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Role of the fibronectin-binding, collagen-binding, T-antigen region during nasopharyngeal infection by Streptococcus pyogenes

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Abstract

Streptococcus pyogenes is a human-specific pathogen that uses a variety of adhesion molecules in order to initiate pharyngeal infection and maintain colonization. One genomic locus that is believed to contribute to adherence is the fibronectin-binding, collagen-binding, T-antigen (FCT) region, primarily due to the inclusion of genes that encode for a pilus (*tee18.1*), collagen-binding protein (*cpa*), and fibronectin-binding protein (*prtF2*). The precise role of these genes and the FCT region as a whole, however, have yet to be elucidated within the context of pharyngeal infection. In this study, we utilize the M18 serotype *S. pyogenes* strain MGAS8232 to demonstrate that the presence of the FCT region is critical in binding collagen type IV and fibronectin, and is additionally required for invasion into human pharyngeal cells, despite not impacting adherence to these cells. Furthermore, using a murine nasopharyngeal infection model, we determine that only *cpa* is expressed *in vivo*, and that removal of the FCT region does not impede the bacteria's ability to establish infection. Overall, this study demonstrates that the FCT region is not involved during acute nasopharyngeal infection by *S. pyogenes* MGAS8232 but may contribute to the pathogenic life cycle through other mechanisms.

Keywords

Streptococcus pyogenes, pilus, collagen-binding protein, fibronectin-binding protein, acute nasopharyngeal infection, transgenic mouse model

Lay Summary

Streptococcus pyogenes is a human-specific bacterium that can cause a multitude of diseases with outcomes that range from mild to fatal. Streptococcal pharyngitis, or more commonly known as 'strep throat', is one of these diseases and affects more than 600 million people annually. Although this disease can be self-limiting or treated with antibiotics, the persistence or recurrence of pharyngeal infection can result in more harmful complications, such as rheumatic heart disease, which can be fatal in severe cases. As such, it is critical to understand the initial infectious state of *S. pyogenes*. A crucial step during its pharyngeal infection is the adhesion to host tissue, which occurs through a variety of molecules that are often attached to the bacteria's surface. One particular genomic locus implicated in adhesion is the fibronectin-binding, collagen-binding, T-antigen (FCT) region, which notably encodes a pilus structure (*tee18.1*), a collagen-binding protein (*cpa*), and a fibronectin-binding protein (*prtF2*). In this study we remove this region from the bacteria's genome and assess the adherence capabilities of this mutant to several components found at the site of infection, including collagen type IV, fibronectin, and human pharyngeal cells and demonstrated that the FCT region is required to bind collagen type IV and fibronectin, but not the pharyngeal cells. Interestingly, we discovered that the ability to invade pharyngeal cells, which may lead to more detrimental diseases, is entirely reliant on the FCT region. In addition, we quantify the expression of *tee18.1*, *cpa*, and *prtF2* in mice that have been infected with *S. pyogenes* and determined that only *cpa* was expressed. Furthermore, using the same mouse nasopharyngeal infection model, we demonstrated that removal of the FCT region does not impede the bacteria's ability to establish infection. This study demonstrates that the FCT region is not involved during acute nasopharyngeal infection by *S. pyogenes* but may contribute to the pathogenic life cycle through other mechanisms. Overall, comprehensively evaluating the life cycle of *S. pyogenes* can provide insight on how to combat this globally-prevalent pathogen.

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Co-authorship Statement

Experiments and molecular cloning procedures completed throughout this study were performed by Akshay Sule with the aid of members in the McCormick lab.

Dr. Katherine Kasper and Jacklyn Hurst taught me how to perform the murine nasopharyngeal infection and assisted with my inoculations and dissections.

A portion of the HLA-DR4/DQ8 murine nasopharyngeal infection and gentamycin invasion assay were performed by Jacklyn Hurst.

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List of Abbreviations

°C	Degrees Celsius
$\times g$	Times gravity
α	Alpha
β	Beta
Δ	Delta/deletion
Ω	Ohm
μg	Microgram
μL	Microlitre
μm	Micrometer
ANOVA	Analysis of variance
ARF	Acute rheumatic fever
BHI	Brain heart infusion media
bp	Base pair
CD(n)	Cluster of differentiation (n)
cDNA	Complementary DNA
CFU	Colony forming unit
cNT	Complete nasal turbinate
CO_2	Carbon dioxide
Cov	Control of virulence
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECM	Extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Erm^R	Erythromycin resistance
FBP	Fibronectin-binding protein
FCT	Fibronectin-binding, collagen-binding, T-antigen
For	Forward
HBSS	Hank's balanced saline solution
HEp-2	Human epithelial type 2 cells
HLA	Human leukocyte antigen
IVIG	Intravenous immunoglobulins
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani broth
LTA	Lipoteichoic acid
Mbp	Megabase pair
MEM	Minimum essential medium
mg	Milligram
Mg^{2+}	Magnesium ion
Mga	multiple gene regulator of group A *Streptococcus*
MHC	Major histocompatibility complex
mL	Milliliter
mM	Millimolar

MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
nm	Nanometer
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	polyvinylidene difluoride
qRT	Quantitative reverse transcriptase
Rev	Reverse
RHD	Rheumatic heart disease
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Spe	Streptococcal pyrogenic exotoxin
STSS	Streptococcal toxic shock syndrome
TAE	Tris-acetate ethylenediaminetetraacetic acid buffer
TBS	Tris buffered saline
TBS-T	TBS with 0.1% (v/v) tween-20
TC	Tissue culture
THY	Todd Hewitt broth with 1% (w/v) yeast extract
TS Ori	Temperature-sensitive origin of replication
TSA	Trypticase soy agar
U	Units
V	Volts
v/v	Volume by volume
Vβ	Variable beta chain domain
w/v	Weight per volume

Chapter 1: Introduction

1.1. *Streptococcus pyogenes*

Streptococcus pyogenes, sometimes referred to as the Group A *Streptococcus*, is a Gram-positive, aerotolerant anaerobe with human-specific tropism. This bacterium typically grows in chains of varying lengths and exhibits the characteristic β-hemolysis of red blood cells. Classification of β-hemolytic streptococci is based on the expression of cell wall polysaccharides and subsequent categorization into Lancefield antigen groups A, B, C, D, etc., wherein *S. pyogenes* is the only species to be classified as a Group A *Streptococcus* (1). Isolates and strains of this species are further classified through examination of the variable (~20) Lancefield T-antigen, as well as the variable (>230) M protein (2). The *S. pyogenes* genome consists of a ~1.8 Mbp circular chromosome with a G+C content of ~38.5%. Many genes encoded within *S. pyogenes*, most notably virulence factors, display great variability between strains, which can be attributed to the inclusion of exogenous genetic elements such as phage, and phage-like insertions, as well as genetic mobility through horizontal gene transfer (3–5). Although *S. pyogenes* can colonize the skin and mucosal surfaces asymptomatically, it can also act as an opportunistic pathogen and cause a multitude of diseases with clinical manifestations ranging from mild to fatal (6, 7). Some of these diseases may be self-limiting such as pharyngitis or impetigo, while others can be more severe such as necrotising fasciitis or toxic shock syndrome. Additionally, *S. pyogenes* has been linked to various post-infection sequelae such as glomerulonephritis, or acute rheumatic fever (ARF) and the subsequent development of rheumatic heart disease (RHD) (8). Accordingly, *S. pyogenes* represents a significant affliction on human health.

1.2. Streptococcal infection and the global burden of disease

S. pyogenes, and the various diseases it can cause, are a major source of worldwide morbidity and mortality. Population studies have estimated that the annual incidence of acute pharyngitis and the superficial skin infection impetigo are at least 616 million and 140 million cases, respectively (9, 10). Symptoms of streptococcal pharyngitis include fever, enlarged lymph nodes, and inflammation of the pharynx with exudations on the tonsil (11, 12). Although the majority of *S. pyogenes* infections can be self-limiting or

treated efficiently with β-lactam antibiotics, the persistence of the bacteria, or invasion past the epithelial barrier, can result in more severe diseases (10, 12).

Invasive streptococcal disease is characterized by the bacteria entering a normally-sterile site, including the bloodstream, fascia, muscle, or lungs. Infection in these areas can then lead to potentially fatal diseases, including bacteremia, streptococcal toxic shock syndrome (STSS), or necrotising fasciitis. Unfortunately, epidemiological data suggests that the prevalence of invasive streptococcal disease is increasing (9, 10). STSS, which is the most dangerous form of invasive streptococcal disease, is believed to be mediated by *S. pyogenes*-produced toxins (13). Early symptoms of STSS are often non-specific but the progression of this disease is associated with early muscle pain or pharyngitis, followed by the onset of fever and hypotension, which may ultimately lead to multiple organ failure, and in ~40% of cases, death (14–16). Treatment of STSS primarily includes supportive therapy, antibiotic treatment with a focus on penicillin and clindamycin, and may include the use of polyclonal intravenous immunoglobulins (IVIG). In recent years, however, an increasing number of studies find that despite the reduction in organ failure and other clinical manifestations, the use of IVIG results in patient survival rates that are statistically no different than the control groups (17–19). Necrotizing fasciitis, otherwise known as "flesh-eating disease," is characterized by necrosis of the subcutaneous tissue and fascia, and presents with symptoms including severe pain, fever, and necrotic tissue with edema (20, 21). The rapid spread of necrotising fasciitis makes it crucial to remove the necrotic tissue and treat the affected area(s) with an antibiotic regimen similar to STSS (20).

Infection with *S. pyogenes* can also induce post-infection sequalae such as ARF or post-streptococcal glomerulonephritis. ARF is a delayed consequence that may occur due to the persistence or recurrence of untreated *S. pyogenes* pharyngeal infection, and can result in damage to the joints, brain, and heart (12, 22). The most substantial manifestation of ARF is repeated immune-mediated damage to the heart valves, which can progress into RHD (23). Globally, there have been estimated to be at least 18.1 million cases of severe *S. pyogenes* infection annually, with 517,000 of these cases resulting in death; the greatest

contributor to mortality is RHD, which results in 345,000 deaths annually, with an additional 116,000 deaths being caused by RHD-mediated complications (9, 10).

The precise incidence of diseases caused by *S. pyogenes*, however, is likely underrepresented given that the bacteria affects low-resource settings significantly more than high-income areas. Aside from the biological and geographical factors influencing prevalence, the burden of *S. pyogenes* can be greatly accredited to socioeconomic factors such as poor sanitation standards and lack of access to healthcare. Coupled with the absence of efficient data managing systems and epidemiological models, these circumstances obscure the actual statistical prevalence, incidence, and mortality of *S. pyogenes* infection in areas where the impact of disease is greatest. The global burden of this organism has caused the World Health Organization to categorize *S. pyogenes* as the ninth leading cause of human mortality with respect to infectious diseases, thus highlighting the importance of understanding the pathogenesis of this organism (10, 24).

1.3. Asymptomatic Carriage

The asymptomatic carriage of *S. pyogenes* is characterized by the presence of bacteria without any signs or symptoms indicative of infection. Indeed, the predominant form of interaction between *S. pyogenes* and humans is asymptomatic carriage, in which persistence in the pharynx is considered the primary reservoir for a variety of *S. pyogenes* strains (25). In addition, *S. pyogenes* is able to asymptomatically colonize the skin, vagina, and rectum, but does so to a lesser degree (26). Concerning asymptomatic carriage, detection of *S. pyogenes* is typically a two-step process that involves a posterior pharynx swab as well as serological testing (27). One meta-analysis found that 37% of children with sore throat presented signs of pharyngitis, and 12% of the children studied were deemed asymptomatic carriers (28). Additionally, it has been documented that approximately 25% of household cohabitants of children with streptococcal pharyngitis were positive for *S. pyogenes* while remaining asymptomatic, therefore suggesting that carriage is linked to living situations within a closed environment (29). Nevertheless, asymptomatic carriers of *S. pyogenes* do not seem to be capable of efficiently transmitting the bacteria. This may be due to several reasons, such as lack of respiratory secretions and symptoms that would

typically help the spread of bacteria, as well as the reduced density of *S. pyogenes* in the pharynx of carriers compared to symptomatic individuals (30, 31). Although it is predicted that host factors and bacterial determinants that assist immune evasion may prompt the development of asymptomatic carriage, the majority of studies detailing this highly common state are epidemiological in nature (27). As such, the exact molecular mechanisms that contribute to asymptomatic carriage of *S. pyogenes* remains unclear.

1.4. *Streptococcus pyogenes* secreted virulence factors

S. pyogenes employs a multitude of secreted virulence factors to mediate the start of infection as well as subsequent colonization of the host. This vast array of factors includes: DNAses, cytolytic toxins, proteases, bacteriocins, complement inhibitors, as well as mitogenic toxins known as superantigens. Although there are many more secreted virulence factors that contribute to the pathogenesis of *S. pyogenes* within its infectious niche, superantigens are of particular relevance to this project (32, 33).

1.4.1. Superantigens

Superantigens refer to a family of mitogenic toxins produced by a limited number of microorganisms. Most notably, these compounds are produced by *S. pyogenes* and *Staphylococcus aureus*, but are also expressed by other bacteria such as *Yersinia pseudotuberculosis* and *Mycoplasma arthritidis*, as well as viruses including the mouse mammary tumour virus (34–37). These unique toxins are characterized by their ability to bridge the connection between the human leukocyte antigen (HLA) major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and the variable-β (Vβ) chain of T cell receptors on T cells; peculiarly, superantigens can undergo this binding without a specific singular peptide (38). Depending on the particular sequence and architecture of each superantigen, it preferentially interacts with a subset of Vβ-specific T cells and certain MHC class II molecule haplotypes (39, 40). Because superantigens are able to circumvent the peptide-specific nature of immunity, they are able to activate up to 20% of a host's T cell population, which is in stark contrast to the 10^{-3}–10^{-4}% of naïve T cells that are activated during classical antigen-specific immunity (37, 40).

Historically, streptococcal superantigens have been implicated in numerous diseases including scarlet fever and STSS, yet their evolutionary function has yet to be solidified. Previous studies from our laboratory have postulated that superantigens are crucial for the initiation of infection by *S. pyogenes* (41, 42). In order to substantiate this concept, we developed a novel murine nasopharyngeal infection model that bypasses the human-specific tropism of *S. pyogenes* by utilizing mice that express HLA MHC class II molecules (alleles DR4/DQ8) (41, 43). Using this model, we verified that the streptococcal pyrogenic exotoxin A (SpeA) was necessary for the mice to consistently develop a robust acute infection (41). Moreover, we demonstrated that vaccination with a toxoid version of SpeA resulted in antibody-mediated protection thereby preventing *S. pyogenes* infection upon bacterial challenge (43). It therefore stands that the relationship SpeA has with the host extends beyond causing severe diseases, and instead may primarily function to initiate acute infection and persist in the nasopharynx. Additionally, because *S. pyogenes* encodes numerous superantigens, this role in pathogenesis may be preserved through redundancy.

1.5. *Streptococcus pyogenes* surface adherence molecules

In addition to the arsenal of secreted factors, *S. pyogenes* also utilizes a variety of surface-linked adhesion molecules in order to avoid bacterial clearance from host-derived mechanical, cellular, and humoral defenses. Some of these predicted factors include lipoteichoic acid (LTA), M protein, the hyaluronic acid capsule, pili, collagen-binding proteins, and fibronectin-binding proteins (FBPs) (44–47). Adhesion in *S. pyogenes* has been hypothesized to be a two-step process, in which LTA initially mediates weak hydrophobic interactions with host cells proceeded by high affinity interactions between specific *S. pyogenes* adhesion molecules and their cognate receptors (48). Interestingly, the expression of these adhesion factors differs dramatically between *S. pyogenes* strains, and is thought to be a significant contributor to stain-specific tissue tropism (25, 49).

1.5.1. M protein

The M protein, encoded by the *emm* gene, is a major surface component of *S. pyogenes*, and remains one of the most studied virulence factors from this bacterium. This protein is

attached to the bacterial cell wall through a conserved LPXTG motif at its C-terminus, while its N-terminus encodes a hypervariable region exposed to the extracellular environment (2, 50). The genetic variability in its N-terminus has resulted in more than 230 *emm* types, which serve as the basis for *S. pyogenes* serotype (2, 51, 52).

This multifunctional protein is primarily known for its function in antiphagocytic immune evasion. It accomplishes this by attaching to various host proteins to prevent the activation and deposition of complement factors on the bacterial surface (53–55). The molecular diversity within its hypervariable N-terminus also presents the opportunity to bind a plethora of human host cells and receptors (44, 54). Studies have demonstrated that M types may play a role in adhesion to various tissue types. For example M1, M5, and M6 serotypes have been shown to adhere HEp-2 cells and the pharyngeal cell line Detroit-562, but, along with M24 and M3, are not responsible for mediating adhesion to buccal epithelial cells (56–58). Moreover, M proteins from various serotypes were found to bind the keratinocyte membrane cofactor protein 46 (CD46); the M6, M12, and M18 proteins, however, displayed weak or no binding to CD46, indicating that this protein is not a universal target of the M protein (59, 60). Therefore, the diverse binding capabilities of different M proteins constitute the potential for a range of serotype-specific host-pathogen interactions.

The M protein has also been potentially implicated in tissue tropism and disease manifestations (61, 62). M proteins are grouped together based on their architecture, as well as their inclusion or exclusion of certain domains. *emm* groups A-C are overwhelmingly obtained from pharyngeal isolates, whereas group D are strongly associated with skin infections. Dissimilarly, strains that are included within group E are considered generalists, and have been obtained from both pharyngeal and skin isolates (45, 62–64).

1.5.2. Hyaluronic acid capsule

The majority of *S. pyogenes* strains produce a high molecular weight polysaccharide capsule composed of hyaluronic acid. Notably, hyaluronic acid is a major component of the human extracellular matrix (ECM), thereby making the capsule immunogenically inert

(7, 65). Although the precise function of the capsule is still debated, multiple studies have implicated a role in adhesion through its interaction with the host ligand CD44, which is involved in adherence to pharyngeal tissue by host cells (66–68).

Of importance, the regulation of the hyaluronic acid capsule seems to be heavily dependant on the nature of infection. For instance, it has been demonstrated in baboons that 1 hour post-inoculation in the pharynx, capsule gene expression is upregulated, suggesting a role during the early stages of infection (69). Moreover, the hyperencapsulated M18 serotype has been shown to persist longer in the nasopharynx of mice compared to a mutant strain that had reduced capsular expression (70). Contrarily, the hyperencapsulation of the virulent M1T1 strain is hypothesized to diminish adhesion to human pharyngeal cells due to its long hyaluronic acid chains physically obstructing other adhesion molecules and their access to host ligands. (71). By producing a molecule identical to a compound found in its host, *S. pyogenes* is able to utilise a possible infection or colonization factor while simultaneously evading the host immune response.

1.5.3. The fibronectin-binding, collagen-binding, T-antigen region

In addition to standalone components that mediate adhesion, *S. pyogenes* also encodes a variable genomic locus, known as the Fibronectin-binding, Collagen-binding, T-antigen (FCT) region. Due to the gene composition of this locus, it is hypothesized to play an important role in the adhesion of *S. pyogenes* to ECM proteins and various epithelial cells. To date, there have been 9 distinct variants of the FCT region identified amongst the many *S. pyogenes* strains, with further strain-specific sequence variation also reported within the encoded genes (49, 72). Although the origin of this ~9–16 kb locus is presently unknown, it is believed to have evolved its genetic organization and allelic variability through intergenomic homologous recombination events, as well as potential transposase activity (72–74). Flanked by two highly conserved genes, the general constituents of this locus are detailed in **Table 1**.

Table 1. Genes encoded within the different FCT region variants

Gene	Description	Presence of gene in FCT region variants [a]
rofA or *nra*	Standalone regulator	All FCT variants
prtF1	Fibronectin-binding protein F1	FCT-1, FCT-4, FCT-5, FCT-7, FCT-8, FCT-9
cpa/fctX	Ancillary pilus protein 1/ collagen-binding protein	FCT-1, FCT-2, FCT-3, FCT-4, FCT-5, FCT-6, FCT-8
lepA/sipA1/sipA2	Putative signal peptidase	FCT-2, FCT-3, FCT-4, FCT-7, FCT-8
tee/*fctA*	Major pilus subunit/T-antigen	All FCT variants
srtB/*srtC2*	Locus-specific sortase	All FCT variants
fctB	Ancillary pilus protein 2/ pilus anchoring subunit	FCT-2, FCT-3, FCT-4, FCT-5, FCT-6, FCT-7, FCT-8, FCT-9
msmR	Standalone regulator; only present in strains with *prtF2*	FCT-3, FCT-4, FCT-7, FCT-8
prtF2	Fibronectin-binding protein F2	FCT-3, FCT-4, FCT-5, FCT-7, FCT-8
inv/*rev*	Putative transposable element	FCT-1, FCT-2, FCT-5, FCT-9

[a] The presence of certain constituents within the 9 distinct FCT variants was organized in this table based on information from various sources (49, 75–79).

1.5.3.1. Pili

The most notable component of the FCT region is the pilus, which is a polymer composed of a major pilus subunit, as well as one or two ancillary proteins. Specifically, ancillary protein 1 is a collagen-binding protein that is usually attached to the pilus tip, and ancillary protein 2 anchors the pilus to the cell wall (80, 81). Interestingly, in some cases, the major pilus subunits are the T-antigens used in serological classification implying that distinct FCT regions encode antigenically distinct pili (47, 82, 83). These subunits are comprised of two immunoglobulin-like domains, which attach to adjacent subunits via a covalent isopeptide bond (84). After numerous rounds of head-to-tail polymerization, the ~2nm wide and >1µm long pilus is anchored to the cell wall via the highly conserved ancillary protein 2 (47, 84). Biochemical and structural analysis of the pilus led to speculation that disruption of the isopeptide bond would substantially compromise the integrity of the pilus. Indeed, the loss of one isopeptide bond causes a weakened ability to withstand stressors such as thermal stability or trypsin-resistance, and this pattern is even more prominent in mutants devoid of both isopeptide bonds (85). Other studies have since supported the stability-conferring properties of the isopeptide bonds and illustrated that its rigid and inextensible nature allows the pilus to withstand mechanical pressures such as coughing (85, 86). This works through the constant breaking of the isopeptide bonds due to mechanical pressure followed immediately by the automatic restoration of the bonds such that the majority of the energy subjected to the pilus is lost as heat, rather than through a more permanent loss of integrity (86–88). The pilus has also been shown to adhere to human tonsil epithelia, which is a major initial site for *S. pyogenes* upper respiratory tract infection (12, 89). Moreover, deletion of the major pilus subunit results in reduced adhesion to pharyngeal cell lines compared to the wildtype strain, with the greatest disparity occurring at earlier time points (<30 minutes), suggesting that the pilus is transiently regulated (90). Despite these findings, there is no evidence directly validating the major pilus subunit as the agent responsible for adhesion; instead, studies predict that ancillary protein 1 interacts with host ligands to mediate adhesion (91–93).

1.5.3.2. Collagen-binding protein

Some strains harbour the pilus ancillary protein 1, otherwise referred to as the collagen-binding protein, Cpa. Most commonly, studies have determined a pilus model in which Cpa is located at the tip of the pilus, thus naming it ancillary protein 1. Depending on the strain, however, *S. pyogenes* can encode *cpa* with the classical LPXTG motif that anchors Cpa to the cell wall, or an alternative (Q/E/V)(V/P)PTG motif that can be attached to the major pilus subunit, ancillary protein 2, or anchored to the cell wall through a locus-specific sortase encoded within the FCT region (47, 94–96). This structural inconsistency can be visualized using immunogold labelling, which has depicted Cpa at the top of the pilus (e.g. M1, M3, and M6), as well as at the base of the pilus (e.g. M49) (47, 97, 98). Despite the sequence variability between *cpa* of different strains, a common feature is the ability to bind collagen type I and type IV, leading to its hypothesized role in adherence during colonization, which is presently unconfirmed (76, 99). It has also been shown to contain a thioester bond, which may mediate an interaction with cognate receptors on host cells (100). In addition, deletion of *cpa* results in an attenuated ability for *S. pyogenes* to adhere to human lung and pharyngeal cell lines (89, 91). Clinical evidence from Cpa-seropositive patients indicated an association with people afflicted with arthritis and osteomyelitis, suggesting the possibility that Cpa interacts with collagen type I found in the bones and synovia (99). Thus, the combination of evidence suggests that Cpa can be utilized by *S. pyogenes* in a variety of ways in order to establish itself in the host, and that for some strains it may be vital in the formation of tissue-specific infections.

1.5.3.3. Fibronectin-binding proteins

The FCT region can also encode genes for one or two FBPs: *prtF1/sfbI* and/or *prtF2/pfbp/fbaB*. These proteins, although structurally distinct, are functionally related in that they facilitate adhesion to fibronectin and are involved with *S. pyogenes* internalization via the $\alpha_5\beta_1$ integrin (101–103). Architecturally, these FBPs consist of one to five tandem repeat domains that are directly proportional to the strength of fibronectin binding, and in some cases, internalization efficiency (104–106). Moreover some strains of *S.pyogenes* share sequence homology between these FCT region-encoded FBPs and other FBPs, such

as *fbaA* or *sof*, which are located near the *emm* genomic cluster (2, 107). The regulation of these FBPs can be remarkably precise, as it has been demonstrated that for some strains the FBPs are upregulated in oxygen-rich conditions and downregulated when carbon dioxide availability increases (106, 108). This may suggest a transient role for FBPs during initial colonization of the skin and respiratory tract, and that they become less involved during late, deep tissue stages of infection at which point the expression of other virulence factors involved in maintaining the infection are prioritized (44). Explorations of the M3 and M18 serotypes have demonstrated that PrtF2 is partially responsible for binding human pharyngeal cells and subsequently mediating bacterial internalization into these host cells. Cumulatively, these data substantiate the idea that the FCT region-encoded FBPs may be critical during initial adhesion to the host, as well as in progressing to an infectious state (109, 110).

1.5.3.4. The relationship between the fibronectin-binding, collagen-binding, T-antigen region and tissue tropism

The type of FCT region (i.e. FCT-1 through FCT-9) has been suggested to play a role in tissue tropism. For example, epidemiological data has shown that approximately 80% of skin isolates (*emm* pattern D) encode the FCT-4 region, whereas less than 15% of throat isolates (*emm* pattern A–C) encode the FCT-4 region (25). Experimental analysis, however, does not appear to demonstrate major differences *in vitro* for adherence of different throat and skin isolates to a series of cell lines (111). Further complexity regarding *S. pyogenes* tissue tropism is introduced when considering that FCT region genes are subject to global regulators that affect their transcriptional expression during different pathogenic scenarios (107).

1.6. Regulation in *Streptococcus pyogenes*

Having evolved alongside humans throughout history, *S. pyogenes* has adapted incredibly well to our various tissues in order to cause infection and subsequently colonize our bodies. In particular, *S. pyogenes* has demonstrated an aptitude for immune avoidance, niche colonization, and in general, mitigating pathology in order to foster an ideal

microenvironment that is conducive to persistence within its host. A key factor in this remarkable feat is the regulation of virulence. In a multifaceted approach, regulation in *S. pyogenes* involves a circuit of coordinated gene regulators that respond to molecular cues from the host and environment. The *S. pyogenes* transcriptome is controlled by at least 13 two-component systems and 30 known stand-alone regulators (>100 including putative gene regulators) (12). As such, for the purpose of this thesis, we will focus on some of the key regulators and how they influence survivability and pathogenesis.

1.6.1. CovRS

A prominent two-component system in *S. pyogenes* is the CovRS system, which primarily responds to environmental stress signals, such as antimicrobial peptides, increased temperature, iron starvation, or excess Mg^{2+} (112–114). Similar to other two-component systems, the process of regulation begins with a membrane-bound histidine kinase sensor, CovS, which then autophosphorylates and subsequently transfers the phosphoryl group to the response regulator, CovR (115, 116). This results in the dimerization of CovR, which is then able to bind DNA and modulate gene expression (117). The CovRS system is known to regulate the transcription of approximately 15% of the *S. pyogenes* genome, with an emphasis on virulence factors. Most commonly, CovRS acts to repress the expression of genes, including DNAses, cytolysins, and the hyaluronic acid capsule (118). Distinctively, the CovRS system appears to activate the transcription of the SpeB protease (119). It has also been documented that a non-functioning CovRS system leads to a hypervirulent phenotype, such as that which occurs in the globally-circulated M1T1 clone (120). Genetic analysis of the M1T1 clone unearthed a frameshift mutation in *covS*, inhibiting action of the CovRS system (121, 122). This hypervirulent phenotype is characterized by increased expression of virulence factors, such as the hyaluronic acid capsule and SpeA, and reduced expression of SpeB (12, 70, 123). The outcome of this mutation imparts CovRS mutants, such as the M1T1 strain, with a proclivity for systemic infection while being resistant to phagocytosis (121).

1.6.2. Mga

Stand-alone regulators account for a considerable portion of *S. pyogenes* regulation, and one of the best-studied is Mga. The chief function of Mga is to control the expression of several genes, particularly the M protein, genes involved in metabolism, and immune evasion genes; however, it has also been shown to regulate the primary regulator of the FCT region, *rofA/nra* (124, 125). All strains of *S. pyogenes* encode for one of the two *mga* alleles, which are suspected to contribute to tissue tropism [*mga-1* (throat isolates) and *mga-2* (skin isolates/generalists)] (124, 126). Regardless of the *mga* variant, this regulator is consistently expressed during the exponential growth phase that most frequently occurs during early stages of infection (126, 127). Therefore, it is likely that Mga transcriptionally upregulates genes involved with the initial acute stage of infection and adhesion factors that prevent early mechanical clearance of *S. pyogenes*.

1.6.3. RofA/Nra

All strains of *S. pyogenes* encode RofA and/or Nra, and may additionally contain RALP3 and/or RivR, which are all members of the RofA-like protein family (119, 128). Typically, these regulators are associated with the transition between the exponential phase and early stationary phase of growth (129). RofA and Nra, which notably share 63% sequence homology, are both encoded within the FCT region and attune the transcription of the genes encoded therein (129). Specifically, RofA directly upregulates transcription of the pilus, *prtF1*, as well as *cpa* and itself concurrently through an intergenic binding sequence (130). Peculiarly, Nra, which is found in a more limited range of *S. pyogenes* serotypes, is repeatedly described as a negative repressor of the aforementioned FCT region genes as well as *prtF2*. A possible explanation for this functional dichotomy may relate to the sites of infection for *rofA*$^+$ and *nra*$^+$ strains, which preferentially infect and colonize the throat and skin, respectively. Thus, these strains may differ in their requirements for spatial or temporal regulation in order to combat site-specific challenges (49, 126, 131).

Although Nra is generally reported as a negative repressor of FCT region genes, its activity appears to be strain-specific as it has also been described as a positive regulator for *cpa* in

an M53 skin infection strain (132). Distinctively, some strains appear to have forfeited their use of Nra while maintaining their pathogenicity. For instance, MGAS315 (isolated from a patient with STSS) naturally produces negligible levels of pili, partially attributed to a non-synonymous mutation in *nra* that results in attenuated regulatory activities (133, 134). Furthermore, introduction of an overexpressed Nra plasmid demonstrates that genes within M3 isolates are categorically upregulated by Nra (133). Relatedly, M18 strains appear to harbour an early stop codon in *nra*, effectively truncating it during translation, and although this mutation has not been studied, it is tempting to speculate that expression of Nra in M18 isolates would also upregulate numerous genes (135). This research suggests that for strains in which Nra acts as a positive regulator, basally high expression levels may be evolutionarily disadvantageous to *S. pyogenes* (133).

Given that there are numerous RofA and Nra binding sites present throughout the genome, these regulators also appear to have a function with regards to global virulence (125, 129, 131). This is further supported by studies that have demonstrated RofA and Nra activity leads to transcriptional downregulation of many genes related to virulence, including the hyaluronic acid capsule, SpeA, the SpeB protease, the cytolytic toxin streptolysin S, as well as Mga (125, 129, 131, 136). The act of downregulating Mga, which typically upregulates both RofA and Nra, further highlights the dynamic interplay between regulatory bodies in *S. pyogenes* and how they communicate to coordinate infection and sustain colonization.

1.6.4. MsmR

The FCT region can also encode the stand-alone regulator *msmR,* but its presence in this locus only occurs in strains that also encode *prtF2* (77, 137). Principally, MsmR is involved in the upregulation of FBPs, regardless of their location within the genome (137). Interestingly, MsmR has been shown to simultaneously upregulate *cpa* and *nra*, which emphasizes the significance of expressing antithetical regulators to fine-tune virulence and colonization. Transcriptional regulation by MsmR also appears to function antipodally to Nra, regardless of the strain; that is, when Nra acts as a repressor, MsmR generally acts as an activator, and vice versa (119, 132). Collectively, the interconnected network of

regulators in *S. pyogenes* exemplifies the necessity to precisely modulate virulence factors during each strain's unique life cycle.

1.7. Rationale and hypothesis

S. pyogenes resides within the throats of several hundred million people, yet many of the bacterial factors that help initiate and maintain pathogenicity remains unelucidated within the context of colonization and an acute pharyngeal infection. The FCT region is of particular interest since studies detailing its role during infection are less intensively studied compared to other surface-linked virulence factors, such as the M protein or hyaluronic acid capsule. Additionally, this study provides an exploratory opportunity to characterize the genes found within the FCT region of a strain that was isolated from an outbreak of ARF, MGAS8232 (135).

During the early stages of pharyngeal infection by *S. pyogenes*, adherence factors aid in the onset of infection by adhering to host tissue to prevent the rapid and initial clearance of bacteria (44, 45). Three components often implicated in this mechanism are the pilus, its commonly associated collagen-binding protein, and FBPs, all of which are encoded within the FCT region of MGAS8232 (**Figure 1**). I hypothesized that these factors would be crucial for *S. pyogenes* to initiate infection in the nasopharynx of mice. Using a genetic deletion of the FCT region (from the start of *cpa* until the end of *prtF2*), I predict that *in vitro* binding of *S. pyogenes* to relevant human tissue will be reduced. Moreover, using our transgenic murine nasopharyngeal infection model, for which MGAS8232 has previously been verified to consistently infect, I speculate that the deletion of the FCT region will inhibit the ability for *S. pyogenes* to establish itself in the nasal turbinates.

Figure 1. The FCT-3 region, and the adjacent upstream and downstream genes, found in *S. pyogenes* MGAS8232.

The genetic organization of the FCT-3 region. The small arrows delineate the flanking boundaries of the portion of the FCT region that was chromosomally deleted in the ΔFCT mutant.

1 kb

| hsp33 | nra | cpa | lepA | tee18.1 | srtC2 | fctB | msmR | prtF2 | spy0136 |

FCT region-flanking genes

Main pilus subunit

Sortase

Regulators

Peptidase

Fibronectin-binding protein

Ancillary pilus proteins
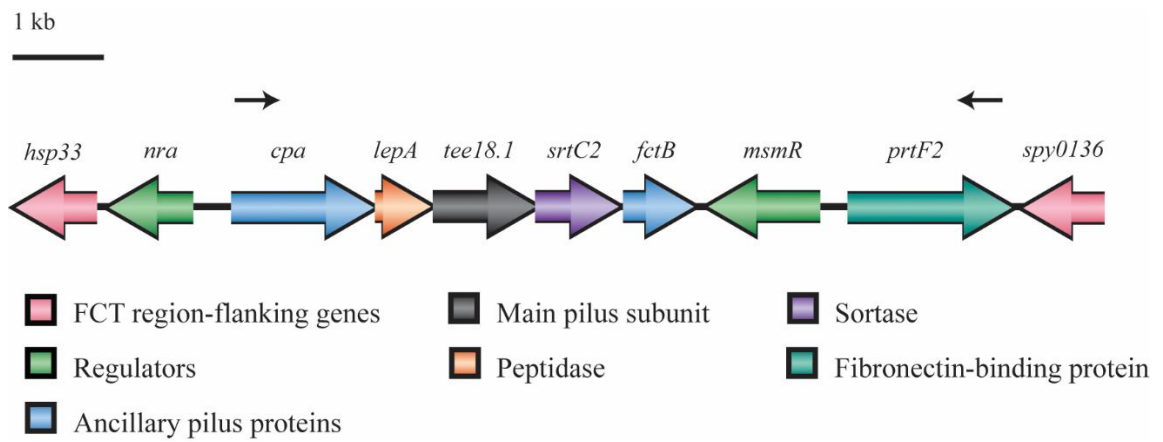
Chapter 2: Methods

2.1. Ethics Statement

All animal experiments were completed under Animal Use Protocol (AUP) Number 2017-024 in compliance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, and was approved by the Animal Use Subcommittee at Western University (London, ON, Canada) (**Appendix I**).

2.2. Bacteria

A complete list of the bacterial strains used in this study can be found in **Table 2**.

2.2.1. *Escherichia coli*

E. coli were grown in Luria-Bertani (LB) broth (Thermo Fisher Scientific; Waltham, MA, USA) aerobically with shaking at 37°C. During molecular cloning, *E. coli* were grown on brain heart infusion (BHI) broth (BD Biosciences; Franklin Lakes, NJ, USA) with 1.5% (w/v) agar (Thermo Fisher Scientific) and 150µg mL^{-1} erythromycin (Sigma-Aldrich Canada; Oakville, ON, Canada) when required. Frozen stock cultures were stored at -80°C in LB broth with 20% (v/v) glycerol.

2.2.2. *Streptococcus pyogenes*

S. pyogenes were grown in Todd Hewitt broth (BD Biosciences) supplemented with 1% (w/v) Bacto yeast extract (BD Biosciences) (THY) statically at 37°C. During molecular cloning, THY media was supplemented with 1.5% (w/v) agar and/or 1µg mL^{-1} erythromycin. Frozen stocks were stored at -80°C in THY broth with 20% (v/v) glycerol.

2.2.3. *Streptococcus pyogenes* growth curves

S. pyogenes MGAS8232 (wildtype and ΔFCT) were grown from frozen -80°C stocks and subcultured at 1:100 for 2 days until day 3, on which 100mL bottles of pre-warmed THY media were inoculated with 6mL from the overnight culture and incubated at 37°C until early exponential phase. Optical densities (OD) were measured at regular intervals using a spectrophotometer (DU 530 Life Science UV/Vis Spectrophotometer, Beckman Coulter

Table 2. Bacterial strains used in this study

Strain	Description	Source
Escherichia coli		
XL1-Blue	Molecular cloning strain	Stratagene
Streptococcus pyogenes		
MGAS8232	M18 serotype isolated from a patient during an acute rheumatic fever outbreak (GenBank accession: AE009949.1)	(135)
MGAS8232 ΔFCT	FCT region deletion mutant in the strain MGAS8232	This study

Canada LP; Mississauga, ON, Canada). Bacterial cultures were then adjusted to an OD_{600} of 0.02 before being loaded into sterile 96-well plates. Bacteria were grown with periodic shaking and OD_{600} measurements were obtained every 10 minutes for 20 hours using the Synergy HTX Multi-Mode Microplate Reader (BioTek; Winooski, VT, USA).

2.3. Molecular cloning

This section details the methodologies that were used to generate the ΔFCT mutant.

2.3.1. DNA visualization

DNA samples were loaded into wells of a 1% (w/v) agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer along with a 1-kb Plus DNA ladder (Invitrogen; Carlsbad, CA, USA). Gels were run at 100V for 1 hour using the PowerPac 200 Electrophoresis Power Supply and Chamber (Bio-Rad Laboratories; Hercules, CA, USA) and subsequently stained in 0.05% (v/v) ethidium bromide in TAE buffer for 20 minutes. DNA gels were then visualized using ultraviolet light on a gel doc (Bio-Rad Laboratories).

2.3.2. Sequencing of DNA

DNA samples (with the inclusion of primers when necessary) were sequenced by the Sequencing Facility at the John P. Robarts Research Institute in London, ON, Canada. Primers used for sequencing can be found in **Table 3**.

2.3.3. Plasmid isolation from *Escherichia coli*

Plasmid DNA was isolated from overnight cultures of *E. coli* using the instructions as outlined by the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). A complete list of plasmids can be found in **Table 4**.

2.3.4. Genomic DNA isolation from *Streptococcus pyogenes*

To isolate total genomic DNA from *S. pyogenes*, 2mL of an overnight culture was centrifuged at $15,000 \times g$ for 2 minutes. Bacteria were washed with 1mL 0.2mM sodium

Table 3. Primers used in this study

Primer	Primer sequence (5' → 3')[a]
Primers used to generate the ΔFCT construct	
FCT up *Kpn*I for	CGC <u>GGTACC</u> GAGGAATGCTAACTGCAAAT
FCT up *Pst*I rev	CGC <u>CTGCAG</u> CCTCTTTTGCAATATATCCTC
FCT down *Pst*I for	CGC <u>CTGCAG</u> TCATCATGTTAAGATAAGCTG
FCT down *Bam*HI rev	CGC <u>GGATCC</u> TCTTCACTTCACTACTAGCAG
Primers used for sequencing and deletion confirmation	
M13 for	GTAAAACGACGGCCAGT
M13 rev	CAGGAAACAGCTATGAC
FCT integration for	CAGCAAGTTTCAATATGCTG
FCT integration rev	AACACAGACATAGTAACGAC
Internal primers used during qRT-PCR	
gyrA RT for	GGTTCGTATGGCTCAGTGGT
gyrA RT rev	TTCTCTTTCGCTTCCATCGT
Int *cpa* for	GCAGAACCATACTCTGTAACAG
Int *cpa* rev	CATTAAAGTCTGGATCAATCG
Int *tee18.1* for	CAGGAGTGATTGATGGTTTAG
Int *tee18.1* rev	AACATCAACTGTCCATTGTTG
Int *prtF2* for	GGAATGGCTTACATTTATTCTG
Int *prtF2* rev	CACCTGAAACAATATCTGGTG

[a] Restriction endonuclease cut sites are underlined

Table 4. Plasmids used in this study

Plasmid	Description	Source
pG⁺host5	*E. coli* → Gram-positive shuttle vector; temperature-sensitive, erythromycin resistant	(138)
pG⁺host5::ΔFCT	FCT region deletion construct chromosomally inserted into *S. pyogenes* MGAS8232	This study

acetate then centrifuged again as described. Cells were then lysed with 500µL of a 50mM EDTA solution with 0.2% (v/v) sodium dodecyl sulphate (SDS) and 0.02µg RNase and were incubated for 1 hour at 70°C. Following, 50µL of 5M potassium acetate was then added for 1 hour at -20°C before being centrifuged as described for 10 minutes. The supernatant was then transferred to a tube with 1mL ice-cold 95% ethanol and left for 1 hour at -20°C. The suspension was centrifuged as described for 10 minutes and the pellet was washed in 1mL ice-cold 70% ethanol. The solution was then centrifuged again, and the supernatant was discarded. The DNA pellet was then air-dried and resuspended in 100µL sterile water from the MilliQ purification filtration system (EMD Millipore; Darmstadt, Germany) before being stored at -20°C.

2.3.5. Polymerase chain reaction

All primers used in this study were obtained from Sigma-Aldrich (**Table 3**). For a 100µL polymerase chain reaction (PCR), the mixture consisted of 5× high-fidelity buffer (Thermo Fisher Scientific), 0.7µL of the high-fidelity DNA polymerase Phusion (Thermo Fisher Scientific), 3mM deoxyribonucleotide triphosphate (dNTP) (Roche; Basel, Switzerland), 2mM magnesium chloride (Thermo Fisher Scientific), 1µM of each primer (forward and reverse), and 2µL of DNA. All reactions were performed in an MJ Mini or MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories) using the DNA amplification parameters as follows: 98°C for 5 minutes for initial denaturation of DNA; 98°C for 30 seconds, 30 seconds at the appropriate melting temperature, 72°C for extension (time: 30 seconds per 1 kb), repeated for 35 cycles; a final extension step at 72°C for 7 minutes before an indefinite 4°C step before sample removal.

2.3.6. Restriction digests and ligations

DNA restriction endonuclease digests were performed as per the manufacturer's instructions (New England BioLabs; Ipswich, MA, USA) before purification using the QIAquick PCR Purification Kit (Qiagen).

Purified endonuclease-digested DNA was ligated into the appropriate vector (10 vector:1 insert) using = T4 DNA Ligase (New England BioLabs) and incubated at 16°C for 1 hour.

2.3.7. Generation of competent *Escherichia coli* cells

To prepare competent rubidium chloride cells, an overnight culture of *E. coli* was subcultured into 100mL PSI broth [2% (w/v) Bacto tryptone (BD Biosciences), 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) magnesium sulphate, pH = 7.6] at a 1:100 dilution and grown at 37°C with shaking to an OD_{600} of 0.5. The bacteria were placed on ice for 15 minutes and then centrifuged at $5,000 \times g$ for 5 minutes at 4°C. The pellet was resuspended in 40mL TfbI buffer [15% (v/v) glycerol, 100 mM rubidium chloride, 30 mM potassium acetate, 10 mM calcium chloride, 50 mM manganese chloride, pH = 5.8] and incubated on ice for 15 minutes. Bacteria were centrifuged as stated above and the resulting pellet was resuspended in 4mL TfbII buffer [15% (v/v) glycerol, 75 mM calcium chloride, 10 mM 3-(N-morpholino) propanesulfonic acid, 10 mM rubidium chloride, pH = 6.5] and stored in 200µL aliquots at -80°C.

2.3.8. Transformation of competent *Escherichia coli* cells

To start, 10µL of plasmid DNA from ligation reactions were added to thawed rubidium chloride competent *E. coli* cells and incubated for 30 minutes on ice. Bacteria were then placed in a 42°C water bath for 45 seconds followed by incubation on ice for 2 minutes. Afterwards, 900µL of LB broth was added to the cells and incubated for 1 hour at 37°C with shaking, and then 150µL of the bacteria were spread over BHI agar supplemented with 150µg mL^{-1} erythromycin and incubated overnight at 37°C.

2.3.9. Generation of competent *Streptococcus pyogenes* cells

To prepare competent *S. pyogenes cells*, an overnight culture was subcultured at a 1:50 dilution into 100mL of THY broth containing 0.6% glycine for 2 hours at 37°C. Following, 5mg of hyaluronidase (Sigma-Aldrich) was then added and the culture was grown to an OD_{600} of 0.3. Bacteria were centrifuged at $6,000 \times g$ for 5 minutes and the pellet was

washed once with 40mL of 15% (v/v) glycerol. The cells were then resuspended in 4mL of 15% (v/v) glycerol and stored in 200µL aliquots at -80°C.

2.3.10. Electroporation of competent *Streptococcus pyogenes* cells

Plasmid DNA (~2µg) were mixed with thawed competent *S. pyogenes* cells and then transferred to a 2mm electroporation cuvette. The cells were pulsed exponentially at 2500V and 600Ω using a GenePulser (Bio-Rad Laboratories). Bacteria were then added to 10mL of THY and incubated for 3 hours at 37°C before centrifugation at $6,000 \times g$ for 5 minutes. The cells were then resuspended in 1mL of THY broth and plated on THY agar (supplemented with 1µg mL^{-1} erythromycin if necessary) and incubated at 37°C overnight.

2.3.11. Generation of *Streptococcus pyogenes* deletion mutant

The temperature-sensitive, erythromycin-selection-marked plasmid pG$^+$host5 was used to delete the FCT region from MGAS8232 via double homologous recombination events (135, 138) (**Table 3**).

Two ~1,000bp segments directly upstream and downstream of the *cpa* and *prtF2* genes, respectively, were amplified from wildtype MGAS8232 genomic DNA, digested using restriction endonucleases, and subsequently ligated into an accordingly-digested pG$^+$host5 vector (**Figure 2A**). The generated deletion construct was then transformed into competent *E. coli* XL1-Blue.

The deletion construct isolated from *E. coli* XL1 Blue was then electroporated into *S. pyogenes* wildtype MGAS8232 and grown on THY agar supplemented with 1µg mL^{-1} erythromycin at 30°C overnight; colonies were subsequently picked and grown in THY liquid media with erythromycin and incubated at 40°C for up to three days. The pG$^+$host5 plasmid is unable to replicate at this temperature, thus, erythromycin-resistance is indicative of chromosomal integration (**Figure 2B**). Clones that had the deletion construct successfully integrated (as verified by PCR) were subcultured 1:100 in THY liquid media

Figure 2: Deletion of the FCT-3 region from *S. pyogenes* MGAS8232.

To genetically remove the FCT region, (**A**) adjacent segments upstream (US) and downstream (DS) were amplified by PCR, digested with restriction endonucleases, and ligated into the temperature-sensitive, erythromycin resistance (erm^R)-marked pG$^+$host5 vector. (**B**) The knockout vector was then electroporated into *S. pyogenes* to remove the FCT region from the host chromosome during homologous recombination events that are contingent on the temperature-sensitive origin of replication (TS Ori) of the pG$^+$host5 vector.

A

S. pyogenes genome

US | FCT Region | DS

PCR amplify and digest

KpnI | US | PstI

PstI | DS | BamHI

Ligate into KpnI and BamHI-digested plasmid

TS Ori | erm^R

pG⁺host5::ΔFCT

US | ΔFCT | DS

B

TS Ori | erm^R

pG⁺host5::ΔFCT

US | ΔFCT | DS

S. pyogenes genome

US | FCT Region | DS

Homologous recombination

S. pyogenes genome

US | ΔFCT | DS

+

TS Ori | erm^R

pG⁺host5::ΔFCT
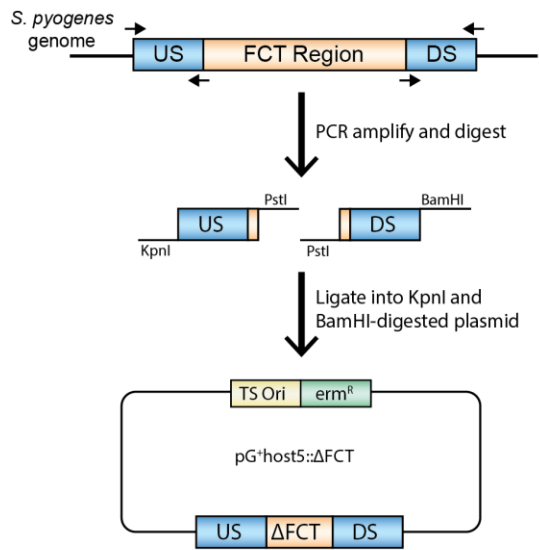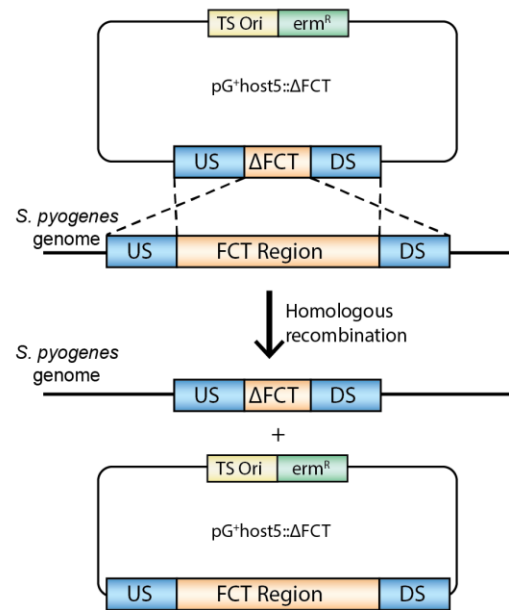
US | FCT Region | DS

without erythromycin at 30°C in order to resolve the plasmid (and the FCT region) from the chromosome. Bacterial cultures were patched onto THY agar with and without 1µg mL^{-1} erythromycin, wherein erythromycin-sensitive colonies had been cured of the pG$^+$host5 plasmid (**Figure 2B**). These clones were then verified by DNA sequencing and PCR to confirm the successful deletion of the ΔFCT mutant.

2.4. Protein visualization

Protein samples were visualized using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blot.

2.4.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Proteins were mixed with Laemmli buffer [125 mM Tris pH 6.8, 50% glycerol, 4% SDS, 5% β-mercaptoethanol, and 0.1% (w/v) bromophenol blue] and boiled for 5 minutes. Samples (20µL) were then run using SDS-PAGE on 12% (v/v) acrylamide gels in electrophoresis buffer [25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS] for 30 minutes at 85V then for 1 hour and 30 minutes at 150V. The gels were then stained with coomassie [0.1% (w/v) coomassie brilliant blue R-250 (Bio-Rad Laboratories), 45% (v/v) methanol, and 10% (v/v) acetic acid], and destained for visualization [10% (v/v) acetic acid, and 45% (v/v) methanol].

2.4.2. Western blot

To detect the superantigen SpeA in the wildtype and ΔFCT strains, samples run through SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond, GE Healthcare; Pittsburgh, PA, USA). This was accomplished by assembling the PVDF membrane and SDS-PAGE gel in a mini trans-blot cell (Bio-Rad Laboratories) and run for 1 hour at 100V in Western transfer buffer [48 mM Tris base, 39 mM glycine, 0.037% (w/v) SDS, and 20% (v/v) methanol]. The PVDF membrane was then blocked using 5% (w/v) skim milk in tris buffered saline (TBS). Subsequently, the membrane was incubated at 4°C overnight with a polyclonal rabbit α-SpeA primary

antibody [(41), produced by ProSci; Poway, CA, USA] at a 1:1,000 dilution in TBS with 1% (w/v) skim milk. The membrane was then washed in TBS with 0.1% (v/v) tween-20 (Amresco, VWR International; Solon, OH, USA) (TBS-T) 3 times for 5 minutes per wash. The membrane was then incubated in darkness at room temperature for 1 hour with a goat α-rabbit IgG conjugated to IRDye800 secondary antibody (Rockland; Limerick, PA, USA) diluted 1:10,000 in TBS with 1% (w/v) skim milk. The membrane was subsequently washed 5 times with TBS-T, as described and then visualized using the LI-COR Odyssey Imaging System (LI-COR Biosciences; Lincoln, NE, USA).

2.5. Cell Surface Extract

To obtain cell wall fractions, wildtype and ΔFCT *S. pyogenes* were grown from frozen stocks into 10mL of THY and incubated overnight at 37°C before subculturing 1:100 into 100mL bottles of pre-warmed THY and grown until an OD_{600} of 0.2–0.4. The bacteria were then adjusted to an $OD_{600} = 0.25$ and centrifuged at $10,000 \times g$ for 10 minutes. The pelleted cells were then washed once in PBS and centrifuged again. The cells were then resuspended in 1mL of cold protoplasting buffer [40% sucrose, 100mM KPO_4 (pH = 6.2), 10mM $MgCl_2$, Complete EDTA-free protease inhibitors (Roche), 2mg ml^{-1} lysozyme (Sigma-Aldrich), and 400U mutanolysin (Sigma-Aldrich)] and incubated at 37°C for 3 hours with constant but gentle movement. The bacteria were then centrifuged at $15,000 \times g$ for 15 minutes at 4°C and the supernatants (cell wall proteins) were collected and stored at -20°C.

2.6. Trichloroacetic acid precipitation

To obtain secreted proteins, wildtype and ΔFCT *S. pyogenes* were grown from frozen stocks into THY media and incubated overnight at 37°C before subculturing 1:100 into 100mL bottles of pre-warmed THY and grown for 13 hours. The bacteria were centrifuged at $10,000 \times g$ for 15 minutes at 4°C and the supernatant was subsequently drawn off and incubated with 50% trichloroacetic acid (TCA) (Sigma-Aldrich) for 30 minutes. The supernatant-TCA precipitations were then centrifuged as described above and the pellets were then washed with ice-cold acetone (Thermo Fisher Scientific) and centrifuged again as described and the supernatant was removed. The proteins were then resuspended in 1mL of 8M urea (BioShop Canada Inc; Burlington, ON, Canada) and stored at -20°C.

2.7. Extracellular matrix binding assay

Wildtype *S. pyogenes* and the ΔFCT mutant were grown from frozen stocks into 10mL of THY media, followed by two days of subculturing. At this time, Corning Costar 9018 high-binding 96-well plates (Corning; Kennebuck, ME, USA) were coated with 1µg of collagen type IV (Sigma-Aldrich) or fibronectin (Calbiochem, EMD Millipore Corporation; Temecula, CA, USA) dissolved in carbonate-bicarbonate buffer (0.2M sodium carbonate anhydrous, 0.2M sodium bicarbonate, pH = 9.6) overnight at 4°C. The day after, 6mL of the overnight *S. pyogenes* cultures were inoculated into separate 100mL bottles of pre-warmed THY and grown until mid exponential phase after which their OD_{600} values were corrected to 0.25 before centrifuging the appropriate volume of bacteria at $15,000 \times g$ and resuspending the pellet in phosphate buffered saline (PBS). While the bacteria were growing, the plates were washed three times using PBS with 0.05% (v/v) tween-20 and blocked for 2 hours with 5% (w/v) skim milk at room temperature before being washed three times as described. Afterwards, 100µL of bacteria ($\sim 1 \times 10^7$ CFUs) were added in triplicate to wells and incubated for 2.5 hours at 37°C. Plates were washed three times as described and fixed with 10% neutral buffered formalin (VWR International; Randor, PA, USA) for 40 minutes before washing three times again as described. Plates were then incubated with 50µL of 0.5% (w/v) crystal violet (Sigma-Aldrich) in 80% (v/v) sterile MilliQ water and 20% (v/v) methanol for 5 minutes at room temperature before being washed five times. Stain was solubilized in 5% (v/v) acetic acid with mild agitation for 10 minutes. Colorimetric analysis was measured at OD_{590} using the Synergy HTX Multi-Mode Microplate Reader (BioTek).

2.8. Human cell culturing

The pharyngeal cell line Detroit-562 (obtained from Dr. Joe Mymryk, Western University) was grown in sterile $75cm^2$ tissue culture (TC)-treated flasks (Falcon, Corning; Corning, NY, USA) in 14mL of minimum essential medium (MEM) (Gibco, Life Technologies; Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 µg mL^{-1} streptomycin (Life Technologies), and 100 U mL^{-1} penicillin (Life Technologies) at 37°C in 5% CO_2 and washed 2 times every other day with

PBS (for ~6 days) to remove cellular debris. To split, flasks with cells at ~75% confluence were dissociated from the flask using 0.25% trypsin-EDTA (Gibco, Life Technologies).

2.8.1. Pharyngeal cell line adhesion assay

Detroit-562 cells were grown to confluence on 12 well TC-treated plates (Falcon, Corning) and then incubated for 2 hours in serum-free MEM. Three wells were dissociated using 0.25% trypsin and counted using a haemocytometer (Hausser Scientific; Horsham, PA, USA) to obtain a representative average for the number of Detroit-562 cells per well, which was used to determine the number of bacterial CFUs to use during the assay [multiplicity of infection (MOI) = 100]. During this time, 6mL of an overnight culture *S. pyogenes* that was previously grown and subcultured for two consecutive days was inoculated into 100mL bottles of pre-warmed THY and grown until mid-exponential phase. The appropriate volume of *S. pyogenes* was centrifuged at $15,000 \times g$ and the pellet was resuspended in serum-, and antibiotic-free MEM and inoculated with the Detroit-562 monolayer for 2.5 hours at 37°C in 5% CO_2. Each well was washed 3 times using PBS to remove non-adherent bacterial cells and the remainder was mechanically lysed in PBS with 0.01% (v/v) Triton X-100 (VWR International) to detach the cells from the plate. The suspension was then serially diluted (neat $\rightarrow$ 10^{-6}) and plated on trypticase soy agar (TSA) supplemented with 5% sheep's blood (BD Biosciences) for bacterial enumeration the next day.

2.8.2. Pharyngeal cell line invasion assay

The invasion assay, which is a modification of the classical gentamycin protection assay, follows the procedure as outlined in section 2.8.1 with an additional step. Briefly, after *S. pyogenes* were incubated with the confluent monolayer of Detroit-562 cells for 2.5 hours, the wells were washed twice with PBS and incubated in MEM supplemented with 100µg mL^{-1} gentamycin (Sigma-Aldrich) for an additional 1.5 hours at 37°C in 5% CO_2 to kill extracellular bacteria. As described above, the cells were then detached from the plates in PBS with 0.01% (v/v) Triton X-100, serially diluted, and plated for bacterial enumeration the next day.

2.9. Mice

Transgenic C57BL/6 mice expressing the MHC class II molecules HLA-DR4/DQ8 or HLA-DQ8 alone were donated from the Kotb laboratory and were since housed and bred in the West Valley Barrier Facility at Western University (41, 139). *S. pyogenes* inoculum preparation and mouse infection were performed as previously described (140).

2.9.1. Murine nasopharyngeal infection

A timeline of the infection model can be found in **Figure 3A**; to begin, mice were given water supplemented with 2mg mL^{-1} neomycin sulphate (VWR International) prior to and throughout the course of infection in order to inhibit their endogenous nasal microbiota.

Wildtype *S. pyogenes* and the ΔFCT mutant were grown from freezer stocks in THY and subcultured 1:100 for two days. On the third day, 6mL of the overnight cultures were then inoculated into separate pre-warmed bottles of THY and grown to an OD$_{600}$ of 0.2–0.4 and the appropriate volume was resuspended in Hank's Balanced Saline Solution (HBSS) (Life Technologies). Mice were anesthetized with isoflurane (USP, Baxter International; Deerfield, IL, USA) and intranasally inoculated with ~5 × 10^7 bacterial CFUs per nostril. Twenty-four or 48 hours post-inoculation, mice were sacrificed and their complete nasal turbinates (cNTs) were harvested (**Figure 3A, Figure 3B**). Subsequently, the cNTs were homogenized in HBSS and a portion of the homogenate was serially diluted (neat → 10^{-7}) and then plated on TSA supplemented with 5% sheep's blood for bacterial enumeration the following day. The remainder of the homogenate was stabilized in RNAprotect Cell Reagent (Qiagen) for 5 minutes at room temperate, centrifuged at 15,000 × *g* for 10 minutes to discard the supernatant, and stored at -80°C until future use.

2.9.2. RNA isolation

S. pyogenes were grown *in vitro* to an OD$_{600}$ of 0.2 (early exponential phase) or 0.8 (late exponential phase) before being incubated with 1mL of RNAprotect Cell Reagent for 5 minutes at room temperature, centrifuged at 15,000 × *g* for 10 minutes to discard the supernatant, and stored at -80°C until future use.

Figure 3. Mouse infection timeline

(**A**) Two days prior to infection, mice were given 2mg mL^{-1} neomycin water. They were then inoculated nasally with the wildtype or ΔFCT strain of *S. pyogenes* MGAS8232 and sacrificed 24 or 48 hours later. (**B**) Their complete nasal turbinates (pink) were then harvested and processed RNA extraction and/or bacterial enumeration.

A

Day

| -2 | -1 | 0 | 1 | 2 |

Neomycin water

Nasal inoculation

Sacrifice

B

1E
3E
5E
6E

T3   T4
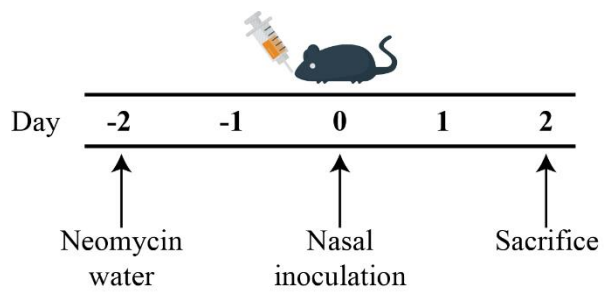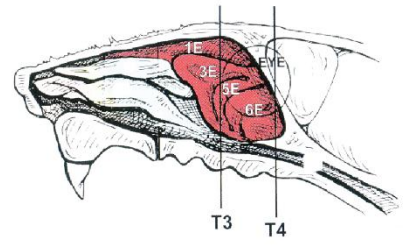
RNA-protected bacterial pellets or murine cNT homogenates were resuspended in 500μL of Tris-EDTA + glucose (2mM EDTA, 10mM Tris, 25% glucose) buffer with 100μg of lysozyme and 50 U of mutanolysin followed by incubation for 1 hour at 37°C. The cells were then centrifuged at 20,000 $\times$ g and the pellet was resuspended in 500μL of streptococcal lysis buffer (50mM EDTA, 0.2% SDS) with 100μg of proteinase K and incubated for 2 hours at 65°C. RNA was then extracted and purified using the RNeasy Kit (Qiagen) as per the manufacturer's instructions.

2.10. Quantitative real-time polymerase chain reaction

Gene-specific primers were designed for *cpa*, *tee18.1*, and *prtF2* with amplicon lengths of ~200bp and amplified from wildtype MGAS8232 genomic DNA to generate standards for each gene (**Table 3**). RNA isolated from murine tissue or *in vitro S. pyogenes* cultures were used to generate cDNA using random primers (Invitrogen) and SuperScript II Reverse Transcriptase (RT) (Invitrogen). The genomic DNA and cDNA were then quantified using a NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). The concentration was adjusted to 5ng μL^{-1} and eight 10-fold serial dilutions were prepared in purified MilliQ water. The quantitative reverse transcriptase (qRT)-PCR reactions were performed with SensiFAST SYBR Green No-ROX Kit (Bioline, Meridian Bioscience Inc; London, ON, Canada) as per the manufacturer's instructions using the previously-designed gene-specific primers. All reactions were performed with their respective standards, a no-template control, and a control excluding RT. Samples were run on the Rotor-Gene 6000 (Corbett Life Sciences; Sydney, NSW, Australia) with the following amplification conditions: 95°C for 5 minutes, denaturation for 10 seconds at 95°C, 10 seconds at 60°C to anneal primers, and a 20 second extension at 72°C (45 cycles).

2.11. Statistical Analyses

Data were analyzed using the unpaired *t*-test or the one-way analysis of variance (ANOVA) with Tukey's post-hoc test, as stated in the figure legends. A *p* value less than 0.05 as determined by Prism software (GraphPad Software; La Jolla, CA, USA) was classified as statistically significant.

Chapter 3: Results

3.1. Confirmation of the *S. pyogenes* ΔFCT deletion mutant

Using the methods described in section 2.3.11, a clean deletion of the FCT region was produced in the *S. pyogenes* strain MGAS8232 and confirmed by DNA sequencing. The deletion was also verified by PCR using integration primers that flanked the excised region (**Figure 4A**). A series of *in vitro* assays were then performed to validate this mutant and ensure no pleiotropic effects occurred due to a theoretical secondary mutation that could have occurred during the mutagenesis procedure. Using a cell surface extract and a TCA precipitation to visualize the expression of cell surface-linked and secreted proteins, respectively, we were able to verify that the ΔFCT mutant retains a similar protein expression profile to the wildtype strain (**Figure 4B, Figure 4C**). As discussed earlier, the superantigen SpeA is necessary to instigate an acute infection in our murine nasopharyngeal model, and thus it was necessary to confirm that production of this toxin was unchanged by the molecular cloning process. The TCA precipitations that were previously performed were used as samples to generate an α-SpeA Western Blot. From this process, we determined that there was no obvious difference in SpeA production in the ΔFCT mutant (**Figure 4D**). Additionally, the wildtype strain and ΔFCT mutant were grown for 20 hours to generate comparative growth curves. Since the two strains did not exhibit differential growth, we were able to ensure that removal of the FCT region did not lead to any discernable growth defects in THY broth (**Figure 4E**).

3.2. *S. pyogenes* FCT region genes mediate adhesion to collagen type IV and fibronectin

Since several genes encoded within the FCT region have been implicated in adhesion, we first sought to evaluate the adhesion capabilities of the ΔFCT mutant compared to the wildtype strain. It has been well-documented that collagen type IV and fibronectin are significant constituents of the nasopharyngeal ECM, and that the ability for bacteria to adhere to them may contribute to streptococcal infection (99, 141–144). In particular, collagen type IV is the most abundant ECM protein that underlays epithelial cells, and although fibronectin is a minor component of the basement membrane, it is constantly being secreted in order to mediate adhesion and migration of host cells (145, 146). In order

Figure 4. Confirmation of the ΔFCT mutant and subsequent *in vitro* analyses.
(**A**) Integration primers flanking the FCT region were amplified using PCR followed by DNA gel electrophoresis to confirm that the FCT region was deleted from the genome. *In vitro* analyses were used to assess (**B**) surface protein expression by cell surface extract, (**C**) secreted protein expression by trichloroacetic acid preparation, and (**D**) SpeA superantigen expression by Western Blot (purified SpeA and SpeC were used as positive and negative controls, respectively). (**E**) An automated BioScreen protocol was used to generate growth curves for the wildtype MGAS8232 and ΔFCT mutant strains to ensure that the ΔFCT mutant did not present any *in vitro* growth defects.
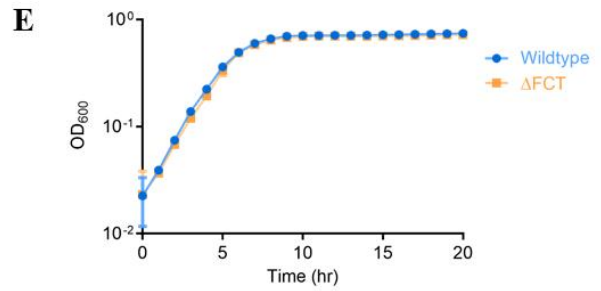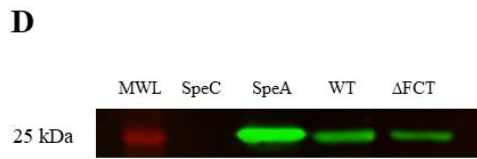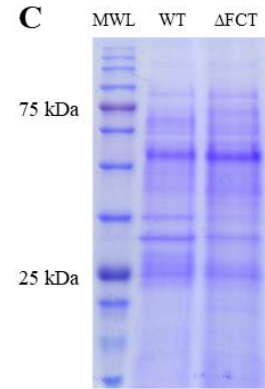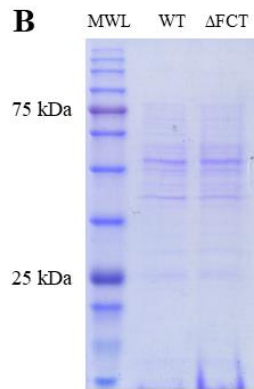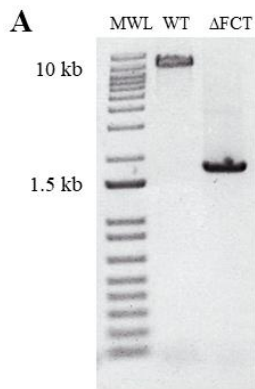
to examine the adhesion properties of the wildtype and ΔFCT strains, a bacterial adhesion assay was performed in which the bacteria were incubated with collagen type IV or fibronectin before being stained by crystal violet. We discovered that wildtype MGAS8232 was entirely reliant on the presence of the FCT region in order to bind collagen type IV, whereas binding to fibronectin was only partially reduced in the ΔFCT mutant (**Figure 5A, Figure 5B**). These results demonstrate that under normal *in vitro* growth conditions, *S. pyogenes* can produce functional FCT region-encoded proteins, most likely Cpa and PrtF2, suggesting that they may be fundamental to the bacteria's natural life cycle.

3.3 The FCT region genes do not mediate adhesion to human pharyngeal cells by *S. pyogenes*

A vital step in the life cycle of *S. pyogenes* is its prompt adherence to a susceptible human host; *S. pyogenes* can accomplish this task by utilizing numerous adhesion molecules. To examine the adherence capabilities of *S. pyogenes* to relevant human tissue, the pharyngeal cell line Detroit-562 was chosen due to its similarity of surface molecules with non-transformed pharyngeal cells, as well as its ability to induce certain superantigens and DNAses that *S. pyogenes* cultured on its own is unable to express (147–149). In short, wildtype MGAS8232 and the ΔFCT mutant were incubated over a confluent monolayer of Detroit-562 cells, and after the non-adherent bacterial cells were washed away, the remainder were plated for bacterial enumeration. We detected no differences in the binding capabilities to Detroit-562 cells between the wildtype and ΔFCT strains (**Figure 6**). These results indicate that the genes encoded within the FCT region are not essential for adherence to these pharyngeal cells. It should be noted, however, that the natural environment of *S. pyogenes* consists of more than one cell type, and adherence to the host is a multifactorial event that needs to be explored further.

3.4 *S. pyogenes* invasion of human pharyngeal cells is dependent on FCT region genes

In addition to adherence, FCT region genes are heavily implicated in the ability of *S. pyogenes* to invade into host cells (101, 150, 151). Compared to other FBPs, PrtF2 (which

Figure 5: The FCT region facilitates adhesion to collagen type IV and fibronectin.

Wells were coated with 1µg of human (**A**) collagen type IV, or (**B**) fibronectin, before incubation with ~ 1×10^7 *S. pyogenes* CFUs at 37°C. Non-adherent cells were washed off and the remaining cells were stained with 0.5% crystal violet before colorimetric analysis. Bars represent the mean ± SEM; unpaired *t*-test was used to determine statistical significance (***p<0.001, ****p<0.0001).
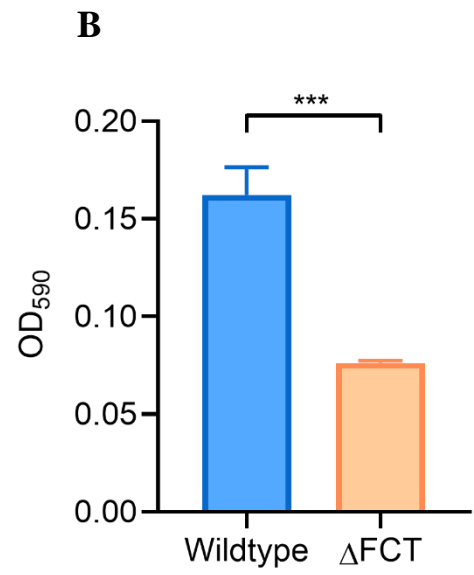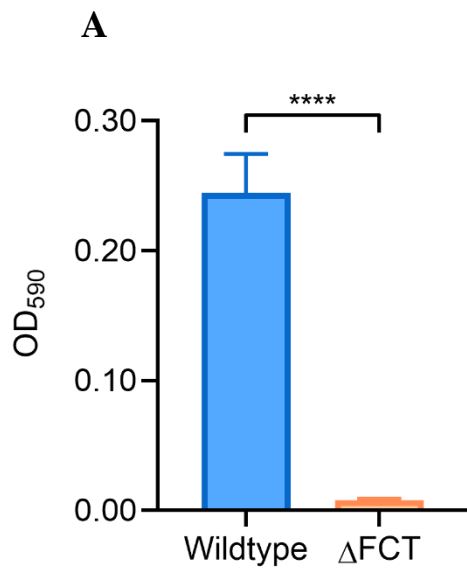
Figure 6. The FCT region does not influence adhesion to Detroit-562 cells.

A confluent monolayer of the human pharyngeal cell line Detroit-562 was grown before incubation with *S. pyogenes* (MOI of 100) at 37°C in 5% CO_2 for 2.5 hours. The non-adherent bacterial cells were then washed away before disrupting the monolayer and plating the suspension for bacterial enumeration. Each data point represents one *in vitro* biological replicate. Error bars represent the mean ± SEM; statistical differences were evaluated using the unpaired *t*-test, ($p>0.05$).
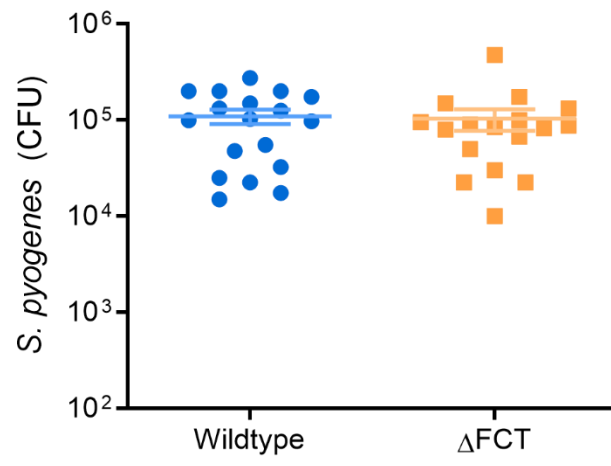
is encoded by MGAS8232) is more commonly associated with severe *S. pyogenes* infection and is more likely to correlate with invasive disease (101, 143, 152, 153). As such, we wanted to characterize the natural invasive proficiency of our wildtype strain and elucidate whether the ΔFCT mutant retained the same invasion potential. To test this query, we utilized Detroit-562 cells in a similar fashion to our adhesion assay, but performed a gentamycin protection assay to eliminate extracellular *S. pyogenes* prior to enumeration. Strikingly, we discovered that removal of the FCT region completely eliminated the ability to invade these pharyngeal cells (**Figure 7**). This assay provides compelling evidence to reinforce the idea that the FCT region plays a significant role during host-cell invasion.

3.5   *S. pyogenes* **expresses cpa, but neither tee18.1 nor prtF2 in** *vivo*

Within the biological context of nasopharyngeal infection, *S. pyogenes* must be able to adhere to the nasopharynx and persist at the site of infection while withstanding an inundation of mucociliary defenses. Success during this stage can be ascribed in part to the repertoire of virulence factors and adhesion molecules. Therefore, we wanted to establish the *in vitro* and *in vivo* expression profiles of the adherence-implicated genes encoded within the FCT region of MGAS8232; this included: the major pilus subunit, *tee18.1*, the collagen-binding protein, *cpa*, and the FBP, *prtF2* (**Figure 1**).

In order to determine the expression of these genes *in vitro*, RNA was isolated from cultures of *S. pyogenes* that were grown to early and late exponential phase. We then used qRT-PCR to measure the transcripts of these genes and normalized their concentration to the internal housekeeping gene, *gyrA*. We observed that *cpa*, *tee18.1*, and *prtF2* were all being transcribed at both early and late exponential phase, with *cpa* being transcribed at levels higher than *tee18.1* and *prtF2* (**Figure 8**). Although not statistically significant, our data depicts a slight trend for higher transcription levels for all FCT region genes during late exponential phase, suggesting the possibility of temporal regulation for these genes. With regards to *in vivo* expression, mice were infected for 48 hours with wildtype MGAS8232 and RNA was subsequently isolated from the cNTs before qRT-PCR analysis.

Figure 7. The FCT region is necessary for invasion into Detroit-562 cells.

A confluent monolayer of the human pharyngeal cell line Detroit-562 was grown before incubation with *S. pyogenes* (MOI of 100) at 37°C in 5% CO_2 for 2.5 hours followed by 1 hour in media supplemented with 100µg mL^{-1} of gentamycin. The non-adherent/internalized bacterial cells were then washed away before disrupting the monolayer and plating the suspension for bacterial enumeration. Each point represents an *in vitro* biological replicate. Error bars represent the mean ± SEM; statistical differences were evaluated using the unpaired *t*-test, (****$p<0.0001$).
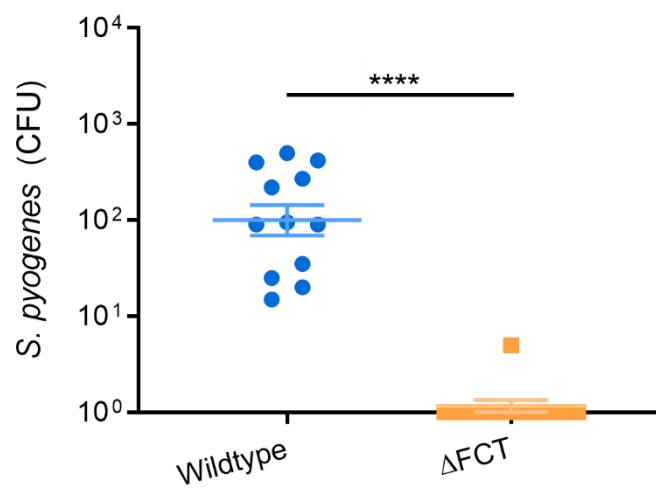
Figure 8. *S. pyogenes* transcribes FCT region genes *in vitro*.

mRNA was isolated from wildtype *S. pyogenes* MGAS8232 that was grown to early (OD_{600} ~0.2) and late (OD_{600} ~0.8) exponential phase. Transcription levels were quantified using qRT-PCR and normalized to the internal housekeeping gene *gyrA* (represented by the dotted line at $y = 1$). Bars represent the mean $\pm$ SEM (n = 4 per growth phase analyzed using duplicate values).
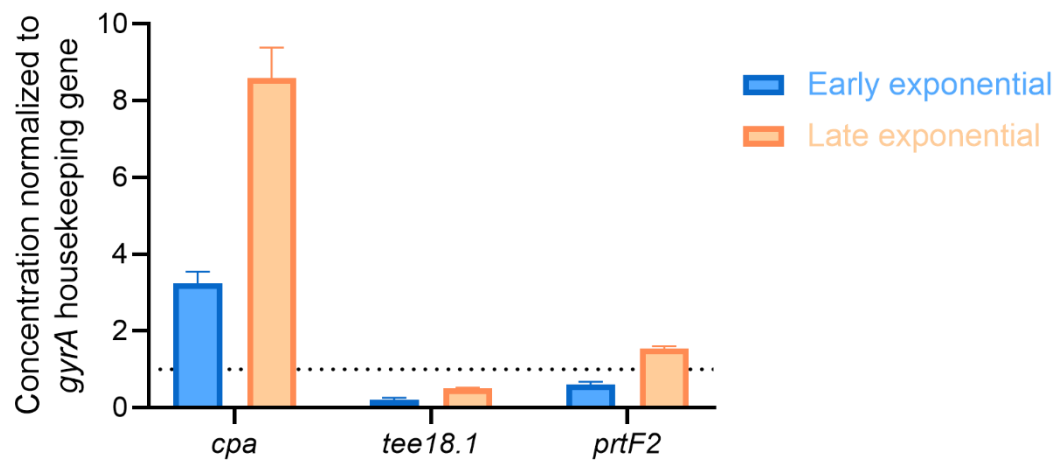
In addition to measuring the absolute concentration of these gene transcripts, we also used the $2^{-\Delta\Delta Ct}$ method to compare the expression of these genes in wildtype-infected mice to their *in vitro* expression (154). Moreover, we infected mice with the ΔFCT mutant and ran qRT-PCR using RNA that was isolated from the extracted cNTS, which resulted in the readouts for all genes below the limit of detection (**Appendix II**). Using mice that were infected for 48 hours with wildtype MGAS8232, we observed that *cpa* transcripts were produced at levels significantly higher than *tee18.1* and *prtF2* transcripts (**Figure 9A**). Surprisingly, *cpa* transcripts *in vivo* were expressed in quantities comparable to *in vitro* samples, whereas *tee18.1* and *prtF2* transcription was considerably downregulated *in vivo* (**Figure 9B**). In fact, *tee18.1* and *prtF2* transcripts were identified at levels similar to what was found in mice infected with the ΔFCT mutant, that is to say, transcription was essentially undetectable (**Figure 9A, Appendix II**). These results suggest two possibilities: (1) the pilus and/or PrtF2 are not expressed throughout the course of acute pharyngeal infection, or (2) these proteins are regulated in a manner such that they are not detected 48 hours post-inoculation. To investigate the second possibility that either *tee18.1* and/or *prtF2* would be transcribed during an earlier timepoint of infection, mice were again inoculated with wildtype MGAS8232, but cNTs were harvested for RNA extraction after 24 hours. Similar to the 48 hour infection, *cpa* transcripts were detected at concentrations several thousand fold higher than *tee18.1* and *prtF2* transcripts (**Figure 10A**). Remarkably, transcription of *cpa* was greatly upregulated during a 24 hour infection compared to *in vitro* samples, and was approximately 36 times greater at this shorter timepoint compared to 48 hours into infection (**Figure 10A**, **Figure 10B**, **Appendix III**). From this finding, we can insinuate that *cpa* is expressed during the earlier stages of nasopharyngeal infection, presumably to mediate adhesion to host tissue, and is subsequently downregulated as infection progresses. With regards to *tee18.1* and *prtF2*, transcription was again exceedingly low and markedly downregulated compared to *in vitro* expression, indicating that the major pilus subunit and FBP were not expressed at this timepoint either (**Figure 10A, Figure 10B**). These data suggest that the expression of certain FCT region constituents are regulated in a manner that differs during *in vitro* expression compared to an *in vivo* acute nasopharyngeal infection.

Figure 9. Expression of FCT region genes during a 48 hour murine nasopharyngeal infection.

Transgenic mice expressing HLA-DR4/DQ8 were inoculated with 1×10^8 CFUs of wildtype *S. pyogenes* MGAS8232 and sacrificed 48 hours later. Their cNTs were then harvested and processed for RNA extraction. qRT-PCR was used to measure the mRNA transcription levels of *cpa*, *tee18.1*, and *prtF2* and were then normalized to the housekeeping gene *gyrA*. Data are presented as (**A**) the concentration of mRNA transcripts (*gyrA* denoted by the dotted line at $y = 1$); and (**B**) the average fold change difference of gene transcription in wildtype-infected mice (n = 5) relative to *in vitro* cultures [n = 4 for both early (OD_{600} ~0.2) and late (OD_{600} ~0.8) exponential growth phases]. Bars represent the mean ± SEM; 1-way ANOVA with Tukey's post-hoc test was used to determine statistical significance (***$p<0.001$).
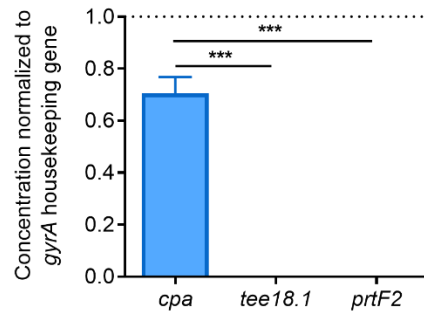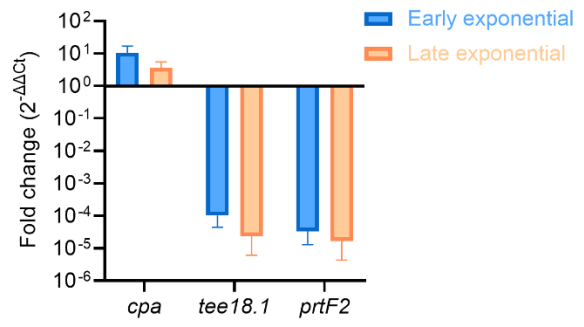
Figure 10. Expression of FCT region genes during a 24 hour murine nasopharyngeal infection.

Transgenic mice expressing HLA-DR4/DQ8 were inoculated with 1×10^8 CFUs of wildtype *S. pyogenes* MGAS8232 and sacrificed 24 hours later. Their cNTs were then harvested and processed for RNA extraction. qRT-PCR was used to measure the mRNA transcription levels of *cpa*, *tee18.1*, and *prtF2* and were then normalized to the housekeeping gene *gyrA*. Data are presented as (**A**) the concentration of mRNA transcripts (*gyrA* denoted by the dotted line at $y = 1$); and (**B**) the average fold change difference of gene transcription in wildtype-infected mice (n = 4) relative to *in vitro* cultures [n = 4 for both early (OD_{600} ~0.2) and late (OD_{600} ~0.8) exponential growth phases]. Bars represent the mean ± SEM; 1-way ANOVA with Tukey's post-hoc test was used to determine statistical significance (***$p<0.001$).

A

Concentration normalized to *gyrA* housekeeping gene

cpa   *tee18.1*   *prtF2*

B

Fold change ($2^{-\Delta\Delta Ct}$)

■ Early exponential
■ Late exponential

cpa   *tee18.1*   *prtF2*
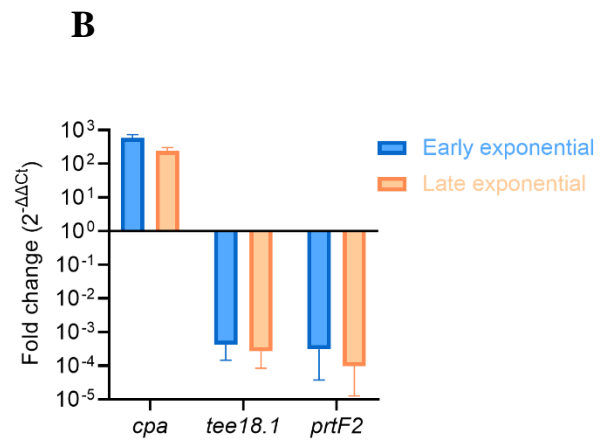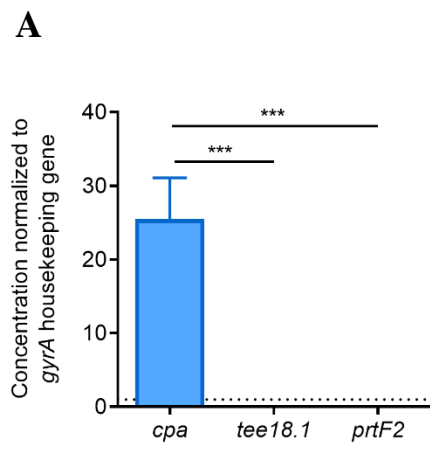
3.6 FCT region genes are not required for infection by *S. pyogenes* in the murine nasopharynx

Ultimately, we sought to ascertain whether deletion of the FCT region would have any bearing on the ability of *S. pyogenes* to stimulate an acute nasopharyngeal infection in mice. Using the previously described murine infection model, cNTs were harvested 48 hours post-inoculation and processed for bacterial enumeration. We discovered that transgenic mice expressing the HLA-MHC II molecule (alleles DQ8 and DR4/DQ8) were infected by the wildtype strain and ΔFCT mutant at comparable magnitudes (**Figure 11**). Taken together, these experiments provide convincing evidence that the FCT region does not play an essential role during acute nasopharyngeal infection by *S. pyogenes* MGAS8232.

Figure 11. Acute nasopharyngeal infection of transgenic mice by *S. pyogenes*. Transgenic mice expressing (**A**) HLA-DQ8, or (**B**) HLA-DR4/DQ8 were infected with ~1×10^8 wildtype *S. pyogenes* MGAS8232 or the ΔFCT mutant for 48 hours before sacrifice. Their cNTs were then harvested and processed for bacterial enumeration. Each data point represents an individual mouse and statistical differences were evaluated by the unpaired t-test ($p>0.05$).

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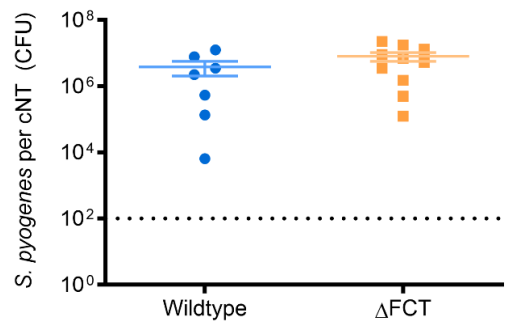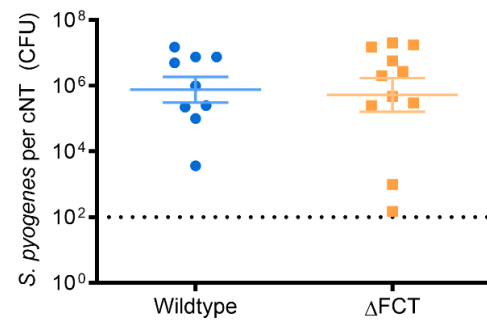
Chapter 4: Discussion

4. Discussion

The evolution of *S. pyogenes* has resulted in a bacterium that is exquisitely adapted to its human host. Its capacity to establish infection, colonize, and persist in the human population can be attributed to its abundance of virulence factors. These molecular elements, and the precise regulation of them, permit *S. pyogenes* to successfully overcome environmental and metabolic challenges, adhere to host tissue, survive intracellularly, and circumvent the host immune system. The FCT region is a genetically diverse genomic locus that encodes such elements, many of which have been implicated in the life cycle of *S. pyogenes* by directly mediating adhesion, invasion, and regulation of locus-specific and chromosomally-distant virulence factors.

In this study, I intended to examine the FCT region of *S. pyogenes* MGAS8232 in order to characterize its properties and functions and how it pertains to pharyngeal infection. I accomplished this aim by generating a mutant with the entire FCT region deleted, rather than single mutants of the investigated genes. However, this presented a challenge to determine which gene(s) were responsible for which results. Therefore, it became necessary to formulate interpretations of our results by carefully analyzing the data with respect to the available literature on how these genes act individually, as well as how they are predicted to behave collectively.

Our first objective was to confirm that *S. pyogenes* possessed the capacity to bind ECM proteins that are found in the nasopharynx (146, 155). Remarkably, we discovered that the ΔFCT mutant had completely lost its ability to bind collagen type IV and had significantly reduced adherence to fibronectin (**Figure 5A, Figure 5B**). The most likely protein candidates responsible for this loss of phenotype are Cpa and PrtF2 as they are the only FCT region constituents known to directly bind ECM proteins (45, 156). With regards to collagen type IV, both Cpa and PrtF2 contain collagen-binding domains; these domains in PrtF2, however, are secluded from the extracellular milieu and are predicted to have a role in conferring structural stability against extremely high temperatures and low pH (109, 157). For this reason, we infer that Cpa is likely mediating adhesion to collagen type IV. Intriguingly, many strains associated with pharyngitis and ARF exhibit weak binding to

collagen type IV, further supporting the notion that a significant portion of strain-specificity originates from within the FCT region. (49, 99). PrtF2 has been described as an important adhesin during infection and colonization by *S. pyogenes* (106, 142). We substantiated this claim by demonstrating a reduction in fibronectin-binding capacity by MGAS8232 upon removal of the FCT region (**Figure 5B**). Although fibronectin-binding decreased substantially, there still remained some residual binding capabilities. This finding was logical, however, as analysis of the MGAS8232 genome illuminated the presence of a putative FBP, *spyM18_0997* (an FbpA-like protein), encoded outside of the FCT region (135). Impressively, for strains that encode *prtF2*, it is typically considered a major factor in determining the tropism and severity of infection for that specific strain, and so, it is possible that in the absence of PrtF2, this putative FBP may be upregulated in an attempt to rescue fibronectin-binding (101, 104, 145, 152, 158, 159). From a regulatory perspective, it would be worthwhile to examine how *S. pyogenes* adapts to the loss of its major FBP(s) both *in vitro* and throughout an *in vivo* infection model. Additional future endeavours can explore the binding capabilities to different collagen types, as well as other ECM proteins, such as laminin, which is a major constituent of the basement membrane (160).

It has previously been demonstrated in a number of serotypes that the FCT region is crucial for binding eukaryotic cells that are associated with different sites of infection (89, 90, 161). We therefore sought to demonstrate this capacity in our strain against the human pharyngeal cell line Detroit-562 and observed that the absence of the FCT region did not impede the ability of *S. pyogenes* to adhere to these cells (**Figure 6**). This result conflicts with previous reports that implicate the FCT region genes in adherence to relevant human tissue (57, 89). It stands to reason that MGAS8232 may not be adept at directly binding human cells without the scaffold of ECM proteins. If this is the case, adhesion in the nasopharynx may transpire through an uncharacterized avenue. That is, it may be necessary for *S. pyogenes* to utilize a mechanism that disrupts the host epithelial barrier to expose the underlying ECM to permit adhesion. This is likely a dynamic process that features several virulence factors such as the hyaluronic acid capsule, which is able to alter cytoskeletal arrangements resulting in an opening of intercellular junctions; the protease SpeB, which

can directly degrade components of intercellular junctions; and/or streptolysin S, which may activate the host protein calpain inducing disruption of cell-to-cell linkages thereby allowing *S. pyogenes* to travel across the epithelial barrier and gain access to ECM proteins (67, 162–164). Conversely, MGAS8232 may indeed mediate adhesion to Detroit-562 cells via its FCT region genes, but the presence of other adhesion molecules, such as the hyaluronic acid capsule, may have rescued the adherence phenotype that we theoretically expected to decrease in the ΔFCT mutant, thereby confounding our results.

Although our adhesion assay did not demonstrate any loss of binding capabilities to Detroit-562 cells, we speculated whether deletion of the FCT region would impact invasion of *S. pyogenes*. We identified an extraordinary phenotype in which invasion by the ΔFCT mutant is completely suppressed (**Figure 7**). This validates previous studies that have implicated the FCT region, and PrtF2 specifically, in invasion (101, 104, 143, 165, 166). Binding interactions between fibronectin and PrtF2 (and homologous FBPs) have been well characterized, and involve the FBP forming an energetically favourable β-zipper with fibronectin (105, 167, 168). Subsequently, *S. pyogenes* FBP can interact with a fragment of fibronectin that is hypothesized to stimulate $\alpha_5\beta_1$ integrin clustering to promote invasion into the host cell (77, 109, 168). Moreover, epithelial cells, such as Detroit-562 cells, have been shown to secrete fibronectin to its apical surface, and together with saliva-derived fibronectin, *S. pyogenes* has ample opportunities to interact with fibronectin and initiate invasion (169–172). Importantly, PrtF2-mediated invasion is likely a major contributor to the intracellular life cycle; in an epidemiological study of *S. pyogenes* isolated from children, 80% of asymptomatic throat carriers were found to be *prtF2*[+] (173). This hypothesis is substantiated by data that identified concurrent presence of *prtF1* and *prtF2* were associated with high erythromycin resistance, suggesting that *S. pyogenes* may internalize into host cells using these FBPs as a means to evade immune detection and antibiotic treatment (159). Coupled with the lack of altered adhesion efficiency from **Figure 6**, our results demonstrate how FCT region-mediated adhesion and invasion are not necessarily linked in *S. pyogenes* as commonly described (101, 145, 174).

In this study, we discovered that during an *in vivo* murine nasopharyngeal infection, *cpa* was unequivocally being expressed both 24, and 48 hours post-inoculation, and that transcription of *cpa* was greater at the 24-hour timepoint (**Figure 9A, Figure 10A**). Accordingly, we can infer that Cpa is critical to the initial attachment of *S. pyogenes* to a susceptible host. Transcripts for *tee18.1*, however, were essentially undetectable (**Figure 9A, Figure 10A**). This result was curious given that Cpa is often, albeit not always, predicted to be the pilus tip adhesin and is typically situated atop the pilus structure (47, 97, 98, 100). Interestingly enough, in MGAS8232 *cpa* contains a VVPTG motif, which is one of the sequences recognized by *srtC2*, a specialized sortase encoded within the FCT region (95, 135). *srtC2* is unique in that its catalytic domain is architecturally flexible thereby allowing it to interact with more variable substrates (96). This feature was likely a consequence of evolution to physically accommodate the strain- and serotype-specific sequences of the major pilus subunit and its ancillary protein(s). Thus, the possibility exists that Cpa is linked directly to the cell wall via an FCT region dedicated sortase. This would suggest that for some strains, it would not benefit *S. pyogenes* to consume energy to produce pili if the associated adhesin is able to be presented in a more efficient manner.

Nevertheless, the lack of *tee18.1* transcript needs to be further investigated since pilus assembly and presentation on *S. pyogenes* M18 serotypes is not well understood (49, 133, 175, 176). This finding entails the possibility that some strains of *S. pyogenes*, such as the one used in this study, do not naturally express the pilus during its infectious life cycle, yet still transcribe the *tee* gene *in vitro* (**Figure 8, Figure 9B, Figure 10B**). A previous analysis of M3 serotypes, which share the FCT-3 variant that MGAS8232 has, determined that the pilus assembly mechanism was effectively nonexistent, and that presentation of the pilus on the bacterial cell was only accomplished by artificially upregulating the genes (133). We can therefore posit that the nature of the pilus may have resulted in some strains, such as MGAS8232, ablating expression of the pilus *in vivo*. This phenomenon could have been caused by the presence of other virulence factors that are sufficient at mediating adhesion, or perhaps as an approach to avoid immune detection. Hence, this finding further illustrates that selective regulation and production of the pilus is a critical factor that is dependant on strain-specific needs *in vivo* during the infectious life cycle. For instance, clinical M3

isolates are regulated in a manner that yields low or negligible levels of pili, which may reduce the adherence of the bacteria from host tissue and allow survival, replication, and dissemination in human blood (133). Disruption of the pilus assembly mechanism in an M1 strain drives the bacteria toward a virulent phenotype, implying that the pilus is active in maintaining colonization rather than promoting disease (98). This concept is further supported by a study that demonstrated how a marked increase in pilus production by an invasive M3 strain resulted in decreased virulence and increased adherence to human epithelial cells (177). The constitutive expression of pili, however, may be detrimental to *S. pyogenes* colonization. M1 serotypes require their pili to establish epithelial infection and colonization, yet due to the immunogenicity of the major pilus subunit, superfluous expression of their pili leads to recognition by phagocytes prompting a neutrophil-driven immune response that elevates *S. pyogenes* killing through neutrophil extracellular entrapment (178). Concerning MGAS8232, immunogold labeling or Western blot using antibodies raised against the major pilus subunit would provide definitive evidence regarding the expression of the pilus both *in vitro* and *in vivo*.

With regards to the expression of *prtF2*, we discovered that no transcripts were being synthesized *in vivo*. Dissimilar to the situation concerning *tee18.1*, we are confident that MGAS8232 is able to transcribe and translate a functional PrtF2 protein, given that *prtF2* transcripts were produced in measurable quantities *in vitro*, and that our fibronectin-binding assay clearly demonstrated that excision of the FCT region attenuates binding to fibronectin (**Figure 5B, Figure 8**). A plausible explanation for not detecting expression of *prtF2 in vivo* revolves around its regulation. Although PrtF2 is heavily implicated in direct adhesion to fibronectin, characterization of this protein has predominantly described its primary function as facilitating invasion into mucosal and skin-associated epithelial cells (101, 102, 152, 159, 174). Therefore, *S. pyogenes* must produce maximal concentrations of this protein when invasion is optimal, which occurs after the bacteria have adhered successfully to their site of infection. Transcription analysis of *prtF2* has shown that the gene is upregulated linearly near the end of exponential phase, and rapidly declines in the early stationary growth phase (101). In relation to our murine nasopharyngeal infection, the precise interval when *prtF2* is being transcribed is currently unknown and may occur

between 24 and 48 hours. To clarify this scenario, a more thorough *in vivo* time course should be performed, one that closely assesses the expression of FCT region genes, including transcription of the FBP-associated regulator *msmR* (137). Alternatively, cNT homogenates can be processed to extract cell surface-linked proteins, including PrtF2, from the bacteria; afterwards, the suspension can be used for immunoblotting against biotinylated fibronectin in order to visualize the presence of translated PrtF2 during an *in vivo* infection model (179).

Collectively, our *in vivo* qRT-PCR analysis reveals that Cpa might be integral during the early stages of pharyngeal infection, whereas the expression status of the pilus and PrtF2 is still unclear. The detection of *tee18.1* and *prtF2* transcripts *in vitro*, however, imply that these genes may be temporally or spatially regulated. Moving forward, we resolved to measure the bacterial burden of *S. pyogenes* during a murine nasopharyngeal infection and anticipated that the ΔFCT mutant would exhibit an attenuated infection phenotype. Using this model, we did not observe lower bacterial counts following inoculation with the ΔFCT mutant, clearly indicating that the FCT region is not essential for MGAS8232 pharyngeal infection (**Figure 11**).

The genetic diversity of the FCT region likely helps aid *S. pyogenes* in its quest to infect and colonize humans successfully. In spite of the genomic diversity, however, the pilus may hold the potential for therapeutic developments. Naturally, since pili are relatively exposed on the bacterial surface, various pilus components are being examined as potential vaccine candidates, the most prominent of which being the major pilus subunit due to its strong immunogenicity (180). This area of research is still quite new and is complicated by the fact that the *tee* gene has massive diversity between *S. pyogenes* strains. Still, the protection conferred by the major pilus subunit is comparable to the protection conferred by the M protein in mice, yet there is significantly more genetic variation within the *emm* gene compared to the *tee* gene (47). One vaccination-based study used *Lactococcus lactis* recombinantly expressing *S. pyogenes* pili as a vector in order to stimulate a protective response against *S. pyogenes* bacterial challenge (176). Specifically, antibodies raised against MGAS8232 pili that were expressed on *L. lactis* inhibited adhesion by *S. pyogenes*

to skin in rabbits, while mice subjected to intranasal immunization were protected against nasopharyngeal infection (176). Even accounting for the diversity in *tee*, estimates suggest that a pilus-based vaccine that incorporates protective epitopes from twelve of the most common major pilus subunits would confer protection against >90% of *S. pyogenes* strains in the United States of America and the European Union (76).

S. pyogenes remains a significant burden on human health, and developing a thorough understanding of its asymptomatic and pathogenic life cycle is vital to prevent and treat complications of infection. The FCT region is a highly recombinatorial locus within the genome of all *S. pyogenes* strains, and encodes numerous genes that may contribute to tissue tropism as well as the propensity to cause invasive disease. In this study, we characterized the binding capacity of *S. pyogenes* to collagen type IV and fibronectin and determined that the FCT region is indeed pertinent to binding these ECM proteins. We additionally characterized the adherence and invasion capabilities to pharyngeal tissue and have demonstrated that adhesion is not influenced by the FCT region, whereas invasion is greatly dependent on its presence. Finally, our work suggests that although the FCT region is not required to establish nasopharyngeal infection in mice, thereby nullifying our hypothesis, we provide transcriptional evidence that *cpa* may have a minor role during the early stages of pharyngeal infection. To conclude, further exploration of this diverse genomic locus holds the potential to comprehensively evaluate the life cycle of *S. pyogenes*, which in turn can provide insight on how to combat this globally-prevalent pathogen.

References

1. Lancefield RC. 1928. THE ANTIGENIC COMPLEX OF *STREPTOCOCCUS HAEMOLYTICUS* : I. DEMONSTRATION OF A TYPE-SPECIFIC SUBSTANCE IN EXTRACTS OF *STREPTOCOCCUS HAEMOLYTICUS*. J Exp Med 47:91–103.

2. Bessen DE. 2016. Molecular Basis of Serotyping and the Underlying Genetic Organization of *Streptococcus pyogenes, Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

3. Beres SB, Musser JM. 2007. Contribution of exogenous genetic elements to the group A *Streptococcus* metagenome. PLoS One 2:e800.

4. Nasser W, Beres SB, Olsen RJ, Dean MA, Rice KA, Long SW, Kristinsson KG, Gottfredsson M, Vuopio J, Raisanen K, Caugant DA, Steinbakk M, Low DE, McGeer A, Darenberg J, Henriques-Normark B, Van Beneden CA, Hoffmann S, Musser JM. 2014. Evolutionary pathway to increased virulence and epidemic group A *Streptococcus* disease derived from 3,615 genome sequences. Proc Natl Acad Sci 111:E1768–E1776.

5. Bessen DE, McShan WM, Nguyen S V., Shetty A, Agrawal S, Tettelin H. 2015. Molecular epidemiology and genomics of group A *Streptococcus*. Infect Genet Evol 33:393–418.

6. Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, Sriprakash KS, Sanderson-Smith ML, Nizet V. 2014. Disease manifestations and pathogenic mechanisms of group A *Streptococcus*. Clin Microbiol Rev 27:264–301.

7. Hurst JR, Kasper KJ, Sule AN, McCormick JK. 2018. Streptococcal pharyngitis and rheumatic heart disease: the superantigen hypothesis revisited. Infect Genet Evol 61:160–175.

8. Nitsche-Schmitz DP, Chhatwal GS. Host–Pathogen Interactions in Streptococcal Immune Sequelae.

9. Sanyahumbi AS, Colquhoun S, Wyber R, Carapetis JR. 2016. Global Disease Burden of Group A *Streptococcus*, p. 661–704. *In* Ferretti, JJ, Stevens, DL,

Fischetti, VA (eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

10. Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–694.

11. Wessels MR. 2011. Streptococcal Pharyngitis. N Engl J Med 364:648–655.

12. Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, Sriprakash KS, Sanderson-Smith ML, Nizet V. 2014. Disease manifestations and pathogenic mechanisms of Group A *Streptococcus*. Clin Microbiol Rev 27:264–301.

13. McCormick JK, Yarwood JM, Schlievert PM. 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol 55:77–104.

14. Nelson GE, Pondo T, Toews K-A, Farley MM, Lindegren M Lou, Lynfield R, Aragon D, Zansky SM, Watt JP, Cieslak PR, Angeles K, Harrison LH, Petit S, Beall B, Van Beneden CA. 2016. Epidemiology of Invasive Group A Streptococcal Infections in the United States, 2005-2012. Clin Infect Dis 63:478–86.

15. Stevens DL. 1995. Streptococcal toxic-shock syndrome: spectrum of disease, pathogenesis, and new concepts in treatment. Emerg Infect Dis 1:69–78.

16. Bartter T, Dascal A. 1990. The "toxic strep syndrome": severe group A streptococcal disease in the absence of bacteremia. Rev Infect Dis 12:1204–1205.

17. Schmitz M, Roux X, Huttner B, Pugin J. 2018. Streptococcal toxic shock syndrome in the intensive care unit. Ann Intensive Care 8:88.

18. Madsen MB, Hjortrup PB, Hansen MB, Lange T, Norrby-Teglund A, Hyldegaard O, Perner A. 2017. Immunoglobulin G for patients with necrotising soft tissue infection (INSTINCT): a randomised, blinded, placebo-controlled trial. Intensive Care Med 43:1585–1593.

19. Kadri SS, Swihart BJ, Bonne SL, Hohmann SF, Hennessy L V., Louras P, Evans HL, Rhee C, Suffredini AF, Hooper DC, Follmann DA, Bulger E, Danner RL. 2016. Impact of Intravenous Immunoglobulin on Survival in Necrotizing Fasciitis with Vasopressor-dependent Shock: A Propensity-Score Matched Analysis from 130 US Hospitals. Clin Infect Dis 64:ciw871.

20. Misiakos EP, Bagias G, Patapis P, Sotiropoulos D, Kanavidis P, Machairas A. 2014. Current concepts in the management of necrotizing fasciitis. Front Surg 1:36.

21. Dahl PR, Perniciaro C, Holmkvist KA, O'Connor MI, Gibson LE. 2002. Fulminant group A streptococcal necrotizing fasciitis: clinical and pathologic findings in 7 patients. J Am Acad Dermatol 47:489–92.

22. Gewitz MH, Baltimore RS, Tani LY, Sable CA, Shulman ST, Carapetis J, Remenyi B, Taubert KA, Bolger AF, Beerman L, Mayosi BM, Beaton A, Pandian NG, Kaplan EL, American Heart Association Committee on Rheumatic Fever, Endocarditis and KD of the C on CD in the Y. 2015. Revision of the Jones Criteria for the diagnosis of acute rheumatic fever in the era of Doppler echocardiography: a scientific statement from the American Heart Association. Circulation 131:1806–18.

23. Guilherme L, Ramasawmy R, Kalil J. 2007. Rheumatic fever and rheumatic heart disease: Genetics and pathogenesis. Scand J Immunol.

24. Organization WH, Others. 2005. Epidemiology and management of common skin diseases in children in developing countries. Geneva World Heal Organ.

25. Bessen DE. 2016. Tissue tropisms in group A *Streptococcus*: what virulence factors distinguish pharyngitis from impetigo strains? Curr Opin Infect Dis 29:295–303.

26. Efstratiou A, Lamagni T. 2016. Epidemiology of *Streptococcus pyogenesStreptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

27. Martin J. 2016. The *Streptococcus pyogenes* Carrier State, p. 587–600. *In* Ferretti, JJ, Stevens, DL, Fischetti, VA (eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

28. Shaikh N, Leonard E, Martin JM. 2010. Prevalence of streptococcal pharyngitis and streptococcal carriage in children: a meta-analysis. Pediatrics 126:e557–e564.

29. Schwartz RH, Wientzen RL, Pedreira F, Feroli EJ, Mella GW, Guandolo VL. 1981. Penicillin V for Group A Streptococcal Pharyngotonsillitis: A Randomized Trial of Seven vs Ten Days' Therapy. JAMA J Am Med Assoc 246:1790–1795.

30. DeMuri GP, Wald ER. 2014. The Group A Streptococcal Carrier State Reviewed: Still an Enigma. J Pediatric Infect Dis Soc 3:336–342.

31. Johnson DR, Kurlan R, Leckman J, Kaplan EL. 2010. The human immune response to streptococcal extracellular antigens: clinical, diagnostic, and potential pathogenetic implications. Clin Infect Dis2010/01/14. 50:481–490.

32. Shannon BA, McCormick JK, Schlievert PM. 2019. Toxins and Superantigens of Group A Streptococci. Microbiol Spectr 7.

33. Hynes W, Sloan M. 2016. Secreted Extracellular Virulence Factors, *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

34. Miyoshi-Akiyama T, Abe A, Kato H, Kawahara K, Narimatsu H, Uchiyama T. 1995. DNA sequencing of the gene encoding a bacterial superantigen, *Yersinia pseudotuberculosis*-derived mitogen (YPM), and characterization of the gene product, cloned YPM. J Immunol1995/05/15. 154:5228–5234.

35. Huber BT, Hsu PN, Sutkowski N. 1996. Virus-encoded superantigens. Microbiol Rev1996/09/01. 60:473–482.

36. Zhao Y, Li Z, Drozd SJ, Guo Y, Mourad W, Li H. 2004. Crystal structure of *Mycoplasma arthritidis* mitogen complexed with HLA-DR1 reveals a novel superantigen fold and a dimerized superantigen-MHC complex. Structure2004/02/14. 12:277–288.

37. Proft T, Fraser JD. 2003. Bacterial superantigens. Clin Exp Immunol 133:299–306.

38. McCormick JK, Schlievert PM. 2001. Toxins and superantigens of group A streptococciGram-positive pathogens. American Society for Microbiology.

39. Fleischer B, Necker A, Leget C, Malissen B, Romagne F. 1996. Reactivity of mouse T-cell hybridomas expressing human Vbeta gene segments with staphylococcal and streptococcal superantigens. Infect Immun 64:987–994.

40. Proft T, Fraser JD. 2016. Streptococcal Superantigens: Biological properties and potential role in disease, p. 445–486. *In* Ferretti, JJ, Stevens, DL, Fischetti, VA (eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

41. Kasper KJ, Zeppa JJ, Wakabayashi AT, Xu SX, Mazzuca DM, Welch I, Baroja ML, Kotb M, Cairns E, Cleary PP, Haeryfar SM, McCormick JK. 2014. Bacterial superantigens promote acute nasopharyngeal infection by *Streptococcus pyogenes* in a human MHC Class II-dependent manner. PLoS Pathog 10:e1004155.

42. Zeppa JJ, Kasper KJ, Mohorovic I, Mazzuca DM, Haeryfar SMMM, McCormick JK. 2017. Nasopharyngeal infection by *Streptococcus pyogenes* requires superantigen-responsive Vβ-specific T cells. Proc Natl Acad Sci 114:10226–10231.

43. Zeppa JJ, Kasper KJ, Mohorovic I, Mazzuca DM, Haeryfar SMM, McCormick JK. 2017. Nasopharyngeal infection by *Streptococcus pyogenes* requires superantigen-responsive Vβ-specific T cells. Proc Natl Acad Sci 114:10226–10231.

44. Rohde M, Cleary PP. 2016. Adhesion and invasion of *Streptococcus pyogenes* into host cells and clinical relevance of intracellular streptococci *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

45. Ryan PA, Juncosa B. 2016. Group A Streptococcal Adherence, p. 521–545. *In* Ferretti, JJ, Stevens, DL, Fischetti, VA (eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

46. Simpson WA, Beachey EH. 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect Immun 39:275–279.

47. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AGO, Maggi T, Taddei AR, Grandi G, Telford JL. 2005. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci 102:15641–15646.

48. Hasty DL, Ofek I, Courtney HS, Doyle RJ. 1992. Multiple adhesins of streptococci. Infect Immun 60:2147–52.

49. Kratovac Z, Manoharan A, Luo F, Lizano S, Bessen DE. 2007. Population genetics and linkage analysis of loci within the FCT region of *Streptococcus pyogenes*. J Bacteriol 189:1299–1310.

50. Metzgar D, Zampolli A. 2011. The M protein of group A *Streptococcus* is a key

virulence factor and a clinically relevant strain identification marker. Virulence 2:402–12.

51. Lancefield RC. 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. J Exp Med 110:271–92.

52. Beall B, Facklam R, Thompson T. 1996. Sequencing emm-specific PCR products for routine and accurate typing of group A streptococci. J Clin Microbiol 34:953–958.

53. Pérez-Caballero D, Albertí S, Vivanco F, Sánchez-Corral P, Rodríguez De Córdoba S. 2000. Assessment of the interaction of human complement regulatory proteins with group A *Streptococcus*. Identification of a high-affinity group A *Streptococcus* binding site in FHL-1. Eur J Immunol 30:1243–1253.

54. Fischetti VA. 2016. M Protein and Other Surface Proteins on Streptococci*Streptococcus pyogenes* : Basic Biology to Clinical Manifestations.

55. Whitnack E, Beachey EH. 1985. Inhibition of complement-mediated opsonization and phagocytosis of *Streptococcus pyogenes* by D fragments of fibrinogen and fibrin bound to cell surface M protein. J Exp Med 162:1983–1997.

56. Courtney HS, Ofek I, Hasty DL. 1997. M protein mediated adhesion of M type 24 *Streptococcus pyogenes* stimulates release of interleukin-6 by HEp-2 tissue culture cells. FEMS Microbiol Lett 151:65–70.

57. Ryan PA, Pancholi V, Fischetti VA. 2001. Group A streptococci bind to mucin and human pharyngeal cells through sialic acid-containing receptors. Infect Immun 69:7402–7412.

58. Frick IM, Schmidtchen A, Sjöbring U. 2003. Interactions between M proteins of *Streptococcus pyogenes* and glycosaminoglycans promote bacterial adhesion to host cells. Eur J Biochem 270:2303–2311.

59. Berkower C, Ravins M, Moses AE, Hanski E. 1999. Expression of different group A streptococcal M proteins in an isogenic background demonstrates diversity in adherence to and invasion of eukaryotic cells. Mol Microbiol 31:1463–75.

60. Feito MJ, Sánchez A, Oliver MA, Pérez-Caballero D, Rodríguez de Córdoba S, Albertí S, Rojo JM. 2007. Membrane cofactor protein (MCP, CD46) binding to clinical isolates of *Streptococcus pyogenes*: Binding to M type 18 strains is

independent of Emm or Enn proteins. Mol Immunol 44:3571–3579.

61.   Cunningham MW. 2000. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13:470–511.

62.   McGregor KF, Spratt BG, Kalia A, Bennett A, Bilek N, Beall B, Bessen DE. 2004. Multilocus sequence typing of *Streptococcus pyogenes* representing most known emm types and distinctions among subpopulation genetic structures. J Bacteriol 186:4285–4294.

63.   Bessen DE, Sotir CM, Readdy TL, Hollingshead SK. 1996. Genetic Correlates of Throat and Skin Isolates of Group A Streptococci. J Infect Dis 173:896–900.

64.   Wannamaker LW. 1970. Differences between Streptococcal Infections of the Throat and of the Skin. N Engl J Med 282:78–85.

65.   Crater DL, Van de Rijn I. 1995. Hyaluronic acid synthesis operon (has) expression in group A streptococci. J Biol Chem 270:18452–18458.

66.   Wessels MR, Bronze MS. 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. Proc Natl Acad Sci U S A1994/12/06. 91:12238–12242.

67.   Cywes C, Stamenkovic I, Wessels MR. 2000. CD44 as a receptor for colonization of the pharynx by group A *Streptococcus*. J Clin Invest 106:995–1002.

68.   Schrager HM, Albertí S, Cywes C, Dougherty GJ, Wessels MR. 1998. Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. J Clin Invest 101:1708–1716.

69.   Gryllos I, Cywes C, Shearer MH, Cary M, Kennedy RC, Wessels MR. 2001. Regulation of capsule gene expression by group A *Streptococcus* during pharyngeal colonization and invasive infection. Mol Microbiol2001/10/27. 42:61–74.

70.   Lynskey NN, Goulding D, Gierula M, Turner CE, Dougan G, Edwards RJ, Sriskandan S. 2013. RocA truncation underpins hyper-encapsulation, carriage longevity and transmissibility of serotype M18 group A streptococci. PLoS Pathog 9:e1003842.

71.   Hollands A, Pence MA, Timmer AM, Osvath SR, Turnbull L, Whitchurch CB,

Walker MJ, Nizet V. 2010. Genetic Switch to Hypervirulence Reduces Colonization Phenotypes of the Globally Disseminated Group A *Streptococcus* M1T1 Clone. J Infect Dis 202:11–19.

72. Bessen DE, Kalia A. 2002. Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. Infect Immun 70:1159–1167.

73. Feil EJ, Holmes EC, Bessen DE, Chan MS, Day NP, Enright MC, Goldstein R, Hood DW, Kalia A, Moore CE, Zhou J, Spratt BG. 2001. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. Proc Natl Acad Sci U S A 98:182–7.

74. Kalia A, Enright MC, Spratt BG, Bessen DE. 2001. Directional gene movement from human-pathogenic to commensal-like streptococci. Infect Immun 69:4858–69.

75. Ibrahim J, Eisen JA, Jospin G, Coil DA, Khazen G, Tokajian S. 2016. Genome Analysis of *Streptococcus pyogenes* Associated with Pharyngitis and Skin Infections. PLoS One 11:e0168177.

76. Falugi F, Zingaretti C, Pinto V, Mariani M, Amodeo L, Manetti AGO, Capo S, Musser JM, Orefici G, Margarit I, Telford JL, Grandi G, Mora M. 2008. Sequence Variation in Group A *Streptococcus* Pili and Association of Pilus Backbone Types with Lancefield T Serotypes. J Infect Dis 198:1834–1841.

77. Ramachandran V, McArthur JD, Behm CE, Gutzeit C, Dowton M, Fagan PK, Towers R, Currie B, Sriprakash KS, Walker MJ. 2004. Two distinct genotypes of prtF2, encoding a fibronectin binding protein, and evolution of the gene family in *Streptococcus pyogenes*. J Bacteriol 186:7601–9.

78. Linke C. 2010. Structural characterisation of adhesins from the human pathogen *Streptococcus pyogenes*.

79. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. 2006. Pili in Gram-positive pathogens. Nat Rev Microbiol 4:509–519.

80. Margarit y Ros I. 2016. *Streptococcus pyogenes* Pili, *Streptococcus pyogenes* : Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

81. Krishnan V. 2015. Pilins in gram-positive bacteria: A structural perspective. IUBMB Life 67:533–543.

82. van Sorge NM, Cole JN, Kuipers K, Henningham A, Aziz RK, Kasirer-Friede A, Lin L, Berends ETM, Davies MR, Dougan G, Zhang F, Dahesh S, Shaw L, Gin J, Cunningham M, Merriman JA, Hütter J, Lepenies B, Rooijakkers SHM, Malley R, Walker MJ, Shattil SJ, Schlievert PM, Choudhury B, Nizet V. 2014. The classical lancefield antigen of group a *Streptococcus* is a virulence determinant with implications for vaccine design. Cell Host Microbe2014/06/13. 15:729–740.

83. Lancefield RC, Dole VP. 1946. THE PROPERTIES OF T ANTIGENS EXTRACTED FROM GROUP A HEMOLYTIC STREPTOCOCCI. J Exp Med 84:449–471.

84. Kang HJ, Coulibaly F, Clow F, Proft T, Baker EN. 2007. Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. Science 318:1625–1628.

85. Kang HJ, Baker EN. 2009. Intramolecular isopeptide bonds give thermodynamic and proteolytic stability to the major pilin protein of *Streptococcus pyogenes*. J Biol Chem 284:20729–20737.

86. Echelman DJ, Alegre-Cebollada J, Badilla CL, Chang C, Ton-That H, Fernández JM. 2016. CnaA domains in bacterial pili are efficient dissipaters of large mechanical shocks. Proc Natl Acad Sci U S A 113:2490–2495.

87. Alegre-Cebollada J, Badilla CL, Fernández JM. 2010. Isopeptide bonds block the mechanical extension of pili in pathogenic *Streptococcus pyogenes*. J Biol Chem 285:11235–11242.

88. Wang B, Xiao S, Edwards SA, Grä F. 2013. Isopeptide Bonds Mechanically Stabilize Spy0128 in Bacterial Pili. Biophysj 104:2051–2057.

89. Abbot EL, Smith WD, Siou GPS, Chiriboga C, Smith RJ, Wilson JA, Hirst BH, Kehoe MA. 2007. Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. Cell Microbiol 9:1822–1833.

90. Manetti AGO, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, Gambellini G, Bensi G, Mora M, Edwards AM, Musser JM, Graviss EA, Telford JL, Grandi G, Margarit I. 2007. *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. Mol Microbiol 64:968–983.

91. Becherelli M, Manetti AGO, Buccato S, Viciani E, Ciucchi L, Mollica G, Grandi G, Margarit I. 2012. The ancillary protein 1 of *Streptococcus pyogenes* FCT-1 pili mediates cell adhesion and biofilm formation through heterophilic as well as homophilic interactions. Mol Microbiol 83:1035–1047.

92. Pointon JA, Smith WD, Saalbach G, Crow A, Kehoe MA, Banfield MJ. 2010. A highly unusual thioester bond in a pilus adhesin is required for efficient host cell interaction. J Biol Chem 285:33858–66.

93. Danne C, Dramsi S. 2012. Pili of Gram-positive bacteria: roles in host colonization.

94. Hendrickx APA, Budzik JM, Oh S-Y, Schneewind O. 2011. Architects at the bacterial surface — sortases and the assembly of pili with isopeptide bonds. Nat Rev Microbiol 9:166–176.

95. Barnett TC, Patel AR, Scott JR. 2004. A novel sortase, SrtC2, from *Streptococcus pyogenes* anchors a surface protein containing a QVPTGV motif to the cell wall. J Bacteriol 186:5865–75.

96. Kang HJ, Coulibaly F, Proft T, Baker EN. 2011. Crystal structure of Spy0129, a *Streptococcus pyogenes* class B sortase involved in pilus assembly. PLoS One 6:e15969.

97. Quigley BR, Zähner D, Hatkoff M, Thanassi DG, Scott JR. 2009. Linkage of T3 and Cpa pilins in the *Streptococcus pyogenes* M3 pilus. Mol Microbiol 72:1379–1394.

98. Nakata M, Köller T, Moritz K, Ribardo D, Jonas L, McIver KS, Sumitomo T, Terao Y, Kawabata S, Podbielski A, Kreikemeyer B. 2009. Mode of expression and functional characterization of FCT-3 pilus region-encoded proteins in *Streptococcus pyogenes* serotype M49. Infect Immun 77:32–44.

99. Kreikemeyer B, Nakata M, Oehmcke S, Gschwendtner C, Normann J, Podbielski A. 2005. *Streptococcus pyogenes* collagen type I-binding Cpa surface protein. Expression profile, binding characteristics, biological functions, and potential clinical impact. J Biol Chem 280:33228–33239.

100. Linke-Winnebeck C, Paterson NG, Young PG, Middleditch MJ, Greenwood DR, Witte G, Baker EN. 2014. Structural model for covalent adhesion of the

Streptococcus pyogenes pilus through a thioester bond. J Biol Chem 289:177–189.

101. Kreikemeyer B, Oehmcke S, Nakata M, Hoffrogge R, Podbielski A. 2004. *Streptococcus pyogenes* fibronectin-binding protein F2: expression profile, binding characteristics, and impact on eukaryotic cell interactions. J Biol Chem 279:15850–15859.

102. Ozeri V, Rosenshine I, Mosher DF, Fässler R, Hanski E. 1998. Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. Mol Microbiol 30:625–37.

103. Jeng A, Sakota V, Li Z, Datta V, Beall B, Nizet V. 2003. Molecular Genetic Analysis of a Group A *Streptococcus* Operon Encoding Serum Opacity Factor and a Novel Fibronectin-Binding Protein, SfbX. J Bacteriol 185:1208–1217.

104. Gorton D, Norton R, Layton R, Smith H, Ketheesan N. 2005. Presence of fibronectin-binding protein gene prtF2 in invasive group A streptococci in tropical Australia is associated with increased internalisation efficiency.

105. Talay SR, Zock A, Rohde M, Molinari G, Oggioni M, Pozzi G, Guzman CA, Chhatwal GS. 2000. Co-operative binding of human fibronectin to SfbI protein triggers streptococcal invasion into respiratory epithelial cells. Cell Microbiol 2:521–535.

106. Jaffe J, Natanson-Yaron S, Caparon MG, Hanski E. 1996. Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. Mol Microbiol 21:373–384.

107. Bessen DE, Kumar N, Hall GS, Riley DR, Luo F, Lizano S, Ford CN, McShan WM, Nguyen S V, Dunning Hotopp JC, Tettelin H. 2011. Whole-genome association study on tissue tropism phenotypes in group A *Streptococcus*. J Bacteriol 193:6651–63.

108. Gibson CM, Caparon MG. 1996. Insertional inactivation of *Streptococcus pyogenes* sod suggests that prtF is regulated in response to a superoxide signal. J Bacteriol 178:4688–4695.

109. Amelung S, Nerlich A, Rohde M, Spellerberg B, Cole JN, Nizet V, Chhatwal GS, Talay SR. 2011. The FbaB-type fibronectin-binding protein of *Streptococcus pyogenes* promotes specific invasion into endothelial cells. Cell Microbiol

13:1200–1211.

110. Nakagawa I, Nakata M, Kawabata S, Hamada S. 2001. Cytochrome *c* -mediated caspase-9 activation triggers apoptosis in *Streptococcus pyogenes* -infected epithelial cells. Cell Microbiol 3:395–405.

111. Loh JMS, Tsai J-YC, Proft T. 2017. The ability of Group A *Streptococcus* to adhere to immortalized human skin versus throat cell lines does not reflect their predicted tissue tropism. Clin Microbiol Infect.

112. Froehlich BJ, Bates C, Scott JR. 2009. *Streptococcus pyogenes* CovRS mediates growth in iron starvation and in the presence of the human cationic antimicrobial peptide LL-37. J Bacteriol 191:673–677.

113. Dalton TL, Scott JR. 2004. CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. J Bacteriol 186:3928–3937.

114. Gryllos I, Levin JC, Wessels MR. 2003. The CsrR/CsrS two-component system of group A *Streptococcus* responds to environmental Mg2+. Proc Natl Acad Sci U S A 100:4227–32.

115. Engleberg NC, Heath A, Miller A, Rivera C, DiRita VJ. 2001. Spontaneous Mutations in the CsrRS Two-Component Regulatory System of *Streptococcus pyogenes* Result in Enhanced Virulence in a Murine Model of Skin and Soft Tissue Infection. J Infect Dis 183:1043–1054.

116. Stock AM, Robinson VL, Goudreau PN. 2000. Two-component signal transduction. Annu Rev Biochem 69:183–215.

117. Gusa AA, Gao J, Stringer V, Churchward G, Scott JR. 2006. Phosphorylation of the group A Streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. J Bacteriol 188:4620–6.

118. Graham MR, Smoot LM, Migliaccio CAL, Virtaneva K, Sturdevant DE, Porcella SF, Federle MJ, Adams GJ, Scott JR, Musser JM. 2002. Virulence control in group A *Streptococcus* by a two-component gene regulatory system: Global expression profiling and in vivo infection modeling. Proc Natl Acad Sci 99:13855–13860.

119. Vega LA, Malke H, McIver KS. 2016. Virulence-Related Transcriptional Regulators of *Streptococcus pyogenes*, p. 337–404. *In* Ferretti, JJ, Stevens, DL,

Fischetti, VA (eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center.

120. Cole JN, Barnett TC, Nizet V, Walker MJ. 2011. Molecular insight into invasive group A streptococcal disease. Nat Rev Microbiol2011/09/17. 9:724–736.

121. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM. 2006. Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. PLoS Pathog 2:e5.

122. Aziz RK, Kotb M. 2008. Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. Emerg Infect Dis2008/10/02. 14:1511–1517.

123. Bao YJ, Liang Z, Mayfield JA, Lee SW, Ploplis VA, Castellino FJ. 2015. CovRS-regulated transcriptome analysis of a hypervirulent M23 strain of group A *Streptococcus pyogenes* provides new insights into virulence determinants. J Bacteriol 197:3191–3205.

124. Kreikemeyer B, McIver KS, Podbielski A. 2003. Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. Trends Microbiol2003/06/05. 11:224–232.

125. McIver KS. 2009. Stand-Alone Response Regulators Controlling Global Virulence Networks in *Streptococcus pyogenes*, p. 103–119. *In* Bacterial Sensing and Signaling. KARGER, Basel.

126. Bessen DE, Manoharan A, Luo F, Wertz JE, Robinson DA. 2005. Evolution of transcription regulatory genes is linked to niche specialization in the bacterial pathogen *Streptococcus pyogenes*. J Bacteriol 187:4163–4172.

127. Virtaneva K, Porcella SF, Graham MR, Ireland RM, Johnson CA, Ricklefs SM, Babar I, Parkins LD, Romero RA, Corn GJ, Gardner DJ, Bailey JR, Parnell MJ, Musser JM. 2005. Longitudinal analysis of the group A *Streptococcus* transcriptome in experimental pharyngitis in cynomolgus macaques. Proc Natl Acad Sci U S A2005/06/16. 102:9014–9019.

128. Granok AB, Parsonage D, Ross RP, Caparon MG. 2000. The RofA binding site in *Streptococcus pyogenes* is utilized in multiple transcriptional pathways. J Bacteriol 182:1529–1540.

129. Podbielski A, Woischnik M, Leonard BAB, Schmidt K-H. 1999. Characterization

of nra, a global negative regulator gene in group A streptococci. Mol Microbiol 31:1051–1064.

130. Kreikemeyer B, Podbielski A, Beckert S, Braun-Kiewnick A. 2002. Group A streptococcal RofA-type global regulators exhibit a strain-specific genomic presence and regulation pattern. Microbiology 148:1501–1511.

131. Kreikemeyer B, Nakata M, Köller T, Hildisch H, Kourakos V, Standar K, Kawabata S, Glocker MO, Podbielski A. 2007. The *Streptococcus pyogenes* serotype M49 Nra-Ralp3 transcriptional regulatory network and its control of virulence factor expression from the novel eno ralp3 epf sagA pathogenicity region. Infect Immun 75:5698–5710.

132. Luo F, Lizano S, Bessen DE. 2008. Heterogeneity in the polarity of Nra regulatory effects on streptococcal pilus gene transcription and virulence. Infect Immun 76:2490–2497.

133. Calfee G, Danger JL, Jain I, Miller EW, Sarkar P, Tjaden B, Kreikemeyer B, Sumby P. 2018. Identification and Characterization of Serotype-Specific Variation in Group A *Streptococcus* Pilus Expression. Infect Immun 86:e00792-17.

134. Beres SB, Sylva GL, Barbian KD, Lei B, Hoff JS, Mammarella ND, Liu MY, Smoot JC, Porcella SF, Parkins LD, Campbell DS, Smith TM, McCormick JK, Leung DY, Schlievert PM, Musser JM. 2002. Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc Natl Acad Sci U S A 99:10078–10083.

135. Smoot JC, Barbian KD, Van Gompel JJ, Smoot LM, Chaussee MS, Sylva GL, Sturdevant DE, Ricklefs SM, Porcella SF, Parkins LD, Beres SB, Campbell DS, Smith TM, Zhang Q, Kapur V, Daly JA, Veasy LG, Musser JM. 2002. Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. Proc Natl Acad Sci U S A 99:4668–4673.

136. Beckert S, Kreikemeyer B, Podbielski A. 2001. Group A streptococcal rofA gene is involved in the control of several virulence genes and eukaryotic cell attachment and internalization. Infect Immun 69:534–537.

137. Nakata M, Podbielski A, Kreikemeyer B. 2005. MsmR, a specific positive

regulator of the *Streptococcus pyogenes* FCT pathogenicity region and cytolysin-mediated translocation system genes. Mol Microbiol 57:786–803.

138. Biswas I, Gruss A, Ehrlich SD, Maguin E. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol 175:3628–3635.

139. Nooh MM, El-Gengehi N, Kansal R, David CS, Kotb M. 2007. HLA transgenic mice provide evidence for a direct and dominant role of HLA class II variation in modulating the severity of streptococcal sepsis. J Immunol 178:3076–3083.

140. Zeppa JJ, Wakabayashi AT, Kasper KJ, Xu SX, Haeryfar SMM, McCormick JK. 2016. Nasopharyngeal infection of mice with *Streptococcus pyogenes* and in vivo detection of superantigen activity. Methods Mol Biol 1396:95–107.

141. LeBleu VS, MacDonald B, Kalluri R. 2007. Structure and Function of Basement Membranes. Exp Biol Med 232:1121–1129.

142. Hanski E, Caparont M. 1992. A *Streptococcus Streptococcus pyogenes* (fibronectin receptor/microbial adherence/virulence).

143. Terao Y, Kawabata S, Nakata M, Nakagawa I, Hamada S. 2002. Molecular characterization of a novel fibronectin-binding protein of *Streptococcus pyogenes* strains isolated from toxic shock-like syndrome patients. J Biol Chem 277:47428–35.

144. Nyberg P, Sakai T, Cho KH, Caparon MG, Fä Ssler R, Bjö Rck L. 2004. Interactions with fibronectin attenuate the virulence of *Streptococcus pyogenes*. EMBO J 23:2166–2174.

145. Hymes JP, Klaenhammer TR. 2016. Stuck in the Middle: Fibronectin-Binding Proteins in Gram-Positive Bacteria. Front Microbiol 7:1504.

146. Kosmehl H, Berndt A, Strassburger S, Borsi L, Rousselle P, Mandel U, Hyckel P, Zardi L, Katenkamp D. 1999. Distribution of laminin and fibronectin isoforms in oral mucosa and oral squamous cell carcinoma. Br J Cancer 81:1071–1079.

147. Broudy TB, Pancholi V, Fischetti VA. 2001. Induction of lysogenic bacteriophage and phage-associated toxin from group a streptococci during coculture with human pharyngeal cells. Infect Immun 69:1440–3.

148. Broudy TB, Pancholi V, Fischetti VA. 2002. The in vitro interaction of *Streptococcus pyogenes* with human pharyngeal cells induces a phage-encoded

extracellular DNase. Infect Immun 70:2805–11.

149. Barthelson R, Mobasseri A, Zopf D, Simon P. 1998. Adherence of *Streptococcus* pneumoniae to respiratory epithelial cells is inhibited by sialylated oligosaccharides. Infect Immun 66:1439–44.

150. Okada N, Watarai M, Ozeri V, Hanski E, Caparon M, Sasakawa C. 1997. A Matrix Form of Fibronectin Mediates Enhanced Binding of *Streptococcus pyogenes* to Host Tissue. J Biol Chem 272:26978–26984.

151. Courtney HS, Ofek I, Simpson WA, Hasty DL, Beachey EH. 1986. Binding of *Streptococcus pyogenes* to soluble and insoluble fibronectin. Infect Immun 53:454–9.

152. Delvecchio A, Currie BJ, McArthur JD, Walker MJ, Sriprakash KS. 2002. *Streptococcus pyogenes* prtFII, but not sfbI, sfbII or fbp54, is represented more frequently among invasive-disease isolates of tropical Australia. Epidemiol Infect 128:391–396.

153. Smoot JC, Korgenski EK, Daly JA, Veasy LG, Musser JM. 2002. Molecular analysis of group A *Streptococcus* type emm18 isolates temporally associated with acute rheumatic fever outbreaks in Salt Lake City, Utah. J Clin Microbiol2002/05/01. 40:1805–1810.

154. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–8.

155. Simpson WA, Beachey EH. 1983. Adherence of Group A Streptococci to Fibronectin on Oral Epithelial CellsINFECTION AND IMMUNITY.

156. Brouwer S, Barnett TC, Rivera-Hernandez T, Rohde M, Walker MJ. 2016. *Streptococcus pyogenes* adhesion and colonization. FEBS Lett 590:3739–3757.

157. Hagan DRM, Björnsson R, McMahon DSA, Schomburg B, Braithwaite V, Bühl PM, Naismith PJH, Schwarz-Linek DU. 2010. NMR Spectroscopic and Theoretical Analysis of a Spontaneously Formed Lys-Asp Isopeptide Bond. Angew Chem Int Ed Engl 49:8421.

158. Henderson B, Nair S, Pallas J, Williams MA. 2011. Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. FEMS Microbiol Rev 35:147–200.

159. Baldassarri L, Creti R, Imperi M, Recchia S, Pataracchia M, Orefici G. 2007. Detection of genes encoding internalization-associated proteins in *Streptococcus pyogenes* isolates from patients with invasive diseases and asymptomatic carriers. J Clin Microbiol 45:1284–7.

160. Kalluri R. 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3:422–433.

161. Smith WD, Pointon JA, Abbot E, Kang HJ, Baker EN, Hirst BH, Wilson JA, Banfield MJ, Kehoe MA. 2010. Roles of minor pilin subunits Spy0125 and Spy0130 in the serotype M1 *Streptococcus pyogenes* strain SF370. J Bacteriol 192:4651–9.

162. Sumitomo T, Nakata M, Higashino M, Terao Y, Kawabata S. 2013. Group A streptococcal cysteine protease cleaves epithelial junctions and contributes to bacterial translocation. J Biol Chem 288:13317–13324.

163. Sumitomo T, Nakata M, Higashino M, Jin Y, Terao Y, Fujinaga Y, Kawabata S. 2011. Streptolysin S contributes to group A streptococcal translocation across an epithelial barrier. J Biol Chem 286:2750–2761.

164. Sumitomo T, Mori Y, Nakamura Y, Honda-Ogawa M, Nakagawa S, Yamaguchi M, Matsue H, Terao Y, Nakata M, Kawabata S. 2018. Streptococcal cysteine protease-mediated cleavage of desmogleins is involved in the pathogenesis of cutaneous infection. Front Cell Infect Microbiol 8:10.

165. Yamaguchi M, Terao Y, Kawabata S. 2013. Pleiotropic virulence factor - *Streptococcus pyogenes* fibronectin-binding proteins. Cell Microbiol 15:503–511.

166. Vlaminckx BJM, Schuren FHJ, Montijn RC, Caspers MPM, Fluit AC, Wannet WJB, Schouls LM, Verhoef J, Jansen WTM. 2007. Determination of the relationship between group A streptococcal genome content, M type, and toxic shock syndrome by a mixed genome microarray. Infect Immun 75:2603–11.

167. Visai L, De Rossi E, Valtulina V, Casolini F, Rindi S, Guglierame P, Pietrocola G, Bellotti V, Riccardi G, Speziale P. 2003. Identification and characterization of a new ligand-binding site in FnbB, a fibronectin-binding adhesin from *Streptococcus* dysgalactiae. Biochim Biophys Acta - Proteins Proteomics 1646:173–183.

168. Schwarz-Linek U, Höök M, Potts JR. 2004. The molecular basis of fibronectin-

mediated bacterial adherence to host cells. Mol Microbiol 52:631–641.

169. Zetter BR, Danielst TE, Quadra-Whitet C, Greenspant JS. 1979. CLINICAL AND MATERIALS SCIENCES LETS Protein in Normal and Pathological Human Oral EpitheliumJ Dent Res.

170. Babu JP, Dabbous' MK. Interaction of Salivary Fibronectin with Oral Streptococci.

171. Gopal S, Veracini L, Grall D, Butori C, Schaub S, Audebert S, Camoin L, Baudelet E, Radwanska A, Beghelli-de la Forest Divonne S, Violette SM, Weinreb PH, Rekima S, Ilie M, Sudaka A, Hofman P, Van Obberghen-Schilling E. 2017. Fibronectin-guided migration of carcinoma collectives. Nat Commun 8:14105.

172. Walia B, Castaneda FE, Wang L, Kolachala VL, Bajaj R, Roman J, Merlin D, Gewirtz AT, Sitaraman S V. 2004. Polarized fibronectin secretion induced by adenosine regulates bacterial-epithelial interaction in human intestinal epithelial cells. Biochem J 382:589–96.

173. Author P, Musumeci R, Lo Bue C, Milazzo I, Nicoletti G, Serra A, Speciale A. 2003. Internalization-Associated Proteins among *Streptococcus pyogenes* Isolated from Asymptomatic Carriers and Children with.

174. Edwards ML, Fagan PK, Currie BJ, Sriprakash KS. 2004. The fibronectin-binding capacity and host cell adherence of *Streptococcus pyogenes* strains are discordant with each other. Microbes Infect 6:1156–1162.

175. Hamada S, Kawabata S, Nakagawa I. 2015. Molecular and genomic characterization of pathogenic traits of group A *Streptococcus pyogenes*. Proc Jpn Acad Ser B Phys Biol Sci 91:539–59.

176. Loh JMS, Lorenz N, Tsai CJ-Y, Khemlani AHJ, Proft T. 2017. Mucosal vaccination with pili from Group A *Streptococcus* expressed on *Lactococcus lactis* generates protective immune responses. Sci Rep 7:7174.

177. Flores AR, Olsen RJ, Cantu C, Pallister KB, Guerra FE, Voyich JM, Musser JM. 2017. Increased Pilus Production Conferred by a Naturally Occurring Mutation Alters Host-Pathogen Interaction in Favor of Carriage in *Streptococcus pyogenes*. Infect Immun 85:e00949-16.

178. Crotty Alexander LE, Maisey HC, Timmer AM, Rooijakkers SHM, Gallo RL, von

Köckritz-Blickwede M, Nizet V. 2010. M1T1 group A streptococcal pili promote epithelial colonization but diminish systemic virulence through neutrophil extracellular entrapment. J Mol Med (Berl) 88:371–381.

179. Wolz C, Pohlmann-Dietze P, Steinhuber A, Chien Y-T, Manna A, van Wamel W, Cheung A. 2000. Agr-independent regulation of fibronectin-binding protein(s) by the regulatory locus sar in *Staphylococcus aureus*. Mol Microbiol 36:230–243.

180. Young PG, Moreland NJ, Loh JM, Bell A, Atatoa Carr P, Proft T, Baker EN. 2014. Structural conservation, variability, and immunogenicity of the T6 backbone pilin of serotype M6 *Streptococcus pyogenes*. Infect Immun 82:2949–2957.

Appendices

Appendix I. Animal ethics approval certification.

Western

2017-024:5:

AUP Number: 2017-024
AUP Title: Bacterial SAgs Play a Key Role in the Pathogenesis of Streptococcus pyogenes
Yearly Renewal Date: 09/01/2019

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2017-024 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

   1) Animals used in this research project will be cared for in alignment with:
         a) Western's Senate MAPPs 7.12, 7.10, and 7.15
          http://www.uwo.ca/univsec/policies_procedures/research.html
         b) University Council on Animal Care Policies and related Animal Care Committee procedures
          http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html
   2) As per UCAC's Animal Use Protocols Policy,
         a) this AUP accurately represents intended animal use;
         b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
         c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
         d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted
         and attended to within timeframes outlined by the ACC.   http://uwo.ca/research/services/animalethics/animal_use_protocols.html
   3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
         a) be made familiar with and have direct access to this AUP;
         b) complete all required CCAC mandatory training (training@uwo.ca); and
         c) be overseen by me to ensure appropriate care and use of animals.
   4) As per MAPP 7.15,
         a) Practice will align with approved AUP elements;
         b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
         c) UCAC policies and related ACC procedures will be followed, including but not limited to:
                  i) Research Animal Procurement
                  ii) Animal Care and Use Records
                  iii) Sick Animal Response
                  iv) Continuing Care Visits
   5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially
    exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/requir ed/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Appendix II. Detection of FCT region genes in mice infected with the MGAS8232 ΔFCT mutant, a representation of zero/undetectable transcription.

Transgenic mice (n = 4) expressing HLA-DR4/DQ8 were inoculated with 1×10^8 CFUs of the *S. pyogenes* MGAS8232 ΔFCT mutant and sacrificed 48 hours later. Their complete nasal turbinates were then harvested and processed for RNA extraction. qRT-PCR was then used to measure the mRNA transcription levels of *cpa*, *tee18.1*, and *prtF2* in these mice normalized to the housekeeping gene *gyrA*. Bars represent the mean ± SEM of 4 mice analyzed using triplicate values.
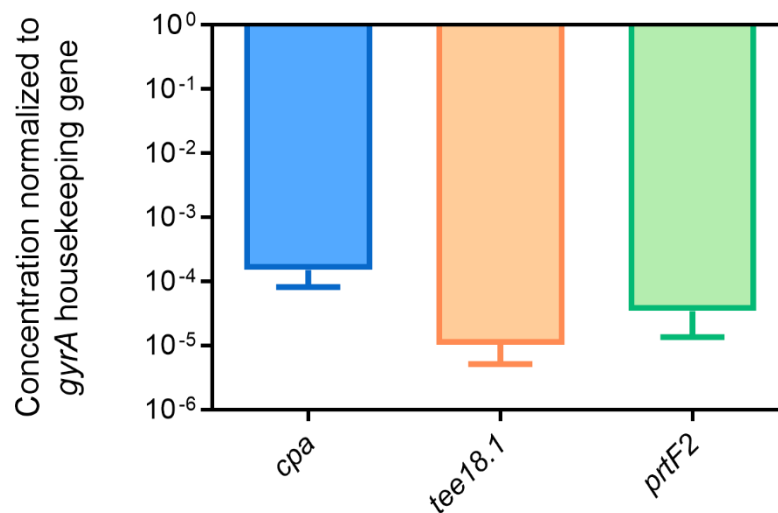
Appendix III. Expression of *cpa* during murine nasopharyngeal infections 24 and 48 hours post-inoculation.

Transgenic mice expressing HLA-DR4/DQ8 were inoculated with 1×10^8 CFUs of wildtype *S. pyogenes* MGAS8232 and sacrificed 24 (n = 4) or 48 (n = 5) hours later. Their cNTs were harvested and processed for RNA extraction. qRT-PCR was then used to measure the mRNA transcription levels of *cpa* in these mice normalized to the housekeeping gene *gyrA*. Bars represent the mean ± SEM; statistical differences were evaluated using the unpaired *t*-test, (**p<0.01).
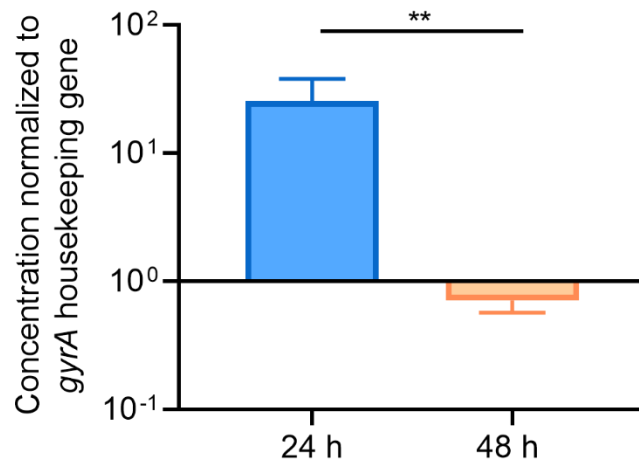
Curriculum Vitae – Akshay Sule

EDUCATION

Master of Science (MSc)   **2019 (Expected)**
Thesis-based Master's of Science in Microbiology and Immunology
Western University

Bachelor of Medical Sciences (BMSc)   **2017 (Graduate)**
Honours Specialization in Microbiology and Immunology
Western University

SCHOLARSHIPS AND OTHER ACCOLADES

Dr. F.W. Luney Travel Award, 2019
Western University, 2019 ($1,000)

Member of the Canadian Society of Microbiologists
2019

Dr. RGE Murray Graduate Scholarship in Microbiology and Immunology
Western University, 2018 ($10,000)

Dr. F.W. Luney Graduate Entrance Scholarship
Western University, 2017 ($2,000)

Dean's Honour List
Western University, 2013 – 2017

Summer Undergraduate Research Fellowship
Western University, May 2016 – August 2016 ($6,000)

The Western Scholarship of Excellence
Western University, 2013 ($2,000)

RESEARCH EXPERIENCE

Bacteriology Laboratory   **May 2017 – Present**
Dr. McCormick, Western University
Master of Science Candidate
- Role of the fibronectin-binding, collagen-binding, T-antigen region during nasopharyngeal infection by *Streptococcus pyogenes*.

Immunology Laboratory Course   **January 2019 – April 2019**
Dr. Summers & Dr. Kim, Western University
Teaching Assistant

Microbiology and Immunology Thesis Course 4970E   **September 2016 – May 2017**
Dr. McCormick, Western University
4th year Honour's Student

Summer Undergraduate Research Fellowship   **May 2016 – August 2016**
Dr. McCormick, Western University
Summer Student

Immunology Laboratory Course 3620G   **January 2016 – April 2016**
Western University

Microbiology Laboratory   **December 2015 – April 2016**
Dr. Heit, Western University
Lab Manual Author & Graphic Artist

Microbiology Laboratory Course 3610F   **September 2015 – December 2015**
Western University

Cognitive Neuroscience Lab   **May 2014 – August 2014**
Dr. Goel, York University
Summer Student

Genetics and Bee Taxonomy Lab   **May 2014 – August 2014**
Dr. Packer, York University
Summer Student

PUBLICATIONS

Hurst JR, Kasper KJ, **Sule AN**, McCormick JK. 2018. Streptococcal pharyngitis and rheumatic heart disease: the superantigen hypothesis revisited. *Infect Genet Evol*. 61:160–175.

Bryan Heit (Ed), **Akshay Sule**, 18 others. A Brief Manual of Microbiological Techniques, First Edition. 2017. *Phagocyte Press*. 159 pages. ISBN: 978-0-7714-3142-5.

PRESENTATIONS

Akshay Sule, Jacklyn Hurst, John K. McCormick. 2019. "*The Role of the Streptococcus pyogenes Adhesion Locus in Nasopharyngeal Colonization.*" Poster presentation for the Canadian Society of Microbiologists; Université de Sherbrooke, Sherbrooke, QC.

Akshay Sule, Jacklyn Hurst, John K. McCormick. 2019. "*The Role of the Streptococcus pyogenes Adhesion Locus in Nasopharyngeal Colonization.*" Poster presentation at London Health Research Day; London Convention Centre, London, ON.

Akshay Sule, John K. McCormick. 2018. "*The Role of the Fibronectin-binding, Collagen-binding, T-antigen locus in Adherence and Nasopharyngeal Colonization by Streptococcus pyogenes*." Poster presentation at the Infection and Immunity Research Forum; The Festival Inn, Stratford, ON.

Akshay Sule, John K. McCormick. 2018. "*The Role of the Fibronectin-binding, Collagen-binding, T-antigen locus in Adherence and Nasopharyngeal Colonization by Streptococcus pyogenes*." Poster presentation at London Health Research Day; London Convention Centre, London, ON.

Hurst JR, Kasper KJ, **Sule AN**, McCormick JK. 2018. Streptococcal pharyngitis and rheumatic heart disease: the superantigen hypothesis revisited. *Infect Genet Evol*. 61:160–175.

Akshay Sule, Delfina M. Siroen, John K. McCormick. 2017. "*The Role of Pili in Nasopharyngeal Colonization by Streptococcus pyogenes*." Presented at the Infection and Immunity Research Forum; Huron College, London, ON.

EXTRA-CURRICULAR ACTIVITIES

Microbiology & Immunology Social Committee   **September 2017 – April 2019**
Western University

Science Student Council Newspaper: The Current   **2013 – 2017**
Western University
Columnist & Copy Editor

Universities Allied for Essential Medicines: Western   **2014 – 2016**
Western University

CERTIFICATIONS

- Basic Animal Care and Use Certification   **May 2017**
- Biosafety Certification   **September 2016**
- Laboratory Safety Certification   **September 2016**
- New WHMIS Certification   **September 2016**