anchorless streptococcal adhesin that binds fibronectin and fibrinogen in soluble and immobilized forms [184]. Adhesion of a M5 GAS strain to human buccal epithelial cells was inhibited by recombinant FBP54, while adhesion to HEp-2 cells was not significantly affected [121]. The authors argue that fibronectin has little effect in GAS adherence to HEp-2 cells; however this is potentially a GAS strain specific effect, as other studies have shown that blocking of fibronectin with other GAS fibronectin binding proteins such as SfbI inhibits GAS adhesion to HEp-2 cells [32]. A more detailed study regarding the role FBP54 in GAS adhesion, colonization and virulence is necessary to determine the relevance of this anchorless adhesin.

Biofilm formation and the interaction of GAS with the microbiota

Many bacteria are capable of switching between single-cell planktonic forms and biofilms. Biofilms are adherent aggregates of bacterial cells that form on biotic and abiotic surfaces, including human tissues. Biofilms formation is well known to play an important role in many chronic bacterial infections. However, the process of biofilm production in GAS has not been extensively characterized and the relevance for pathogenesis has only recently been recognized. There is an increasing appreciation that the biofilm phenotype of GAS is of clinical relevance contributing to oto-nasopharyngeal and skin infection [16].

The fact that GAS does not exclusively live a planktonic lifestyle is well documented by several studies (both *in vitro* and *in vivo*), but there is also a high variability in biofilm formation by different GAS serotypes [185,186]. It is

evident that the M or M-like proteins play a crucial role in the establishment of biofilms [187], and there are several other factors which affect the course of biofilm development including adhesins such as pilli [28,188], AspA [87], Scl1 [53] and SpeB [189]. In contrast, the involvement of the GAS capsule has not been fully elucidated and remains a point of contention [16]. To date, relatively limited data is available on the nature of GAS biofilm. However a transcriptomic analysis has been conducted on biofilm gene expression. In that study, mRNA expression profiles of the M14 GAS strain HSC5 showed differential regulation of approximately 25% of the genome upon adapting to biofilm growth *in vitro* [190]. This profile, however, differed from that of GAS biofilm-like structures present in infected zebrafish tissue and, thus, may not accurately reflect the *in vivo* situation [190]. A recent study, using the M3 GAS strain MGAS315, reported differing data concerning the expression of virulence genes such as *speB* and *sagA* in biofilm-adapted cells grown in association with live keratinocytes [191]. Such differences may represent a level of strain or serotype specificity in biofilm formation, and suggests it may be difficult to fit different data into a general profile related to GAS biofilm.

A limitation of *in vitro* and *in vivo* models to study mono-species biofilm formation is that they do not necessarily mimic the situation in humans, where an established and complex microflora exists. GAS must compete with and penetrate this established community of microbial species colonizing the upper respiratory tract or skin, in order to establish infection. Fiedler and colleagues [192] demonstrated *in vitro* that GAS can form mixed-species biofilms with other respiratory tract streptococci. Interestingly, the early colonizer *S. oralis* consistently formed the bottom layer of these biofilms triggering colonization by even poor biofilm producers such as GAS serotype M49. However, established biofilms of *S. oralis* and *S. salivarius* on HEp-2 cells abolished adherence and invasion by GAS, suggesting probiotic properties of the resident oral microbiota. The study nicely highlights the complex interspecies interactions that may occur in the human airway. There is also *in vivo* evidence that GAS is a member of multi-species biofilms in root canals and the nasopharynx [193,194].

Given the complexity of the process involved in biofilm formation, it is unsurprising that transcriptional regulation of biofilm-associated genes is sophisticated and complex, and GAS gene regulation occurs in response to environmental triggers such as pH, temperature and oxygen saturation that control the fate of GAS infection [17,188]. Mutation in the two-component Control of Virulence (CovRS, also referred to as CsrRS) gene regulatory system has been shown to alter both GAS adherence and biofilm formation [195]. In the globally disseminated serotype M1T1, mutation in the gene encoding covS resulted in reduced adhesion and biofilm formation both *in vitro* and *in vivo* [195].

Quorum sensing in GAS

Gram-positive cell-cell communication systems typically make use of small peptide pheromones. These signalling peptides are exported as pro-peptides and are then post-translationally processed to generate mature pheromones. Upon re-entry into the bacterial cell, these bind to a cognate member of the RRNPP family of transcriptional regulators to control transcriptional regulator-mediated activation of target genes [196]. The Rgg pheromone-responsive transcriptional regulators are members of one of the most relevant and widespread quorum sensing (QS) families in streptococci [197]. The past few years have witnessed significant interest in the four Rgg QS systems identified in GAS, all of which regulate multicellular behaviours such as biofilm formation or coordinated expression of virulence factors.

The two Rgg/SHP pairs, Rgg2/SHP2 and Rgg3/SHP3 were the first conserved QS systems identified in GAS [198]. The regulators of both systems compete for binding to specific promoters of the two adjacent *shp* genes along with several other co-regulated genes. Rgg3 acts as a repressor and Rgg2 has an activator function. Although the regulated genes are not directly involved in adherence, the Rgg2/3 pathway was found to increase biogenesis of biofilms in the M49 GAS strain NZ131 [198]. For the M1 serotype GAS strain SF370, *rgg2* mutation

increased biofilm formation, but abolished growth in human blood and virulence in mice [199]. Rgg2/3 signalling has been shown to contribute to resistance to lysozyme as a physiological response to metal limitation and utilisation of alternate carbon sources [200]. This effect was observed across multiple serotypes and supports a role for the Rgg2/3 regulatory circuit in protection from host defence and early colonization. Recent studies significantly extend our understanding of Rgg-SHP-mediated QS in GAS demonstrating that the SHP signalling peptides enable interspecies communication between streptococci, affecting the dynamics of GAS biofilm development [201,202]. This is an important observation, considering that GAS is able to grow in multi-species biofilms with oral streptococci [192].

Another intriguing finding is the presence of an internal control system in GAS, whereby the endopeptidase PepO is capable of degrading the signalling molecules SHP2 and SHP3 [203]. The CovRS-regulated PepO allows fine-tuning of Rgg/SHP signalling in both M49 GAS strain NZ131 and M1T1 GAS strain 5005. However, it appears that this role of PepO in GAS QS is serotype-specific, where PepO proved to be ineffective in disrupting the Rgg2/3 QS pathway in the M14 GAS strain HSC5 [203].

There exists a third Rgg QS system in GAS that is fundamentally distinct from the aforementioned Rgg/SHP QS systems. Although the regulator of proteinase B (RopB/Rgg1) shares structural homology to the RRNPP family of QS regulators, no pre-peptide encoding ORF has been identified to date [197,204]. Instead, the RopB regulator was shown to bind an amino-terminal signal peptide of the Vfr protein, repressing RopB-dependent *speB* expression [205,206]. Production of the cysteine protease SpeB, in turn, is linked to GAS biofilm dispersal [207]. However, it has long been hypothesized that RopB may use an unknown secreted peptide as a signal to induce *speB* expression [197]. Makthal and colleagues recently provided experimental evidence of such a low molecular weight proteinaceous secreted factor that induced RopB-dependent *speB* expression during high cell density, although the identity of this peptide remains elusive [204]. Additionally, the activity of RopB is under catabolic control via direct

protein-protein interaction with the aldolase LacD1, that results in the repression of *speB* transcription [208]. As these examples illustrate, there are complex regulatory circuits that control *speB* expression and thus affect biofilm development in GAS.

ComR is the fourth Rgg paralogue present in GAS, and like the other Rgg regulators it plays an important role in biofilm formation. Marks and colleagues recently observed that ComR is necessary for planktonic bacteria to adopt the biofilm lifestyle and is crucial for the ability of biofilm GAS grown on human SCC-13 squamous carcinoma cells to take up exogenous DNA [191].

Conclusions

Recent years have witnessed a better understanding of the molecular basis and mechanisms of GAS colonization. Prevention of bacterial adhesion and/or biofilm formation is a promising phenotype to target for GAS vaccine development. Successful colonization depends on both intra- and inter-cellular regulatory networks controlling the expression of adhesins and other virulence determinants contributing to colonization. These networks, however, may vary between different GAS serotypes, which make general assumptions regarding their precise role during GAS colonization difficult. Recent studies on bacteria-bacteria interactions within the epithelial microbiota further highlight the requirement for a better understanding of the interactions and dynamics between GAS and mixed bacterial communities and how such interactions govern pathogenesis.

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Figure Legends

Figure 1: *S. pyogenes* adherence and invasion of host cells. a) Adherence to and induction of cytoskeletal rearrangements by non-PrtF1/SfbI expressing *S. pyogenes*, and b) internalization of host cell via fibronectin and PrtF1/SfbI [32,127,209,210].

1433 **Figures**

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