The Group A Streptococci Bacteriocins Facilitate a Competitive Advantage During Nasopharyngeal Infection

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Abstract

Pathogenic streptococci have evolved specific systems to eliminate bacterial competitors within their biological niche. In microbial environments, niche competition is often driven by the production of short antimicrobial peptides called bacteriocins; this provides a mechanism by which *Streptococcus pyogenes* may compete for ecological stability and establish infection. Recent findings from our laboratory have identified two novel Class IIb bacteriocin systems – *Streptococcus pyogenes* bacteriocin (Spb) JK and MN in the M18 serotype *S. pyogenes* strain MGAS8232 – that may contribute to nasopharyngeal infection. Here, we show that galactose and CO$_2$ are distinct regulatory cues which induce antimicrobial activity. Under these conditions, bacteriocin-producing strains inhibit the growth of other Gram-positive bacteria *in vitro*. We also demonstrate that acute infection in the nasopharynx of mice is dependent on the expression of at least one functional bacteriocin system. When mice are challenged intranasally with a double-bacteriocin knockout, the bacterial burden is dramatically reduced compared to the wild-type and single bacteriocin mutant controls. Furthermore, we show that nasal coinfection with *S. pyogenes* wild-type and ΔspbJKMN results in a wild-type dominant population, suggesting that bacteriocins enhance bacterial fitness in a polymicrobial environment. Taken together, these observations demonstrate that *S. pyogenes* secretes bacteriocins which contribute to the establishment of nasopharyngeal infection.

Keywords

*Streptococcus pyogenes*, bacteriocins, antimicrobial peptides, bacteriocin immunity, colonization, competition, nasopharynx, transgenic mice
Co-Authorship Statement

Studies presented in this thesis were completed by Lana Estafanos with the assistance of past and present members in the laboratory of Dr. John McCormick as listed:

Dr. Brent Armstrong generated all *S. pyogenes* MGAS8232 Δ*spb* deletion mutants and pTRKL2 plasmid constructs containing *pgyrA* and *spbMNI*.

Dr. Katherine Kasper taught me how to perform the nasal infections and cNT extractions and assisted with the time-course experiments.

Kathleen Qu assisted with the patching of *S. pyogenes* colonies recovered from the cNTs of mice for antimicrobial testing.
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List of Abbreviations

α alpha
β beta
γ gamma
μg microgram
μL microliter
Ω ohm
° C degrees Celsius
× g times gravity
ABC adenosine triphosphate binding cassette
ATP adenosine triphosphate
BHI brain heart infusion media
blp bacteriocin-like peptide
bp base pair
CaCO₃ calcium carbonate
CcpA catabolite control protein A
CCR carbohydrate catabolite repression
CD(n) cluster of differentiation (n)
cDNA complementary DNA
CFU colony forming unit
cNT complete nasal turbinate
CO₂ carbon dioxide
Cov control of virulence
cre catabolite responsive elements
DHAP dihydroxyacetone phosphate
DNA deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxyribonucleotidetriphosphate
EDTA ethylenediaminetetraacetic acid
EI enzyme I
EII enzyme II
EMP Ebden-Meyerhof-Parnas pathway
erm™ erythromycin resistance
FBP fructose 1,6-bisphosphate
FCT fibronectin-binding, collagen-binding, T-antigen region
For forward
G3P glyceraldehyde-3-phosphate
GalM17 M17 media with 0.5% galactose
GAS Group A Streptococcus
HBSS Hank’s balanced saline solution
HCl hydrogen chloride
HEp-2 human epithelial type 2 cells
His histidine residue
HK histidine protein kinase
HLA human leukocyte antigen
HPr histidine-containing phosphoryl carrier protein
HPrK/P phosphocarrier protein kinase/phosphorylase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>Vβ</td>
<td>β-chain variable domain</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Chapter 1: Introduction
1.1 *Streptococcus pyogenes*

*Streptococcus pyogenes*, also known as group A *Streptococcus* (GAS), is a Gram-positive extracellular bacterial pathogen with strict human tropism. This aerotolerant anaerobe grows in chains of varying lengths and is often identified by the unique β-hemolytic morphology it exhibits when grown on blood agar (1). The core-genome of *S. pyogenes* is composed of a single circular chromosome that is ~1.8 Mbp with a low G+C content of ~39%, and encodes genes mainly involved in pathogenicity and virulence (2). Inside the pan-genome, there is repertoire of virulence genes that are highly variable among bacterial strains. These differences in non-essential genes are acquired via horizontal transfer of exogenous genetic elements; a process fundamental to streptococcal evolution (3–5). With a highly diverse genome, *S. pyogenes* is able to colonize human skin and mucosal surfaces, including the throat, vagina and rectum, but is also able to cause a wide range of diseases. These illnesses often manifest into mild cases of pharyngitis and impetigo but may also lead to invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome during severe infection. *S. pyogenes* has also been implicated in the development of post-infection sequelae, including acute rheumatic fever, rheumatic heart disease and glomerulonephritis. The Lancefield serological classification system based on surface carbohydrate expression has identified *S. pyogenes* as the only member of the group A streptococci. However, *emm* typing, which involves sequencing of the hypervariable region of the M protein gene, has emerged as the dominant method of classification, identifying over 200 GAS serotypes to date (1, 6, 7).

1.2 Global disease burden of group A *Streptococcus*

*S. pyogenes* is the etiological agent of a broad spectrum of disease. Despite its major contributions to human morbidity and mortality, the global importance of GAS remains largely under-recognized. Systematic reviews over the last decade have estimated the global burden of GAS disease using population-based analyses where possible. However, due to unreliable disease surveillance – particularly in regions where *S. pyogenes* has the greatest impact (i.e., developing countries) – the absolute burden of streptococcal diseases has been widely under-estimated (8).
Superficial *S. pyogenes* skin (impetigo and pyoderma) infections and acute pharyngitis account for more than 111 million and 616 million cases of disease per year, respectively. Although mild diseases caused by *S. pyogenes* are seemingly benign, these infections impose considerable health and economic burdens worldwide, with the highest rate of incidence among children 5 to 15 years of age (1, 8). These infections can be easily treated with first-line β-lactam antibiotics (8), however, if left untreated or with penetration of the epithelial surface, *S. pyogenes* may also trigger a variety of severe invasive diseases. This includes bacteremia, necrotizing fasciitis and streptococcal toxic shock syndrome, among other less common clinical manifestations (9).

Invasive *S. pyogenes* infections are often life-threatening and carry a high mortality rate (>50%) in both the developed and developing world (1, 9). Globally, invasive diseases affect over 18.1 million individuals, with an incidence of 1.78 million cases (8). In developed countries, the highest burden of GAS-related mortality results from invasive disease, despite the steady decline in incidence (9). While this may account for the greatest number of deaths in affluent countries, ~97% of the 660,000 estimated cases of invasive GAS infections occur in less developed countries, accounting for nearly 163,000 deaths each year. Of the cases identified, low- to middle-income areas within Sub-Saharan Africa and Asia, and indigenous-minority groups in the developed world, are predominately affected by invasive streptococcal diseases. Within these regions, ~29% of all bacteremia isolates in children are caused by GAS (8, 9).

Acute rheumatic fever and its sequelae, rheumatic heart disease, continue to be the leading causes of GAS-associated morbidity and mortality worldwide. With an estimated 15.6 million people living with rheumatic heart disease, ~233,000 premature deaths, and another 116,000 deaths each year from additional complications, rheumatic heart disease is recognized as a global health problem (8). Studies have identified overcrowding and poverty as risk factors of acute rheumatic fever, with ~60% of cases leading to rheumatic heart disease. In endemic regions such as Africa, the Pacific, Latin America, the Middle East, Asia, and indigenous populations of Australia and New Zealand, children, young adults and pregnant women are particularly vulnerable to acute rheumatic fever and rheumatic heart disease. To reduce the risk of acute rheumatic fever and mitigate valve
damage, prophylactic antibiotic treatment for GAS pharyngitis is recognized as the primary prevention strategy. However, if acute rheumatic fever progresses to rheumatic heart disease, surgical intervention may be required (8, 10).

The global distribution of *S. pyogenes* contributes to an immense burden of human illness. However, prevalence, incidence, and mortality of disease are unequally distributed throughout the world. Studies suggest that disease is strongly correlated with seasonal variability, geographic location, and socio-economic status. Therefore, developing countries are disproportionately impacted by GAS due to poor sanitary standards and minimal access to healthcare and resources (8), making it increasingly difficult to establish the true burden of GAS disease. Without accurate high-quality data from low-resource settings where the burden of disease is greatest, we are unable to design appropriate prevention and treatment strategies to control *S. pyogenes* outbreaks and ultimately reduce the global burden of streptococcal disease (8, 10).

### 1.3 Asymptomatic carriage

The GAS carrier state is described as having a prolonged bacterial presence that does not appear to be causing overt signs of disease. Under these circumstances, a physical examination – swab of the posterior pharynx – and serological testing have been used to identify the presence of *S. pyogenes*. If rapid diagnostic testing and/or throat culture for *S. pyogenes* are positive without the classical clinical signs and symptoms of acute pharyngitis (i.e., sore throat, inflammation of tonsils, fever), individuals are considered asymptomatic carriers (11, 12).

GAS primarily colonizes the naso- and oropharynx in the human host (13). However, it is unclear whether individual genetic factors and type-specific bacterial characteristics influence host-pathogen interactions that contribute to streptococcal colonization. Therefore, despite what we know about *S. pyogenes*, the predominant and benign asymptomatic state is poorly understood. (11, 13, 14).

Many studies suggest that carriage within the human population is dependent on a number of factors: age-group, geography, climate, repeated environmental contact and seasonal
trends (5). For example, a 4-year longitudinal study in Pittsburgh on school-aged children 5 to 15 years of age isolated *S. pyogenes* from 27% to 32% of the cohort over the course of one school year. In this study, asymptomatic children typically carried a single *emm* type that persisted between 3 to 123 weeks, and those that sporadically cleared GAS were more likely to acquire recurrent infections with a different *emm* type and demonstrate new clinical symptoms (12). A meta-analysis on the prevalence and carriage of GAS in the United States in the past 35 years found that 37% of children under 18 years of age presenting signs of bacterial pharyngitis were positive for *S. pyogenes*. However, only 12% of children surveyed were considered asymptomatic carriers of GAS (15). In Melbourne, Australia, one study reported GAS carriage rates between 0% to 10% for children under 18. These findings were similar to the carriage rates of healthy children surveyed in both Croatia (6%) and Iran (11%) (16, 17), but varied greatly from a study in Victoria, Australia, which previously described carriage rates as high as 16% due to seasonal and geographic variability (18, 19). In a family-based study in Australia, the incidence of *S. pyogenes* infection increased with household exposure to primary cases of GAS pharyngitis. It was determined that household contacts were almost twice (43%) as likely to acquire secondary GAS infections (20). This association had been previously noted by Schwartz *et al.* (21), who reported that ~25% of household contacts of children with streptococcal pharyngitis were asymptomatic but positive for GAS. Therefore, the carrier state of GAS is both complex and very common among school aged-children, but despite decades of research, the underlying molecular mechanisms contributing to this dominant state remain an enigma.

### 1.4 Mechanisms of colonization and disease

#### 1.4.1 Mechanisms of adhesion

The nasopharynx and the skin are major sites of colonization for GAS. In order to cause infection or establish asymptomatic colonization, *S. pyogenes* requires strong adherence mechanisms to subvert a variety of mechanical, chemical, and biological barriers present in humans. Surface adhesins allow *S. pyogenes* to bind to specific receptors on host cells, penetrate intact barriers, and colonize in unfavourable environments with low pH and moisture content, and among antimicrobial compounds (i.e., defensins, lysozymes) highly prevalent on epidermal and mucosal surfaces (1, 22). While many adhesions have been
described in the literature, expression of streptococcal adhesions are tissue-specific. Therefore, although *S. pyogenes* utilizes a multi-adhesin approach that is often functionally redundant to colonize host cells, different surface adhesins provide selectivity for the adherence of different streptococcal strains (1, 23).

### 1.4.1.1 LTA

The adhesion process for GAS is often described using a two-step process. The first step of adhesion involves a relatively weak hydrophobic interaction mediated by lipoteichoic acid (LTA). This glycolipid moiety is a major cell surface component of Gram-positive bacteria that plays an important role in adhesion and biofilm formation. In this model, LTA allows *S. pyogenes* to overcome electrostatic repulsion and attach to a number of cell types including buccal epithelial cells, HEp-2 cells, and polymorphonuclear cells, lymphocytes, and erythrocytes (24–26). To mediate adherence of *S. pyogenes* to the epithelial layer, LTA binds to its cognate host-receptor, fibronectin, forming a complex with other streptococcal surface proteins (1, 26, 27). This complex stimulates multiple high-affinity, tissue-specific interactions formed by other adhesins, including M protein, the pili, and fibronectin-binding proteins (1, 5, 27, 28).

### 1.4.1.2 M protein

The M protein, encoded by the *emm* gene, is one of the best studied surface molecules of *S. pyogenes* (27). Composed of typically four repeat regions designated A through D, this multi-domain alpha-helical coiled-coil structure extends as hair-like projections from the surface of the bacteria. With the conserved C-terminus region anchored to the cell wall and the hypervariable N-termini protruding from the surface, the M protein is able to adhere to a number of eukaryotic receptors found on skin and mucosal surfaces (23, 27, 29).

Recognized for its prominent role in immune evasion and adhesion, the M protein is considered a major virulence factor of *S. pyogenes*. To subvert the innate immune response, the streptococcal M proteins possess anti-phagocytic properties rendering the bacteria resistant to opsonization and neutrophil recognition (1, 5). However, the molecular mechanisms involved in adherence are less understood. Much of the evidence suggests that different M proteins bind to particular eukaryotic cells to mediate adherence. For example,
some of the earliest work demonstrates that the M protein is required for binding to human buccal cells (30). Alternatively, work by Courtney and colleagues (25) has showed that certain serotypes including M1, M5, M6, and M24 are not involved in attachment to buccal epithelial cells, but are required for adherence to the human laryngeal epithelial cell line HEp-2. Other studies have found that specific parental M types attached to human pharyngeal epithelial cells in greater numbers than both buccal or tongue epithelial cells. Compared to the parental strains, the M protein-deficient strains in this study were unable to efficiently bind epithelial cells, demonstrating the role of M protein in throat-mediated adherence (31).

Based on these findings, it is evident that the streptococcal M proteins attach selectively to host receptors on specific cell types. Therefore, the role of the M protein in mediating adherence to eukaryotic cells is dependent on the M-serotype and tissue tropism. There are four different subfamilies of \textit{emm} genes that may play a partial role in determining tissue tropism (1, 23, 27, 28, 32, 33). The chromosomal arrangement of the \textit{emm} genes are divided into five distinct patterns denoted A to E and are distinguished by the peptidoglycan-spanning domain. Strains with patterns A to C have tissue site preference for the pharynx; whereas strains with pattern D are recovered more often from cases of impetigo. Unlike patterns A to D, strains with pattern E are not tissue specific, and have been isolated from both the skin and the pharynx (32).

Much of the work done on M proteins suggests that multiple membrane bound proteins may serve as cellular receptors for the M protein ligand (1, 27). There have been reports of streptococcal M proteins binding to: fibronectin on the surface of oropharyngeal cells (34); membrane cofactor protein CD46 on epidermal keratinocytes (35); sialic acid-containing receptors on mucin lining the pharyngeal epithelium (36); and fucose-containing oligosaccharides found on cultured HEp-2 cells (37). Despite these findings, there remains a lot of controversy over the M protein and its role in \textit{S. pyogenes} adherence (27).

1.4.1.3 \textit{Pili}

Gram-positive and Gram-negative bacteria are decorated with long pilus-like structures protruding from the bacterial cell wall (38, 39). These surface appendages are encoded on
a pathogenicity island known as the fibronectin-binding, collagen-binding, T-antigen (FCT) region, and are described as vital structures for adherence, biofilm formation, and virulence in group A and group B streptococci. Many studies demonstrate that the GAS pilus mediates efficient attachment to multiple human cell types, including primary and cultured keratinocytes, as well as epithelial cells derived from human tonsils (40); the skin (41), and the pharynx (38). To date, there have been nine different FCT islands identified. Although these islands are highly variable in *S. pyogenes*, the structural integrity of the pilus is highly conserved across serotypes. Pilus-like appendages in *S. pyogenes* are composed of polymerized backbone protein covalently attached to one or two ancillary protein caps. These structural subunits are assembled by pili-associated sortase enzymes which are also encoded in the FCT operon (5, 27, 38). Despite our understanding of the structure and assembly of the pilus, the complex processes involved in pili-mediated adherence and colonization have yet to be elucidated (38).

1.4.1.4 *Hyaluronic acid capsule*

*S. pyogenes* produces a capsule comprised of hyaluronic acid; a linear polymer of N-acetylglucosamine and glucuronic acid, that yields distinct mucoid colonies when grown on blood agar. Encoded by the 4.2 kb *has* operon, the hyaluronic acid capsule is highly conserved across most clinical isolates of GAS, except M4 and M22 serotypes, which lack the capsular genes and in turn produce hyaluronidase. As a major component of mammalian tissue, the hyaluronic acid polysaccharide produced by *S. pyogenes* is structurally identical, and therefore, poorly immunogenic in the human host (5, 42, 43).

Hyaluronic acid capsule production varies among different strains of GAS. In *S. pyogenes*, capsule biosynthesis is regulated by growth conditions and in response to changes in the host environment (5). Testing *in vitro* has demonstrated that capsule production is rapidly induced during early- to mid-exponential phase (44). These observations are further supported *in vivo*. During invasive infection in the peritoneum of mice, and in pharyngeal colonization using a non-human primate model, high levels of capsule gene expression were induced within 1-2 h after the introduction of GAS. This suggests that the hyaluronic acid capsule is essential during the initial stages of colonization (45). Although it is widely believed that the capsule allows GAS to persist in the pharynx, the role of the hyaluronic
acid capsule in mediating adherence remains controversial. Many studies have demonstrated that GAS attachment to epithelial cells occurs through binding to the cell surface ligand CD44 (46–48). While this may serve as a critical point of attachment to pharyngeal and epidermal cells, there is evidence to suggest that encapsulated strains of GAS bind the epithelium with similar (49), and in some cases, reduced efficiency to acapsular strains (46, 48). Despite these findings, encapsulated streptococcal strains seem to provide a greater advantage in infection models. In both murine and primate models of pharyngeal colonization, capsule-deficient strains of GAS were unable to persist in the pharynx, and cleared rapidly by host phagocytes when compared to the wild-type strains (49, 50). Therefore, the structural and anti-phagocytic properties of the capsule allow S. pyogenes to circumvent the host immune response, demonstrating a critical role for the hyaluronic acid capsule in GAS virulence and colonization.

1.4.2 Secreted virulence factors

S. pyogenes secretes a number of extracellular proteins that play an important role in colonization and the initiation of disease. These secreted toxins are considered virulence factors and are responsible for a diverse range of functions essential to the success of the pathogen. This impressive arsenal of virulence factors includes: proteinases, DNases, complement-inhibitors, plasminogen-binding proteins, hemolysins, superantigens, and bacteriocins, which allow S. pyogenes to facilitate invasion, resist host defence mechanisms, and outcompete endogenous microbiota within a given biological niche (1, 28, 51). Although each of these virulence factors plays a critical role disease pathogenesis, this chapter will review the hemolysins, superantigens, and bacteriocins secreted by S. pyogenes during infection.

1.4.2.1 Hemolysins

S. pyogenes secretes two major extracellular toxins called the streptococcal cytolysins, streptolysin O (SLO) and streptolysin S (SLS). These potent pore-forming toxins target a number of eukaryotic cells (51–53), which contribute to GAS virulence through a variety of poorly understood mechanisms (51, 54, 55). The first of these hemolysins, SLO, is an oxygen-labile and cholesterol-dependent cytolysin capable of forming large pores in the host cell membrane to induce apoptosis (54–56). In a pore-dependent manner (57), SLO
enhances GAS pathogenicity through the translocation of NAD\(^+\)-glycohydrolase (NADase). This toxin, which is co-expressed with SLO, is delivered to the cytoplasm of host cells through a process known as cytolysin-mediated translocation, where it functions to modulate host cell signalling pathways. SLO-mediated delivery of NADase interferes with cellular repair mechanisms and prevents acidification of the phagolysosome, thereby blocking internalization and destruction of the invading bacterium. Murine models of GAS infection demonstrate that in the absence of SLO, \textit{S. pyogenes} is significantly impaired in its ability to survive in macrophages. These findings suggest that together, SLO and NADase play a critical role in enhancing intracellular survival and proliferation of GAS (52, 58–60).

In addition to SLO, \textit{S. pyogenes} produces a second hemolysin known as SLS. This oxygen-stable, non-immunogenic toxin is encoded by the nine-gene \textit{sag} operon, which is remarkably similar to the genetic organization of bacteriocin-gene clusters encoded by other bacteria (56, 61). SLS is secreted by many strains of \textit{S. pyogenes} and is responsible for the distinct \(\beta\)-hemolytic phenotype surrounding GAS colonies grown on blood agar. Similar to SLO, SLS possesses a broad cytotoxic spectrum that targets erythrocytes (62, 63), leukocytes (53), and platelets (64) by the formation of a transmembrane pore that disrupts osmotic balance, resulting in cell lysis (63). However, recent studies suggest a more complex role for SLS in facilitating GAS pathogenesis. Flaherty \textit{et al.} (65) demonstrate that SLS is capable of manipulating host cell signalling pathways, thereby triggering a rapid proinflammatory response that leads to programmed cell death of epithelial cells. Other groups have reported SLS-induced apoptosis of immune cells through caspase-dependent and independent mechanisms (65, 66). These processes implicate SLS as a critical factor for innate immune evasion by GAS (67–69). Further evidence suggests that SLS-deficient mutants are attenuated in their ability to induce cell death during infection (68), and are significantly less virulent than their wild-type counterparts \textit{in vivo} (54, 65, 70).

1.4.2.2 \textbf{Superantigens}

Streptococcal superantigens represent a large fraction of the family of mitogenic exotoxins that are capable of inducing an exaggerated T lymphocyte response (71, 72). These highly
potent pyrogens are best studied in *Staphylococcus aureus* and *S. pyogenes*, but are also present in other bacterial pathogens, including group C and G streptococci, coagulase-negative staphylococci, *Mycoplasma arthritidis* and *Yersinia pseudotuberculosis*. Together, the streptococcal and staphylococcal superantigens comprise a group of structurally and functionally-related toxins recognized for their key role in the pathogenesis of toxic shock syndrome (71–76).

Superantigens have a remarkable ability to simultaneously bind host major histocompatibility complex (MHC) II molecules on antigen presenting cells and the β-chain variable domain (Vβ) on T cell receptors of CD4+ and CD8+ T cells in a peptide-independent manner. Unlike conventional T cell activation, superantigens bind outside the designated peptide-binding groove and are not restricted by polymorphic determinants of MHC class II. Therefore, superantigens can bind to multiple Vβ chains in the T cell repertoire and stimulate up to 25% of an individual’s T cell population, compared to classical peptide presentation which activates only one in 10^5 to 10^6 naïve T cells (73, 75). Recent evidence suggests that a second signalling molecule may also be required for T cell activation by superantigens. Induction of human inflammatory cytokine gene expression may require direct superantigen binding to co-stimulatory molecule CD28 (77) and its co-ligand B7-2 (CD86) (78), which are constitutively expressed on T cells and antigen presenting cells, respectively. As a critical component of the immune response, the superantigen-CD28-B7-2 interaction drives a hyper-active T helper 1 (Th1) response (77, 79) characterized by the overproduction of inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-2, and interferon (IFN)-γ (23, 73, 78, 80).

Evidence suggests that other factors may influence the T cell response to superantigens. The most important consideration is the strength and stability of the immune complex (superantigen-MHC-T cell interaction). Although all complex formations are able to potently activate T lymphocytes, different superantigens have unique preference or affinity towards different binding sites, which may affect the proliferation response. For example, nine out of fourteen streptococcal superantigens bind the MHC II β-chain in a zinc-dependent manner, which is referred to as the “high-affinity” binding site. In contrast, the
MHC class II invariant α-chain is recognized as a “generic” or “low-affinity” binding site for streptococcal superantigens (73). Furthermore, superantigens preferentially bind to human leukocyte antigens (HLA) class II molecules, indicating that HLA polymorphisms may also affect the binding affinity of the immune complex. High-affinity interactions between the superantigen, MHC, and T cell receptor are thought to enhance the potency of the resulting inflammatory response (73, 75, 81).

Although superantigens have been implicated in the development of severe streptococcal diseases such as necrotizing fasciitis and toxic shock syndrome (73, 79), the evolutionary advantage of superantigens is only now beginning to be understood. Recent evidence from our laboratory has demonstrated that these exotoxins play a critical role in the initial stages of colonization by S. pyogenes (82, 83). Using a murine model of acute nasopharyngeal infection adapted from Park et al. (84), we show that expression of human MHC class II molecules HLA-DR4/DQ8 and the streptococcal pyrogenic exotoxin A (SpeA) results in a robust infection phenotype observed in transgenic mice compared to wild-type C57BL/6 mice (82). We also demonstrated that vaccination with a toxoid SpeA molecule reduced the bacterial burden and provided antibody-mediated protection against subsequent nasopharyngeal challenge with wild-type S. pyogenes (82, 83). Moreover, these immunization experiments show that in the absence of Vβ-specific CD4+ and CD8+ T cells, S. pyogenes is unable to drive the proinflammatory environment necessary for nasopharyngeal colonization. Therefore, there is strong evidence to suggest that 1) bacterial superantigens and host MHC II molecules are required for acute infection in the nasopharynx (82) and 2) superantigens utilize Vβ-specific T cells to remodel the nasopharyngeal environment to establish colonization (83).

1.4.2.3 Bacteriocins

Niche competition is often driven by the secretion of potent antimicrobial toxins called bacteriocins. Bacteriocins are ribosomally-synthesized polypeptides produced by both Gram-positive and Gram-negative bacteria, which inhibit the growth of close genetic relatives or non-related (broad spectrum) bacteria within an ecological niche. Due to the diverse and abundant nature of bacteriocins, families are often grouped by their mode of action, mechanism of secretion, structure, genetics, immunity mechanism, and microbial
targets. In complex microbial environments such as the skin, gastrointestinal tract, and the nasopharynx, bacteriocins are thought to be used to eliminate other bacterial species competing for the same limited space and resources. Importantly, bacteriocin-producing strains encode additional genes which provide protection against their own antimicrobial peptides (85, 86).

Gram-positive bacteriocins can be classified into four distinct groups based on the classification system first proposed by Klaenhammer (87) and illustrated in Figure 1. Although this may be the most accepted classification scheme, only three groups are universally acknowledged in literature. Due to the increasing body of knowledge on the structure and chemistry of bacteriocins, and inconsistency in the parameters used for classification, there remains some criticism of the current classification approach (88). Despite these discrepancies, all established classes will be reviewed: (I) Lantibiotics are small (<5 kDa) membrane-active antimicrobial peptides that derive their name from the addition of post-translationally modified amino acids such as lanthionine and β-methyllanthionine. Depending on their molecular structure, these peptides are grouped as linear (type A) or globular (type B) lantibiotics. (II) Class II bacteriocins are small (<10 kDa), unmodified, heat-stable toxins that permeabilize target-cell membranes through an influx of ions. In their precursor form, class II bacteriocins contain a double-glycine N-terminal leader motif, which is a hallmark of class II secretion. This motif is cleaved by a dedicated ATP binding cassette (ABC)-transporter to produce the active secreted form. Class II bacteriocins can also be structurally divided into multiple subclasses, including: type-IIa (pediocin-like peptides), -IIb (two-component peptides), and -IIc (cyclic peptides). Other subdivisions have also been considered due to differences in peptide structure. (III) The third class of bacteriocins, recognized as the group of large (>30 kDa) heat-labile proteins, possess a lytic- (IIIa) or non-lytic (IIIb) mode of action. Due to their enzymatic nature, they are sometimes referred to as bacteriolysins (85, 87, 88). Lastly, a fourth class of bacteriocins has also been proposed (87, 89, 90). This group consists of antimicrobial peptides containing lipid or carbohydrate moieties. For example, bacteriocins with post-translational cysteine S-glycosylation modification have been identified in Lactobacillus plantarum (90). However, due to difficulties in peptide purification, class IV bacteriocins have typically been dismissed (88). To illustrate the different classes and their mode of
Figure 1. Classification of bacteriocins produced by Gram-positive bacteria.

There are four classes of Gram-positive bacteriocins based on the original classification system proposed by Klaenhammer (87). Class IV bacteriocins are typically omitted from the current classification scheme (85).
Bacteriocins of Gram-positive Bacteria

Class I
Lantibiotic peptides
- Type A Linear
- Type B Globular

Class II
Small (<10 kDa)
non-modified peptides
- Type Iia Pediocin-like
- Type Iic Cyclic

Class III
Large (>30 kDa)
proteins
- Type IIIa Lytic
- Type IIIb Non-lytic

Class IV
Peptides containing
non-proteinaceous moieties

Type IIb
Two-peptide
action, Figure 2 provides a summary of Gram-bacteriocin activity (91).

1.5 *S. pyogenes* bacteriocins

Many members of the *Streptococcus* genus produce bacteriocins to mediate intra- and interspecies colonization in a given biological niche. Examples include pathogenic and benign streptococci such as *Streptococcus pneumoniae* and *Streptococcus salivarius* that utilize bacteriocins to eliminate bacterial competitors within the oral mucosa (92). Although various streptococci have evolved mechanisms to compete for niche space, the bacteriocins produced by *S. pyogenes* are poorly characterized in comparison.

Antimicrobial activity by GAS was first observed in 1971 by the *S. pyogenes* strain FF22 (93). The name, streptococcin A-FF22 (SA-FF22), was ascribed to the 26-amino acid bacteriocin, which was later classified as a type A lantibiotic for its similarity to the prototypical lantibiotic nisin produced by *Lactococcus lactis* (94). SA-FF22 is predominately active against other streptococci, and a wide range of Gram-positive bacteria including *L. lactis, Bacillus cereus,* and *Bacillus subtilis* (92–94). However, unlike other linear lantibiotics, SA-FF22 possesses unique structural (i.e., different proteolytic cleavage site) and regulatory components (i.e., two-component system [TCS]) that are essential for bacteriocin production (95).

Streptin is class A lantibiotic originally isolated and characterized from *S. pyogenes*. Although the streptin locus is highly conserved across different streptococci, only 10% of *S. pyogenes* strains produce a biologically active form of streptin. There are two major forms of streptin with comparable inhibitory and autoinducing properties. The fully processed form of streptin, referred to as streptin 1, is a 23-amino acid peptide with a broad activity spectrum against streptococci. The second form of the peptide – streptin 2 – has similar antimicrobial activity, yet contains three additional amino acids (TPY) at the N-terminal region (92, 96, 97). A comparison of the biosynthetic gene clusters in nisin and subtilin demonstrate high sequence homology to the structural gene of streptin (srtA) among other open reading frames for streptin immunity (srtI), regulation (srtR/K), transport (srtT), prepeptide modification (srtC/B), and self-protection (srtF/E/G). The arrangement of the operons, however, differ considerably. While most lantibiotics encode a dedicated
Figure 2. Mode of action of Gram-positive bacteriocins.

Bacteriocins produced by Gram-positive bacteria can be grouped into three distinct classes based on their mode of action. Some members of class I (or lantibiotic) bacteriocins have a dual mode of action. They typically function as pore-forming toxins, but in some cases, they may also prevent correct cell wall synthesis. Both mechanisms involve an interaction with lipid II molecules that leads to cell lysis. Class II (small, unmodified peptides) bacteriocins have an amphiphilic helical structure that allows them to insert into the cell membrane of target cells. This process disrupts the membrane potential, which also results in cell lysis. The third class of bacteriocins, referred to as bacteriolysins, directly degrades the cell wall of target bacteria (91).
protease for prepeptide processing, removal of the leader sequence in streptin is dependent on the general streptococcal cysteine protease SpeB (96).

The only non-lytic and plasmid-borne bacteriocin system identified in *S. pyogenes* is the large 179-amino acid polypeptide streptococcin A-M57 (SA-M57). This class III bacteriocin system derives its name from the unique association to one serotype-specific lineage of *S. pyogenes*, M57 (98, 99). SA-M57 possesses a particularly unusual activity spectra that targets a diverse range of non-streptococcal Gram-positive bacteria, including *Micrococcus luteus, L. lactis, Bacillus megaterium, Staphylococcus simulans*, and all tested species of *Listeria*. Although the exact mechanism of killing remains unclear, SA-M57 secretion is thought to occur through the Sec-dependent transport pathway (85, 99).

Recent findings from our laboratory suggest that *S. pyogenes* also produces two class IIb bacteriocin systems termed the *S. pyogenes* bacteriocins (*spb*) which have not been previously characterized. To avoid confusion between the two systems, we designated the open reading frames of the first system as the *spbM* and *spbN* genes, and those of the second putative system as the *spbJ* and *spbK* genes (100; unpublished data) (Figure 3). Although the latter system has not been explored in detail, we demonstrate through recombinant expression of the SpbM and SpbN peptides that Spb possesses broad spectrum activity against other group A and G streptococci, and Gram-positive bacteria such as *M. luteus* and *L. lactis*. Further analysis of the structural genes *spbM* and *spbN* reveal that these peptides are homologous to the bacteriocin-like peptide (Blp) M and N in *S. pneumoniae*, respectively (100). These peptides can be found in all sequenced strains of *S. pneumoniae* and may contribute to nasopharyngeal colonization (101, 102). Similarly, most sequenced strains of *S. pyogenes* contain complete sequences for *spbM* and *spbN*, suggesting that the Spb locus provides a potential advantage during colonization (100). Since there are no prior reports of functional two-peptide bacteriocin systems in *S. pyogenes*, the next section will provide an overview of the structural and intrinsic properties common to all class IIb bacteriocins.
1.5.1 *Class IIb bacteriocins*

Class IIb bacteriocins consist of two different peptides that function together as a single antimicrobial unit. Although the individual peptides may display low antimicrobial properties, the production of two complementary peptides in equal amounts is required to induce optimal activity (103, 104). The structural genes encoding the two peptides are adjacent in the operon and found next to the immunity gene—which offers protection from the bacteriocins in an unknown mechanism—and genes for bacteriocin export. In their immature form, each peptide is synthesized with a double-glycine N-terminal leader sequence ~15-30 amino acids in size. This motif is cleaved off at the C-terminal side by a dedicated ABC-transporter, resulting in export of the mature peptides out of the cell. Together, the two peptides—often referred to as α and β—form a transmembrane helical-helical structure that penetrates into the hydrophobic core of target cell membranes. Through the highly conserved GxxxG-motifs present on the peptides, the double helix structure remains stabilized in a parallel orientation, which allows for formation of the pore (103, 105).

Aside from their unique structural features, class IIb bacteriocins have pore-forming abilities that differ in specificity. With few exceptions, the members of this group possess a relatively narrow inhibitory spectrum that is limited to their own bacterial species. This specificity is predominately determined by the N-terminal amino acid sequence of the β-peptide, and is displayed with respect to the molecules conducted across the cell membrane (104, 106). For example, lactococcin G produced by *L. lactis* disrupts the membrane potential by increasing permeability to a number of monovalent cations such as sodium, potassium, and choline, with the exception of protons. But it appears that other two-peptide systems, such as plantaricin E/F and plantaricin J/K produced by *L. plantarum*, transport monovalent ions, including protons, efficiently across the cell membrane to induce membrane-leakage and cell death (105).

To regulate bacteriocin expression, most two-peptide systems employ a sophisticated intracellular communication system known as quorum-sensing. Quorum-sensing is bacterial cell-to-cell communication that relies on changes in cell density to regulate expression of a wide variety of genes within the cell (107). This system is usually comprised
Figure 3. Map of *sil-spb* loci in the M18 serotype *S. pyogenes* strain MGAS8232.

Open reading frame maps of the *Streptococcus* invasion locus (*sil*) and the *Streptococcus pyogenes* bacteriocin (*spb*) operons in *S. pyogenes* MGAS8232 (accession: NC_003485.1). The *spbJK* locus, located downstream of the *sil* operon, encodes the bacteriocin structural genes, *spbJ* and *spbK*, and an associated immunity protein, *spbL*, for degradation of the bacteriocins through a putative membrane-bound metalloprotease. The *spbMN* locus, located upstream of the *sil* operon, encodes two adjacent bacteriocin genes, *spbM* and *spbN*, and a functional immunity protein, *spbI*, with a frameshift mutation.
of three components: an intracellular response regulator (RR), a membrane-associated histidine protein kinase (HK), and a peptide pheromone (104, 105). Peptide pheromones, also referred to as induction peptides, are small, typically unmodified peptides that are highly related to class IIb bacteriocins in structure. They contain the same N-terminal signal motif that is recognized by the bacteriocin export machinery during secretion. Once the inducing peptide reaches a threshold concentration, the bacteria respond accordingly to collectively regulate gene expression within their environment (107). Therefore, signal transduction is initiated when the peptide pheromone interacts with the HK. This triggers a phosphorylation event of the RR that subsequently activates transcription of the bacteriocins and associated genes, as well as the induction peptide (104). After a certain amount of time, the genes are downregulated, resulting in the inactivation of quorum-sensing based bacteriocin production (108).

1.6 **S. pyogenes** metabolism

Different sites of infection represent a nutritionally unique landscape for *S. pyogenes* that differ in carbohydrate availability and abundance. To thrive in these diverse microenvironments, *S. pyogenes* has adapted numerous pathways to generate energy in the cell and maintain metabolic fitness (109). While many pathways contribute to the success of *S. pyogenes* as a pathogen, this chapter will focus on carbohydrate metabolism with respect to glucose and lactose utilization.

1.6.1 **Glucose metabolism and transport**

Like most bacteria, the preferred carbohydrate source for *S. pyogenes* is glucose. In glucose-rich environments, *S. pyogenes* generates energy through the classical glycolytic pathway known as the Ebden-Meyerhof-Parnas (EMP) pathway. This pathway consists of twelve coupling enzymes that are activated by divalent cations such as magnesium (Mg$^{2+}$) and manganese (Mn$^{2+}$). Once activated, the enzymes bind to negatively-charged, high-energy phosphorylated substrates to catabolize glucose into pyruvate. This conversion yields immediate energy in the form of two molecules of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH$_2$) (109).
While glucose may be the favoured substrate, *S. pyogenes* must adapt to specific nutritional microenvironments depending on the site of infection. For example, during colonization of the oral cavity or the skin where glucose is present in only scarce amounts, *S. pyogenes* must rely on alternative substrates and metabolic pathways to generate energy for growth and survival (109). However, in the presence of glucose, the genes responsible for catabolizing complex carbohydrates are repressed. This conserved regulatory phenomenon, referred to as carbohydrate catabolite repression (CCR), enables streptococci to cope with alternating periods of carbohydrate starvation and excess glucose availability by the differential regulation of genes (109, 110).

In *S. pyogenes*, CCR is regulated by the phosphoenolpyruvate (PEP)-dependent phosphotransferase (PTS) system which catalyzes the uptake and transport of various carbohydrate sources though phosphorylation-dependent mechanisms. The PTS is composed of two cytoplasmic components, enzyme I (EI) and a histidine-containing phosphoryl carrier protein (HPr), in addition to one membrane spanning protein known as the sugar-specific permease (EII) (109, 111, 112). Briefly, a phosphorylation cascade is initiated when the phosphoryl donor PEP phosphorylates a conserved histidine residue in EI. Phosphorylated EI then transfers the high-energy group to the His-15 site of HPr, which serves as an intermediate phosphoryl donor to the EIIA and EIIB domains of the sugar-specific permease. In the final step, the phosphoryl group of EIIB is transferred to a cognate carbohydrate molecule bound to the membrane-spanning EIIC domain of the EII complex. The resulting phosphorylated carbohydrate is then released into the cytoplasm and processed as glycolytic intermediate (112). In addition to PEP-mediated phosphorylation of HPr, the phosphocarrier protein can also be phosphorylated by a HPr kinase/phosphorylase (HPrK/P) at an alternative site. Regulation of the HPrK/P is dependent on the concentrations of fructose 1,6-bisphosphate (FBP) and inorganic phosphate. In general, the kinase function of HPrK/P is induced in response to high levels of FBP. This results in phosphorylation of HPr at the Ser-46 residue, which is then able to interact with catabolite control protein A (CcpA). Together, the CcpA-HPrSer-46 dimer stimulates the repressor function of CCR in the presence of glucose by binding to specific catabolite responsive elements (cre) present upstream of catabolic genes. Over time, utilization of intracellular ATP and FBP leads to an increase in inorganic phosphate, which
modulates phosphatase activity of the HPrK/P enzyme. Therefore, alterations in the phosphorylated state of the PTS are dependent on the metabolic demands of the cell and the relative concentrations of FBP and inorganic phosphate (109, 112).

1.6.2 Lactose metabolism and transport

All strains of *S. pyogenes* possess two *lac* gene clusters designated *lac.1* and *lac.2* that encode components of the tagatose-6-phosphate pathway for lactose and galactose metabolism. These operons have likely evolved from gene duplication yet serve distinctly separate functions in *S. pyogenes*. The Lac.1 locus contains several truncated genes, making it unable to catabolize the appropriate sugars. It has instead adapted a novel regulatory role via LacD.1 as a transcriptional regulator of virulence genes, while maintaining its enzymatic aldolase capacity. In contrast, the Lac.2 operon encodes eight full-length genes dedicated for lactose and galactose utilization, but does not have any reported regulatory activity (113, 114).

Lactose and galactose catabolism begin through PTS-mediated uptake and phosphorylation. Using the lactose-specific PTS transporter encoded by the *lac.2* operon, exogenous lactose and galactose are released into the cytoplasm in the form of phosphorylated lactose, and subsequently broken down into either energy-rich glucose or galactose intermediates. The former molecule serves as a substrate for the canonical EMP pathway, while the latter molecule is channeled into the tagatose-6-phosphate pathway. Following a series of catalytic events, tagatose bisphosphate aldolase (LacD.2) converts the galactose intermediate into two three-carbon sugars, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). These sugars are utilized through EMP pathway for the formation of pyruvate (109).

1.7 Virulence gene regulators of *S. pyogenes*

The ability of *S. pyogenes* to colonize and persist in multiple tissues is dependent on its capacity to adapt to a dynamic host environment. During infection, *S. pyogenes* regulates a varied repertoire of virulence factors to cope with changes in its microenvironment. The spatial and temporal expression of these virulence factors are controlled by a complex network of regulators that are capable of integrating environmental cues such as nutrient
availability, pH, osmolarity, temperature, with information on the pathogen’s own metabolic state, to determine the appropriate response (115). This highly coordinated response promotes nutrient acquisition and the evasion of host immune factors, which facilitates disease progression by *S. pyogenes* (115, 116).

While *S. pyogenes* possesses 13 TCS and more than 100 stand-alone response regulators (28), the following section will explore a few of the best characterized virulence-associated transcriptional regulators of global gene expression.

### 1.7.1 CovRS

Two-component signal transduction systems in bacteria employ a common stimuli-response coupling mechanism to sense and respond to changes in the external environment. This system is composed of two conserved components: a membrane-bound HK and a corresponding RR. The former consists of a sensor domain that monitors environmental stimuli, and a cytosolic kinase domain that serves to modulate activity of the HK. When a stimulus interacts with the sensor domain of the HK this triggers autophosphorylation of the cytosolic domain. The phosphoryl group is then transferred from the HK to the RR, which binds to DNA to elicit a cellular response (117).

The control of virulence (CovRS) TCS in *S. pyogenes* serves to regulate genes associated with the general stress response. When cells are subjected to environmental stressors such as elevated temperature, environmental Mg$^{2+}$, low pH, LL-37, and iron starvation, CovS signals for a change in the phosphorylation state of its cognate RR CovR (28, 118–120). While this mechanism is thought to enhance DNA binding and transcription of downstream effectors, it has been proposed that the non-phosphorylated state of CovR also regulates a specific subset of genes. Therefore, CovR acts as a global transcriptional repressor of virulence genes in both a phosphorylated and non-phosphorylated state (115).

The *covRS* regulon modulates transcription of ~10-15% of genes in the *S. pyogenes* genome (121); many of these genes are essential for infection and evasion of the host immune response. Although this list is not exhaustive, CovRS has been characterized as a transcriptional repressor of virulence factors such as the hyaluronic acid capsule,
streptolysins, DNases, streptokinase, and immunoglobulin (Ig)-degrading enzymes. While CovR functions primarily as a global repressor of virulence genes, it also acts as a transcriptional activator of speB cysteine protease expression (28, 115).

Spontaneous mutations that inactivate either CovR or CovS enhance the virulence of this pathogen. In murine models of skin and soft tissue infection, mutations in the covRS genes correlate to development of severe invasive disease by S. pyogenes. This hypervirulent phenotype is characterized by an increase in the size and abundance of necrotic lesions, which results from overexpression of virulence factors (i.e., capsule, C5a peptidase, DNase Sda1) and decreased production of SpeB (28, 122–124). This renders covRS mutants highly resistant to phagocytosis and neutrophil-mediated killing (28, 124).

1.7.2 Mga

One of the best-characterized stand-alone regulators in S. pyogenes is Mga (115). Mga is a global response regulator that controls expression of multiple virulence genes involved in colonization (M-protein, M-like proteins, and fibronectin- and collagen-binding proteins) and immune evasion (C5a peptidase, complement inhibitors, and Ig-binding proteins). The mga regulon is activated during the exponential phase of growth, and in response to elevated CO₂, increased temperature, and iron-limiting conditions (125–127). Under these growth conditions, Mga binds to upstream promoter sites of its “core” gene targets and its own encoding gene to upregulate transcription (128). In addition, indirect regulation by Mga may also alter expression of other virulence gene targets, including the hyaluronic acid capsule, SpeB, and genes involved in carbohydrate metabolism (28, 115).

All GAS isolates carry either one of two allelic variations of the mga gene which give rise to throat (mga-1) or skin/non-specific (mga-2) infections by S. pyogenes. To adapt to such distinct microenvironments there are strain-specific differences in the regulation of Mga and its associated gene targets. This is considered a major driving force of tissue tropism (129). Furthermore, recent studies demonstrate that there is a direct link between carbohydrate utilization and Mga-dependent expression of virulence genes in S. pyogenes. In the mga operon, there are two PTS-regulatory domains that may be phosphorylated by the PTS. Mga appears to be phosphorylated in a carbohydrate-deficient state, suggesting
that Mga and its associated adherence factors are preferentially downregulated to promote tissue dissemination. However, in a carbohydrate-rich environment, *mga* is not phosphorylated and able to upregulate factors that promote colonization. Therefore, virulence factor gene expression by Mga is regulated in a temporal fashion that is coupled to nutrient availability (28, 130).

### 1.7.3 **CcpA**

In *S. pyogenes*, CCR is predominately controlled by the catabolite response regulator CcpA. CcpA serves to regulate activity of the PTS, and expression of several key virulence factors in a carbohydrate-dependent manner. At high intracellular concentrations of glycolytic intermediates such as FBP and inorganic phosphate, HPr interacts with CcpA to form a dimer. In its active form, CcpA binds to cre sites located in the promoter region of catabolic operons to repress transcription of alternative carbohydrate utilization genes [refer to section 1.6.1 for more detail] (115).

Global transcriptional analyses of *S. pyogenes* have identified CcpA as an important regulator of virulence gene expression. It controls expression of many glucose-related virulence genes in a growth-phase dependent manner. While many of these virulence genes (*speB* [encoding the cysteine protease], *sagA* [for streptolysin S production], and *cfa* [encoding CAMP factor]) are linked to carbohydrate availability, a specific subset of CcpA gene targets are regulated independently of glucose. This suggests that multiple catabolite-sensing pathways likely contribute to the global regulation of virulence factors in *S. pyogenes* (110, 131). Furthermore, it has been shown that loss of *ccpA* attenuates virulence in murine models of skin ulcers (110), oropharynx colonization, and invasive intraperitoneal infection (132).

### 1.7.4 **LacD.1**

LacD.1 was initially identified as a global regulator of virulence gene expression for its role in negative regulation of the secreted SpeB cysteine protease. Global profiling of the *S. pyogenes* transcriptome has demonstrated that LacD.1 represses SpeB expression in response to pH levels above 6.5, high salt concentrations, and carbohydrate availability (114). As mentioned previously, LacD.1 and its paralogous counterpart, LacD.2, possess
aldolase activity to metabolize lactose and galactose. However, LacD.1 has a unique regulatory role that has been adapted for virulence gene expression [refer to section 1.6.2 for more detail] (113, 114).

The mechanism by which LacD.1 regulates virulence gene expression in *S. pyogenes* is poorly understood (110). Regulation appears to function independently of LacD.1 catalytic activity but requires binding to glycolytic substrates, G3P and DHAP. Therefore, LacD.1-mediated regulation is likely coupled to nutrient availability (110, 114). To validate this hypothesis, the contribution of LacD.1 on CCR was explored with respect to glucose utilization. Analyses have revealed that 15% of genes in *S. pyogenes* are altered in response to glucose, and together, CcpA and LacD.1 may control up to 60% of this subset in various combinations. Regulation of affected virulence genes can occur through CcpA- and LacD.1-independent mechanisms (for example, CcpA alone regulates *lctO* [encoding lactate oxidase]), co-regulation (*speB* and *ackA* [encoding a putative arginine deaminase]), and through pathways independent of glucose (*ackA* regulation by CcpA). These findings demonstrate that differential expression of virulence genes requires the integration of temporal and environmental cues from numerous catabolite-sensing pathways during infection (110, 131).

### 1.7.5 Sil

A peptide-based quorum sensing system designated the streptococcal invasion locus (*sil*) has been identified in ~18% of *S. pyogenes* strains. Of these strains, many harbor mutations that render the Sil system non-functional (115, 133). However, in the M14 serotype *S. pyogenes* strain JS95 isolated from patient with necrotizing fasciitis, Sil was discovered as a key component of virulence. In this strain, Sil is associated with rapid GAS dissemination and invasion in a murine model of human soft-tissue infection. Other findings suggest that Sil mediates DNA transfer between different M-types (134).

The genetic organization of *sil* resembles two separate regulatory networks used to activate competence (*com*) and bacteriocin production (*blp*) in *S. pneumoniae* (135). It contains six genes that encode a TCS (*silA/B*), an ABC transporter (*silD/E*), and two signalling peptides (*silCR* and *silC*) which form a novel regulatory circuit that controls the transcription of *sil*
Interestingly, this signal peptide can be sensed across different streptococci, which suggests a unique role for sil in facilitating colonization (135).

Activation of sil plays a critical role in GAS adherence and competition during infection. Recent work has demonstrated that SLO- and SLS-mediated membrane damage triggers an endoplasmic reticulum (ER) stress response that results in the production of asparagaine. The released asparagaine is sensed by an alternative TCS that activates sil to modulate transcription of bacteriocin production. The resulting population-wide production of bacteriocins mediates killing of bacterial competitors and allows S. pyogenes to establish niche dominance in the host (137, 138).

1.8 Rationale and Hypothesis

Coordination of metabolism and virulence factor expression has proven to be critical for immune evasion and the establishment of infection by S. pyogenes. However, despite continuous efforts to understand the dominant asymptomatic state, little is known about the molecular processes employed by S. pyogenes to outcompete the endogenous microbiota and stably colonize the nasopharynx.

In polymicrobial environments, intra- and interspecies competition is often driven by the production of short antimicrobial peptides called bacteriocins; this provides a mechanism by which S. pyogenes may compete for ecological stability in its host (86, 138). Using a recombinase-based in vivo expression technology system developed in our laboratory by Dr. Brent Armstrong, we previously demonstrated that spb bacteriocin expression is upregulated upon introduction of GAS in the mouse nasopharynx (100). Therefore, we hypothesize that the Spb bacteriocin systems contribute to the establishment of nasopharyngeal infection. To explore this hypothesis, we sought to 1) identify the regulatory cues and mechanisms utilized by S. pyogenes to induce bacteriocin production; 2) characterize the inhibitory spectra of the Spb bacteriocins; and 3) assess the potential competitive advantage of the bacteriocins during nasopharyngeal infection. The work presented here will characterize the novel Spb two-peptide bacteriocins systems and provide greater insights into the mechanisms utilized by S. pyogenes to establish dominance in a model of its biological niche.
Chapter 2: Materials and Methods
2.1 Bacterial strains, media, and growth conditions

A complete list of bacterial strains used in this study can be found in Table 1. Molecular cloning experiments utilized the *Escherichia coli* XL1-Blue strain cultured at 37 °C in Luria-Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA, USA) with agitation, or Brain Heart Infusion (BHI; BD Biosciences, Franklin Lakes, NJ, USA) media containing 1.5% (w/v) agar (Thermo Fisher Scientific). Media was supplemented with 150 μg mL⁻¹ erythromycin (Sigma-Aldrich Canada, Oakville, ON, Canada) as necessary. Streptococcal strains were grown in Todd-Hewitt broth (BD Biosciences) supplemented with 1% (w/v) Bacto Yeast Extract (BD Biosciences) (THY) and incubated at 37 °C under static conditions. When appropriate, 1.5% agar and/or 1 μg mL⁻¹ erythromycin were added to the media. For bioactivity experiments utilizing galactose and glucose supplementation, filter sterilized 10% (w/v) stock solution of sugar (Sigma-Aldrich) was added at a final concentration of 0.5% to M17 (Oxoid, Hampshire, UK) broth (GalM17). For solid media preparation, M17 broth was supplemented with 1.5% agar and 0.1% (w/v) calcium carbonate (CaCO₃). Indicator strains *M. luteus* and *L. lactis* spp. were grown in THY at 37 °C under aerobic and static conditions, respectively. THY agar tubes were prepared with 0.75% (w/v) agar.

2.2 DNA manipulations

2.2.1 *S. pyogenes* genomic DNA extraction

For total genomic DNA extractions from *S. pyogenes*, 1.5 mL of overnight culture was centrifuged at 15,000 × g for 1 minute and washed with 0.2 mM sodium acetate. Cells were centrifuged again and resuspended in 500 μL lysis buffer (50 mM EDTA, 0.2% SDS) with 0.02 μg RNase. Following an hour-long incubation at 70 °C, 50 μL of 5 M potassium acetate was added to precipitate proteins in a 1-hour incubation at −20 °C. Cells were then pelleted at 15,000 × g for 10 minutes. For DNA precipitation, the supernatant was transferred to a new tube with 1 mL ice-cold 95% ethanol and incubated at −20 °C for 1 hour. Centrifugation was repeated, and the pellet was washed once in 1 mL ice-cold 70% ethanol. The resulting DNA was air-dried, resuspended in 100 μL Qiagen Elution Buffer (Toronto, ON, Canada), and stored at −20 °C until use.
Table 1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain/Strain No.</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>Cloning Strain: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’proABlacZΔM15 Tn10 (Tet’)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Streptococcus pyogenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGAS8232</td>
<td>M18 serotype isolated from a patient with acute rheumatic fever (GenBank accession: NC_003485.1)</td>
<td>(139)</td>
</tr>
<tr>
<td>MGAS8232 ΔspbJK</td>
<td>spbJKL deletion mutant derived from MGAS8232</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>MGAS8232 ΔspbMN</td>
<td>spbMNI deletion mutant derived from MGAS8232</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>MGAS8232 ΔspbJKMN</td>
<td>MGAS8232 containing in-frame deletions of spbJKL and spbMNI operons</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td><strong>Indicator strains for bacteriocin typing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Micrococcus luteus</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>2</td>
<td>Streptococcus pyogenes M52</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>3</td>
<td>Streptococcus constellatus</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>4</td>
<td>Streptococcus uberis</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>5</td>
<td>Streptococcus pyogenes M4</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>6</td>
<td>Lactococcus lactis spp.</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>7</td>
<td>Streptococcus pyogenes M28</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>8</td>
<td>Streptococcus pyogenes M87</td>
<td>Burton Lab</td>
</tr>
<tr>
<td>9</td>
<td>Streptococcus dysgalactiae</td>
<td>Burton Lab</td>
</tr>
</tbody>
</table>
2.2.2  Polymerase chain reaction

All polymerase chain reaction (PCR) primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) or New England Biolabs Tm Calculator (https://tmcalculator.neb.com/#!/main) (Table 2) and supplied by Sigma-Aldrich. Reactions utilized Phusion High-Fidelity DNA polymerase (0.7 µL /100 µL reaction) and 1 x High-Fidelity buffer (Thermo Fisher Scientific), 2 mM magnesium chloride (MgCl₂; Thermo Fisher Scientific), and a 3 mM deoxyribonucleotide triphosphate (dNTP) mixture (Roche, Basel, Switzerland). Forward and reverse primers were added at a final concentration of 1 µM, and 1 µL template DNA was added per 100 µL total reaction volume. All reactions were performed in the MJ Mini or MJ Research PTC-200 Thermal Cyclers (Bio-Rad Laboratories, Hercules, CA, USA) using the following gene amplification conditions: 98 °C for 5 minutes to preheat the thermocycler; 98 °C for 30 seconds for initial denaturation; 30 seconds at the calculated melting temperature (T_m) to anneal primers; 36 cycles of gene amplification at 72 °C (extension time: 30 seconds per 1 kb); and a final 7-minute extension step at 72 °C. The thermocycler was held at 4 °C to indicate completed PCR reactions.

2.2.3  DNA visualization

DNA was visualized on 1% (w/v) agarose (Thermo Fisher Scientific) in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. DNA samples were mixed with loading dye buffer (40% [v/v] glycerol, 0.25% [w/v] bromophenol blue [International Biotechnologies, New Haven, CT, USA]) in a 5:1 ratio and loaded into pre-cast wells in the gel, along with a standard 1 kb DNA molecular ladder (Invitrogen, Carlsbad, CA, USA). The PowerPac 200 Electrophoresis Power Supply and Chamber (Bio-Rad) was used to run the gel at 100 V for 1 hour. Agarose gels were stained with 0.05% (v/v) ethidium bromide in TAE for 15 minutes and visualized under ultraviolet light (Gel Doc; Bio-Rad Laboratories).

2.2.4  Plasmid isolation from E. coli

Plasmid DNA was isolated from an overnight culture of E. coli using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions. All plasmids used in this study can be found in Table 3.
### Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for generating deletion constructs</strong></td>
<td></td>
</tr>
<tr>
<td>spbJK up XhoI for</td>
<td>GCG CTGAGAA ACTCATTAAGCCATTCTTT</td>
</tr>
<tr>
<td>spbJK up BamHI rev</td>
<td>GCG GGATCC TGATTCTACACAGATAATATCTCTCT</td>
</tr>
<tr>
<td>spbJK down BamHI for</td>
<td>GCG GGATCC TGGTTTTAAATTTAGGACATATTTAAG</td>
</tr>
<tr>
<td>spbJK down XbaI rev</td>
<td>GCG TCTAGA TCAAAATTGACATCGACAGAAG</td>
</tr>
<tr>
<td>spbMN up PstI for</td>
<td>GCG GTCGAG TCTATTAGTCACTTTACTCGG</td>
</tr>
<tr>
<td>spbMN up SalI rev</td>
<td>GCG GTCGAG AAGAACAGTATCCTGTTTTCAAAA</td>
</tr>
<tr>
<td>spbMN down SalI for</td>
<td>GCG GTCGAG CCAACATCATCATAGGATTTTCAGA</td>
</tr>
<tr>
<td>spbMN down BamHI rev</td>
<td>GCG GGATCC TGAATACCTGCAAAATAAACAATACC</td>
</tr>
<tr>
<td>covR up KpnI for</td>
<td>GCG GTTACC AGTGGTTGAGAAATCTGTTTATT</td>
</tr>
<tr>
<td>covR up SalI rev</td>
<td>GCG GTCGAC TTTCTTTTGTTATCTACACCCAACCTTT</td>
</tr>
<tr>
<td>covR down SalI for</td>
<td>GCG GTCGAC ATTCGTAAGAAATAGTCATAT</td>
</tr>
<tr>
<td>covR down BamHI rev</td>
<td>GCG GGATCC GACTGATATCTCGGATATG</td>
</tr>
<tr>
<td>mga up PstI for</td>
<td>GCG CTGAGAG CAGTAATGTTGAGTAAATTGACTGA</td>
</tr>
<tr>
<td>mga up SalI rev</td>
<td>GCG GTCGAC ACTACATGATACCTGCTCTTTC</td>
</tr>
<tr>
<td>mga down SalI for</td>
<td>GCG GTCGAC GCAACATCATCATAGGATTTTCAGA</td>
</tr>
<tr>
<td>mga down BamHI rev</td>
<td>GCG GGATCC ACTCCGTTGTTTAAATAGAAAGGCAC</td>
</tr>
<tr>
<td>ccpA up KpnI for</td>
<td>GCG GTTACC GTTCTTTGTTGTGGTGGTGGA</td>
</tr>
<tr>
<td>ccpA up SalI rev</td>
<td>GCG GTCGAC TCCCCCTTCTCTCTCTCTCTCTCTCTCTCTCT</td>
</tr>
<tr>
<td>ccpA down SalI for</td>
<td>GCG GTCGAC ACTAGTAACCTGTGACTTATTAGAAGGCAC</td>
</tr>
<tr>
<td>ccpA down PstI rev</td>
<td>GCG CTGAGAG TCCTTCTTCTTCTCTCTCTCTCTCTCTCTCT</td>
</tr>
<tr>
<td>sil up BamHI for</td>
<td>GCG GGATCC GTAGTTTTTTGAAAAAATCATTT</td>
</tr>
<tr>
<td>sil up SalI rev</td>
<td>GCG GTCGAC GACAAAAATATTCATTTTTAATACAAAACT</td>
</tr>
<tr>
<td>sil down KpnI for</td>
<td>GCG GTTACC CGTTCTCTTACCTATATCA</td>
</tr>
<tr>
<td>sil down SalI rev</td>
<td>GCG GTCGAC ATAGGATATCATTTGTTGAAAAAGAC</td>
</tr>
<tr>
<td>silDE up KpnI for</td>
<td>GCG GTTACC CTGGTCTCTTTACTGTTA</td>
</tr>
<tr>
<td>silDE up SalI rev</td>
<td>GCG GTCGAC ATAGGATATCATTTGTTGAAAAAGAC</td>
</tr>
<tr>
<td>silDE down SalI for</td>
<td>GCG GTCGAC GTAGTTTTTTGAAAAAATCATTT</td>
</tr>
<tr>
<td>muDE down BamHI rev</td>
<td>GCG GGATCC TGGTAAACAGATATTAATTGGTT</td>
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<tr>
<td>rgg up KpnI for</td>
<td>GCG GTTACC CATGATACCTGTGGTCTGTCTA</td>
</tr>
<tr>
<td>rgg up SalI rev</td>
<td>GCG GTCGAC TCCCCCTTCTCTCTCTCTCTCTCTCTCTCTCT</td>
</tr>
<tr>
<td>rgg down SalI for</td>
<td>GCG GTCGAC GCAGTTTTATGAGTTGCTTTTCTATC</td>
</tr>
<tr>
<td>rgg down BamHI rev</td>
<td>GCG GTTACC CTGGTAAACAGATATTAATTGGTT</td>
</tr>
<tr>
<td><strong>Primers for generating the complement plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>PgY A XhoI for</td>
<td>GCG CCGGCG GCAACATCACCAGCAAGAGTA</td>
</tr>
<tr>
<td>PgY A KpnI rev</td>
<td>GCG GGATCC CATTAAAGATGCTCTTTTTCTAGTCAA</td>
</tr>
<tr>
<td>spbMN comp KpnI for</td>
<td>GCG GGATCC ATGGAGATAAAAGAAACTGGAACATTTTC</td>
</tr>
<tr>
<td>spbMN comp BamHI rev</td>
<td>GCG GGATCC AAAAAGACACCAGACTCGTGCCACTTTTCTCAA</td>
</tr>
<tr>
<td>terminator SalI for</td>
<td>GTTGATAA GTCGAC TTAGTAAAGGCCAGATATATTTTGAC</td>
</tr>
<tr>
<td><strong>Primers for sequencing and screening</strong></td>
<td></td>
</tr>
<tr>
<td>M13 for</td>
<td>GTAAACGACGAGCCAG</td>
</tr>
<tr>
<td>Primers for qRT-PCR analysis</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--</td>
</tr>
<tr>
<td><strong>M13</strong> rev</td>
<td>GTCATAGCTGTTTCTG</td>
</tr>
<tr>
<td><strong>spbJKL</strong> integration for</td>
<td>CTCATCATCTGTTGTAGGATAGG</td>
</tr>
<tr>
<td><strong>spbJKL</strong> integration rev</td>
<td>GTTTGGCTTCAAGAAGACC</td>
</tr>
<tr>
<td><strong>spbMNI</strong> integration for</td>
<td>GAGATCGCTGTTTAAAAATAAGAGG</td>
</tr>
<tr>
<td><strong>spbMNI</strong> integration rev</td>
<td>CTTGATACCATGTGAGCACTTCT</td>
</tr>
</tbody>
</table>

| **gyrA** RT for             | GGTTCGTATGGCTCAGTGGT |
| **gyrA** RT rev             | TTCTCTTTTCGCTTCATCGT |
| **speC** RT for             | TTTCAGCAGGCATATTCTC |
| **speC** RT rev             | TTTAAGGAAGACATGGAAAC |
| **spbJ** RT for             | GAAATATGGATGAGGCC |
| **spbJ** RT rev             | TTACCACCAACAGTCG |
| **spbK** RT for             | GGAACAATTTGAAGTGATG |
| **spbK** RT rev             | CCATTACACGCATTAGGT |
| **spbM** RT for             | GGAGATAAGGAAGACTGAAA |
| **spbM** RT rev             | CCCAAAAGTAAATAGCTGT |
| **spbN** RT for             | GAAACAAAAGGAAGACTAAG |
| **spbN** RT rev             | GTATATCCAAAATATGCTCC |

* Restriction endonuclease sites are underlined in the primer sequence.
Table 3. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pG(^{+})host5</td>
<td>Temperature-sensitive Gram-positive/E. coli shuttle vector; Erm(^r)</td>
<td>(140)</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔspbJK</td>
<td><em>spbJK</em> deletion plasmid inserted into the genome of MGAS8232</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔspbMN</td>
<td><em>spbMN</em> deletion plasmid inserted into the genome of MGAS8232</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔspbJKMN</td>
<td>Plasmid used to delete <em>spbJK</em> and <em>spbMN</em> operons in MGAS8232</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔcovR</td>
<td>Plasmid used to delete <em>covR</em> gene in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pG(^{+})host5::Δmga</td>
<td>Plasmid used to delete <em>mga</em> gene in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔccpA</td>
<td>Plasmid used to delete <em>ccpA</em> gene in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pG(^{+})host5::Δsil</td>
<td>Plasmid used to delete the <em>sil</em> operon in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pG(^{+})host5::ΔsilDE</td>
<td>Plasmid used to delete the <em>silD</em> and <em>silE</em> genes encoding an ABC-transporter in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pG(^{+})host5::Δrgg</td>
<td>Plasmid used to delete <em>rgg</em> gene in MGAS8232</td>
<td>This study</td>
</tr>
<tr>
<td>pTRKL2</td>
<td>Low-copy Gram-positive/E. coli shuttle vector; Erm(^r)</td>
<td>(141)</td>
</tr>
<tr>
<td>pTRKL2::PgyrA</td>
<td>pTRKL2 plasmid carrying the constitutive <em>gyrA</em> promoter</td>
<td>McCormick Lab</td>
</tr>
<tr>
<td>pTRKL2::PgyrA::spbMNI</td>
<td>pTRKL2 containing <em>spbMNI</em> driven by the <em>gyrA</em> promoter and rrnB terminator fused to <em>spbI</em></td>
<td>McCormick Lab</td>
</tr>
</tbody>
</table>

*Abbreviations: Erm\(^r\), erythromycin resistance.*
2.2.5 Preparation of E. coli competent cells

For preparation of rubidium chloride competent cells, an overnight culture of E. coli XL1-Blue was subcultured 1:100 in PSI broth (0.5% [w/v] Bacto Yeast Extract [BD Biosciences], 2% [w/v] Bacto Tryptone [BD Biosciences], 0.5% [w/v] magnesium sulphate, pH 7.6) and grown aerobically at 37 °C to an optical density at 600 nm (OD\textsubscript{600}) of 0.5. The bacteria were incubated on ice for 15 minutes followed by 5 minutes of centrifugation (5,000 \times g) at 4 °C. The pellet was resuspended in 0.4 \times the original volume of TfbI buffer (100 mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride, 15% [v/v] glycerol, pH 5.8) and chilled on ice for another 15 minutes. Cells were centrifuged and resuspended in 0.04 \times the original volume of TfbII buffer (75 mM calcium chloride, 10 mM rubidium chloride, 10 mM 3-(N-morpholino)propanesulfonic acid, 15% [v/v] glycerol, pH 6.5) and stored in 200 µL aliquots at -80 °C.

2.2.6 E. coli transformation

Recombinant plasmids were transformed into competent E. coli by heat-shock. Briefly, competent cells were thawed, 10 µL of the ligation reaction was added, and cells were incubated on ice for 30 minutes. Cells were shocked at 42 °C for 45 seconds and placed on ice for an additional 2 minutes. Cells were transferred to a new tube with 900 µL LB and incubated at 37 °C with aeration for 1 hour. The bacteria were plated on BHI agar with appropriate antibiotics and incubated overnight at 37 °C. Clones were verified via sequencing by the Sequencing Facility at the John P. Robarts Research Institute at Western University, London, ON, Canada.

2.2.7 Preparation of S. pyogenes competent cells

An overnight culture of S. pyogenes was used to inoculate THY broth supplemented with 0.6% glycine (1:50). Hyaluronidase (1 mg mL\textsuperscript{-1}) was added within two hours of incubation and bacteria were grown to an OD\textsubscript{600} of ~0.3. Cells were pelleted at 7,000 \times g for 5 minutes, washed once in 0.4 \times the original volume with ice-cold 15% [v/v] glycerol, and resuspended again in 0.04 \times 15% [v/v] glycerol. Competent cells were stored in 200 µL aliquots at -80 °C until use.
2.2.8  *S. pyogenes* electroporation

Correct plasmids were introduced into *S. pyogenes* by electroporation. Briefly, 2 μg of DNA were thoroughly mixed with electrocompetent cells thawed to room temperature. Cells were transferred to a 2 mm electroporation cuvette and pulsed exponentially at 2500 V and 600 Ω using the Bio-Rad GenePulser. Bacteria were then transferred to 10 mL THY and recovered at 37 °C. After 3 hours, cells were pelleted at 6,000 × g and resuspended in 1 mL THY. Cells were plated on THY and incubated at 37 °C until colonies were visible. When appropriate, erythromycin was added to solid media.

2.2.9  Construction of deletion mutants

References to genomic loci are based on the genome of *S. pyogenes* MGAS8232, an M18 clinical isolate associated with acute rheumatic fever (139). In-frame deletion mutants in the genes encoding SpbJKL, SpbMNI, CovR, CcpA, Mga, Rgg, SilDE, and Sil were generated in the Gram-positive/E. coli shuttle vector, pG* host5* (140) (Table 3). Using the appropriate PCR primers (refer to Table 2), two ~500 bp fragments flanking the upstream and downstream regions of the gene of interest were amplified from MGAS8232. PCR products, as well as pG* host5*, were digested using restriction endonucleases (New England Biolabs, Ipswich, MA, USA) and purified using the QIAquick PCR Purification Kit (Qiagen). Purified products were ligated together using T4 DNA Ligase (New England Biolabs). Ligations were performed with an insert:vector molar ratio of 5:1 and incubated overnight at room temperature. The resulting constructs were transformed into competent *E. coli* XL1-Blue and verified by DNA sequencing.

Using the pG* host5* system which contains a temperature-sensitive origin of DNA replication and an erythromycin selection marker, target wild-type genes were replaced with a mutant allele via homologous recombination (Figure 4). This process has been previously described in detail (140). Briefly, knockout constructs were electroporated into *S. pyogenes* MGAS8232 and grown at 30 °C on THY agar supplemented with erythromycin. Visible colonies were cultured in THY broth with erythromycin at 30 °C for up to four days. Bacteria was streak plated onto THY with erythromycin and incubated at 40 °C. At this temperature, the plasmid is unable to replicate, and cells that have integrated
Figure 4. Molecular cloning strategy for generating gene deletions in the *S. pyogenes* MGAS8232 genome.

In-frame genetic deletions were generated in *S. pyogenes* MGAS8232 using the pG<sup>+</sup>host5 plasmid, which contains a temperature-sensitive origin of DNA replication and an erythromycin resistance marker. Briefly, bacteria were grown in THY supplemented with erythromycin at 30 °C to enable plasmid replication. At the non-permissive temperature (40 °C), plasmid-integrated colonies (i.e., single-crossover clones that have undergone the first recombination event) were screened. Selected clones were next cultured in THY without erythromycin at 30 °C to stimulate the double cross-over event that leads to plasmid excision. Correct clones were verified by loss of erythromycin resistance and PCR.
the plasmid remain resistant to erythromycin. To verify the first integration event into the chromosome, genomic DNA was extracted from individual colonies grown at 40 °C and plasmid integration was confirmed by PCR. Correct clones were then subcultured 1:100 in THY broth without antibiotics at 30 °C. Bacteria were streaked daily on THY to isolate individual colonies. Patching of individual colonies on THY with and without erythromycin was used to identify clones that lost resistance to erythromycin, indicating that the plasmid was cured. Sensitive clones were next verified by PCR and DNA sequencing.

2.2.10 Complementation of spb mutants

For complementation of the spb in-frame deletions, DNA fragments containing the spbMNI genes were amplified from MGAS8232 without its native promoter element and inserted under control of the gyrA promoter in pTRKL2 as previously described (141). The resulting plasmid was introduced into MGAS8232 ΔspbJKMN for constitutive expression of the spbMN operon.

2.3 Bioactivity assays

Bacteriocin-typing or “P-typing” is a method used to characterize bacteria based on their production of bacteriocins or sensitivity to a range of different bacteriocins. Patterns of inhibitory activity are determined using two fundamental assays commonly referred to as the simultaneous- and deferred-antagonism tests. These assays, which are often performed on solid media, are used to identify indicator organisms that are sensitive to bacteriocin production (142). In particular, typing of streptococci utilizes a set of nine indicators (I1-I9) (143), which are listed in Table 1.

The simultaneous antagonism assay produces an environment where test strains (i.e., bacteriocin-producing strains) and selected indicators are in direct competition (142). Briefly, overnight cultures of MGAS8232 wild-type, ΔspbJK, ΔspbMN and ΔspbJKMN were spotted (10 µL) on 0.5% GalM17 agar buffered with 0.1% CaCO3 and incubated overnight at 37 °C under atmospheric and elevated (5%) levels of CO2. A THY agar tube inoculated 1:100 with an overnight culture of the target indicator strain was poured on the
pre-inoculated plate and incubated for 24–48 hours at 37 °C to form a confluent bacterial lawn. However, bacteriocins are also diffusible inhibitors that are released during the initial stages of bacterial growth (142). Therefore, to investigate diffusion of inhibitory substances into the media, the deferred antagonism assay was utilized. Test strains were streaked from one end of the plate to the other using a sterile cotton swab and incubated under similar conditions above. Following overnight incubation, bacterial growth was mechanically removed, and all remaining viable cells were exposed to chloroform. An overnight culture of the target indicator was streaked perpendicular to the test strain, and bacteria were incubated at 37 °C for 24 hours.

2.4 Growth curves

*S. pyogenes* MGAS8232 wild-type and isogenic mutant strains were grown from frozen stocks and subcultured twice (1:100 dilution) in THY broth at 37 °C. On day 3, 6 mL overnight culture was used to inoculate 100 mL pre-warmed THY to early log-exponential phase. Bacterial samples were adjusted to an OD$_{600}$ of 0.02, and loaded in triplicate, along with blank controls, in a sterile 96-well plate. Bacteria were grown under appropriate conditions and OD$_{600}$ measurements were recorded every 15 minutes for 18 hours using the Synergy HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

2.5 In vivo experiments

2.5.1 Ethics Statement

All animal experiments were conducted in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The animal protocol (2017-024) was approved by the Animal Use Subcommittee at Western University (London, ON, Canada) (Appendix 1).

2.5.2 Murine nasopharyngeal infection model

Mice were bred and housed in the West Valley Barrier Facility at Western University. All experiments were performed using transgenic C57BL/6 (B6) mice expressing the major histocompatibility complex (MHC) II human leukocyte antigen (HLA)-DQ8 donated from the Kotb Laboratory (144, 145). As previously described (82, 83, 146), HLA-DQ8 mice 9–
13 weeks of age were intranasally inoculated with \( \sim 10^8 \) CFU of \( S. \) pyogenes grown to early exponential phase (OD\(_{600} \sim 0.2–0.4 \) and resuspended in Hanks’ Balanced Saline Solution (HBSS; Life Technologies, Carlsbad, CA, USA). Mice were anaesthetized with Forane (isoﬂurane, USP; Baxter International, Deerfield, IL, USA) and nasally infected with a 7.5 µL dose of bacteria per nostril. At 48 hours post-infection, mice were sacrificed and their complete nasal turbinates (cNTs), including the nasal-associated lymphoid tissue (NALT), nasal turbinates (NT), and maxillary sinuses (MS), were extracted (Figure 5). The cNTs were homogenized in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) buffer, serially diluted, and plated on Trypticase Soy Agar (TSA) supplemented with 5% sheep’s blood (BD Biosciences) for bacterial enumeration. Counts less than 25 CFU per 100 µL of cNT were considered below the theoretical limit of detection (147).

2.5.3 **Time-course of S. pyogenes infection**

Mice were inoculated intranasally with 15 µL HBSS-vehicle or wild-type \( S. \) pyogenes MGAS8232 on day 0 as previously described (see above). To examine whether the length of infection affected microbial abundance in the nasopharynx, mice were anesthetized and sacrificed at 24-hour time points for up to 96 hours post-infection. Mice infected with the vehicle control were sacrificed of day 0. All cNTs were processed on TSA, BHI, and TSA with 5% sheep’s blood for bacterial enumeration, and stabilized in RNAprotect Cell Reagent (Qiagen) for storage at -80 °C.

2.1.1 **Nasopharyngeal competition experiments**

To determine whether Spb production outcompetes \( spb \)-deficient strains of streptococci, \( S. \)pyogenes MGAS8232 and the \( \Delta spbJK \), \( \Delta spbMN \), and \( \Delta spbJKMN \) mutants were grown to early exponential phase and prepared individually for intranasal inoculation. An equal volume of wild-type MGAS8232 was mixed with each of the isogenic mutants for a colonization ratio of 1:1. These strains were then diluted 1:2 with HBSS to the appropriate inoculum dose. Control mice were infected with either \( 10^8 \) CFU MGAS8232 wild-type or the respective mutant strain. Mice were euthanized 48 hours post-inoculation and cNTs were collected and homogenized in 500 µL TE buffer, serially diluted in HBSS, and plated.
Figure 5. Isolation of murine complete nasal turbinates.

(A) A cross-section view of a mouse head to visualize the nasal passage. (B) Front view of the nasal passage, including the maxillary sinuses (MS), nasal turbinates (NT) and the nasal-associated lymphoid tissue (NALT) [Image modified (148)]. The collective tissue environment is known as the complete nasal turbinates (cNT).
for bacterial enumeration on TSA with 5% sheep’s blood. In some experiments, mice were administered 2 mg L\(^{-1}\) neomycin sulphate in their drinking water two days prior and throughout the length of infection to reduce variability in the nasal microbiota. Other experiments utilized clean water to examine the effects of background nasal microbiota on colonization efficiency. cNTs collected from non-antibiotic treated mice were stabilized in RNAprotect Cell Reagent and stored at -80 °C for future analysis.

### 2.1.2 Complementation of spbMNI

The expression plasmid pTRKL2, containing spbMNI under control of the gyrA promoter (pTRKL2::P\(_{gyrA}::spbMNI\)), was transferred to MGAS8232 ΔspbJKMN by electroporation. To determine whether plasmid-based complementation of one functional bacteriocin system was able to restore antimicrobial activity, mice were inoculated with \(~10^8\) CFU of \(S.\) pyogenes grown to early exponential phase. Nasal inoculations were performed as described above.

### 2.1.3 Co-colonization ratio

Hemolytic colonies recovered from control-treated mice and mice inoculated with competing bacterial strains were patched on GalM17 agar supplemented with 0.1% CaCO\(_3\) and incubated overnight at 37 °C in 5% CO\(_2\). Colonies with inhibitory activity, or lack thereof, against \(M.\) luteus were quantified by clearance zones or overgrowth, respectively.

### 2.2 \(S.\) pyogenes RNA extraction

\(S.\) pyogenes cells, from frozen or fresh overnight cultures (OD\(_{600}\) ~0.2–0.8), were incubated with 1 mL RNAprotect Cell Reagent for 5 minutes at room temperature. The cells were centrifuged for 10 minutes at 15,000 \(\times g\) and resuspended in 500 µL Tris-EDTA Glucose (10 mM Tris, 2 mM EDTA, 25% glucose) buffer with 5 µL of 20 mg mL\(^{-1}\) lysozyme and 5 µL of 10 U µL\(^{-1}\) mutanolysin. Cells were incubated in 37 °C water bath for 1 hour. Following centrifugation at 21,000 \(\times g\), cells were resuspended in 500 µL lysis buffer (50 mM EDTA, 0.2% SDS) with 10 µL 10 mg mL\(^{-1}\) proteinase K and incubated at 65 °C for 2 hours. RNA was isolated using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions.
2.3 Quantitative reverse transcriptase PCR

RNA was used to generate cDNA using SuperScript II Reverse Transcriptase (RT) and Random Primers purchased from Invitrogen Life Technologies. Sequence-specific primers were designed for \textit{spb} antimicrobial genes with an amplicon length of \textasciitilde200 bp. All gene samples, including the \textit{gyrA} housekeeping gene and \textit{speC} positive control, were first amplified from wild-type MGAS8232 genomic DNA and quantified using the NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Purified products were adjusted to a starting concentration of 5 ng \( \mu \text{L}^{-1} \) \( (10^0) \) and serially diluted to \( 10^{-8} \) in Qiagen Elution Buffer. The quantitative reverse transcriptase (qRT)-PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) according to manufacturer’s recommendations. All reactions were performed using the appropriate primers (refer to Table 2) and template, with an additional no template and minus RT control. Samples were run on a Corbett Life Sciences Rotor-Gene 6000 (Sydney, Australia) using the following gene 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 (\( \times 45 \) cycles).
Chapter 3: Results
3.1 Confirmation of \textit{spb} deletion mutants

In-frame deletions of the \textit{spbJK} and \textit{spbMN} operons in \textit{S. pyogenes} MGAS8232 were previously designed and generated by Dr. Armstrong of the McCormick laboratory (Table 3; Figure 6A). Deletion mutants were validated using PCR amplification with primers in the flanking regions of the deleted regions. This analysis demonstrated that compared to wild-type, the MGAS8232 \textit{A}spb\textit{JK} strain was found to have a 1854 bp deletion in the \textit{spbJK} region, the \textit{A}spb\textit{MN} strain featured a 1049 bp deletion, and the double mutant was shown to include both these deletions (Figure 6B). Growth in THY media was compared to ensure that the mutations had no detrimental effects on the growth rate of \textit{S. pyogenes}. MGAS8232 \textit{A}spb\textit{JK}, \textit{A}spb\textit{MN}, and \textit{A}spb\textit{JKMN} mutants were found to have no major growth difference when compared to wild-type (Figure 6C).

3.2 \textit{In vitro} expression of \textit{spb} genes is induced by CO$_2$ and galactose

Virulence factor gene expression in \textit{S. pyogenes} is coregulated by multiple regulatory loci in a growth phase-dependent manner (110, 131). With regard to bacteriocins produced by other Gram-positive bacteria, patterns of gene expression have been linked directly to the growth cycle and changes in environmental conditions (149, 150). For example, it has been shown that environmental cues such as pH (150, 151), temperature (152, 153), and the accumulation of specific quorum-sensing molecules (149) are able to stimulate bacteriocin synthesis. To determine the optimal conditions required to induce Spb production by \textit{S. pyogenes}, wild-type and isogenic mutant strains of MGAS8232 were grown on M17 media supplemented with various carbohydrate sources and cultivated under different growth parameters; antimicrobial activity was detected by growth inhibition of indicator strains. Here, we show that growth in the presence of 5% CO$_2$ and 0.5% galactose are distinct regulatory cues that appear to activate \textit{spb} expression \textit{in vitro}. Under these conditions, \textit{S. pyogenes} strains encoding both or one of the \textit{spb} gene clusters produced an antimicrobial compound that was active against \textit{M. luteus}. To confirm that the \textit{spb} operons were responsible for the antimicrobial activity of MGAS8232, the double-bacteriocin knockout strain was also screened for activity. As anticipated, the \textit{A}spb\textit{JKMN} mutant showed no detectable antimicrobial activity. Furthermore, \textit{M. luteus} formed a confluent bacterial lawn
Figure 6. Verification and evaluation of spb deletion mutants in *S. pyogenes* MGAS8232.

(A) To confirm in-frame deletions of the *spbJK* and *spbMN* operons in MGAS8232, primers outside of the homologous recombination site were used to amplify genomic DNA extracted from wild-type and isogenic mutants. (B) PCR products were visualized on a 1% agarose gel stained with ethidium bromide. (C) Growth of *S. pyogenes* MGAS8232 and Δ*spbJK*, Δ*spbMN*, and Δ*spbJKMN* mutants in THY at 37 °C was measured by OD₆₀₀ over the course of 18 hours. Data is presented as mean ± SD of each culture analyzed in triplicate.
in the absence of CO₂, suggesting that Spb activity is CO₂-dependent (Figure 7A, B).

Next, we sought to examine the effect of incubation time on bacteriocin production. To determine the optimal incubation period, *S. pyogenes* strains were grown at 12-hour intervals for up to 48 hours before simultaneous antagonism testing against *M. luteus*. No differences in antimicrobial activity between incubation periods were observed, as zones of clearance surrounding *spb*-producing strains were comparable to those produced after 24 hours as illustrated in Figure 7A. Lastly, to investigate the effect of different carbon sources on Spb production, test strains were cultured on M17 agar supplemented with 0.5% glucose under atmospheric and elevated levels of CO₂. Notably, all MGAS8232 strains, including the double bacteriocin-deficient strain, produced an inhibitory substance that was active against *M. luteus* under CO₂ (Figure 7C). These findings suggest Spb bacteriocin production is induced in response to galactose and CO₂, and that glucose and CO₂ may activate other unidentified bacteriocin-like inhibitory substances by *S. pyogenes*.

### 3.3 Inhibitory spectra of the Spb bacteriocins

To identify the inhibitory spectra of the Spb bacteriocins, *S. pyogenes* MGAS8232 wild-type and isogenic mutant strains were tested against a set of defined indicators listed in Table 1. Test strains were spotted on M17 media supplemented with 0.5% galactose and cultured under atmospheric and elevated levels of CO₂. Using the simultaneous assay, we demonstrate that *S. pyogenes* strains encoding either one or both of the *spb* loci exhibit antimicrobial activity against all M-types of *S. pyogenes*, *S. dysgalactiae*, and *M. luteus* when grown with 5% CO₂ (Table 4). In the absence of CO₂, visible zones of clearance were not observed. These results suggest that the Spb bacteriocins produced by *S. pyogenes* possess a narrow range of killing that is limited to closely related bacterial species.

### 3.4 Time-course analysis

To examine whether microbial abundance is affected by bacteriocin production over time, an experiment using the murine model of *S. pyogenes* acute nasal infection was designed to analyze the time course of infection in the presence of the microbiota. Previous time-course experiments utilizing this model have been performed with neomycin-treated drinking water to reduce the microbiota to assist in the establishment of acute infection.
Figure 7. Spb production by *S. pyogenes* MGAS8232 is induced by galactose and CO₂.

Bioactivity assays for detecting antimicrobial activity by *S. pyogenes* MGAS8232 wild-type and isogenic mutant strains. (A) Cells from overnight cultures of wild-type and *spb*-deficient strains ΔspbJK, ΔspbMN, and ΔspbJKMN were spotted on M17 media supplemented with 0.5% galactose and 0.1% CaCO₃ and incubated overnight at 37 °C with atmospheric and elevated levels of CO₂. A second agar layer inoculated with *M. luteus* was grown overtop, forming a confluent bacterial lawn. (B) All strains and plates were prepared as described in Panel A. Overnight cultures of *S. pyogenes* were streaked across agar plates. Cells were mechanically removed and treated with chloroform. *M. luteus* was streaked perpendicular to the test strains. (C) Cells from overnight cultures of wild-type and isogenic mutants were spotted on M17 media supplemented with 0.5% glucose and 0.1% CaCO₃ and incubated for 24 hours at 37 °C under atmospheric and elevated levels of CO₂. Antimicrobial activity was tested against *M. luteus*. 
### Table 4. Antimicrobial spectra of the Spb bacteriocins.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Elevated CO₂ (5%)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MGAS8232</td>
<td>ΔspbJK</td>
<td>ΔspbMN</td>
<td>ΔspbJKMN</td>
</tr>
<tr>
<td>M. luteus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. pyogenes M52</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. constellatus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. uberis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. pyogenes M4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. lactis spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. pyogenes M28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. pyogenes M87</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. dysgalactiae</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, zone of clearance; -, no clearance zone.
However, in the current study, neomycin was not administered to examine potential interactions between *S. pyogenes* and the nasopharyngeal microbiota. HLA-DQ8 transgenic mice were intranasally inoculated with *S. pyogenes* MGAS8232 and sacrificed at 24, 48, 72, and 96-hour time-points. Control mice received saline solution and were sacrificed on day 0 for microbiome analysis. Murine cNTs were removed, homogenized and the *S. pyogenes* burden in the nasopharynx was determined by growth of hemolytic colonies on TSA with 5% sheep’s blood agar. Consistent with previous findings from our laboratory (82), we show that mice sacrificed at the 48-hour endpoint had the highest load of *S. pyogenes* in the nasopharynx (Figure 8A). By 96 hours, CFU of *S. pyogenes* were dramatically reduced but not completely eliminated from the nasopharynx. However, clearance has been shown to take up to 6 days (82). Furthermore, through the administration of water lacking antibiotics, these data demonstrate that *S. pyogenes* is able to establish an acute infection. In addition to TSA with 5% sheep’s blood agar, the endogenous murine nasal microbiota was enumerated on various nutrient-rich mediums. The nasopharyngeal burden recovered on TSA and BHI agar were comparable to the total bacterial counts recovered on TSA with 5% sheep’s blood agar (Figure 8B), and therefore, not shown. Of note, counts of total bacteria and *S. pyogenes* in the nasopharynx were remarkably similar, indicating that non-streptococcal bacteria were only detected when CFU of *S. pyogenes* were low. These results indicate that another approach, such as 16S small subunit ribosomal RNA (rRNA) sequencing, may be required to identify changes in microbial diversity and abundance in the nasopharynx in response to bacteriocin production.

### 3.5 Evaluation of Spb systems during nasopharyngeal infection

Bacteriocins are considered one of the major driving forces of intraspecies bacterial competition within a biological niche (92, 101, 154). In humans, the composition of upper respiratory tract microbiome is particularly complex and frequently colonized by both commensal and pathogenic bacteria, including many species of streptococci (155, 156). Therefore, it is likely that organisms that exist within this environment produce bacteriocins to promote colonization. To test whether the Spb bacteriocin systems play a role in the establishment of nasopharyngeal infection by *S. pyogenes*, mice were infected with MGAS8232 wild-type and the ΔspbJK, ΔspbMN, and ΔspbJKMN mutants. cNTs were
Figure 8. Time-course analysis of mice infected with *S. pyogenes* MGAS8232.
HLA-DQ8 transgenic mice were intranasally inoculated with ~10⁸ CFU of *S. pyogenes* MGAS8232 and sacrificed at 24, 48, 72, and 96 hours post-infection. Control mice received saline solution and were sacrificed on day 0 (n ≥ 3 for each group). The nasopharyngeal burden of (A) *S. pyogenes* and (B) total bacteria were enumerated at each time point. Data points represent CFU recovered from an individual mouse. Horizontal bars represent the median. The theoretical limit of detection (25 CFU/100 µL) is denoted by the dotted horizontal line.
collected at 48 hours post-infection and used to assess the bacterial burden. Mice infected with wild-type MGAS8232 or either of the single-bacteriocin deficient mutants were able to establish an acute infection in HLA-DQ8 mice more effectively than the double-bacteriocin knockout mutant. Overall, a significant reduction in the bacterial burden recovered from the cNTs of ΔspbJKMN-treated mice was observed compared to the wild-type and the single bacteriocin-producing strains, suggesting that infection of the nasopharynx by *S. pyogenes* requires at least one functional bacteriocin system (Figure 9A).

In addition, to confirm that the wild-type and mutant strains retained their antimicrobial phenotypes, hemolytic colonies from the cNTs were cultured and spot plated onto 0.5% GalM17 agar buffered with 0.1% CaCO₃ (Figure 9B). Notably, the colonies recovered from an *in vivo* environment recapitulate the *in vitro* phenotype of bacteriocin activity. Taken together, these results suggest an important role for Spb bacteriocin production in nasopharyngeal infection by *S. pyogenes*.

### 3.6 *In vivo* competition analyses between MGAS8232 wild-type and spb mutants

From an ecological perspective, bacteriocin production is considered an important trait in the context of colonization and bacterial fitness (157). To explore whether wild-type *S. pyogenes* could interfere with nasal infection by mutant strains of *spb*, a competition model was employed *in vivo*. In this way, the application of the *spb* mutants allowed us to use these bacteria as a proxy for competing organisms during colonization and infection. In turn, this would give us an indication of whether the *spb* bacteriocin systems provide *S. pyogenes* with a competitive advantage over other streptococcal strains and species. Again, using the murine model of acute nasal infection, HLA-DQ8 transgenic mice were inoculated with mixtures of MGAS8232 wild-type and ΔspbJK, ΔspbMN, or ΔspbJKMN for a period of 48 hours. As shown in Figure 9A, when the *S. pyogenes* MGAS8232 wild-type, ΔspbJK and ΔspbMN strains were instilled individually, they were able to establish infection unlike the bacteriocin-deficient strain ΔspbJKMN. Notably, co-inoculation appeared to impair all *spb*-producing strains from establishing infection (Figure 10A). When MGAS8232 ΔspbJK, ΔspbMN, or ΔspbJKMN were co-inoculated with wild-type,
Figure 9. One functional bacteriocin system is required for efficient nasopharyngeal infection by *S. pyogenes*.

Transgenic mice were intranasally infected with ~10^8 CFU of *S. pyogenes* MGAS8232 wild-type, ΔspbJK, ΔspbMN, or ΔspbJKMN for 48 hours (n ≥ 10 per group). (A) The nasopharyngeal burden was assessed. Each data point represents CFU from the cNT of one mouse. Horizontal solid lines are the median of 10 independent experiments. The dotted horizontal line is used to indicate the theoretical limit of detection (25 CFU/100 μL). Statistical differences between groups were determined using the Mann-Whitney U test (* P < 0.05, ** P < 0.01). (B) CFU recovered from mice were tested for bacteriocin activity using the simultaneous antagonism assay against an *M. luteus* indicator lawn.
Figure 10. Nasal coinfection with *S. pyogenes* MGAS8232 wild-type and *spb* mutants reduces the bacterial burden in the nasopharynx but appears to promote a wild-type dominant population.

Transgenic mice were co-inoculated in a 1:1 ratio with MGAS8232 wild-type and either of the *spb*-deficient strains: ΔspbJK (*n* = 7), ΔspbMN (*n* = 6), or ΔspbJKMN (*n* = 21 without neomycin; *n* = 10 with neomycin) (10⁸ total CFU). (A) The nasopharyngeal burden was assessed at 48 hours from 10 independent experiments. Data points represent CFU recovered from the cNT of individual mice. The horizontal bars and dotted line represent the median of each group and the theoretical limit of detection (25 CFU/100 µL), respectively. (B) Colonies from wild-type (*n* = 395), ΔspbJKMN (*n* = 394), and coinfectected (*n* = 541) mice were patched on GalM17 agar supplemented with 0.1% CaCO₃ and tested for bacteriocin activity against *M. luteus*. Bacterial recovery from mice with CFU counts below the limit of detection were omitted. (C) The ratio between MGAS8232 wild-type and ΔspbJKMN was determined using the simultaneous antagonism assay. Clearance and overgrowth phenotypes were quantified and used to distinguish strains. Bars represent the mean ± SD. Significant differences were determined by the Chi-squared test (** **P < 0.001, **** P < 0.0001).
significantly less CFU of *S. pyogenes* were retrieved from the mice, suggesting that competitive interactions between the two strains were able to block infection. To evaluate whether the presence of the *spb* bacteriocin systems provided a competitive advantage during nasopharyngeal colonization, the simultaneous antagonism assay was used to quantify the ratio of wild-type to Δ*spbJKMN* bacteria that colonized coinfected mice (Figure 10B). Phenotypic analysis of the bacteria recovered from the coinfected mice revealed a significantly higher rate of recovery for wild-type versus the Δ*spbJKMN* mutant (Figure 10C). This striking difference in recovery indicates that the *spbJKMN* systems gives *S. pyogenes* a competitive advantage against streptococcal species that may not encode these systems.

### 3.7 Plasmid-based complementation of SpbMN

To further confirm that Spb is involved in establishing infection, we genetically complemented the MGAS8232 Δ*spbJKMN* isogenic mutant using the Gram-positive/*E. coli* shuttle vector pTRKL2 to generate strain MGAS8232 Δ*spbJKMN* containing pTRKL2::*PgyrA*::*spbMNI* (Table 3), and confirmed transformation by PCR amplification (Figure 11A). Despite successful transformation, the MGAS8232 Δ*spbJKMN* containing pTRKL2::*PgyrA*::*spbMNI* strain did not rescue the antimicrobial phenotype *in vitro* (data not shown). Although antimicrobial activity was not restored to the double-bacteriocin deficient strain *in vitro*, we utilized the complement strain in our acute infection model using HLA-DQ8 transgenic mice. Consistent with our *in vitro* data, MGAS8232 Δ*spbJKMN* containing pTRKL2::*PgyrA*::*spbMNI* proved to be ineffective at restoring antimicrobial activity following murine nasopharyngeal passage. It is important to note that although there was no statistical difference between the bacterial burden in mice infected with the *S. pyogenes* MGAS8232 Δ*spbJKMN* and Δ*spbJKMN* containing pTRKL2::*PgyrA*::*spbMNI* strains, there was a trend towards higher CFU recovery in the Δ*spbJKMN* + pTRKL2::*PgyrA*::*spbMNI*-treated group compared to the non-complemented mutant (Figure 11B). It also appears as though many of the colonies recovered from murine cNTs were found to be sensitive to erythromycin, indicating that the plasmid may have been lost during infection (Figure 11C). Based on these findings, plasmid-based complementation may be effective at restoring the colonization phenotype, but due to the
Figure 11. Plasmid-based complementation of SpbMN in *S. pyogenes* MGAS8232 ΔspbJKMN does not increase the bacterial burden in the nasopharynx or restore antimicrobial activity.

(A) Integration of the pTRKL2 plasmid containing *spbMNI* under control of the *gyrA* promoter into *S. pyogenes* MGAS8232 ΔspbJKMN. Genomic DNA was amplified via PCR and visualized on a 1% agarose gel. (B) Transgenic mice were intranasally infected with 10⁸ CFU of either *S. pyogenes* MGAS8232 ΔspbJKMN or MGAS8232 ΔspbJKMN containing pTRKL2::PgyrA::spbMNI. The nasopharyngeal burden was assessed from murine cNTs 48 hours post-infection. Data points represent CFU of individual mice. Horizontal bars represent the median. The horizontal dotted line indicates the theoretical limit of detection (25 CFU/100 µL). Significance determined by Mann-Whitney U test (ns, not significant). (C). Colonies recovered from murine cNTs were tested for bacteriocin activity against *M. luteus* using the simultaneous antagonism assay on GalM17 agar supplemented with 0.1% CaCO₃ and erythromycin.
high rate of excision throughout the length of infection, this approach could not reliably restore the wild-type phenotype. Together this suggests that an alternative method to plasmid complementation for spbJKMN should be explored to fully validate the role of these bacteriocins in establishing infection by *S. pyogenes*.

### 3.8 *In vitro* and *in vivo* detection of spb expression

Recent findings from our laboratory have demonstrated that transcription of *spbM* is highly upregulated at 48 hours following infection in the mouse nasopharynx. In comparison, *spbM* transcripts were hardly detectable *in vitro*. This suggests that bacteriocin-encoding genes are transcribed in an *in vivo* environment, where bacteriocin production may be advantageous for mediating competition against other bacteria (100). Because GAS infection of the nasopharynx is capable of inducing *spbM* expression, we examined whether infection would increase other *spb* transcripts similarly. Using qRT-PCR, *speC, spbJ, spbK* and *spbM* transcripts were analyzed from wild-type *S. pyogenes* MGAS8232 grown under *in vitro* (OD$_{600}$ ~0.2 or ~0.8) and *in vivo* (following 48-hour infection) conditions. All data were normalized to internal housekeeping gene *gyrA*, and expression fold changes were determined using the $2^{-\Delta \Delta Ct}$ method (158). The *speC* gene was used as a positive control. In this study, we demonstrate that *spbM* transcription is 8-fold greater *in vivo* compared to wild-type MGAS8232 grown at early and late exponential phase. These findings support previous data reported from our laboratory (100). Similarly, expression of *spbJ* and *spbK* genes were upregulated under *in vivo* growth conditions. Notably, *spbJ* transcripts were significantly increased in the nasopharynx, with a 2000- and 500-fold difference in gene expression relative to early and late *in vitro* conditions, respectively (Figure 12). These findings provide strong evidence to suggest that the *spb* genes are specifically induced in an *in vivo* nasopharyngeal environment.
Figure 12. Transcription of bacteriocin-encoding genes are upregulated *in vivo* during nasopharyngeal infection.

Expression analyses of *spb* bacteriocin-encoding genes. Total RNA was isolated from cultures of *S. pyogenes* MGAS8232 grown to OD$_{600}$ ~0.2 or ~0.8 (early and late *in vitro* conditions, respectively; n = 4) and from HLA-DQ8 mice intranasally infected with MGAS8232 for 48 hours (n = 2; *in vivo* conditions). Transcription levels of *speC* (positive control), *spbJ*, *spbK*, and *spbM* were determined by SYBR green qRT-PCR and normalized to the housekeeping gene *gyrA*. Data are presented as fold change from wild-type *in vivo* relative to *in vitro* growth conditions. Data represent the mean of four independent experiments with samples analyzed in duplicates.
Fold change ($2^{ΔΔCt}$)

- Early exponential
- Late exponential

(spec, spbJ, spbK, spbM)
Chapter 4: Discussion
4.1 Discussion

*S. pyogenes* is a globally prominent bacterial pathogen with the propensity to colonize the skin and upper respiratory tract of a human host (1, 23). Successful adaptation to different tissues is, in part, due to *S. pyogenes*’ ability to sense variations in the external environment and rapidly alter its gene expression profile. Due to *S. pyogenes*’ remarkable versatility, ongoing research efforts are focused on identifying the spatial and temporal patterns of regulation that control virulence factor gene expression and immune-evasion mechanisms utilized by *S. pyogenes* to establish infection (110, 131, 132, 137). In comparison, the processes governing ecological stability, bacterial fitness, and competition against the microbiota, which are vital for colonization, remain poorly understood.

Microbial communities within humans are often characterized as competitive and complex environments that play an important role in determining health and disease. Resident microbiota are involved in many human biological processes, including modulation of the immune system, metabolism, epithelial development, as well as the prevention of colonization by opportunistic pathogens (159, 160). In any given biological niche, competition between microorganisms is driven by the need to acquire finite resources such as space and nutrients. In order to meet these demands, bacteria have adapted evolutionary traits and tools (i.e., degradative enzymes, bacteriocins and antimicrobial compounds, and mechanisms to disrupt quorum sensing-mediated signalling) to compete with one another (159, 161).

Opportunistic pathogens such as streptococci must compete with a variety of pre-existing bacteria to colonize the upper respiratory tract of humans. In many cases, direct interactions between bacteria of the same or closely related species are mediated through the production of bacteriocins (101, 159, 162); a mechanism widely used by streptococci. For example, bacteriocins salivaricin (Sal) A and SalB produced by *S. salivarius* – a well-characterized colonizer of the oral cavity – are able to inhibit growth of pathogenic streptococci, including *S. pyogenes* (163, 164). However, bacteriocins are not exclusively produced by commensal bacteria. Invaders of the upper respiratory tract and the oral cavity, such as *S. pneumoniae* and *Streptococcus mutans*, also utilize bacteriocins to mediate intraspecies competition (92, 101, 161, 162). While these groups have all demonstrated an improved ability to
colonize when compared to their bacteriocin-deficient counterparts, *S. pyogenes* also encodes a number of bacteriocin genes that have received little recognition. In this study, we demonstrate that *S. pyogenes* utilizes two novel class IIb bacteriocin systems to establish infection and strain dominance within the polymicrobial environment of the mouse nasopharynx.

Recently published findings from our laboratory have identified and characterized a novel two-component class IIb bacteriocin in *S. pyogenes*, which has been designated as the *spbMN* locus (100). Based on unpublished data, *S. pyogenes* appears to encode a second proximal type IIb system with potential genetic redundancy to *spbMN*. In turn, we designated these open reading frames as the *spbJ* and *spbK* genes. To characterize SpbJK and SpbMN in terms of their inhibitory spectrum, multiple approaches, including recombinant peptide expression and bioactivity testing, have been utilized. Using recombinant expression of SpbM and SpbN, a number of different streptococcal species, including *S. pyogenes*, *S. dysgalactiae*, *S. uberis*, as well as other Gram-positive bacteria such as *M. luteus* and *L. lactis* were shown to be susceptible to antimicrobial activity (100). To determine the range of SpbJ and SpbK activity, a similar approach was attempted; however, because SpbK appears to be toxic in *E. coli*, recombinant peptide expression proved to be less feasible. A goal of this project was thus to determine the regulatory cues required to induce expression of *spb* in vitro. This would allow us to test for antimicrobial activity using the standard simultaneous and deferred antagonism assays. Through empirical analysis, we show that *spb*-mediated inhibition is induced in response to galactose and CO₂. To formally demonstrate that the *spb* operons are functionally active in *S. pyogenes*, the double-bacteriocin knockout strain was also screened for antimicrobial activity. Under the same conditions, we show that the Δ*spbJKMN* strain is unable to inhibit grown of the Spb-sensitive indicator *M. luteus* (Figure 7A, B). Since most bacteriocins have a narrow range of killing, it is widely thought that they play a role in mediating intraspecies competition (157, 162). To investigate this assumption, we tested the wild-type and isogenic mutant strains of MGAS8232 for bacteriocin production against a defined set of nine indicator strains listed in Table 1. Although there was significant variability between experiments, the putative inhibitory spectrums have been reported in Table 4. We speculate that SpbJK and SpbMN are active against several *S. pyogenes* serotypes, including M52,
M4, M28, M87 strains, *M. luteus* and *S. dysgalactiae*, and that the Spb systems possess a similar inhibitory range. Interestingly, both susceptibility testing methods have reported slightly different antimicrobial targets. We predict that the observed disparities are due to differences in protein expression levels. Based on these findings, further testing is required to determine the range of targets susceptible to SpbJK and SpbMN. Moreover, it would be of interest to identify the antimicrobial compound that is activated in response to glucose and CO$_2$ (Figure 7C).

It is possible that other regulatory signals may also induce expression of *spb* by *S. pyogenes*. It has been recently reported that in the M179 serotype *S. pyogenes* strain JS12, bacteriocin production is stimulated through a series of sequential events involving the sil locus. In this strain, which contains a fully intact *sil*, host cell membrane-triggered stress caused by the delivery of SLS and SLO is able to upregulate asparagine synthesis (137, 138). Asparagine appears to mediate activation of *sil* in a SilCR-dependent manner, which upregulates bacteriocin production (138). This proposed mechanism for Spb regulation and transport is possible in strains encoding an intact *sil* locus; however, it is currently unknown what replaces *sil* function in *S. pyogenes* strains with mutations in this operon, such as MGAS8232 (i.e., truncation in *silD*). This is a major question that has yet to be answered, but our work demonstrates that additional pathways independent of *sil* are capable of inducing *spb* expression. Based on the regulatory cues we have identified in this study, we predict that multiple regulators may be involved in transcriptional regulation of *spb*, including CovRS, Mga, CcpA, and LacD.1. These genes were chosen as putative regulators for their role in virulence factor gene expression, sugar metabolism, or in terms of their activation signals, which correspond with the regulatory cues required to induce *spb*. To test the possible involvement of these genes in *spb* regulation, we intend to generate deletions in each of these genes (refer to Figure 4); at this time, only the plasmid-based deletion constructs have been generated (Table 3). In addition, ongoing experiments in our laboratory are focused on elucidating the potential regulators and transporters involved in Spb production through transposon-mediated random mutagenesis. This involves the development of a transposon mutant library in MGAS8232 using the kanamycin-resistant *in vivo* delivery plasmid pKRMIT (165).
Asymptomatic carriers of *S. pyogenes* are typically infected with one strain which persists in the nasopharynx for an extended period of time. In some cases, the bacteria are passively cleared, or in others, replaced by another GAS strain. Although serotype switching is a common phenomenon among group A and group B streptococci, the mechanisms which determine strain dominance remain unclear (12, 166). One likely mechanism used to explain these patterns of colonization are bacteriocins. In this study, we demonstrate that expression of one functional Spb system is required to establish acute infection in our transgenic mouse model. Loss of either SpbJK or SpbMN did not impair nasopharyngeal infection (Figure 9A) or ablate antimicrobial activity (Figure 9B). This is not surprising because we believe that these systems are redundant and used to mediate competition in the nasopharynx similar to other streptococci. In contrast, the bacteriocin-deficient strain ΔspbJKMN failed to establish infection consistently in the mice. Inoculation with ΔspbJKMN elicited a bimodal phenotype, by which only a fraction of mice had a nasopharyngeal burden similar to that of wild-type (Figure 9A). These findings suggest that in some cases, host factors such as the composition of the microbiota in the nasopharynx, allow the bacteria to establish an infection. However, a significant decrease in the bacterial burden recovered from murine cNTs indicates that deletion of both spb operons reduces the fitness of *S. pyogenes* in a polymicrobial environment.

Bacteriocins play a crucial role in altering the composition of the microbiota in humans. In a particular niche, such as the gastrointestinal tract, they are able to modulate the microenvironment in ways that are advantageous or determinantal to certain groups of bacteria (167). To examine differences in microbial abundance and diversity due to Spb production in the nasopharynx, an *in vivo* time course analysis of wild-type *S. pyogenes* in the presence of the background microbiota was performed. Our analysis was limited to the identification of different bacteria by plating on nutrient-rich mediums, which proved not to be feasible. This is because differences in bacterial counts of *S. pyogenes* and total bacteria recovered from murine cNTs were very similar (Figure 8A, B), and did not provide the means to comprehensively evaluate all bacterial species colonizing the nasopharynx, including those that are unculturable in the laboratory. Therefore, it would be of interest to assess how infection by *S. pyogenes* may change the microbial composition in the nasopharynx overtime using a more reliable approach such as 16S rRNA gene sequencing.
Although there may be limitations to the approach we have utilized, our data has revealed an important finding. When *S. pyogenes* is able to establish infection, as observed by high bacterial loads, it appears as though no other bacteria were able to colonize the mice. This finding suggests that the establishment of *S. pyogenes* in a niche may preclude other bacteria from colonizing. However, it is important to note that although this mouse model is used to mimic acute nasopharyngeal infection, microbial population dynamics differ substantially in humans. Therefore, it may be advantageous to collect nasopharyngeal wash samples from individuals colonized with *S. pyogenes*, and process these samples using 16S rRNA sequencing. This approach has been previously utilized to characterize changes in the nasopharyngeal composition that contribute to asthma (168), and may provide important insights on population dynamics during *S. pyogenes* infection in humans.

The BlpM and BlpN peptides produced by *S. pneumoniae*, which are known homologs of SpbM and SpbN (100), have also been studied in the context of colonization. Interestingly, in *S. pneumoniae*, previous work by Dawid *et al.* has shown that mutations in the *blp* operon do not impair nasopharyngeal infection when the mutant strain was instilled individually. However, when wild-type and *blp*-deficient strains were co-inoculated in equal numbers, the parent strain was able to outcompete the mutant, indicating that Blp production could interfere with colonization of *blp*-negative strains (101). In contrast, we have shown that co-inoculation impairs all *spb*-producing strains from establishing infection (Figure 10A). These results were highly unexpected and may suggest a less prominent role for Spb in mediating microbial interactions and a more important role in mediating GAS adherence compared to Blp. Although there is no exact mechanism to explain this phenomenon, one study has reported a similar finding. In a study by Lee *et al.*, interactions between the gut microbiota and *Bacteroides fragilis* were examined using a germ-free murine model of gut colonization. They demonstrated that mice infected with one *B. fragilis* strain were resistant to subsequent colonization by another strain of the same species. Remarkably, however, when mice were infected with *B. fragilis* and challenged with another *Bacteroides* species, both able to efficiently colonize. These results suggest that certain bacteria colonize the gut by saturating a specific and limited niche to effectively exclude similar strains from establishing colonization. This observation of colonization resistance, which they have coined as the “saturable niche hypothesis”, is used to describe how two closely related
bacteria may only co-exist if they have their own particular niches (169). Based on these findings, we may speculate that individual *S. pyogenes* strains may colonize the nasopharynx in a similar species-specific and saturable manner. In the current study, our mice were challenged with two *S. pyogenes* strains at the same time; both of which would theoretically occupy the same niche. Therefore, we can presume that neither strain was able to effectively displace the other to establish infection. In cases where the nasopharyngeal burden is high, we predict that *S. pyogenes* wild-type was able to exclude the ΔspbJKMN strain and infect the mice (Figure 10A). To support this notion, we show that more than 75% of colonies retrieved from coinfected mice have antimicrobial activity against *M. luteus* (Figure 10B, C). Thus, these results suggest that expression of *spb* may be critical for the establishment of nasopharyngeal infection and dominance in the host.

We next investigated whether complementation *in trans* could restore the ability of the bacteriocin-deficient strain to establish nasopharyngeal infection. Despite successful transformation of the *spbMNI* genes into MGAS8232 ΔspbJKMN (Figure 11A), this strain was unable to restore colonization in transgenic mice. There was no significant difference between the bacterial burden of mice infected with ΔspbJKMN containing pTRKL2::PgyrA::spbMNI compared to ΔspbJKMN; however, an increased trend in CFU was observed (Figure 11B). Furthermore, it appears as though many of the colonies recovered for antimicrobial testing against *M. luteus* were sensitive to erythromycin, indicating that the plasmid may have been lost during infection (Figure 11C). Therefore, colonization defects of ΔspbJKMN could not be restored through plasmid-based complementation. Future work will aim to complement *S. pyogenes* MGAS8232 ΔspbJKMN with *spbJKL* or *spbMNI in cis* to rescue the infection and antimicrobial phenotypes.

Analysis of the Spb systems using qRT-PCR demonstrates that *spbJ*, *spbK*, and *spbM* expression are highly upregulated *in vivo* compared to *in vitro* growth conditions. This data, which is similar to previous findings from our laboratory (100), further supports the idea that these bacteriocins are important for mediating competition within a polymicrobial environment of the nasopharynx (100). Interestingly, with regard to *spbJK* expression, our preliminary data seems to suggest that the α and β-encoding genes are not transcribed in a
a 1:1 ratio. While they may be released in equal amounts for optimal activity, these findings suggest that the SpbK structural peptide is sequestered and/or degraded compared to SpbJ. To our knowledge, this is first instance which may suggest that bacteriocins of the same class IIb system could be transcriptionally regulated in a different manner. However, a more likely explanation for this phenomenon involves the immunity protein. In lantibiotics, such as nisin, the immunity protein is involved in sequestering the bacteriocin on the cell membrane (170, 171). The likely immunity protein for SpbJK, which encodes a putative membrane-bound metalloprotease, may also sequester SpbK in a similar mechanism to nisin. However, it is also possible that SpbL may directly degrade excess SpbK, similar to the metalloprotease immunity protein identified in Enterococcus faecalis (170, 172). Although it is not certain why spbK is transcribed in a greater quantity, these data provide strong evidence to suggest that S. pyogenes utilizes SpbJK and SpbMN bacteriocins to colonize the host.

Host genetic factors and the composition of the microbiota contribute to a predisposition for developing S. pyogenes infection. In this work, we provide strong evidence that demonstrates that antimicrobial products, SpbJK and SpbMN, are critical for establishing infection in the nasopharynx. Although the mechanism has yet to be elucidated, our findings suggest that S. pyogenes produces bacteriocins to challenge closely related competitors. If this bacterium is successful, it may be able to saturate the nasopharyngeal niche. Our expression analyses further validate this hypothesis and show that Spb is produced only under in vivo growth conditions that are used to mimic a competitive microbial environment. In summary, our study has identified and characterized two novel class IIb bacteriocins that are used by S. pyogenes to establish infection and strain dominance in a host.
References


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Appendix 1. Animal ethics approval.

Appendix

Western

2017-024:

AUP Number: 2017-024
AUP Title: Bacterial bags play a key role in the pathogenesis of Streptococcus pyogenes
Yearly Renewal Date: 09/01/2020

The YEARLY RENEWAL for 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 RMPS 7.12, 7.13, and 7.15
      http://www.unc.ca/organizational/research/animal_use_and_welfare.html
   b) University Council on Animal Care Polices and related Animal Care Committee procedures
      http://www.unc.ca/organizational/research/animal_use_and_welfare.html

2) As per UGAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approval associated with this AUP, including permits and scientific/developmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken without the protocol author's (requestor) approval, is approved by the ACC, and
      (iii) the AUP is appropriately implemented and attended to within timelines outlined by the ACC.
      http://www.unc.ca/organizational/research/animal_use_and_welfare.html

3) As per RMPS 7.15 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 UCAC mandatory training (https://ofrh.universityofcanada.ca/); and
   c) be overseen by me to ensure appropriate care and use of animals.

4) As per RMPS 7.15, each study will align with approved AUP elements;
   a) centralized access to all animal areas will be given to UCAC veterinarians and ACC Leaders;
   b) UCAC policies and relevant ACC procedures will be followed, recruiting but not limited to:
      i) Research Animal Procurement
         a) Animal Care and Use Records
      ii) Sick Animal Response
      iii) Continuing Care Visits

5) As per institutional ORBS 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 ORBS training, facility-level training, and reviewed related PERISDS Sheets, http://www.unc.ca/health/learning/representatives.html

Submitted by: Cindy E. Laur
on behalf of the Animal Care Committee
University Council on Animal Care
Curriculum Vitae

Lana Estafanos, B.Sc. (Hons)

Education

2016 – Present  Master of Science (M.Sc.) Candidate
Department of Microbiology and Immunology
Schulich School of Medicine and Dentistry
Western University, London, Ontario

2011 – 2016  Bachelor of Science (B.Sc.), Honours Specialization in Kinesiology;
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Research Experience

2016 – Present  M.Sc. Candidate, Department of Microbiology and Immunology
Western University, London, Ontario
• Thesis: The group A streptococci bacteriocins facilitate a competitive advantage during nasopharyngeal infection
• Research advisor: Dr. John McCormick

2015 – 2016  Work Study Student, Division of Restorative Dentistry
Schulich School of Medicine and Dentistry
Western University, London, Ontario
• Research advisor: Dr. Les Kalman

2015 – 2016  Volunteer, Microbiome, Probiotics, and Urology Sciences Laboratory
Lawson Health Research Institute, London, Ontario
• Research advisor: Dr. Jeremy Burton

2015 – 2016  Research Assistant, Department of Kinesiology
Western University, London, Ontario
• Research advisor: Dr. Glen Belfry

2014 – 2015  Honors Thesis Project, Department of Kinesiology
Western University, London, Ontario
• Thesis: Effects of high-intensity versus continuous training on power output, performance and VO2max in elite rowers
• Research advisor: Dr. Glen Belfry

Publications and Presentations


Honours and Awards

2018 Dr. Frederick W. Luney Graduate Travel Award in Microbiology and Immunology, Western University, London, Ontario ($1,000)

2017 Dr. Frederick W. Luney Graduate Travel Award in Microbiology and Immunology, Western University, London, Ontario ($1,000)

2016 – 2018 Western Graduate Research Scholarship, Western University, London, Ontario ($1,500/term)

2015 – 2016 Dean’s Honour List, Western University, London, Ontario

2011 Queen Elizabeth II Aiming for the Top Scholarship ($3,500)
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**Teaching Experience**

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<td>2018</td>
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**Volunteer Experience**

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<td>2015–2016</td>
<td>Events Coordinator for the Undergraduate Microbiology and Immunology Student Association</td>
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