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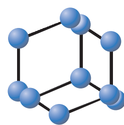

Current Understanding of Group A *Streptococcal* Biofilms

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Abstract: ***Background***: It has been proposed that GAS may form biofilms. Biofilms are microbial communities that aggregate on a surface, and exist within a self-produced matrix of extracellular polymeric substances. Biofilms offer bacteria an increased survival advantage, in which bacteria persist, and resist host immunity and antimicrobial treatment. The biofilm phenotype has long been recognized as a virulence mechanism for many Gram-positive and Gram-negative bacteria, however very little is known about the role of biofilms in GAS pathogenesis.

Objective: This review provides an overview of the current knowledge of biofilms in GAS pathogenesis. This review assesses the evidence of GAS biofilm formation, the role of GAS virulence factors in GAS biofilm formation, modelling GAS biofilms, and discusses the polymicrobial nature of biofilms in the oropharynx in relation to GAS.

Conclusion: Further study is needed to improve the current understanding of GAS as both a mono-species biofilm, and as a member of a polymicrobial biofilm. Improved modelling of GAS biofilm formation in settings closely mimicking *in vivo* conditions will ensure that biofilms generated in the lab closely reflect those occurring during clinical infection.

Keywords: *Streptococcus pyogenes*, Group A *Streptococcus*, biofilms, biofilm formation, antibiotics, virulence factors, biofilm modelling, polymicrobial.

1. INTRODUCTION

Group A *Streptococcus* (GAS) is a Gram-positive pathogen, with the human population serving as its only known reservoir. GAS is known to cause an array of diseases ranging from mild, superficial infections such as impetigo, tonsillitis and pharyngitis, to serious invasive infections including necrotizing fasciitis, *Streptococcal* toxic shock syndrome, cellulitis, and autoimmune sequelae (including rheumatic heart disease, acute rheumatic fever, and glomerulonephritis) [1, 2]. Globally, GAS causes 700 million infections and accounts for half a million deaths per year [3].

Increased use of macrolides for the treatment of GAS infections has seen an increase in resistance; however, there have been no reports of resistance to penicillin among clinical GAS isolates [4-6]. Despite GAS remaining sensitive to penicillin, a number of studies have indicated antibiotic treatment failure rates of 20-40% [5, 7]. Numerous hypotheses explaining antibiotic treatment failure have been proposed, including biofilm formation [4, 5, 8]. The biofilm phenotype provides an increased survival advantage, enabling bacteria to persist and resist both host immune defenses and antimicrobial treatment [4, 9, 10]. Although the biofilm phenotype has been studied extensively for other bacteria, little is known about GAS biofilm *in vitro* or *in vivo*. This review provides an overview of the current knowledge of the GAS biofilm.

2. BIOFILMS IN GAS PATHOGENESIS

Biofilm formation is recognized as a virulence mechanism for a variety of Gram-positive and Gram-negative bacterial species [11-14]. The presence of GAS biofilms in a variety of clinical infections suggests this phenotype may play a role in GAS pathogenesis [15, 16]. While there have been numerous studies that have characterized the role of GAS virulence factors in biofilm formation, studies assessing the role of biofilm formation in GAS pathogenesis have been limited. To understand GAS as a biofilm, it is first important to understand the biofilm phenotype.

2.1. Bacterial Biofilms

Bacteria can exist either in planktonic or biofilm states. Planktonic cells are singular 'free floating' entities existing in a liquid environment [17], whereas in biofilms, bacteria exist as sessile aggregates encased in a self-produced matrix of Extracellular Polymeric Substances (EPS) attached to a biological or non-biological surface [18]. Biofilm cells vary

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drastically in their physiology, growth rate, and gene expression when compared to planktonic cells. Furthermore, it has become clear that bacteria frequently flux between planktonic and biofilm states, which is central to biofilm formation and development [19, 20].

2.1.1. Biofilm Formation and Development

Biofilm formation is a dynamic, multifaceted process triggered by environmental cues that prompt changes in gene expression. This results in a re-organization of the spatial and temporal arrangement of bacterial cells leading to a transition from the planktonic phenotype, to a biofilm [21]. Overall, biofilm formation and development has been well-defined, and can be simplified into 4 steps: i) reversible bacterial attachment; ii) irreversible bacterial attachment; iii) biofilm maturation and iv) biofilm dispersal [21]. In brief, initial reversible attachment of free-floating planktonic cells to a host tissue surface is driven by environmental cues (*e.g.* pH, temperature, ions, nutrients, and gas/O_2 availability), forces of gravity, Brownian motion, and local environmental hydrodynamics [21-23]. Some motile bacteria may use appendages (*e.g.* pili and flagella) for migration [24, 25]. Upon irreversible attachment, EPS, consisting of polysaccharides, proteins, nucleic acids, and lipids are produced and this meshwork provides the scaffold for a complex, three-dimensional biofilm architecture [11, 26]. At this stage, initial micro-colonies begin to form [27]. Biofilm maturation can be induced by quorum sensing signals prompting phenotypic changes and genetic diversification. This process results in enhanced structural defense against biological, physical, and chemical stress, which assists nutrient acquisition by forming complex water/nutrient channels, and facilitates reproductive ability [28]. Dispersal of bacterial cells into the host environment is the final stage of the biofilm cycle which can occur passively through physical forces such as fluid shear or abrasion, or can be induced in response to various environmental conditions (*e.g.* pH, nutrient levels, and gas concentrations), highly regulated signal transduction pathways, and effectors. Dispersal is key for re-colonization and re-establishment of the biofilm at other sites [19, 29].

2.1.2. Biofilm Resistance to Immune Clearance and Antimicrobial Agents

It has been suggested that 99% of the world's bacteria exist in a biofilm state, highlighting an adaptive advantage of this phenotype [30]. Biofilms are highly resistant to both antimicrobial treatment and immune action [31]. Biofilm communities are ~10-1000 fold more resistant to antimicrobials when compared to their planktonic bacterial cell counterparts [32, 33]. Resistance displayed by biofilm communities is attributed to, but not limited to: i) changes in gene expression; ii) sharing of resistance genes *via* horizontal gene transfer or adaptive mutations; iii) active release of antibiotic-degrading enzymes; iv) three-dimensional structure; v) the physical EPS barrier (to both antibiotics/antimicrobials and immune cells) and vi) reduced metabolic activity among some bacterial cells found deep within the biofilm [34-36].

2.2. Evidence of GAS Biofilm Formation

GAS typically infects the skin and mucosal surface of the oropharynx. The earliest evidence for the presence of GAS biofilm *in vivo* was found in impetigo lesions, where glycocalyx encapsulated micro-colonies resembling early stage biofilm formation were detected *via* FITC-ConA staining and visualized by confocal laser scanning microscopy (CLSM) [37]. A more recent clinical case study described a previously healthy male presenting with a Necrotizing Soft Tissue Infection (NSTI) persisting over 24 days. The surgeon noted the presence of "thick layer biofilm" in the patient's fascia [16]. This observation prompted the team to further investigate the biofilm as a constituent of GAS NSTI. A multicenter study revealed that 32% of patient tissue biopsies (n=31) contained GAS biofilm. The biofilms found in these NSTIs exacerbated inflammation and led to severe tissue damage at the site of the NSTI infection; moreover, the presence of bacterial loads far exceeded those of wound biopsies lacking biofilm. Taken together, these studies highlight the need for further consideration of GAS biofilm as a complicating factor in NSTIs.

GAS enters the throat *via* the oral cavity and typically colonizes the oro-nasopharynx. Asymptomatic and persistent oropharyngeal GAS carriage has been attributed to biofilm formation, complicating, and contributing to oropharyngeal disease [38]. Although penicillin resistant GAS has not been reported, it has been hypothesized that GAS biofilm may be a contributing factor to the antibiotic treatment failure rate of 20-40% associated with GAS infection [7, 39]. A number of clinically relevant GAS serotypes (M2, M6, M14, and M18) have been found to form biofilm *in vitro* on both uncoated surfaces, and surfaces coated with human fibronectin, human fibrinogen, human collagen types I and IV, and human laminin [39]. In a study observing GAS pharyngitis and antibiotic treatment failure, all 99 GAS isolates collected from patients suffering from GAS pharyngitis displayed biofilm forming abilities to varying degrees [5]. The Minimum Biofilm Eradication Concentrations (MBECs) were overall higher than the Minimum Inhibitory Concentration (MIC) values for all GAS isolates, with resistance to penicillin demonstrated by 60% of the GAS isolates when in the biofilm phenotype. This study is one of the earliest to demonstrate a link between *in vitro* GAS biofilm formation and penicillin insensitivity, highlighting the protective advantages offered by the biofilm phenotype against antibiotics like penicillin [5].

Another study investigated 289 differing clinical GAS isolates from carriers, pharyngitis cases, and invasive/non-invasive infections in an attempt to understand the link between clinical source, biofilm-forming ability, and antibiotic insensitivity [4]. Although 90% were found to be biofilm forming *in vitro*, some *emm* types displayed a greater propensity for biofilm formation than others, this was especially apparent among *emm* 6 strains when assessed for the biofilm biomass. However, intra-strain variability within an M-type suggested that the biofilm formation was strain specific rather than an overall characteristic offered by the serotype. Moreover, this study found that GAS strains which were otherwise macrolide sensitive utilized the biofilm phenotype to resist β-lactam treatment [4]. A more recent study explored biofilm formation by 15 differing GAS *emm* types frequently isolated from patients suffering from recurrent GAS pharyngitis [8]. Findings indicated that *emm* types 1, 12 and 28 were most abundant; however, the *emm 6* strains

produced significantly more biofilm. These GAS biofilms were 10 times more resistant to penicillin, erythromycin, and clindamycin used individually or in combination when compared to MICs generated for their planktonic cell counterparts.

An *in vivo* study by Roberts*, et al.* [15] revealed GAS residing within the tonsillar crypts of sufferers of recurrent GAS tonsillopharyngitis using fluorescence microscopy and Scanning Electron Microscopy (SEM). The group also observed cocci chains typical of GAS arranged in three-dimensional communities resemblant of biofilm in an *ex vivo* pig epithelium model [15].

Overall, it is apparent that both the skin and the throat are able to cultivate GAS biofilms from the earlier stages of micro-colony formation, to the more mature biofilm communities. Moreover, these findings highlight the clinical relevance of GAS biofilms and their potential role in antibiotic treatment failure in recurrent GAS infections. Taken together, this reinforces the need for further investigation into GAS biofilm formation *in vitro*, *in vivo* and *ex vivo*.

2.2.1. Virulence Factors Involved in GAS Biofilm Formation

GAS expresses multiple virulence factors involved in adherence to host tissue surfaces. GAS virulence factor expression is variable between individual strains, and differs between GAS serotypes, with expression and regulation impacted by both host and environmental stimuli [2, 40]. Several GAS virulence factors have been implicated in various stages of biofilm phenotype development (Table **1** and Fig. **1**).

2.2.1.1. M Protein Family and Lipoteichoic Acid

The M protein is highly expressed on the surface of GAS, and numerous studies have demonstrated a role for M protein in adherence, as it facilitates the attachment of GAS to host epithelial cells [41-43]. It has also been proposed that the M protein mediates initial cell-surface interactions during biofilm formation. Cho and Caparon [44] demonstrated this using an isogenic M protein deficient GAS HSC5 (*emm* 14) mutant grown on abiotic polystyrene surfaces under static conditions. The mutant displayed a decrease in biofilm biomass relative to wild-type by safranin staining. Courtney*, et al.* [45] also observed significantly reduced biofilm biomass relative to wild-type for isogenic M protein mutants of *emm* 1, 5, 6 and 24 isolates under similar growth conditions. A correlation has been shown between M protein expression and the levels of membrane-bound lipoteichoic acid (LTA), surface hydrophobicity, and ability to form biofilms for *emm* types 1, 5, 6 and 24. Isogenic M protein deficient GAS mutants displayed diminished bacterial hydrophobicity, decreased membrane-bound LTA, and decreased biofilm biomass relative to wild-type strains. It was suggested that complex interactions between M protein and LTA expose the ester fatty acids of LTA, increasing bacterial hydrophobicity, ultimately favoring LTA-host cell interactions [45]. Conversely, no significant reduction in biofilm biomass, hydrophobicity or membrane-bound LTA was observed for isogenic M2, M4 and M49 protein deficient GAS. Notably, a key difference between the strains displaying M-protein-LTA mediated adherence and those that did not, is their *emm* pattern classification. Specifically, *emm*1, 5, 6, and 24 strains all belong to the *emm* pattern A classification, which display a single M protein family member on their surface whilst the *emm* 2, 4 and 49 belong to the pattern D and E classification, which express other surface proteins of the M protein family, such as the M-related protein (*mrp*) and M-like protein (*enn*) on their surface. Thus, it appears that the M-protein-LTA interaction may be specific to strains expressing only one M protein family member. Courtney*, et al.* [45] also investigated if the Mrp and Enn proteins participated in LTA complex mediated biofilm formation and found a significant decrease in hydrophobicity, membrane-bound LTA, and biofilm formation relative to wild-type using an isogenic *mrp4* deficient GAS mutant of the *emm*4 expressing strain. However, an isogenic *enn*4 deficient GAS mutant was also examined and there was no difference in hydrophobicity, membrane-bound LTA, or biofilm formation compared to the wild type. The M protein has previously been shown to be expressed at significantly higher levels than Mrp and Enn in a CS101 GAS (*emm*49) strain [46]. Expression levels may play a part in the role of these proteins in LTA complex initiation and biofilm formation; however this has yet to be determined. Collectively, this research highlights the need to study GAS biofilm formation using strains from diverse genetic backgrounds.

2.2.1.2. Pili

Pili are long filamentous structures that exist on the surface of GAS, and numerous other bacterial species [58, 59]. Pili are encoded by the FCT (fibronectin-binding protein, collagen-binding protein, and trypsin-resistant antigen) genomic region of GAS. This is a highly variable 11kb pathogenicity island which contains genes for a pilus backbone protein, at least one matrix protein binding ancillary protein, sortases (SrtB/SrtC2), and a signal peptidase [1, 40, 60]. GAS can be classified into nine FCT subtypes based on the diversity of gene content and nucleotide sequence [47, 61, 62]. Pili are considered a major adhesin of GAS, as their involvement in the adherence of GAS to human tonsillar tissue, keratinocytes, lung, and throat epithelial cells is well characterized [63-65]. Numerous studies have demonstrated that pili play an integral part in GAS biofilm formation and have shown associations between several different FCT types and the capacity of GAS to form bacterial biofilms *in vitro* [1, 8, 47, 58, 60].

Manetti*, et al.* [1] confirmed that GAS FCT-2 pili are essential for efficient attachment of the GAS M1 strain SF370 to human epithelial cells, as GAS pilus negative mutants, constructed by either deletion of the pilus backbone structural protein (Δspy0128) or the sortase C1 (Δspy0129) gene which are essential for pili assembly, were unable to attach to epithelial cells. The same mutants did not efficiently aggregate in liquid culture and did not form sufficient biofilms relative to the wildtype strain on polystyrene as determined by crystal violet biomass staining, or epithelial cells as observed by confocal microscopy [1]. Visualization of the wildtype, Δspy0128 and Δspy0129 strains demonstrated that the EPS was virtually absent for the mutant strains. This dramatically contrasted to the wild-type GAS SF370 strain and the complementation mutant for which the biofilm phenotype was restored. Together, these results demonstrate that FCT-2 pili are important in the transition to the biofilm

Table 1. Summary of GAS biofilm studies assessing the effect of different virulence factors and growth conditions on biofilm formation. The following molecules have been shown to contribute to GAS biofilm formation (BF): Lipoteichoic acid (LTA) and M protein (*emm*), M-related protein (*mrp*), and M-like protein (*enn*) complexes, pili, *Streptococcal* collagen-like protein 1 (*scl-1*), short hydrophobic peptides (SHP 1 and 2), the hyaluronic acid (HA) capsule, Group A *Streptococcus* protein A (*aspA*), *Streptococcal* regulator of virulence (*srv*) and cysteine protease (*speB*), and *Streptococcal* invasion locus peptide (*silC*).

Virulence Factor	Mutation	Genetic Background		Biofilm Model	Growth Substratum	References
		Role in BF	No Role in BF			
M protein-LTA complex	Δ *emm*	*emm*14	-	Flow model (34 µm s-1) Static model	Glass coverslips Uncoated polystyrene	[44]
	Δ *emm*	*emm*1 *emm*5 *emm*6 *emm*24	*emm*2 *emm*4	Static model	Uncoated polystyrene	[45]
MRP-LTA complex	Δ *mrp*	*emm*4 *emm*49	*emm*2	Static model	Uncoated polystyrene	[45]
ENN-LTA complex	Δ *enn*	-	*emm*2 *emm*4	Static model	Uncoated polystyrene	[45]
Pili	Δ *spy0128* (pili backbone) Δ *spy0129* (C1 sortase)	*emm*1	-	Static model	Polylysine-coated glass coverslips	[1]
	Δ *tee6* (pilus backbone) Δ *fctX* (ancillary protein) Δ *srtA* (sortase) Δ *srtB* (sortase)	*emm*6	- -	Static model	Glass coverslips Uncoated polystyrene	[47]
	Δ *ALP-1*(ancillary protein 1)	*emm*6	-	Static model	Uncoated polystyrene	[48]
Scl-1	Δ *scl-1*	*emm*1 *emm*28 *emm*41	-	Static model	Glass cover slips	[49]
	Δ *scl-1*	*emm*3	-	Static model	Polystyrene coated with fibronectin and laminin	[50]
HA capsule	Δ *HasA (*Hyaluronan synthase)	*emm*14	-	Static model	Uncoated polystyrene	[44]
	Δ *CovS* (sensor kinase)	*emm*18	-	Static model	Uncoated + fibronectin/collagen coated polystyrene	[51]
AspA	Δ *aspA*	*emm*28	-	Static model	Saliva-coated coverslips	[52]
Srv and SpeB	Δ *srv*	*emm*1	-	Flow model (0.7 ml/min)	Polystyrene chamber	[53]
SilC	Δ *silC*	*emm*14 *emm*18	- -	Flow model (1.2 ml/min) Static model	Fibronectin/ collagen IV coated plastic coverslips Uncoated, fibronectin, fibrinogen, laminin, collagen I and IV coated polystyrene	[39]

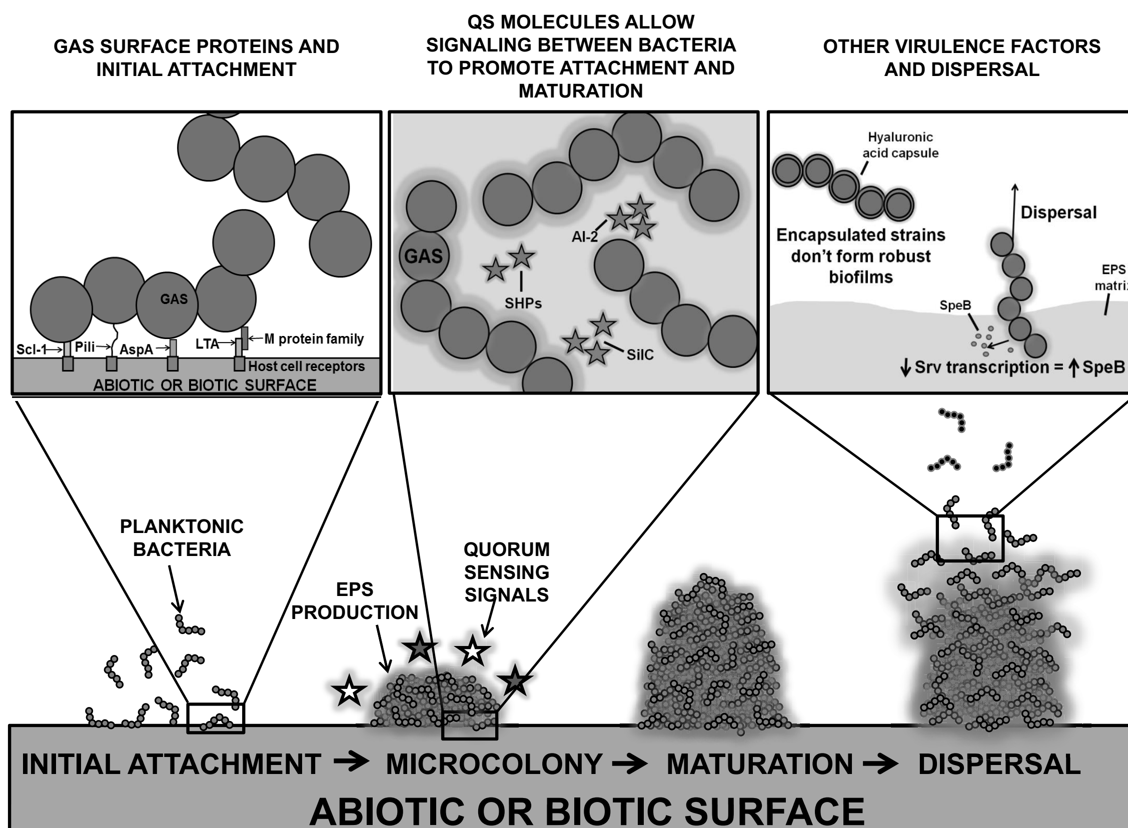

Fig. (1). Role of GAS virulence factors in biofilm formation. Many bacterial surface molecules aid in the initial attachment of planktonic GAS to abiotic/biotic surfaces. This includes *Streptococcal* collagen-like protein (Scl-1), pili, Group A *Streptococcus* protein A (AspA), as well as members of the M protein family such as the M protein and M-related protein, which form complexes with LTA to facilitate adherence [1, 44, 45, 47, 48, 50, 52, 54]. Communication between bacteria may utilize quorum sensing (QS) systems, secreting QS molecules such as *Streptococcal* invasion locus protein (SilC), Short Hydrophobic Peptides (SHP) and Autoinducer-2 (Al-2) of the *Streptococcal* invasion locus (Sil), Regulatory gene of glucosyltransferase (Rgg), and LuxS/autoinducer-2 QS systems, respectively to facilitate adherence and maturation of biofilms which includes the production of the extracellular polymeric matrix (EPS) [39, 55-57]. Studies have also demonstrated strains that are encapsulated by hyaluronic acid do not form biofilm as readily as un-encapsulated strains, and it has been suggested that the hyaluronic acid capsule decreases biofilm forming capacity [9, 44, 51]. Decreased transcription of the *Streptococcal* regulator of virulence has also been shown to increase production of SpeB, a cysteine protease which may play a role in biofilm dispersal [53].

phenotype for this strain. Following these findings, Köller*, et al.* [60] assayed biofilm formation under an array of different growth conditions for 183 isolates that were *emm* and FCT-typed in an effort to demonstrate novel correlations between FCT-type and biofilm formation. Whilst novel associations between FCT-type and biofilm formation were demonstrated for multiple isolates, the study did not support a direct link between biofilm formation and FCT-type. This is most likely because there are many other virulence factors that play a role in biofilm formation by GAS [60].

There is also data to suggest that the role of individual FCT types in biofilm formation may be dependent on environmental conditions during infection. In a study of clinical isolates obtained from the University Hospital in Rostock, Germany, between 2001 and 2006, an association was observed between environmental conditions (acidity) and biofilm formation for different FCT-types [66]. Biofilm formation on abiotic surfaces and micro-colony formation on epithelial cells for FCT-types 2, 3, 5 and 6, and a subset of FCT-4 strains increased as a result of acidification caused by fermentative sugar metabolism. The subsequent decrease in environmental pH was associated with an enhanced expression of the pilus components and transcriptional regulators including the RofA-Like Protein (RALP) regulator family proteins RofA and Nra, and the AraC/XylS type transcriptional regulator family protein, MsmR. Manetti*, et al.* [66] speculated that a decreased pH at the cell surface due to sugar metabolism may favor biofilm formation during colonization; however, this requires further investigation, particularly to determine if pH plays a role in biofilm formation in the oropharynx [66].

As an extension of the study by Köller*, et al.* [60] that demonstrated that *emm* type 6 strains with the FCT-1 type pili form high levels of biofilm *in vitro*, Kimura*, et al.* [47] characterized the role of FCT-1 pili in the GAS strain TW3558 (*emm*6). In-frame deletions of the pilus backbone (*tee6*), ancillary protein (*fctX),* and sortases *(srtB and srtA)* were used to produce isogenic pili deficient GAS mutants. Use of the mutants in biofilm growth assays demonstrated that deletion of the *tee6* gene compromised the ability of the strain to form a biofilm on an abiotic surface, with deletion of *fctX* and *srtB* genes (pilus ancillary protein and class C

pilus-associated sortase, respectively) further decreasing the capacity for biofilm formation. It was also noted that assembly of the pili, rather than individual pili components, is required for biofilm formation. The FCT-1 pilus region (*fctX* operon) was then ectopically expressed in M1 strain SF370, and an increase in biofilm formation was observed, substantiating the role of FCT-1 pili in biofilm formation. A similar study was conducted by Becherelli, *et al.* [48], whereby isogenic mutants of ancillary protein 1 (ALP-1) of FCT-1 type GAS were examined for biofilm formation. Wild-type bacteria produced substantial biofilms, whilst the mutant strain exhibited impaired biofilm forming capacity [48]. Taken together, the data from Becherelli, *et al.* [48] and Kimura, *et al.* [47] suggests both structural, and assembly components of pili are important in adherence and biofilm formation of FCT-1 type GAS. However, the authors highlight that the role of pili should not be considered in isolation, as GAS biofilm formation is likely to be dependent on multiple factors [47].

2.2.1.3. Streptococcal Collagen-like Protein

Streptococcal collagen-like protein-1 (Scl-1) is expressed on the surface of GAS as a homotrimeric protein consisting of a N-terminal variable region, a collagen-like region containing Gly-X-Y repeats, and a cell-wall-anchored C-terminal region which contains a linker region connecting the collagen-like region to the cell wall and membrane associated portions of the protein [54, 67, 68]. Transcription of Scl-1 is positively regulated by the multiple gene activator regulon, suggesting it may be co-expressed with many other GAS virulence factors such as the M protein family [49]. Scl-1 is thought to be expressed by all strains of GAS and is regarded as a major cell surface adhesin [67, 68]. It is also recognized as a contributor to the biofilm forming capacity of GAS on abiotic surfaces. Isogenic *scl1* mutants have been shown to have a significantly reduced capacity for adhesion and biofilm development, as well as variation in surface morphology and EPS production [49]. In this study, the expression of the Scl-1 protein in a heterologous *Lactococcus lactis* system facilitated biofilm formation, further implicating this protein in biofilm production. A study by Bachert, *et al.* [50] assaying biofilm formation in M3 GAS, supported these earlier findings. They identified 47 M3 GAS strains containing a nonsense mutation in the 11th Gly-X-Y repeat of the collagenous region. Biofilms of the M3 strains (MGAS315 and MGAS10870) grown on abiotic surfaces coated with fibronectin and laminin exhibited a significantly reduced capacity to form biofilm. Moreover, the M3 GAS mutant was not able to form micro-colonies when introduced into a skin infection model. When the nonsense mutation was repaired in two M3 strains (MGAS315 and MGAS10870) through homologous complementation, restoration of expression increased biofilm formation upon fibronectin and laminin coated surfaces [50]. The nonsense mutation within the 11th Gly-X-Y repeat of the collagenous region had previously been shown to be present in 98.7% of 479 sequenced invasive M3 isolates [69]. Another study by Lukomski, *et al.* [67] also demonstrated this polymorphism in five M3 strains, but not in any of the other 20 M-types tested. Bachert, *et al.* [50] therefore suggested this mutation is highly conserved and unique to M3 isolates and that Scl-1 promotes stable biofilm formation for M3 GAS.

2.2.1.4. Hyaluronic Acid Capsule

The Hyaluronic Acid (HA) capsule, a major virulence factor of GAS, is highly conserved and surface-exposed, and made from a polymer of repeating units of glucuronic and N-acetylglucosamine [70]. Some studies suggest that HA is an adhesin that aids in the attachment of GAS strains to host cells [71, 72]. Conversely, it has been reported that HA capsule can decrease *Streptococcal* adherence by preventing the surface-exposed adhesins from attaching to host cell receptors [73]. The capsule is also central to immune evasion upon colonization of host tissues, and generally, encapsulated strains display a greater propensity for virulence than those with a reduced/absent capsule [74]. Investigations into the role of capsule in GAS biofilm formation have implicated HA in biofilm maturation. When Cho and Caparon [44] assessed the biofilm forming capacity of a wild-type HSC5 GAS strain and an isogenic capsule deficient mutant whereby the Hyaluronate synthase (HasA) gene was abolished, they found biofilm forming ability of the mutant was not affected under static conditions. This suggests HA does not affect the initial bacterial attachment that is required for subsequent biofilm formation. To illustrate the role of HA in later stages of biofilm maturation, flow chambers were utilized, and although the mutant was able to adhere to the surface of the chamber it was unable to propagate the biofilm phenotype, and cells seemed to appear dispersed across the substratum evenly, suggesting that HA has a role in biofilm maturation [44]. A more recent study observed a decrease in HasA transcription in biofilms of MGAS315 (*emm*3) grown on live keratinocytes (SCC13) in comparison to planktonic bacteria. Marks, *et al.* [9] suggested other factors must affect the role of HA in biofilm formation. Indirect evidence that the amount of capsule inhibits GAS biofilm formation was reported by Sugareva, *et al.* [51]. CovS is a sensor kinase involved in regulation of hyaluronic acid synthesis. Deletion of *covS* was shown to lead to increased capsule production but lower biofilm biomass for *emm*2, 6, 18, and 49 [9]. Furthermore, Sugareva, *et al.* [51] suggested strains with less HA formed biofilm more readily. It could therefore be suggested that the reduction in capsule production upon change from planktonic to biofilm phenotypes may facilitate biofilm maturation. Overall, the role of HA in GAS biofilm has yet to be fully elucidated.

2.2.1.5. Regulators of Gene Expression

Most oral streptococci express *Streptococcal* antigen I/II (Agl I/II) family polypeptides, which have demonstrated roles in adhesion to human salivary glycoproteins, other microbial cells, and calcium to facilitate colonization in the oral cavity [52, 75]. An Agl I/II polypeptide produced by M28 GAS called Group A *Streptococcus* protein A (AspA) has been shown to have a role in biofilm formation in two independently isolated M28 serotypes [52]. Deletion of the *aspA* gene in these strains abolished their ability to propagate the biofilm phenotype on saliva-coated surfaces. The biofilm developing capacity of the strain was reinstated upon trans-complementation of the *aspA* deletion. Additionally, expression of the AspA protein in *L. lactis* allowed for biofilm development in this species, relative to wild-type *L. lactis*. Whilst AspA is a relatively understudied GAS virulence factor, these results indicate AspA plays a role in adhesion and biofilm propagation in GAS.

The *Streptococcal* regulator of virulence (Srv) is a transcriptional regulator required for GAS virulence, as it controls the transcription of many extracellular proteins [53, 76]. There is also some evidence indicating participation of this virulence factor in biofilm formation. An isogenic *srv* deficient GAS strain (MGAS5005) and its complementation mutant were used to demonstrate that Srv-mediated transcription is an important contributor to biofilm formation [77]. A decreased capacity to form biofilm was observed for the *srv* deficient strain which was restored to levels comparable to the wild-type strain following complementation. It was suggested that Srv may control the transcription of genes necessary for adherence and micro-colony formation. One of the extracellular proteases controlled by Srv is the cysteine protease SpeB [77]. In the absence of *srv*, overproduction of SpeB is associated with decreased biofilm formation [77]. Complementation decreased SpeB production and restored biofilm formation comparably to the wild-type. A subcutaneous model of skin infection in mice that were infected with the *srv* deficient strain (MGAS5005) supported previous findings as it demonstrated increased detection of SpeB, decreased detection of biofilm, and overall increased virulence relative to wild-type GAS. Furthermore, the model demonstrated that inactivation of SpeB in the *srv* deficient strain was subsequently shown to reduce GAS virulence relative to wild-type and promote biofilm formation [75]. Doern*, et al.* [76] and Connolly*, et al.* [75] both proposed a model for the Srv-mediated biofilm dispersal, suggesting SpeB may degrade the components required for biofilm formation such as the extracellular DNase (Sda1). Taken together these data demonstrate a potential mechanism that GAS may utilize to transition from mild to severe infections. Whilst this model is preliminary, further investigations into the downstream effects of the *srv* mutation on the expression of other extracellular virulence factors such as the *Streptococcal* inhibitor of complement (Sic) and its effect on GAS biofilm formation and virulence could provide new insights into the role of biofilm dispersal in invasive GAS infection [77].

2.2.2. Quorum Sensing

Quorum Sensing (QS), a hallmark feature of many bacterial biofilms, has also been noted for GAS biofilms. Three GAS quorum sensing systems have been described: *Streptococcal* invasion locus (Sil), regulatory gene of glucosyltransferase (Rgg), and LuxS/autoinducer-2.

Sil has been linked to regulating genes involved in invasive disease [56]. SilC is a signaling peptide of Sil, and has been linked to the ability of GAS to spread in soft tissues [78]. Importantly, SilC has been shown to have a role in QS for GAS biofilm formation [39, 57]. Using safranin-staining, an isogenic *emm*14 *silC* mutant showed reduced adherence to surfaces coated with fibronectin, fibrinogen, and polystyrene surfaces, relative to the wild-type strain, but this was not statistically significant [39]. An isogenic *emm*18 *silC* mutant exhibited reduced adherence to collagen type I and IV substrates, relative to the wild-type strain, but this was also not statistically significant. SEM analysis of the *emm*14 and *emm*18 *silC* mutants revealed phenotypic changes in the strain relative to the wild-type. The *emm*14, *silC* mutant biofilm surface displayed more clefts than the wild-type analogue whilst the *emm*18 *silC* mutant displayed a thinner biofilm with a patchy appearance, whilst its wild-type comparator formed a solid and thick biofilm [39, 79].

Conversely, in a study by Thenmozhi*, et al.* [57], it was suggested that strains from different serotypes form biofilms regardless of the presence of the *sil*C gene. Crystal violet staining of biofilms grown from strains of 11 different serotypes distinguished M56, M65, M74, M89, M100, and st38 as biofilm formers and M49, M63, M88.3, M122, and st2147 as non-biofilm formers. These biofilms were grown under static conditions on uncoated polystyrene. These strains were screened for the presence of *sil*C which present in only M100, M74, and st38 from the biofilm formers and the non-biofilm former M122. Serotypes lacking *silC* were the most proficient biofilm producers under these conditions suggesting that the involvement of SilC in QS is not required for biofilm formation in all strains.

The Rgg QS system is one of the most conserved systems among Firmicutes, universally existing among all species of *Streptococcus*. Within this large family of regulatory proteins, many paralogs exist. For GAS, biofilm formation, biofilm development, and virulence is controlled by the Rgg2/Rgg3 system in response to the short hydrophobic peptides SHP2 and SHP3 [80]. The effects of SHP have been witnessed in GAS biofilm development, with potentiation of surface-associated biofilms forming in concentrations of synthetic SHP pheromone as low as 5 nM [55]. Rgg-SHP-mediated QS has also been hypothesized to promote inter-species signaling between common residents of the nasopharynx; *S. pneumoniae*, *S. dysgalactiae*, and GAS [80]. Interspecies signaling utilizing the orthologous Rgg/SHP systems has been further investigated and shown to be bidirectional between GAS, Group B *Streptococcus*, and *Streptococcus dysgalactiae* subsp. *Equisimilis.* However, the role of this particular bidirectional communication using QS systems in multispecies biofilm formation and virulence warrants further analysis [81].

Lastly, Autoinducer-2 synthesized by LuxS, an enzyme expressed by several biofilm-forming bacteria, including GAS, is necessary for biofilm formation [56]. Although this QS system has yet to be thoroughly investigated, it is thought to control some virulence mechanisms (*e.g.* hemolytic and proteolytic activity), and importantly *emm* gene expression and SpeB production, which have both been shown to affect biofilm formation and development [82-85].

2.2.3. Modelling GAS Biofilms

Current knowledge of biofilm formation, basic hallmark characteristics, physiology, and antimicrobial resistance has come primarily from *in vitro* biofilm modelling. *In vitro* models aim to depict conditions similar to those *in vivo* considering variables such as growth rate, flow rate, nutrient availability, gas concentrations, and substratum. The simplest and earliest biofilm model was liquid growth media inoculated with bacteria left to colonize a solid surface [86]. However, higher throughput models have since been developed such as multi-well systems using various abiotic growth surfaces (glass, plastic, silicone, and polystyrene). Although these are cost-effective and somewhat easy to implement, the biofilms formed are limited in their ability to truly mimic those in an *in vivo* setting [9]. Moreover, while *in vitro* GAS biofilms have provided important insight into GAS biofilm formation, GAS colonization and infection *in vivo* is a complex process involving multiple interactions

with the host. Such interactions initiate and alter GAS gene expression in a way that is difficult to mimic in most *in vitro* models. This was demonstrated in a study showing that a lack of host factors resulted in highly differential virulence gene expression between biofilms produced *in vitro* to those present *in vivo* [44]. The study highlights the importance of mimicking the host environment as closely as possible. Alternative approaches to biofilm formation include the use of epithelial cells as the substratum for GAS biofilm growth to compensate for host factor presence. However, the toxicity of broth grown GAS to cultured eukaryotic cells renders long term biofilm-epithelial modelling difficult [87]. A more recent study that utilized both live and prefixed epithelial substrata successfully integrated aspects of the necessary environment, closely mirroring *in vivo* GAS colonization [9]. Specifically, GAS was able to form micro-colonies resemblant of biofilms *in vivo*, as opposed to dense biofilm sheets typical of biofilms grown on abiotic surfaces. Additionally, biofilms were non-toxic to the live human keratinocyte substratum. Overall, this study confirmed that an epithelial substratum potentiates biofilm formation, with bacterial morphology resemblant of *in vivo* GAS biofilms, a result not achievable for GAS biofilms grown on abiotic surfaces like glass or plastic. Another *in vitro* study investigated the ability of 2 dominating *emm*-types involved with NSTI, (*emm* 1 strain 8157, and *emm* 3 strains 5626 and 8003) to form biofilm using standard polystyrene and glass surfaces [16]. Both *emm* 3 strains formed biofilm on uncoated and fibronectin-coated polystyrene surfaces. In contrast, only strain 5626 was able to form a biofilm on glass surfaces. The *emm* 1 strain 8157 did not form biofilm under the tested plate conditions. A 3D organotypic skin model was also generated using human keratinocyte cells (N/TERT-1) and normal human dermal fibroblasts (NHDF) to mimic key anatomical/functional features of the skin. This model had the advantage of dermal and epidermal layers being formed, and key epidermal structural proteins included. The *emm* 1 strain 8157 and *emm* 3 strains 5626 and 8003 were all able to initiate tissue infection in this model, and after 8 hours of incubation, the bacteria were found to predominate the stratum corneum. Upon further incubation, the bacteria were dispersed throughout the whole tissue, with bacterial aggregates that developed typical of biofilm upon immunostaining. Further investigation of these aggregates utilizing confocal laser scanning microscopy confirmed the presence of hallmark biofilm features of the EPS including; exopolysaccharides, lipids and extracellular DNA associated with the bacterial community [16]. At present, GAS existing in the biofilm phenotype is not recognized as a potential component of NSTIs. This comprehensive study supports the need for further investigation into the role biofilms play in GAS NSTIs, and by extension, other GAS associated skin infections. Future studies using similar epithelial-GAS biofilm models are necessary, with a focus on establishing the interaction between GAS and the local resident microbiome to provide a more accurate understanding of the underlying mechanisms in which GAS may adhere, colonize and persist in the host.

2.3. The Oropharyngeal Microbiome

Over 700 different bacterial species have been recovered from the microbiome of the oral cavity and contiguous regions of the tonsils, pharynx, and oesophagus which cumulatively play an important role in human health and disease [88]. As found by Lemon, *et al.* [89], the phylum-level composition of bacterial residents of the oropharynx is distinct from the nasal cavity. Moreover, differences in relative abundance profiles of phyla vary between the pharynx and saliva. These differences highlight how each niche environment, although quite interconnected, supports discrete microbial populations [90].

Commonly found species that predominate this niche are from the genera *Prevotella*, *Capnocytophaga*, *Campylobacter*, *Veillonella*, *Streptococcus*, *Neisseria, Rothia, Actinoomyces,* and *Haemophilus* [90, 91]. This site is also colonized by important human pathogens such as GAS, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Moraxella catarrhalis* [92, 93]. However, due to the limited number of studies, much remains unknown about the interspecies interactions that occur in this rich, polymicrobial ecosystem.

2.3.1. Polymicrobial Biofilms of the Oropharynx

Currently, very little is known regarding the interactions between species of the oropharyngeal microbiome, and the opportunistic bacterial pathogen GAS [38, 94]. It is possible that the overall biofilm architecture/organization and relative species abundance within these polymicrobial communities may alter in a strain-specific manner [95-97]. Additionally, the polymicrobial biofilm may offer GAS numerous survival and competitive advantages such as; metabolic cooperation, complex QS systems, an increased gene pool, efficient DNA sharing, by-product sharing, cross-protection, protective advantages to host immunity and antimicrobials, alongside numerous other synergies [98]. This is especially important when we consider the antibiotic treatment failure rate, and persistence/recurrence of GAS pharyngitis. An example of this is the co-operation between GAS and co-pathogenic organisms such as *Haemophilus parainfluenzae*, *S. aureus*, and *Moraxella catarrhalis*. It has been theorized that these bacteria collectively contribute to antibiotic insensitivity through their combined β-lactamase production when in a polymicrobial biofilm [5]. Another study demonstrated that in a polymicrobial biofilm of *Streptococcus oralis* and GAS, *S. oralis* was the initial colonizer and formed the bottom layer of the biofilm, while the GAS M49 strain, which was otherwise a poor biofilm former, was more able to form a biofilm on top. This study additionally found that when both *S. oralis* and *Streptococcus salivarius* formed the bottom biofilm layer, they collectively displayed a growth inhibitory effect on GAS *via* bacteriocin secretion [38]. Overall, efforts are needed toward designing models enabling the assessment of how pathogens like GAS interact with the local microbiome of the throat. Characterizing these interactions may provide a greater understanding of the GAS colonization process, and may also help to identify more targeted treatments or preventative strategies.

CONCLUSION

Whilst it is evident that there have been some advances in the understanding of GAS biofilms, it is clear that further characterization of the biofilm phenotype is needed in order to define its role in GAS pathogenesis. Specifically, further

study is needed to improve the current understanding of GAS as a mono-species biofilm, particularly with respect to the virulence factors involved, and their roles in the GAS biofilm phenotype. Improved models of GAS biofilm formation that closely mimic *in vivo* conditions will ensure that biofilms generated in the lab more accurately reflect those occurring during a clinical infection. Moreover, an improved understanding of the way in which GAS interacts, and forms biofilms with a broader array of bacterial species typical of the local host environment is also necessary. Taken together, this valuable information may provide better insight into GAS antibiotic treatment failure and the mechanisms utilized by GAS biofilms that may underpin recurrent and persistent GAS infections. In turn, such findings will serve as potential avenues for better treatment modalities and therapeutics.

AUTHORS PERSPECTIVE

Group A *Streptococcus* (GAS) is a Gram-positive human pathogen, known to cause an array of diseases ranging from mild, superficial infections, to more serious invasive infections, and numerous autoimmune sequelae. There is evidence to suggest that GAS forms biofilms both *in vitro* and *in vivo*. Current understanding of the biofilm phenotype in GAS pathogenesis and disease progression is limited and further characterization is needed. A greater understanding of GAS biofilms, may support the development of novel therapeutics and treatment strategies that specifically target GAS in the biofilm phenotype.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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