RECOGNITION AND EVASION OF THE *STREPTOCOCCUS* MILLERI GROUP

MECHANISMS OF HOST RECOGNITION AND IMMUNE EVASION OF MEMBERS OF THE *STREPTOCOCCUS* ANGINOSUS/MILLERI GROUP

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Lay Abstract

The *Streptococcus* Miller/Anginosus Group (SMG) is a group of bacteria comprised of three species. Members of this group are recovered from roughly one-third of healthy individuals. However, the SMG are also found in samples collected from patients with invasive disease. It is not well understood why some human-SMG relationships are pathogenic and others are not. However, it is likely that the combination of both human and SMG factors determine the nature of the relationship formed between the two. In this study, the human and SMG factors that contribute to infection were investigated. The ways by which human cells recognize members of the SMG and defend themselves from damage was explored. Additionally, SMG factors that potentially contribute to infection were probed to discover their effect on human cells. By investigating both the bacterial and host factors that lead to infection, disease treatments and preventative strategies can be tailored to individual cases.

Abstract

The *Streptococcus* Anginosus/Milleri Group (SMG) is made up of three closely related but distinct bacterial species: *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus*. The SMG are recovered from about one-third of healthy, asymptomatic individuals. Despite this, the SMG cause more incidences of invasive streptococcal disease than Group A and Group B *Streptococcus* combined. Members of this group are somehow able to live a dual lifestyle. Little work has been conducted on the molecular pathogenicity of the SMG and host factors that contribute to host susceptibility to this group have been under-investigated. My research works towards discovering how the host recognizes the SMG as well as what enables the SMG to evade clearance by the immune system. I hypothesize that: 1) recognition of the SMG by toll-like receptor 2 (TLR2) plays a key role in triggering a cytokine response by the innate immune branch (which coordinates the immune response to the SMG), 2) the expression of cytolysins and extracellular polysaccharides by members of the SMG enables evasion of innate immune recognition and cytokine responses.

hTLR2 reporter and monocyte-like cell lines as well as human blood samples from healthy donors were used to investigate the host factors that contribute to SMG infection. Five clinical reference SMG strains and a transposon mutant library were used to probe the contributing bacterial factors. It was found that TLR2 activation plays an important role in the cytokine response to the SMG, but there is heterogeneity between strains in their ability to activate TLR2. It was also found that intermedilysin expression by *S. intermedius* strains enables evasion of recognition; however, different hosts display varying susceptibility to this cytolysin. This study reveals that investigation of both host and microbial factors is essential to build an understanding of the mechanisms of SMG transition from commensalism to pathogenicity.

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Abbreviations and Symbols

AI-2	Autoinducer-2
adcA	Zinc-binding lipoprotein
Akt	Protein kinase B
ATCC	American Type Culture Collection
CBA	Columbia Blood Agar
CD59	Cluster of Differentiation 59 or Complement Regulatory Protein
ccpA	Catabolite Control Protein A Gene
ccpA	Catabolite Control Protein A
CF	Cystic Fibrosis
cps	Capsular Polysaccharide Synthesis
cps 3U	UDP-glucose Pyrophosphorylase Gene
cps A	Putative Transcriptional Regulatory Protein Gene
cps B	Putative Phosphotyrosine-protein Phosphatase Gene
cps C	Putative Regulatory Protein Gene
cps D	Putative Protein-tyrosine Kinase Gene
cps E	Undecaprenylphosphate Glucosyl Transferase Gene
cps F	β-1,4-rhamnosyltransferase Gene
cps G	N-acetylglucosamine Transferase Gene
cps I	Glucosyl Transferase Gene
cps K	Putative Galf Transferase Gene
cps M	Putative Glycosyltransferase Gene
cps N	Putative Glycosyltransferase Gene
cre	Catabolite Repressible Element
DMEM	Dulbecco's Modified Eagle Medium
EPS	Extracellular Polysaccharide

FBS	Fetal Bovine Serum
GAS	Group A *Streptococcus*
gbpB	Glucan Binding Protein B Gene
GBS	Group B *Streptococcus*
GCGS	Group C and Group S *Streptococcus*
H_2O_2	Hydrogen peroxide
HB2	HEK-Blue-hTLR2 cell line
hCBA	Columbia Blood Agar supplemented with 5% v/v Human Blood
hTLR2	Human Toll-like receptor 2
IL	Interleukin
IL-1β	Interleukin-1beta
ily	Intermedilysin Gene
ILY	Intermedilysin
lacA	Putative Galactose-6-phosphate Isomerase Subunit LacA Gene
lacR	Lac Repressor Gene
LacR	Lac Repressor
LPS	Lipopolysaccharide
luxS	S-ribosylhomocysteinase Gene
LuxS	S-ribosylhomocysteinase
MAC	Membrane Attack Complex
MOI	Multiplicity of Infection
NFκB	Nuclear Factor Kappa B
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-3-kinase
PRR	Pattern Recognition Receptor

pulA2	Putative Alkaline Amylopullulanase Gene
RBC	Red Blood Cell
RPM	Rotations per Minute
RPMI	Roswell Park Memorial Institute medium
sCBA	Columbia Blood Agar supplemented with 5% v/v Sheep Blood
SEAP	Secreted Embryonic Alkaline Phosphatase
SMG	*Streptococcus* Anginosus/Milleri Group
THY	Todd Hewitt Broth supplemented with Yeast extract
TLR2	Toll-like receptor 2
TNFα	Tumour Necrosis Factor alpha
WT	Wildtype
wzx	Flippase

Declaration of Academic Achievement

Karissa Giraldi contributed to the conception and design of experiments in this study, performed all the experiments – except for the transmission election microscopy, and analyzed and interpreted data. Research assistant Marcia Reid, from the McMaster University Electron Microscopy Facility, conducted the transmission election microscopy experiment.

Drs. Michael Surette and Dawn Bowdish contributed to the conception and design of the experiments in this study and the interpretation of the results.

All experiments involving human samples were approved by the McMaster Research Ethics Board and written informed consent was obtained from all participants.

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Chapter 1

Introduction

The current understanding of host-microbe relationships is that they fall along a spectrum that ranges from mutualism to commensalism to pathogenicity (Hooper and Gordon, 2001; Leimbach *et al*., 2013). Of particular interest are the host-microbe relationships that fall between commensalism and pathogenicity on the spectrum. Certain microbes, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, are capable of living a dual lifestyle: a commensal in some hosts and a pathogen in others (Kumar *et al*., 2014; Laub *et al.*, 2011). These organisms can be recovered from a quarter to one-third of asymptomatic, healthy individuals (Whiley *et al.*, 1992; Witzel *et al*., 2014), but they are also significant contributors of infectious disease (Ahmed *et al.*, 2006). Investigators are working to discover the mechanisms by which this dual lifestyle is possible (Peres and Madrenas, 2013).

Among the cohort of microbes capable of living a dual lifestyle is the *Streptococcus* Anginosus/Milleri Group (SMG), which is made up of three distinct, but closely related, species: *Streptococcus anginosus, Streptococcus intermedius,* and *Streptococcus constellatus* (Jensen *et al.*, 2013). Members of the SMG have been found to act as commensals and as pathogens. They are recovered from roughly one-third of healthy adults from diverse mucosal surfaces: the upper respiratory, gastrointestinal, and urogenital tracts (Gossling, 1988). Yet, the SMG cause more invasive streptococcal disease than group A and group B *Streptococcus* combined (Laupland *et al*., 2006; Siegman-Igra *et al.*, 2012). The SMG are significant contributors to human disease, but, relative to other common human pathogens, little investigation has been done on the molecular pathogenicity of the SMG (Asam and Spellerberg, 2014).

1.1 The Changing Understanding of Host-Microbe Relationships

Through the years, the perception of the ways microbes can interact with a host has changed. It was in 1876 that Robert Koch demonstrated that a bacterium is the causative agent of anthrax (Blevins and Bronze, 2010). Building on the work of Koch, scientists and members of the medical community were able to identify the many microbes that were at the root of various diseases (Blevins and Bronze, 2010). These correlations led to a widely held belief by the general population that bacteria are detrimental to human health. Yet, right around the time Koch made the connection between *Bacillus anthracis* and anthrax, microbes were being discovered in the human intestine by Billroth and Escherich (Smith *et al.*, 2007). Louis Pasteur was the first to hypothesize that microbes play an essential role in maintaining human health (Falk *et al.*, 1998). However, there was a lag between the time this was proposed and the development of techniques that could be used to investigate what roles microbes play in the human body. As findings that supported Pasteur's hypothesis grew (Gorbach *et al.*, 1988; Reid *et al.*, 1990; Tabaqchali and Booth, 1967), this led to a dichotomous view of microbes. Microbes could either be "good" or "bad" for human health.

More recently, evidence provided from research has caused further changes to the scientific community's views on host-microbe relationships. As research on this topic has grown, appreciation has been gained for the impact microbes have on virtually every aspect of host health. Microbes not only cause infectious disease (Krzyściak *et al.*, 2013), they also affect host metabolism (Baruch *et al.*, 2014), physiology (Yang *et al.*, 2015), and even neurology (Rosenfeld, 2015). These findings have led to the belief that the relationship between a microbe and a host can fall anywhere on a spectrum that ranges from mutualism to commensalism to pathogenicity (Hooper and Gordon, 2001; Leimbach *et al.*, 2013).

It has been observed that some bacterial species are able to lead a dual lifestyle, behaving as a commensal or a pathogen in different contexts. For example, *Streptococcus pneumoniae* is a potent pathogen and major cause of childhood and elderly morbidity and mortality (Feldman and Anderson, 2014). However, the carriage rate of *S. pneumoniae* is high; studies have shown that approximately one-third of healthy subjects carry *S. pneumoniae* in their nasopharynx (Kumar *et al.*, 2014; Neves *et al.*, 2013). Similarly, *Staphylococcus aureus* - capable of causing toxic shock syndrome (Stingley *et al.*, 2014), endocarditis (Chirouze *et al.*, 2014), and pneumonia (Zhang *et al.*, 2014) – has been recovered from the nasal passages of one-fifth (Witzel *et al.*, 2014) to one-third (Laub *et al*., 2011) of healthy individuals. Organisms that are capable of transitioning between commensalism and pathogenicity have been dubbed "pathobionts" (Round and Mazmanian, 2009): pathogens that colonize the host, but only cause infection under particular circumstances.

Though it is known that host-microbe relationships are dynamic, we do not have a thorough understanding of which factors affect these dynamics and in what way they come together. A burgeoning area of research is investigation into why some individuals form commensal relationships with certain pathobionts while others form pathogenic relationships (Fanning *et al.*, 2012; Larsen *et al.*, 2015). If measurable factors are discovered which trigger transitions from commensalism to pathogenicity, detection of these factors can be used to predict host susceptibility to particular pathobionts and disease progression.

1.2 The *Streptococcus* Anginosus/Milleri Group (SMG)

The SMG is among the curious collection of organisms recognized as pathobionts. DNA-DNA hybridization (Whiley and Hardie, 1989), 16S rRNA sequence comparison (Grinwis *et al.*,

2010a), and, more recently, whole genome sequence comparisons (Olson *et al.*, 2013), have demonstrated that the SMG is made up of three distinct, yet closely related, species. These are: *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* - *S. intermedius* and *S. constellatus* being more closely related to one another than they are to *S. anginosus* (Jensen *et al.*, 2013; Whiley and Beighton, 1991). Within *S. constellatus* are the subspecies *S. constellatus* subsp. *pharyngis* and *S. constellatus* subsp. *viborgensis* (Whiley *et al.*, 1999) and within *S. anginosus* is the subspecies *S. anginosus* subsp. *whiley* (Jensen *et al.*, 2013). As a result of the genotypic and phenotypic heterogeneity in the SMG - and even within each species of the group - it is likely that more subspecies will be identified in the future (Lal *et al.*, 2011).

Unifying traits of members of the SMG are that they are facultative anaerobes, their small colony size, and a characteristic butterscotch smell on agar (Asam and Spellerberg, 2014). However, other phenotypic traits displayed by this group are far more heterogeneous. For example, at one time it was thought that the group F Lancefield antigen was exclusive to the SMG (Brogan *et al*., 1997), though now it is known that the SMG can express group A, C, G, or no Lancefield antigen (Clarridge *et al*., 1999). The SMG have also been found to express a number of common virulence factors including DNase, hyaluronidase, hemolysins, and capsule (Asam and Spellerberg, 2014; Olson *et al*., 2013). Again, the expression of these factors is heterogeneous within the SMG and within the species making up this group.

In the past, the SMG was thought to only behave as a commensal organism. This is because it is recovered from roughly one-third of healthy individuals, colonizing the upper respiratory, gastrointestinal and urogenital tracts (Gossling, 1988; Whiley *et al*., 1992). More recently, a greater appreciation has been gained for the prevalence of the SMG in patients with

invasive disease. During the five-year population surveillance study conducted by Laupland *et al*, the incidence of invasive streptoccal disease caused by the SMG, Group A *Streptococcus* (GAS), Group B *Streptococcus* (GBS), and Group C and S *Streptococcus* (GCGS) was monitored from 1999 – 2004 (Figure 1.1) (Laupland *et al.*, 2006). As seen in Figure 1.1, from 1999 – 2001, no incidences of invasive streptococcal disease were attributed to the SMG (Laupland *et al.*, 2006). This is partially because clinical microbiologists believed that members of the SMG recovered from samples of clinical infections were only present because they lived as commensal organisms at the sampled sites – not because they were causing disease (Gossling, 1988). The notorious phenotypic heterogeneity of the SMG also made identification using biochemical assays difficult (Summanen *et al*., 2009). Overlap of phenotypes with other streptococcal species often led to mischaracterization of the SMG or inability to identify strains as a consequence of ambiguous characterization assay results. Without the ability to correctly identify members of the SMG, it was difficult to attribute this group as the cause of invasive streptococcal diseases.

Currently, 16S rDNA sequencing (Sibley *et al*., 2010) and real-time PCR (Olson *et al*., 2010) have proven to be much more useful for the identification of the SMG from clinical samples. As use of molecular methods for identification has increased, the SMG have been increasingly recognized as a causative agent of infection. In fact, the data from 2001 – 2004 in Figure 1.1 reveals that the SMG cause more invasive streptococcal disease that GAS and GBS combined (Laupland *et al*., 2006). The SMG are capable of causing a variety of infectious diseases. They are the most commonly isolated organism from brain abscess (Deutschmann *et al*., 2013; Gossling, 1988), cause 30-40% of pleural empyemas (Ahmed *et al.*, 2006; Maskell *et al*., 2005), and have more recently been found to cause cystic fibrosis (CF) pulmonary

exacerbations (Parkins *et al*., 2008; Sibley *et al.*, 2010). The SMG also cause other infections at lower frequencies, such as meningitis (Stelzmueller *et al*., 2009; Verma *et al.*, 2013), pneumonia (Porta *et al.*, 1998; Shinzato and Saito, 1995; Wong *et al*., 1995), and urinary tract infections (Weightman *et al.*, 2004; Whiley *et al.*, 1992). As awareness to look for the presence of the SMG in streptococcal invasive disease has increased, appreciation for the pathogenic potential of the SMG has increased.

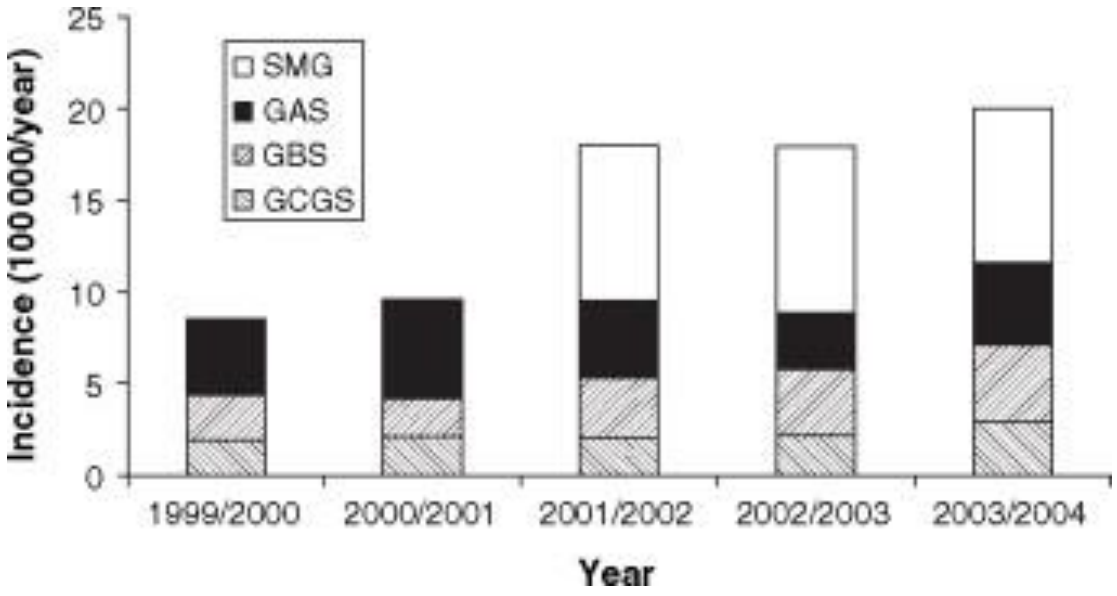

Figure 1.1: The SMG cause more incidences of invasive streptococcal disease than Group A *Streptococcus* and Group B *Streptococcus* combined.

A 5-year population surveillance study published in 2006 revealed that the SMG cause more invasive streptococcal disease that Group A and Group B *Streptococcus* combined (Laupland *et al.*, 2006).

These findings demonstrate that members of the SMG are more accurately described as pathobionts. They are somehow able to live a dual lifestyle. Even though the SMG are significant contributors to human disease, relative to other common human pathogens, little investigation has been done on the molecular pathogenicity of the SMG (Asam and Spellerberg,

2014). As with other pathobionts, a key part of understanding how the SMG forms relationships with the host is understanding how the SMG interact with the human immune system.

1.3 Innate Immunity is the Orchestrator that sets the Tone of the Host Immune Response

Investigators are working to discover the mechanisms by which the pathobiont lifestyle is possible. In many cases, the focus of this investigation is placed on microbe-immune cell interactions. A special focus is placed on microbe interactions with components of the innate branch of the immune system (Parker *et al*., 2014; Wong *et al.*, 2009).

Essentially, the immune system is a group of physiological and biochemical mechanisms that maintain homeostasis within the body of an organism and restore balance when homeostasis is disturbed (Cruvinel *et al.*, 2010). An example of the maintenance of homeostasis occurs within the gut. When components of the immune system and commensal members of the microbiota interact in healthy individuals, the resulting molecular signals enable host tolerance of these microbes, preventing chronic inflammation (Mu *et al*., 2015). Disturbed homeostasis is restored by eliminating and repairing the damage inflicted by non-self materials (ie: microbes, parasites, foreign tissue, biological molecules, and non-living substances) (Cruvinel *et al.*, 2010). In vertebrates, the immune system has been divided into two branches: innate immunity and adaptive immunity (Tomar and De, 2014). The innate immune system is pre-formed; its response to non-self materials is the same regardless of how many times it is exposed to them (Medzhitov and Janeway, 2000). Components of innate immunity include epithelial and endothelial cells, monocytes, macrophages, dendritic cells, and neutrophils (Portou *et al.*, 2015). These cells

recognize and respond to intruders in a non-specific manner (Janeway and Medzhitov, 2002). Therefore, innate immunity has no molecular memory of infection.

All innate immune cells express pattern recognition receptors (PRR) on their plasma and intracellular membranes, which recognize pathogen associated molecular patterns (PAMP) - or non-self molecules (Kumar *et al*., 2011). Binding of PRRs to PAMPs elicits release of different molecular signals from immune cells, depending on the PAMP (Mogensen, 2009). These signals are cytokines and chemokines, which allow innate immune cells to chemically communicate to the adaptive immune system that homeostasis has been disrupted (Gandhi and Vliagoftis, 2015). Generally, an innate immune response is required to activate the adaptive branch of the immune system (Janeway and Medzhitov, 2002). The cytokines and chemokines released from innate immune cells alter the gene expression of adaptive immune cells, altering their behavior in the ways required to restore homeostasis (Friedl and Storim, 2004).

Adaptive immunity is made up of B cells, which produce antibodies that specifically bind to intruders (Perera and Huang, 2015), and T cells, the effector cells of this branch of immunity (von Andrian and Mackay, 2000). Adaptive immune cells cannot respond to a microbe upon primary exposure. It is only by receiving signals from innate immunity that adaptive immunity is able to recognize that something that is non-self is in the body and respond to this intrusion (Eisenbarth and Flavell, 2009). Pro-inflammatory signals activate adaptive immune cells, attracting them to the site of infection, enabling a concerted effort to clear the infection (Gandhi and Vliagoftis, 2015). After this primary exposure, B cells produce antibodies against the intruder; this is the component of adaptive immunity that provides the specificity of its response and enables memory of exposure (Kurosaki *et al*., 2015). In addition, repeat intrusions improve the response of the adaptive branch, further distinguishing it from innate immunity (Kurosaki *et*

al., 2015). Anti-inflammatory signals are also produced by innate immune cells, which inhibit activation of adaptive immunity (Knolle *et al*., 1998). This occurs when an infection has been cleared in order to end the inflammatory response (to allow the body to heal) and to prevent an immune response against material that has not been recognized by innate immunity as an intruder (Gilroy and De Maeyer, 2015).

When a microbe enters the body, it interacts with innate immunity first, giving rise to a rapid, general response (Dranoff, 2004). This initial response will determine whether a concerted immune response will be launched against the microbe and the nature of that response (Li *et al.*, 2013). This is why study of pathobiont-innate immune cell interactions is essential in the research of the transition from commensalism to pathogenicity.

1.3.1 The Host's Innate Immune Response to a Microbe Impacts the Type of Host-Microbe Relationship Formed

The impact of innate immune responses on host-microbe relationships is well exemplified by the interactions between *Staphylococcus aureus* and macrophages. As previously mentioned, the carriage rate of *S. aureus* in healthy individuals is high (Laub *et al*., 2011). However, super antigen-encoding genes are widespread in *S. aureus* strain genomes (Wongboot *et al.*, 2013). Super antigens are exotoxins that are potent activators of T cells, causing them to release great amounts of pro-inflammatory cytokines, resulting in severe, systemic inflammation (ie: toxic shock syndrome) (Stingley *et al.*, 2014). Why isn't toxic shock syndrome widespread in the human population? Madrenas and colleagues have tackled this question; their extensive research has revealed that the relationship formed between *S. aureus* and the host is heavily

reliant upon toll-like receptor 2 (TLR2) signalling (Mele and Madrenas, 2010). TLR2 is a PRR found on innate immune cells; it plays an important role in the recognition of Gram positive microbes, as it binds diacylated lipoproteins and lipoteichoic acids, components commonly incorporated in Gram positive cell walls (Elson *et al.*, 2007; Kang *et al.*, 2009). It has been found that there is heterogeneity among *S. aureus* strains, in their ability to either colonize a host or to elicit an inflammatory response (Peres *et al*., 2015). One of the factors that contributes to this heterogeneity is the expression of different proportions of two types of TLR2 ligand (Li *et al.*, 2013; Peres *et al.*, 2015).

Certain strains express more of one type of TLR2 ligand in their cell wall, which, upon binding TLR2, triggers the MyD88-dependent canonical NFκB (Nuclear Factor Kappa B) signalling pathway (Li *et al*., 2013) (Figure 1.2). At the end of this pathway, pro-inflammatory cytokines (ex: IL-8, IL-1β, and IL-6) are secreted by innate immune cells (Li *et al*., 2013). Pro-inflammatory cytokines trigger an inflammatory response and communicate to the adaptive branch of immunity that the inflammatory strains must be cleared from the host (Janeway and Medzhitov, 2002). If an *S. aureus* strain expresses a higher proportion of this type of ligand, it is less likely it will remain in the host for long before being eliminated (Mele and Madrenas, 2010).

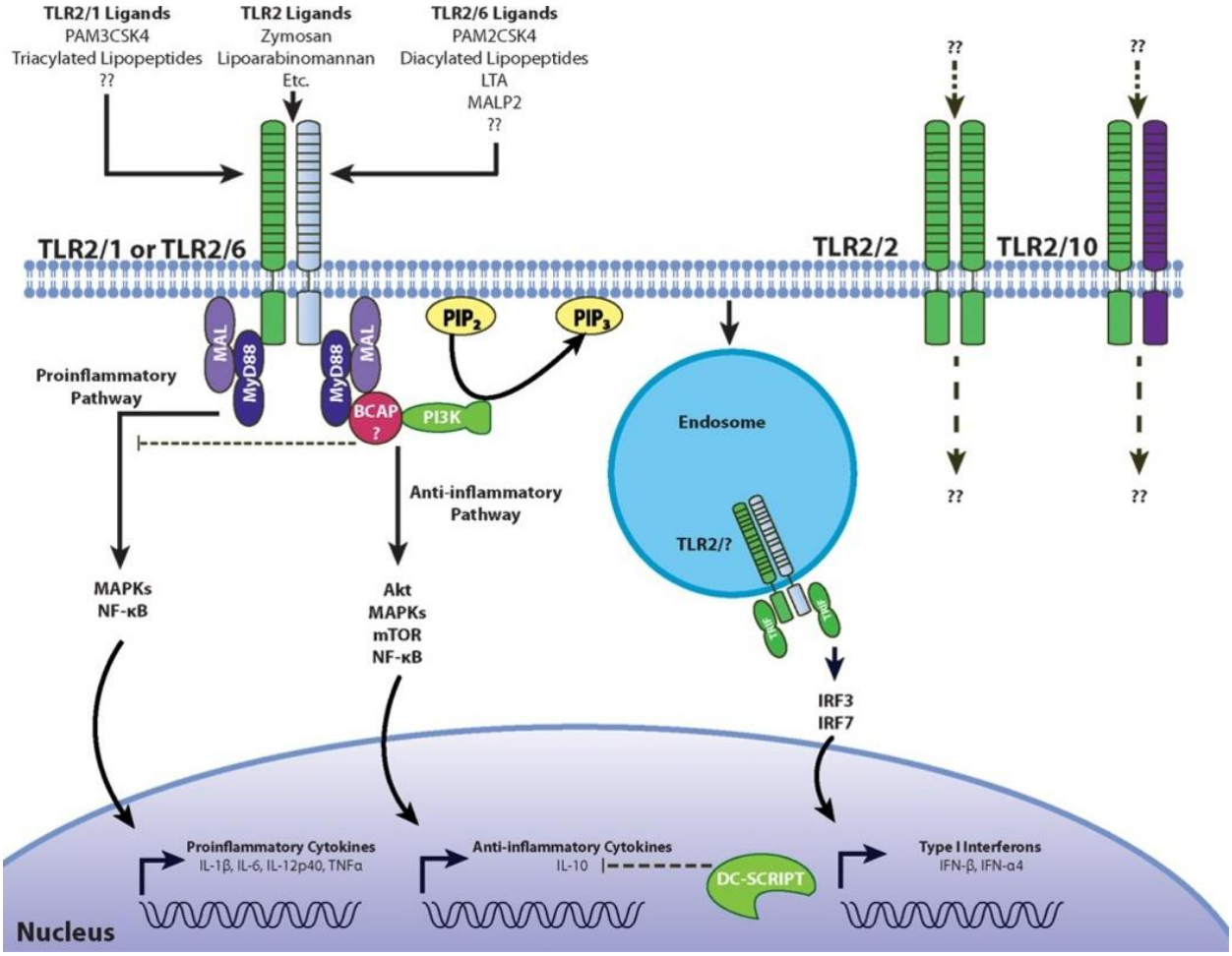

Figure 1.2: Pro- and anti-inflammatory TLR2 signalling pathways.

One type of *S. aureus* TLR2 ligand triggers the activation of a pro-inflammatory signalling pathway, resulting in the activation of NFκB and the release of pro-inflammatory cytokines by innate immune cells. The other type of TLR2 ligand activates an anti-inflammatory pathway that concludes in the NFκB-activated transcription of anti-inflammatory cytokines. (Li *et al*., 2013)

Other strains express greater proportions of the second type of TLR2 ligand, which turns on the PI3K (phosphatidylinositol-3-kinase)/Akt (protein kinase B) signalling pathway, concluding in the NFκB-activated transcription of IL-10, an anti-inflammatory cytokine (Li *et al.*, 2013) (Figure 1.2). IL-10 directly down-regulates T cells, thereby down-regulating the adaptive immune branch (Frodermann *et al.*, 2011). In this way, anti-inflammatory *S. aureus*

strains can prevent their own clearance, allowing them to persist. These strains can colonize and form a commensal relationship with the host (Mele and Madrenas, 2010). However, these strains may also cause infection when presented with an opportunity to do so and can cause more severe bacteremia as a result of T cell inhibition (Peres and Madrenas, 2013).

TLR2 signalling ultimately affects the nature of the relationship formed between the host and *S. aureus*. This example demonstrates how formative the initial molecular interactions that occur with innate immune cells are in pathobiont-host relationships.

1.4 The Study of Host-Pathobiont Relationships Requires Researchers to Reform their Perspective

As the body of data on how microbes cause infection in the host has grown over the years, insight has been gained on the factors that contribute to infection. Infection is the summation of a multitude of elements and the timing at which they come together. The genomes of many microbes encode a considerable arsenal of virulence factors; the interactions that take place with members of the host microbiota and with various host factors influence the expression of these virulence factors. This insight must be applied to the study of the transition of pathobionts from commensalism to pathogenicity.

1.4.1. The Presence of Virulence Factors Does not Always Result in Virulence

In the case of some pathogens, explicit connections can be made between the presence of virulence genes and pathogenicity. For example, the ability of *Salmonella enterica* serovar Typhi to cause severe, systemic infection (ie: typhoid fever) has been attributed to the expression of typhoid toxin – which is absent non-typhoidal *Salmonella* (Song *et al.*, 2013). And the

prerequisite for the production of cholera toxin - the cause of cholera diarrhea - by *Vibrio cholerae* is the presence the CTXϕ prophage and Toxin Co-regulated Pilus (Robins and Mekalanos, 2014). In spite of this, a genotype-phenotype connection cannot always be made by identifying particular genes in pathbiont genomes. For example, though *mecA* has been attributed to conferring methicillin resistance in *Staphylococcus aureus*, *mecA*-negative methicillin resistant *S. aureus* strains have been identified (Cortimiglia *et al*., 2015; Ludden *et al.*, 2015). Conversely, many *S. aureus* strains that have been isolated from healthy, asymptomatic individuals have many or all of the virulence genes the pathogenic *S. aureus* strains have (Peres *et al.*, 2015). In addition, virulence factors and antibiotic resistance have been found in commensal *Escherichia coli* (Bok *et al.*, 2015). In the case of the SMG, the cytolysin-encoding *ily* gene is ubiquitous in *Streptococcus intermedius* strains (Goto *et al.*, 2002), but expression of the cytolysin is heterogeneous (Nagamune *et al*., 2000).

Members of the SMG have been found to express various common virulence factors, as previously mentioned. However, there is heterogeneity in the expression of all these virulence factors, both between and within each species in the group. Except for intermedilysin, a cytolysin expressed exclusively by *S. intermedius* strains (Goto *et al.*, 2002), the expression of the other virulence factors found in the SMG overlaps between SMG species. In addition, no correlations have been made between the presence of certain virulence genes in SMG genomes and pathogenicity (Grinwis *et al.*, 2010a). Based on these results, no conclusions can be made about whether the virulence factors found in the SMG are "true" virulence factors. It is not known whether the presence of certain virulence genes in the SMG genome is a prerequisite for infection. However, recent results from host-microbe interaction research imply that the

microenvironment of a microbe provides a great contribution to pathogenicity by impacting gene regulation, and, therefore, influencing the production of virulence factors.

1.4.2 Microbe-Microbe Interactions Affect Virulence Gene Expression

The SMG are often recovered from polymicrobial infections (Belko *et al*., 2002; Sibley *et al*., 2012). In fact, it has been found by multiple research groups that the SMG and various anaerobes, such as *Prevotella intermedia* (Shinzato and Saito, 1994) and *Fusobacterium nucleatum* (Kuriyama *et al*., 2000), in combination are more likely to cause invasive disease than each species alone (Robertson and Smith, 2009). Findings on the regulation of SMG virulence factors provide possible explanations for why this occurs. For example, intermedilysin (ILY) is a human-specific, soluble cytolysin expressed by *S. intermedius* strains (Jacobs *et al*., 2000). ILY is recognized by the human membrane protein CD59 (cluster of differentiation 59 or complement regulatory protein), which is ubiquitously expressed in tissues and circulating cells (Johnson *et al*., 2013). Normally, CD59 functions as a vital inhibitor of killing by the host's complement system (Cai *et al*., 2014). However, binding of ILY to CD59 ultimately leads to pore formation in the membrane, resulting in human cell lysis (Johnson *et al*., 2013; Wickham *et al*., 2011). The gene encoding ILY – *ily* – is widespread in *S. intermedius* strains. So much so that *ily* can be used as a marker to identify strains that belong to this species (Goto *et al*., 2002). Expression of ILY has been found to be a key factor enabling cell invasion (Sukeno *et al.*, 2005) and contributes to abscess formation (Nagamune *et al.*, 1996) and deep-seated infection (Nagamune *et al*., 2000).

To date, there are three genes known to be involved in *ily* regulation: *ccpA*, *lacR*, and *luxS*. Tomoyasu *et al* showed that catabolite control protein A (ccpA), which monitors extracellular glucose concentration, binds to the catabolite-repressible element (*cre*) in the *ily* promoter region (Tomoyasu *et al*., 2010a). They demonstrated that addition of glucose into growth medium decreased the amount of ILY secreted by *S. intermedius* cells (Tomoyasu *et al.*, 2010a). In a separate study, this research group found that point and/or insertion mutations in *lacR* (Lac Repressor) correlated with higher levels of ILY production in transposon mutants and clinical isolates (Tomoyasu *et al*., 2013). These investigators demonstrated that LacR binds the promoter region of *ily* and that addition of lactose into growth medium increases *ily* transcription (Tomoyasu *et al*., 2013). Pecharki *et al* found that disruption of the *luxS* gene in *S. intermedius* resulted in a five-fold decrease in the hemolytic activity of their mutant (Pecharki *et al*., 2008). LuxS (S-ribosylhomocysteinase) generates the precursor of autoinducer-2 (AI-2), a quorum sensing molecule used by many bacterial species to chemically communicate with one another. It was found by these researchers that addition of the AI-2 precursor restored the hemolytic activity of their *luxS* mutant (Pecharki *et al*., 2008).

The involvement of *ccpA, lacR,* and *luxS* in *ily* regulation suggests that other members of a host's flora impact gene expression. Upon colonization by the SMG in proximity of other microbes which catabolise glucose, repression of *ily* would be relieved. In addition, AI-2 produced by surrounding bacteria would up-regulate *ily* expression. Perhaps the expression of other SMG virulence genes is affected by microbial factors in similar fashion. In fact, it has also been found that *luxS* decreases SMG susceptibility to antimicrobials through biofilm formation (Ahmed *et al*., 2009). The virulence of SMG strains may be increased if they are part of a microbial community that increases expression of their virulence genes.

1.4.3 Host-Microbe Interactions Affect Virulence Gene Expression

Host factors have been found to affect virulence factor expression as well. In certain contexts, they indirectly affect microbial gene expression. Extracellular polysaccharide (EPS) is an important virulence factor commonly expressed by pathogens (Li *et al*., 2014) that enables bacteria to evade elimination by the host immune system (Agarwal *et al.*, 2014; Cress *et al.*, 2014; Miajlovic and Smith, 2014). In the case of many pathogens, a single key regulatory protein is responsible for activating signalling pathways that regulate various different virulence genes within the genome – including EPS biosynthesis genes (Bagchi, 2015; Jones *et al*., 2010). These regulatory proteins respond to environmental factors within the host, such as oxygen availability (Geno *et al*., 2014), carbon source(s) (Wendler *et al*., 2014), and temperature (Elsholz *et al.*, 2014). The host environment influences whether or not a pathobiont expresses particular virulence genes.

Host and microbial cells can also directly interact with one another, influencing gene expression in one another. This was elegantly demonstrated by Baruch *et al* (Baruch *et al.*, 2014). This group found that Group A *Streptococcus* (GAS) release streptolysin toxins into host cells during adherence. These toxins cause endoplasmic reticulum stress, increasing the expression of asparagine synthetase and, consequently, levels of asparagine. The asparagine released by the host cells is sensed by GAS, leading to altered expression of almost one-fifth of GAS genes. This change results in increased GAS proliferation. (Baruch *et al*., 2014) Molecular interactions with the host have been found to alter virulence gene expression in other bacterial species (Dey *et al.*, 2013) as well as in fungi (Gupta *et al*., 2013).

To date, no genetic or molecular host factors have been determined to influence SMG virulence gene expression. However, Nagamune *et al* recently discovered that levels of ILY produced by *S. intermedius* strains vary, depending on where the strains are recovered from (Nagamune *et al*., 2000). Isolates from brain and liver abscesses were found to produce 6 and 10 times more ILY, respectively, than isolates from the mouth. Yet, a single copy of *ily* was found in the genomes of all the isolates studied. (Nagamune *et al*., 2000) These findings suggest that, in the brain and liver, host factors select for genetic variants with increased expression of *ily*. This is an area of SMG research yet to be explored.

1.5 Experimental Questions and Hypothesis

The SMG are significant contributors to human disease, but the mechanisms by which pathogenicity occurs in this group have gone largely unexplored. Work on the SMG in the Surette laboratory focuses on the bacterial as well as the host factors that lead to disease. One finding that has arisen from recent work in the Surette laboratory is that there is heterogeneity in the host cytokine response to different strains of the SMG (Figure 1.3) (Kaiser *et al*., 2014). Kaiser *et al* stimulated peripheral blood mononuclear cells (PBMC) from three healthy donors with 35 SMG strains and measured the cytokine response of the PBMCs to each strain (Kaiser *et al.*, 2014). These 35 strains included three ATCC (American Type Culture Collection) typed strains along with clinical isolates from acute infections, including hip abscess (1), brain abscesses (2), empyema (3), blood (7), and unknown invasive infections (3). There were also strains isolated from chronic infections, including isolates from CF patients in times of stability

(13) and exacerbation (3) (isolated at an abundance of $\geq 10^7$ CFU/mL). All of these strains have been phenotypically characterized in the Surette laboratory (Grinwis *et al*., 2010a, 2010b).

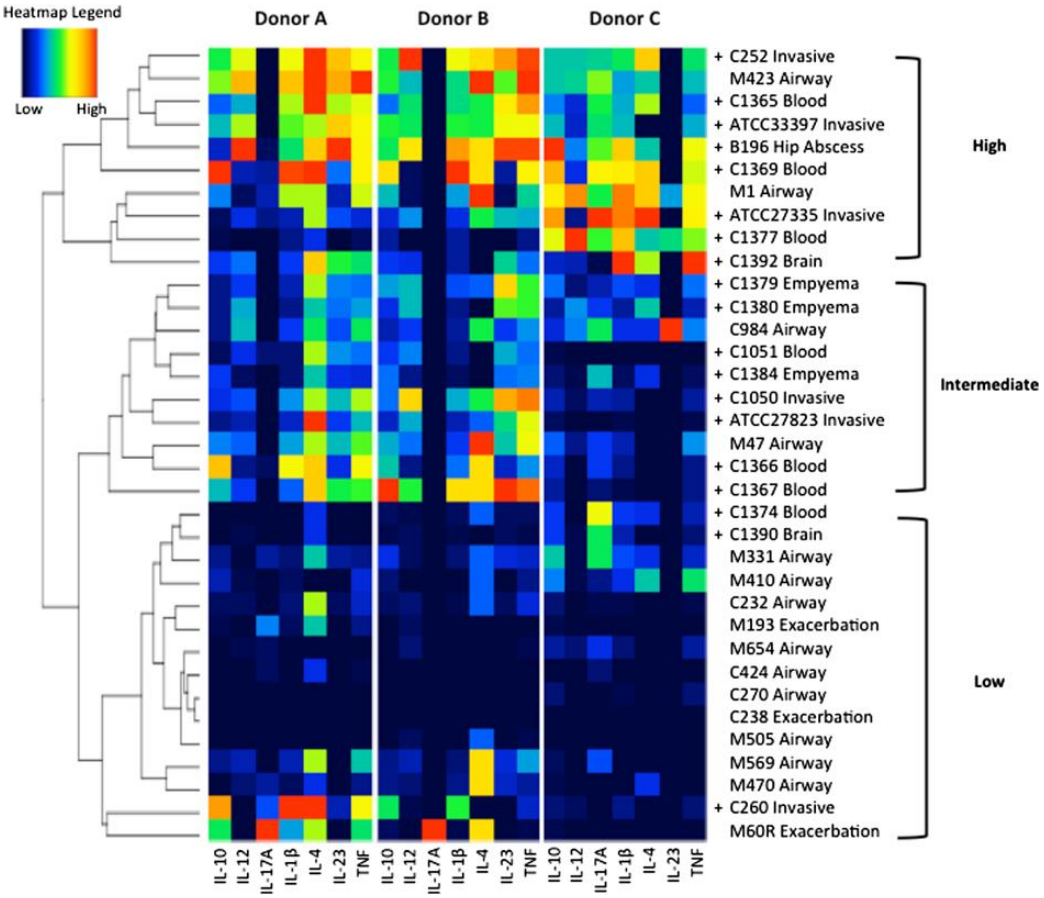

Figure 1.3. There is heterogeneity in the host cytokine response to different strains of the SMG. Kaiser *et al* stimulated peripheral blood mononuclear cells (PBMC) from three healthy donors with 35 SMG strains and measured the cytokine response of the PBMCs to each strain (Kaiser *et al*., 2014). It was found that the SMG strains could be split into three groups: strains that elicited a high release of cytokines, those that triggered an intermediate cytokine response, and strains that caused a low release of cytokines (Kaiser *et al*., 2014).

Kaiser *et al* found that the SMG strains could be split into three groups based on host cell response: strains that elicited a high release of cytokines, those that triggered an intermediate cytokine response, and strains that caused a low release of cytokines (Kaiser *et al*., 2014) (Figure

1.3). For my thesis project, I aim to learn what is occurring upstream of this response, to understand what host-microbe molecular interactions are causing these different cytokine responses. This contributes to gaining understanding on how human-SMG relationships are formed.

The ultimate goal of my work is to discover how and under what circumstances the SMG are recognized and treated like pathogens and to uncover what enables the SMG to evade clearance by the immune system. Triggering heterogeneous host cytokine responses is not a phenomenon unique to the SMG. There is also heterogeneity in the cytokine responses to *S. aureus* strains, as discussed earlier (Peres *et al.*, 2015). In the case of *S. aureus*, it is TLR2-ligand interactions – and the signalling pathways triggered by these interactions - that ultimately determine the type of relationship formed between *S. aureus* and the host (Mele and Madrenas, 2010; Peres and Madrenas, 2013). In general, TLR2 activation plays an important role in activating an immune response to Gram positive microbes (Elson *et al*., 2007; Li *et al*., 2013). Additionally, streptococcal pathogens express a variety of virulence factors in order to evade the innate immune response (Krzyściak *et al*., 2013). Two common virulence factors expressed by Streptococci are cytolysins (Littmann *et al*., 2009; Timmer *et al.*, 2009) and extracellular polysaccharide (Hyams *et al*., 2010; Lakkitjaroen *et al*., 2014), factors that are also expressed by members of the SMG (Asam and Spellerberg, 2014).

Taking this background information into account, I have set out to answer two specific questions for my thesis research project:

1) Does TLR2 activation play a significant role in triggering innate immune cytokine responses (which will, in turn, orchestrate the immune response to the SMG)?

2) Does the expression of cytolysins and extracellular polysaccharide affect human innate immune cell responses to the SMG?

To answer these questions, experiments were conducted with five reference SMG strains: B196, C1365, and M60R (*S. intermedius*), C1392 (*S. constellatus*), and C984 (*S. anginosus*). To contribute towards answering these questions and to advance understanding of the molecular pathogenicity of the SMG, further characterization was done with a B196 transposon mutant library. Experiments were designed to test the following hypotheses:

1) Recognition of the SMG by TLR2 plays a key role in triggering a cytokine response by the innate immune branch.

2) Expression of cytolysins and extracellular polysaccharide by members of the SMG enables evasion of innate immune recognition and cytokine responses.

Chapter 2

Materials and Methods

2.1 Bacterial Strains

Five reference SMG strains were selected to conduct experiments with based on their ability to cause a range of cytokine responses from PBMCs, as reported by Kaiser *et al* (Kaiser *et al*., 2014). B196, C1365, and M60R are *Streptococcus intermedius* strains. B196 is an isolate from a hip abscess and C1365 was isolated from the blood. M60R was isolated from the sputum of a CF patient – between exacerbation periods. C984 is an *S. anginosus* strain, also from the airway of a CF patient between exacerbations. C1392 is an *S. constellatus* strain from a brain abscess. Each of these strains is naturally competent, except for C1365, which can be made

electrocompetent. The Surette laboratory also has the genome sequence of each of these strains. The SMG are usually grown in 5% CO_2 at 37ºC; strains were streaked onto THY plates (Todd Hewitt media + 0.5% w/v yeast extract + 1.5% w/v agar) and one-day old colonies were used to inoculate THY broth. When used in assays, overnight cultures of the SMG were centrifuged at 12 500 rpm for 5 min and washed twice in 1X sterile PBS (phosphate buffered saline). Dead cells were prepared by aliquoting washed cells into 1.5mL Eppendorf tubes and heating them at 65ºC for 10 mins.

2.2 hTLR2 NFκB:SEAP Reporter Assay

The HEK-Blue-hTLR2 (HB2) cell line, part of the InvivoGen PlasmoTest kit, is stably transfected with hTLR2 (human Toll-like receptor 2) and a reporter gene: a secreted embryonic alkaline phosphatase (SEAP). SEAP expression is induced by the transcription factors NF-κB and AP-1. Cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) (ie: DMEM plus 2mM L-glutamine, 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin) with the addition of 350 μg/mL hygromycin, 5 μg/mL blasticidin, and 25 μg/mL zeocin. Cells were allowed to grow at 37ºC in 5% CO_2 until they reached roughly 80% confluency. 2 x 10^4 cells/well were seeded in complete DMEM in a 96-well flat bottom plate and incubated overnight. The media was then removed and either 1 x 10^6 cells of SMG or the positive control Pam_3Csk_4 (InvivoGen) (highest concentration 50 ng/mL), diluted in HEK-Blue Detection media (InvivoGen), was added to the cells. Each sample was performed in triplicate. Absorbance was read at 630 nm after 24 hours of stimulation on the SpectroMax M3 plate reader and relative activation was calculated relative to the unstimulated negative control.

2.3 Cell Viability Assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega) was used to measure HB2 cell viability following stimulation with SMG reference strains. The HB2 stimulation was conducted in a very similar fashion to the hTLR2 NFκB:SEAP Reporter Assay. However, the cell numbers were decreased by half (ie: $1x10^4$ HB2 cells/well and $5x10^5$ SMG cells/well), which yielded better signals in the assay. All of the dilutions for the stimulation were made with antibiotic-free DMEM. After the 24-hr stimulation period, the Cell Proliferation Assay was conducted according to the manufacturer's instructions. A standard curve of viable HB2 cells was constructed and the number of viable cells in each experimental and control group was interpolated from this curve. The percentage of viable cells was then calculated.

2.4 Generation of B196 Transposon Library + B196 Transformation

Transposon mutations within the B196 genome were generated *in vitro* using the TnSeq method developed by van Opijnen and Camilli (van Opijnen and Camilli, 2010). The transposon used in this technique, *magellan6*, is derived from the Himar1 Mariner transposon and contains a spectinomycin resistance marker. In addition, the inverted repeat sequences of *magellan6* contain an MmeI recognition site. Multiple identical reactions were conducted to enhance transposon library variety. Each reaction was used in separate transformations of wildtype (WT) B196.

The protocol for the transformation of WT B196 was as follows: 5 mL of THY were inoculated from a 24-hr old THY plate and incubated overnight at 37ºC in 5% CO_2. The next day, a 1:100 dilution was made of the overnight culture; this was incubated for 4 – 5 hrs at 37ºC with 5% CO_2. This culture was spun down for 15 min at 4 000 rpm, then the pellet was resuspended in 500 µL THY and transferred to a 1.5 mL Eppendorf tube. 5 ng of Competence

Stimulating Protein and the transposon library reaction were pipetted into the bacterial suspension. This was left to incubate for 1 hr at 37ºC in 5% CO_2. After this incubation period, the bacterial cells were spun down for 15 sec at 13 000 rpm in a tabletop centrifuge; all but 100 µL were removed. The cells were re-suspended in the remaining 100 µL and the suspension was plated on a THY + 75 µg/mL spectinomycin agar plate. The plate was incubated for 48 hrs at 37ºC in 5% CO_2.

After this 48-hr incubation period, B196 transposon mutants were picked from THY agar plates and arrayed into 384-well plates, each well containing 65 µL THY broth, using the Hudson Robotics Automated Colony Picker. Twenty 384-well plates were inoculated. These plates were incubated at 37ºC in 5% CO_2 for 48 hr. Each well was then filled with glycerol to a final concentration of 10% v/v using the MicroFill Dispenser (BioTek). The plates were stored at -80ºC.

2.5 Phenotype Screening of B196 Transposon Mutant Library

When phenotype screening of the B196 transposon library was conducted, the library was stamped from the -80ºC 384-well plate collection into 384-well plates containing THY broth using a 384 Pin Replicator. These plates were incubated for 48 hrs at 37ºC in 5% CO_2 and then the library was stamped from broth onto various selective media. Hemolysis was screened for by stamping the transposon library on Columbia Blood Agar supplemented with either 5% v/v human blood or 5% v/v sheep blood and monitoring plates for zones of clearing following incubation in 5% CO_2 at 37ºC. Hydrogen peroxide (H_2O_2) production was screened for by stamping mutants onto Prussian Blue media and checking for a zone of blue precipitate around growth (Saito *et al*., 2007). The absence of DNase production was screened for by stamping

mutants onto Brain Heart Infusion Agar + DNA (2 mg/mL) plates; after 24 hrs the plates were flooded with 1 M HCl for 10 min (Porschen and Sonntag, 1974). The absence of a zone of clearing indicates that DNase is not produced. Individual frozen stocks of transposon mutants with phenotypes of interest were made using 10% sterile skim milk.

2.6 Identifying Sites of Transposon Insertion

The procedure used in the van Opijnen and Camilli 2010 publication was modified to sequence the site of transposon insertion in B196 mutants of interest (van Opijnen and Camilli, 2010). The protocol used in this study is as follows: sequencing the *magellan6* insertion site requires an adaptor. In this study, the oligonucleotides used for the adaptor in the van Opijnen and Camilli paper (van Opijnen and Camilli, 2010) (Table 2.1) were utilized; however, any adaptor with a random two-base overhang (ie: 'NN') at the 3' end of the complementary strand (ie: 'oligonucleotide B') would be suitable. In addition, the 5' and 3' ends of the coding strand (ie: 'oligonucleotide A') of the adaptor were phosphorylated (Table 2.1). 5' phosphorylation allowed the adaptor to be ligated to the dephosphorylated genomic DNA fragments; 3' phosphorylation prevented the formation of adaptor-adaptor products during the ligation step of this procedure (van Opijnen and Camilli, 2010). Both oligonucleotides of the adaptor were diluted to 0.2 mM with 1 mM Tris Cl, pH 8.3. Equal volumes of both were mixed in a microcentrifuge tube and placed in a heat block at 96ºC for 2 min. The heating component of the heat block was then placed on the bench and allowed to cool to room temperature, to allow the oligonucleotides to anneal. The adaptor can be stored at -20ºC until use.

Next, genomic DNA was isolated from selected mutants using the Genomic DNA Purification Kit (Fisher Scientific). The genomic DNA of each mutant was digested with MmeI

in microcentrifuge tubes; the inverted repeat sequence of *magellan6* contains an MmeI recognition site. This endonuclease cleaves 20 bases downstream of its recognition site, producing DNA fragments that contain *magellan6* with 16 bp of B196 genomic DNA at either end. Each 50 µL MmeI digestion required 1 µL MmeI/1 µg genomic DNA, 50 µM S-adenosylmethionine (SAM), 1X Cut Smart® Buffer, and pure H_2O up to final reaction volume. The reaction mixture was incubated at 37ºC for 2.5 hr. Then, 0.5 µL CIP was added to each tube and incubated at 37ºC for 1 hr. This way, only digest fragment-adaptor ligations will occur during the ligation step. The digested DNA was then purified using a PCR Purification Kit from purchased from Life Technologies.

Table 2.1: Adaptor oligonucleotide and primer sequences utilized in the transposon insertion site sequencing protocol.

Oligonucleotide/Primer	DNA Sequence 5’ to 3’
Oligonucleotide A[a]	5Phos/GTG TGA TCG TCG GAC TGT AGA ACT CTG AAC CTG TC/3Phos
Oligonucleotide B[b]	GTT CAG AGT TCT ACA GTC CGA CGA TCA CAC NN
P1_M6_MmeI	AGA CCG GGG ACT TAT CAT CCA ACC TGT
P2_adaptor	GAC AGG TTC AGA GTT CTA CAG TCC GA

[a] 5’ phosphorylation of oligonucleotide A allowed the adaptor to be ligated to the dephosphorylated genomic DNA fragments; 3’ phosphorylation prevented the formation of adaptor-adaptor products during the ligation step of this procedure

[b] The ligation step of the protocol is possible because MmeI digestion produces a two-base 3’ overhang in the coding strand of each digest fragment and the adaptor has a two-base 3’ overhang in its complementary strand.

MmeI recognizes many sites in the B196 genomic DNA for cleavage, so to isolate the desired fragment containing *magellan6*, the digest fragments were then ligated to the adaptor. This ligation is possible because MmeI digestion produces a two-base 3' overhang in the coding strand of each digest fragment and the adaptor has a two-base 3' overhang in its complementary strand. The ligation mixtures consisted of: 25 µL pure digested genomic DNA, 1 µL of the adaptor, 1 µL 400 U/µL T4 DNA ligase, and 3 µL T4 DNA ligase buffer. The mixtures were incubated at 16ºC overnight. Next, PCR was performed on each ligation, using a forward primer that annealed to the inverted repeat sequence of *magellan6* (ie: 'P1_M6_MmeI') and a reverse primer that annealed to the ligated adaptor (ie: 'P2_adaptor') (van Opijnen and Camilli, 2010) (Table 2.1).

Since the inverted repeat sequence is at both ends of the transposon and the adaptor ligates to both ends of each digest fragment, each PCR produced two 78-bp PCR products. In order to isolate one product for sequencing, each reaction was purified using the DNA Clean & Concentrator™-25 (Zymo Research) and cloned into the pCR™4-TOPO® vector using the TOPO TA Cloning Kit for Sequencing (Life Technologies). Only one PCR product can be ligated into each vector molecule. Rubidium chloride competent cells were transformed with each cloning reaction; cells that did not contain recombinant vector died due to the presence of the lethal *Escherichia coli* gene *ccdB* in the vector – ligation with a PCR product disrupts this gene. Colony PCR was conducted on transformants; M13R binds 73 bp upstream of the ligation site and M13F anneals 60 bp downstream, producing a 211 bp fragment. This product was purified using a PCR Purification Kit (Life Technologies) and sent to the MOBIX lab at McMaster University for Sanger sequencing. Each 16 bp sequence of B196 genomic DNA was then mapped back to the WT B196 genome to determine the site of transposon insertion.

2.7 THP-1 Stimulation & ELISAs

THP-1s are non-adherent monocyte-like cells grown in Roswell Park Memorial Institute medium 1640 (RPMI) supplemented with 2mM L-glutamine, 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. They can be differentiated into adherent macrophage-like cells. Cells are centrifuged at 1500rpm for 5 min and resuspended in fresh media to a concentration of $2x10^5$ cells/mL. Phorbol 12-myristate 12-acetate is added to the cell suspension to a final concentration of 100 nM, which stimulates differentiation. 4×10^4 cells/well were seeded into a 96-well flat bottom plate and allowed to differentiate for three days before use in experiments. After this period, the media was removed from each well. Either antibiotic-free RPMI or anti-hTLR2-IgA (InvivoGen) antibody, diluted in antibiotic-free RPMI, was added to designated wells and the plate was incubated for 1 hr. After pre-blocking, live and washed C984, B196, and C1392 cells were added to wells at an MOI (multiplicity of infection) of 50. 1 ng/mL Pam_3Csk_4 was used as a positive control for TLR2 activation and 5 ng/mL lipopolysaccharide (LPS) was used as a negative control. Dilutions were made in antibiotic-free RPMI. The plate was incubated for 18 hr in 5% CO_2 at 37ºC. After this period, supernatant was removed from each well and levels of tumour necrosis factor alpha (TNFα) were measured using the ELISA MAX™ kit purchased from Biolegend, following the manufacturer's recommendations.

2.8 Cytotoxicity Assay

To determine the cytotoxicity of the β-hemolytic B196 transposon mutants isolated from phenotype screening, the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used. WT B196 and all of the β-hemolytic mutants were streaked from frozen stocks onto THY

agar. The H_2O_2-producing mutants were incubated in 5% CO_2 while the WT and the remaining β-hemolytic mutants were incubated in anaerobic conditions. After 24 hrs, these plates were used to inoculate THY broth cultures; the WT and mutants were incubated in the aforementioned atmospheric conditions for 20 hrs. Before dilution, all bacterial cultures were washed once with sterile PBS. Dilutions were made in DMEM that did not contain either antibiotics or FBS. HB2 cells seeded in a 96-well flat bottom plate were stimulated with live cell suspensions of WT B196 and each transposon library mutant at an MOI of 50 for 24 hrs in 5% CO_2 at 37ºC. After this period, the Cytotoxicity Assay was conducted according to the manufacturer's protocol. Percent cytotoxicity was calculated relative to the 100% lysed control.

2.9 Electron Microscopy

The extracellular structures of B196, C984, and C1392 were analyzed using Transmission Electron Microscopy. Aliquots of THY broth cultures of each strain were sent to the McMaster University Electron Microscopy Facility for preparation and analysis.

Chapter 3

Results

In this study, human-SMG interactions were investigated from both the human and bacterial perspective. Two hypotheses were tested: 1) recognition of the SMG by TLR2 plays a key role in triggering a cytokine response by the innate immune branch, and 2) expression of cytolysins and extracellular polysaccharide by members of the SMG enables evasion of innate immune recognition and cytokine responses. These hypotheses were tested by conducting

experiments with human cell lines and human blood samples along with five SMG reference strains and a transposon mutant library. The results from the human and the bacterial side of this investigation are outlined below.

3.1 Investigation of the Contribution hTLR2 Activation makes to the Cytokine Response of Innate Immune Cells

The following sections contain the results of experiments conducted to investigate the role hTLR2 activation plays in the innate cytokine response to reference SMG strains.

3.1.1 The hTLR NF-κB:SEAP Reporter Assay Revealed that Different SMG Strains Activate hTLR2 to Different Extents. There are Pronounced Differences Between Activation by Live versus Dead Cells in some Strains.

To test the hypothesis that hTLR2 activation plays a key role in triggering a cytokine response by innate immune cells to the SMG, experiments first needed to be done to determine whether the SMG can trigger significant levels of hTLR2 activation. To measure the ability of the reference SMG strains to activate hTLR2, the HEK-Blue-hTLR2 (HB2) cell line was used. HB2 cells are stably transfected with hTLR2 and inherently express only hTLR1 and hTLR6 (which dimerize with hTLR2). In the hTLR2 NFκB:SEAP reporter assay, HB2 cells were stimulated in triplicate with live and dead SMG cells at an MOI of 50 for 24 hours at 37°C in 5% CO_2. All dilutions were done in HEK Blue Detection media. The positive control was known hTLR2 agonist, Pam_3Csk_4, and the negative control was media without any ligand.

Five reference SMG strains were chosen for this assay: B196, C1365, M60R, C984, and C1392. B196, C1365, and M60R are *Streptococcus intermedius* strains. B196 is an isolate from a

hip abscess and C1365 was isolated from the blood. M60R was isolated from the sputum of a CF patient – between exacerbation periods. C984 is an *S. anginosus* strain, also from the airway of a CF patient between exacerbations. C1392 is an *S. constellatus* strain from a brain abscess. These strains were chosen based on their ability to cause a range of cytokine responses from PBMCs, as reported by Kaiser *et al* (Kaiser et al., 2014). The Surette laboratory also has the genome sequence of each of these strains.

SEAP, the NFκB-induced reporter in the HB2 cell line, catalyzes a colour change in the HEK Blue Detection media, which enabled indirect measurement of hTLR2 activation. When SEAP was induced by NF-κB activation (which is triggered by hTLR2 activation), this could be measured spectrophotometrically. The absorbance of supernatant from each well of the assay plate was measured at 630 nm following the stimulation period. Relative hTLR2 activation was calculated for each positive control and experimental group by calculating the fold change in absorbance relative to the negative control.

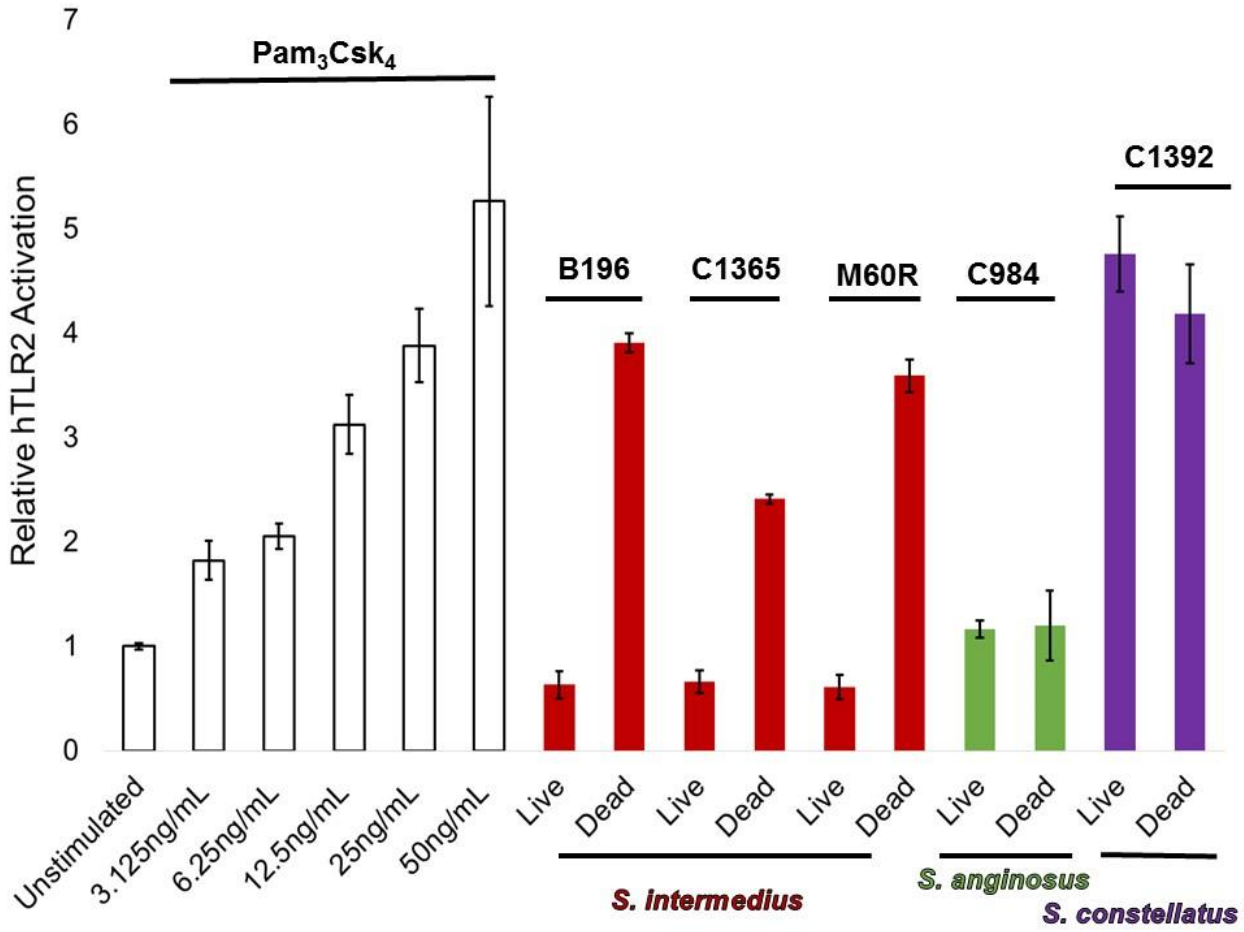

Figure 3.1: The hTLR NF-κB:SEAP reporter assay reveals that different SMG strains activate hTLR2 to different extents. There are pronounced differences between activation by live versus dead cells in some strains.

The HB2 cell line was stimulated in triplicate with live and heat-killed bacterial cells at an MOI of 50; Pam_3Csk_4 was used as the positive control. The negative control was not stimulated with any ligand. HB2 cells were stimulated for 24 hours at 37ºC in 5% CO_2 and the absorbance of the supernatants was measured at 630nm on the SpectroMax M3 plate reader. Relative hTLR2 activation was calculated for each positive control and experimental group by calculating the fold change in absorbance relative to the negative control. The trends observed in these data are representative of three biological replicates. Error bars represent standard deviations.

Figure 3.1 shows that all of the five SMG reference strains were able to activate hTLR2 (as demonstrated by activation of NF-κB). However, the magnitude of activation by each strain varied. C1392 activated hTLR2 to the greatest extent, dead B196, dead C1365, and dead M60R elicited intermediate activation, and C984 consistently activated hTLR2 to the least extent (Figure 3.1). Additionally, it was observed that the *S. intermedius* SMG strains elicited a higher level of hTLR2 activation when dead compared to when they were alive. The hTLR2 response to dead B196 was 6 times higher than live B196. hTLR2 activation by dead C1365 and dead M60R was 4 and 7 times higher, respectively, than activation by live C1365 and live M60R. hTLR2 activation by live and dead C984 and by live and dead C1392 was similar. (Figure 3.1)

3.1.2 Treatment of THP-1 Cells with Anti-hTLR2-IgA Decreases their Release of TNFα in Response to Stimulation with Live C984, B196, and C1392

The HB2 cell line was a useful tool for determining the capability of the SMG reference strains to activate hTLR2. However, this cell line only expresses hTLRs 1 and 6 (which dimerize with hTLR2) and is stably transfected with hTLR2. To determine whether hTLR2 signalling contributes significantly to the innate cytokine response to the SMG, it was imperative that a different cell line that expresses all TLRs be used. The THP-1 cell line was chosen for this investigation; THP-1s are monocyte-like cells that can be differentiated into macrophage-like cells which express all pattern recognition receptors. Differentiated THP-1s were stimulated in the presence and absence of anti-hTLR2-IgA for 18 hours at 37°C in 5% CO_2. The differentiated cells were stimulated with live C984, B196, and C1392 at an MOI of 5; these strains were chosen because they gave the lowest, an intermediate, and the highest response in the hTLR2

NFκB:SEAP reporter assay, respectively. 1 ng/mL Pam_3Csk_4 was used as a positive control for hTLR2 activation and 5 ng/mL LPS – a TLR4 agonist - was used as the negative control.

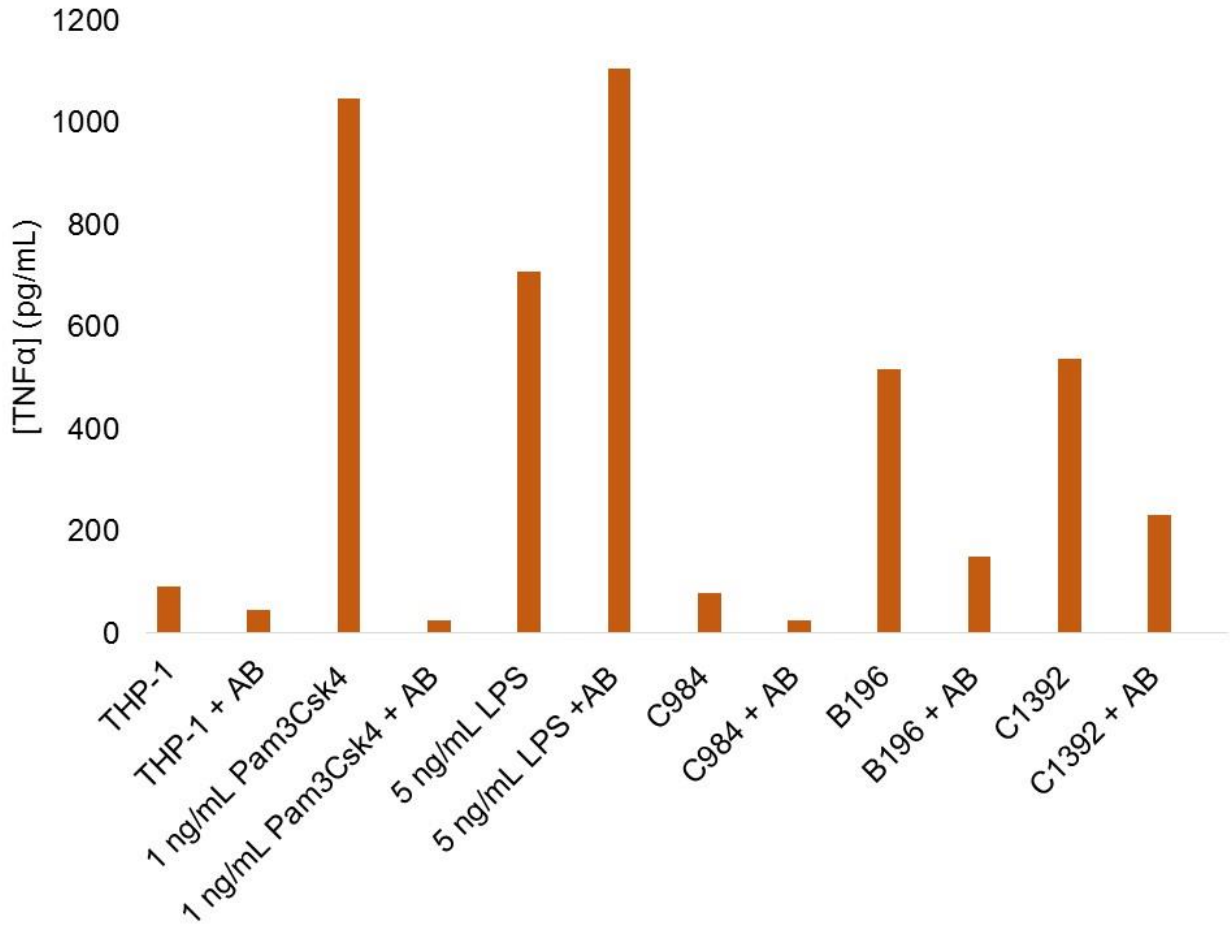

Figure 3.2: Blocking hTLR2 with anti-hTLR2-IgA decreases the amount of TNFα released by differentiated THP-1s following stimulation with C984, B196, and C1392.

Differentiated THP-1 cells were stimulated in 5% CO_2 at 37ºC with suspensions of live C984, B196, and C1392 for 18 hrs in the presence and absence of anti-TLR2 antibody. Supernatant concentrations of TNFα were measured in duplicate by ELISA. These data are representative of the three biological replicates with C984 and C1392; stimulation with B196 was conducted once.

Following the stimulation period, tumour necrosis factor-α (TNFα) concentrations in the supernatants were measured by sandwich ELISA in duplicate. As seen in Figure 3.2, stimulation with C1392 triggered a release of 538 pg/mL TNFα. Pre-treatment of THP-1s with anti-hTLR2 antibody caused the TNFα concentration to decrease to 232 pg/mL – a 230% decrease. Blocking hTLR2 resulted in the decrease of the TNFα response to B196 from 516 pg/mL to 149 pg/mL – a 346% decrease. C984 triggered the release of very low amounts of TNFα: 79 pg/mL. However, it was observed that blocking hTLR2 resulted in a decrease in TNFα concentration to 25 pg/mL, which is a 316% decrease. (Figure 3.2) In the positive control, the anti-hTLR2 antibody caused a decrease in TNFα concentration from 1048 pg/mL to 24 pg/mL. Blocking with anti-hTLR2 antibody did not decrease the release of TNFα following stimulation with LPS, as would be expected (Figure 3.2).

3.2 Investigation of the Capacity of SMG Cytolysins and Extracellular Polysaccharide to Enable Evasion of hTLR2 Recognition and Innate Cytokine Responses

The following sections contain the results of experiments conducted to investigate SMG cytolysins and EPS and their ability to enable innate immune evasion.

3.2.1 *S. intermedius* Strains Kill HB2 Cells

As previously mentioned in section 3.1.1, *S. intermedius* strains B196, C1365, and M60R all activate hTLR2 to greater extents when dead *vs* alive. This, however, was not observed with *S. anginosus* strain C984 or *S. constellatus* strain C1392. To investigate this phenomenon, HB2 cell viability was measured using the CellTiter 96® Non-Radioactive Cell Proliferation Assay kit

(Promega) following 24-hour stimulation with each of the control and experimental groups used in the hTLR2 NF-κB:SEAP reporter assay. Figure 3.3 shows that HB2 cell viability was approximately 40% in the presence of live B196, about 30% when stimulated with live C1365, and roughly 10% with live M60R. However, HB2 cell viability was 100% or greater when stimulated with almost all of the other groups. The only exception was the control group stimulated by 25ng/mL Pam_3Csk_4, for which the cell viability was 80%. From these data, it is apparent that the live *S. intermedius* strains kill HB2 cells (Figure 3.3).

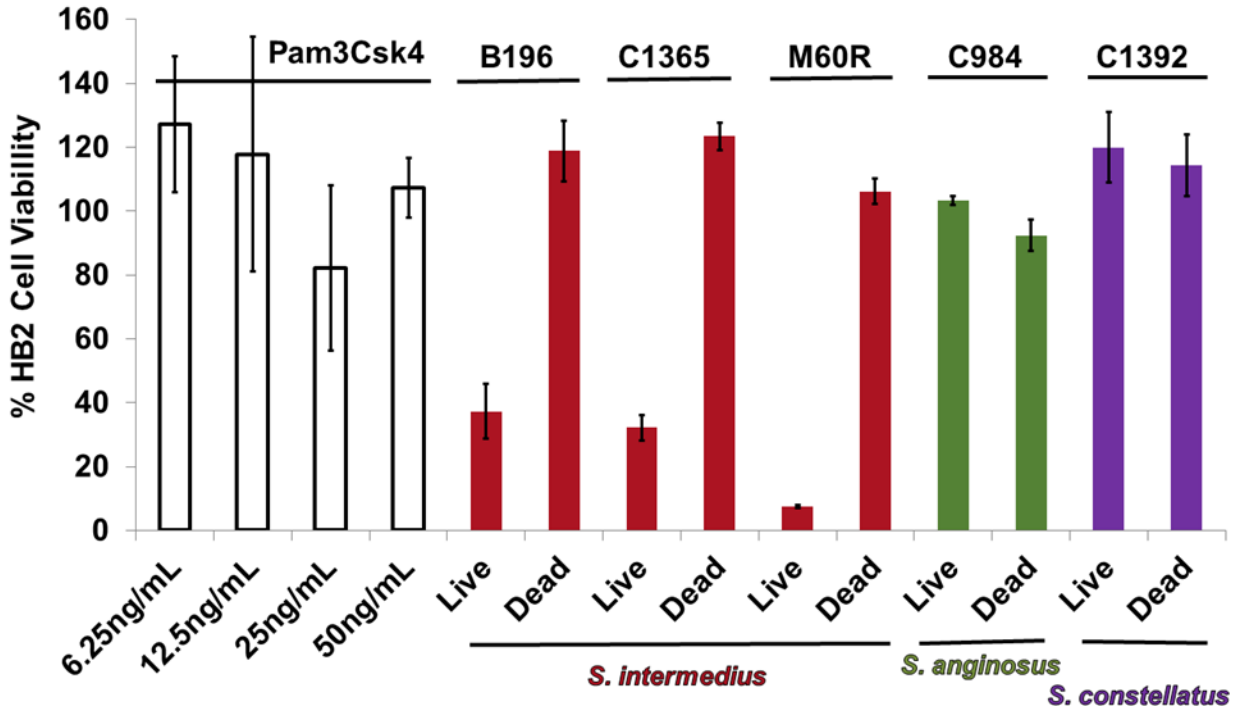

Figure 3.3: *S. intermedius* strains kill HB2 cells.

HB2 cell viability was measured using the CellTiter 96® Non-Radioactive Cell Proliferation Assay following 24 hr stimulation in triplicate with each of the control and experimental groups used in the hTLR2 NF-κB:SEAP reporter assay. The trends observed in these data are representative of three biological replicates. Error bars represent standard deviations.

3.2.2 β-Hemolytic Mutants were Isolated during *S. intermedius* B196 Transposon Mutant Library Phenotype Screening

The body of work on mechanisms of SMG pathogenicity is small in comparison to work on other streptococcal species. Previously published work is a valuable aid in planning informative experiments; since data on the SMG is lacking, designing experiments to elucidate mechanisms of pathogenicity is challenging. Working with a wildtype SMG strain is not the most efficient way to glean information about SMG pathogenicity. A transposon mutant library is a powerful molecular tool that provides an abundance of information from each experiment conducted. Therefore, a transposon mutant library was generated using the *magellan6* mini-transposon (van Opijnen and Camilli, 2010). The library was generated in *S. intermedius* B196 because the genome of this strain has been sequenced and annotated and a body of work has already been generated with this strain in the Surette laboratory. The transposon mutant library was arrayed into twenty 384-well plates. This number of mutants was collected in hopes that mutants in all of the 1800 B196 genes would be obtained and that multiple mutants in each gene would be within the collection. The arrangement of the mutants into well plates enabled high-throughput screening of transposon mutants on selective media. Mutants with phenotypes of interest were isolated and the sites of transposon insertion were determined. This enabled correlations between gene and phenotype and also revealed genetic elements responsible for gene regulation.

The *S. intermedius* B196 transposon library was screened on Columbia Blood Agar (CBA) + 5% v/v human blood (hCBA), CBA + 5% v/v sheep blood (sCBA), and Prussian Blue agar. Figure 3.4 shows the hemolysis of WT B196 and 41 transposon mutants found to be β-hemolytic on hCBA. It must be noted that, to generate enough plates to screen the library and

isolate mutants of interest, blood was collected from multiple donors and pooled. WT B196 (circled in black) was α-hemolytic on pooled blood while the transposon mutants were β-hemolytic to various extents (refer to Table A.1 for the position of each mutant). In the sCBA screen, two transposon mutants were found to be β-hemolytic (Figure 3.5). Additionally, these mutants generated hydrogen peroxide, as detected by a blue precipitate on Prussian Blue agar (Figure 3.5).

3.2.3 There is Variation in the Cytotoxicity of β-Hemolytic B196 Transposon Library Mutants against HB2 Cells

The data in Figures 3.4 and 3.5 show that a collection of transposon mutants are β-hemolytic on blood agar. To determine whether these mutants are specifically hemolytic or are cytotoxic to all human cells, a cytotoxicity assay was conducted. The release of lactate dehydrogenase from lysed HB2 cells was measured in triplicate using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit following a 24-hour infection with WT B196 and the 41 β-hemolytic mutants isolated from phenotype screening. Percent cytotoxicity was calculated using the signals from the 0% and 100% lysed controls. As seen in Figure 3.6, WT B196 cytotoxicity was 25%. The cytotoxicity of the H_2O_2-producing mutants that are β-hemolytic on sCBA, 9-D10 and 18-C14, was roughly 7% (Figure 3.6). The cytotoxicity of the remaining 39 mutants ranged from below 10% to above 100% (Figure 3.6).

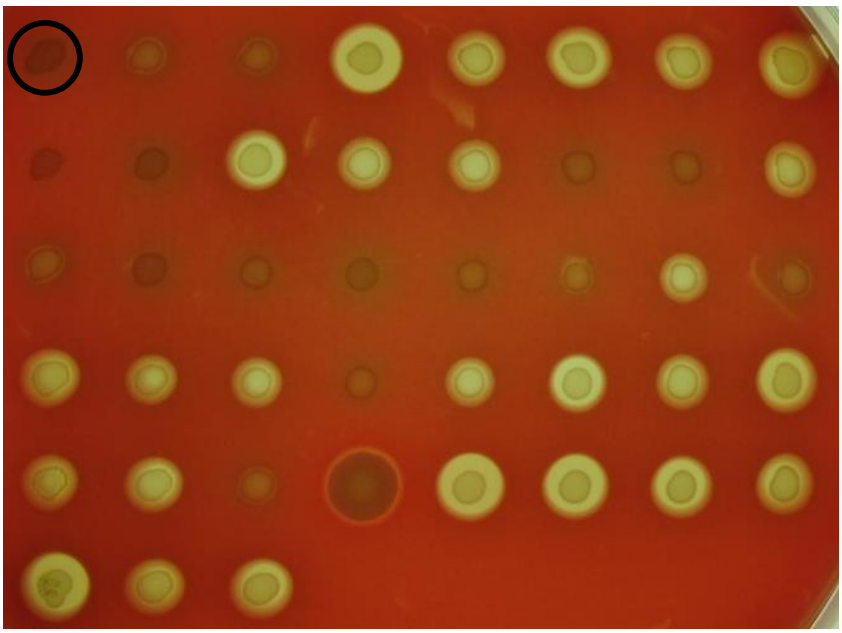

Figure 3.4: The hemolysis of WT B196 and 41 transposon mutants found to be β-hemolytic on hCBA. The mutant at each position on the stamped plate can be found in Table A.1.

WT *S. intermedius* B196 and 41 tranposon mutants of interest were stamped on Columbia Blood Agar + 5% v/v pooled human blood from overnight broth cultures and grown for 48 hours in 5% CO_2 at 37ºC. WT B196 is located in the upper left-hand corner (in black circle). Different transposon mutants displayed varying degrees of β-hemolysis.

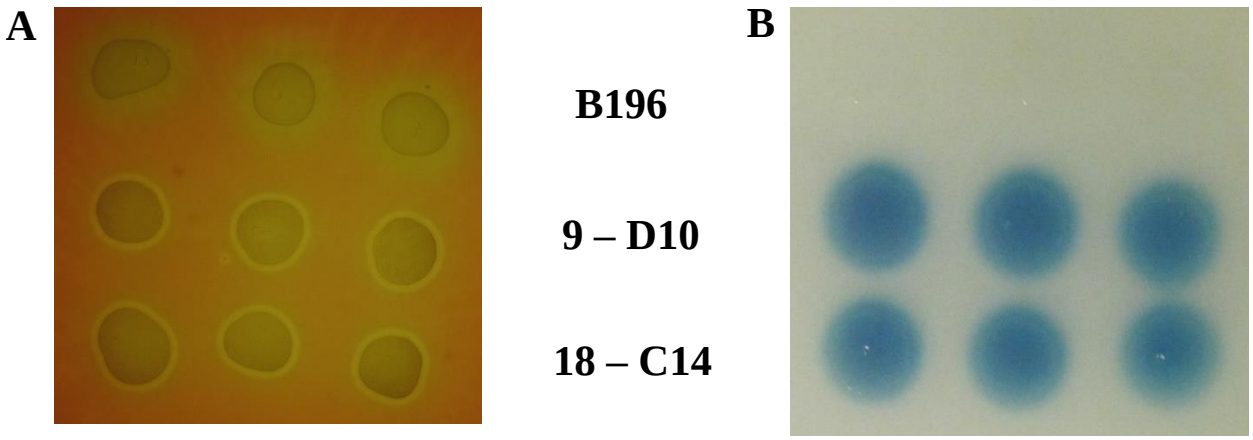

Figure 3.5: Two transposon mutants are β-hemolytic on sCBA and produce H_2O_2.

A) On sCBA, 9-D10 and 18-C14 were found to be β-hemolytic. B) On Prussian Blue agar, these two mutants generated a blue precipitate, indicating H_2O_2 production.

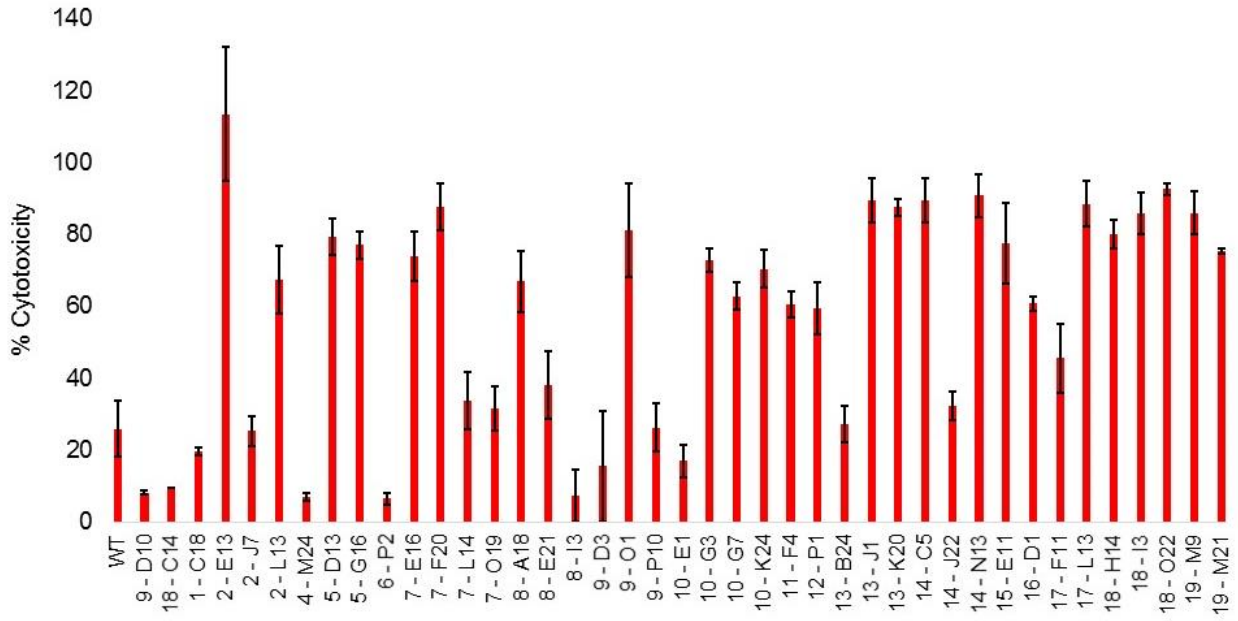

Figure 3.6: The cytotoxicity of WT B196 and 41 transposon library mutants.

The release of lactate dehydrogenase from lysed HB2 cells was measured in triplicate using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit following a 24-hour infection with WT B196 and the 41 β-hemolytic mutants isolated from phenotype screening. Percent cytotoxicity was calculated using the signals from the 0% and 100% lysed controls. Error bars represent standard deviation. The level of cytotoxicity of the WT and mutants observed in these data is representative of three biological replicates.

3.2.4 The Sites of Transposon Insertion in the *S. intermedius* B196 Transposon Library Mutants of Interest have been Identified

In order to discover which B196 genes were responsible for the phenotypes observed during the transposon library phenotype screens, the sites of transposon insertion in the mutants isolated were identified. The method used was adapted from van Opijnen and Camilli, 2010 (van Opijnen and Camilli, 2010), except Illumina sequencing was not utilized. An adaptor was ligated to digested mutant genomic DNA, allowing PCR to be done to isolate the desired digest

fragment containing the transposon. The PCR product was then cloned into the pCR™4-TOPO® vector (Life Technologies), enabling Sanger sequencing. As a result of using MmeI to digest genomic DNA, each PCR product contained 16 base pairs of mutant DNA; this provided enough sequencing data to map back to the WT B196 genome and identify the site of transposon insertion. Table 3.1 shows the site of transposon insertion in each transposon mutant of interest. In the transposon mutants that were β-hemolytic on hCBA, insertions were found in the gene *adcA* (zinc-binding lipoprotein), at three separate locations, as well as 51 bp downstream of this gene (Table 3.1). Insertions were also found in the putative lactose phosphotransferase system transcriptional repressor, *lacA* (putative galactose-6-phosphate isomerase subunit LacA), and *pulA2* (putative alkaline amylopullulanase) (Table 3.1). In addition, transposon insertions were found approximately 40, 200, and 300 bps upstream of the intermedilysin-encoding gene *ily* (Table 3.1 and Figure 3.7). In the H_2O_2-producing mutants that were β-hemolytic on sCBA, the 9-D10 genome had an insertion between base pairs 875 889 and 875 890 and 18-C14 had an insertion in *ahpF* (alkyl hydroperoxide reductase subunit F) (Table 3.1).

Table 3.1: Sites of transposon insertion in *S. intermedius* B196 transposon mutants of interest.

Mutant(s)	Insertion Site	Observed Phenotype in Transposon Library Screen
2 - E13[a], 10 - G7	119 648 - 119 649 bp *adcA* (zinc-binding lipoprotein)	β-hemolytic on hCBA
7 - L14, 7 - O19, 9 - D3, 9 - P10	120 679 - 120 680 bp *adcA* (zinc-binding lipoprotein)	β-hemolytic on hCBA
16 - D1	119 720 - 119 721 bp *adcA* (zinc-binding lipoprotein)	β-hemolytic on hCBA

13 - B24	120 797 - 120 798 bp 51 bps downstream of *adcA*	β-hemolytic on hCBA
2 - J7	420 534 - 420 535 bp putative lactose phosphotransferase system transcriptional repressor	β-hemolytic on hCBA
4 - M24 14 - J22	422 314 - 422 315 bp *lacA* (putative galactose-6-phosphate isomerase subunit LacA)	β-hemolytic on hCBA
8 - I3	1 693 968 - 1 693 969 bp *pulA2* (putative alkaline amylopullulanase)	β-hemolytic on hCBA
13 - K20[b]	116 767 - 116 768 bp 41 bps upstream of *ily*	β-hemolytic on hCBA
5-G16, **17 - L13**, **18 - O22**	116 769 - 116 770 bp 43 bps upstream of *ily*	β-hemolytic on hCBA
1-C18, 5 - D13, **6-P2**, 12 - P1, **14 - C5**	116 929 - 116 930 bp 203 bps upstream of *ily*	β-hemolytic on hCBA
11- F4	116 938 - 116 939 bp 212 bps upstream of *ily*	β-hemolytic on hCBA
8 - E21	116 942 – 116 943 bp 216 bps upstream of *ily*	β-hemolytic on hCBA
10 - E1	116 943 – 116 944 bp 217 bps upstream of *ily*	β-hemolytic on hCBA
8 - A18	117 019 - 117 020 bp 293 bps upstream of *ily*	β-hemolytic on hCBA
7 - F20, 10 - G3, 13 - J1, **14 - N13**, **15-E11**	117 028 -117 029 bp 302 bps upstream of *ily*	β-hemolytic on hCBA
18-I3, **19-M9**, 19-M21, 7 - E16, 10 - K24	117 044 -117 045 bp 318 bps upstream of *ily*	β-hemolytic on hCBA
2 - L13	117 052 - 117 053 bp 326 bps upstream of *ily*	β-hemolytic on hCBA
9 - D10	875 889 - 875 890 bp	H_2O_2 production & β-hemolytic on sCBA

18 - C14	1 409 685 - 1 409 686 bp *ahpF* (alkyl hydroperoxide reductase subunit F)	H_2O_2 production & β-hemolytic on sCBA

[a] Different transposon mutants were found to be β-hemolytic at different time points. Black font = 24 hours, green font = 48 hours, and purple font = 72 hours.

[b] Some mutants were more β-hemolytic than others. Bolded font indicates pronounced β-hemolysis.

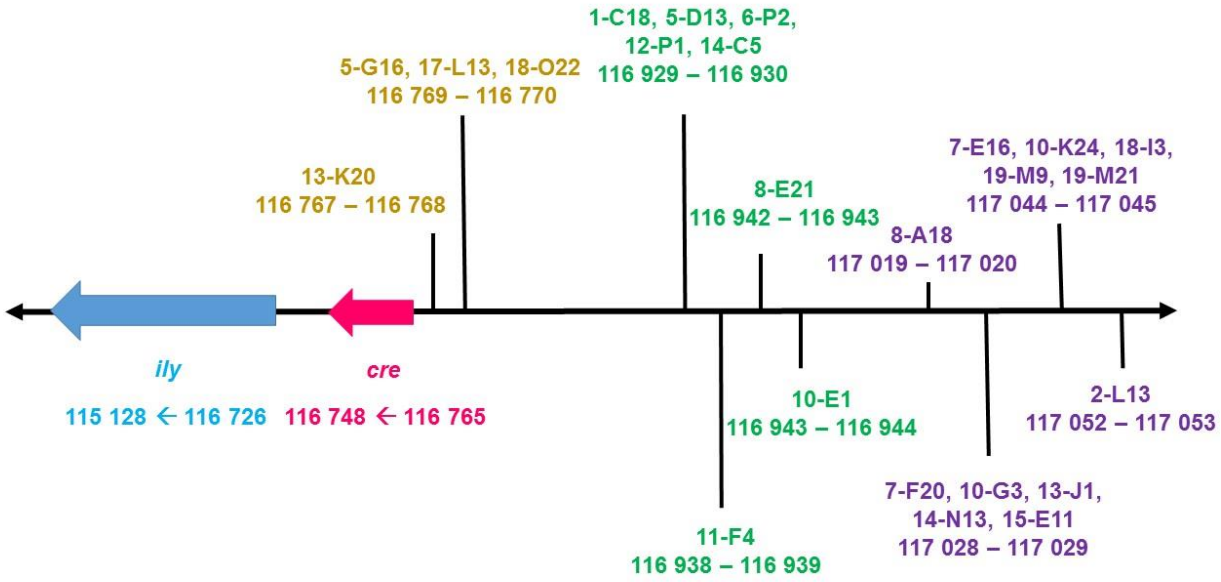

Figure 3.7: Transposon insertions were found approximately 40, 200, and 300 bps upstream of the intermedilysin-encoding gene, *ily*.

The sites of transposon insertion in the B196 transposon mutants that were β-hemolytic on pooled human blood are shown above. There were two separate insertions roughly 40 bp upstream of *ily* and four separate insertions in both of the regions approximately 200 and 300 bps upstream of this gene. *cre* = catabolite repressible element. Diagram is not drawn to scale.

3.2.5 Different Blood Donors Displayed Different Hemolytic Profiles

An unexpected observation was made during follow-up experiments on the isolated β-hemolytic B196 transposon library mutants. As mentioned in section 3.2.2, the phenotype screen of the transposon library on human blood plates was conducted with pooled blood from multiple donors. When the collection of β-hemolytic mutants and WT B196 were stamped on new hCBA plates made using blood from an individual donor, the hemolysis data was quite different from that of the hemolysis previously observed on the pooled blood. As seen in Figure 3.8, the blood of different individual donors varied in susceptibility to hemolysis. WT B196, M60R, and 41 B196 transposon library mutants were stamped onto the blood of seven individual donors; WT B196 is circled in black and M60R is circled in purple. The donors can be split into one of three groups, depending on the hemolytic profile of their blood. The first is the group that is sensitive to hemolysis by all of the strains and mutants tested (Figure 3.8A). The second group displays intermediate resistance; these donors resist hemolysis by WT B196 and several mutants, but are still susceptible to M60R and most of transposon the mutants (Figure 3.8B). The third group is resistant to hemolysis by WT B196, M60R, and many of the transposon mutants (Figure 3.8C).

The strain or mutant in each position of the stamped plate can be found in Table A.2. It should be noted that 9-D10 and 18-C14 are only β-hemolytic on the blood of donors most susceptible to hemolysis (Figure 3.8). In addition, 18-O22 was found to give a very large zone of clearing on all donors – even the resistant ones (Figure 3.8). When the blood of the seven donors was mixed and the strains and mutants tested were stamped on this mixture, the hemolysis observed resembled that on the plates made from the donors who displayed intermediate resistance (Figure 3.8D)

3.2.6 Extracellular Polysaccharide Biosynthesis Genes were Identified in the B196 Genome

Many streptococcal species evade the host immune system through the expression of EPS, such as capsule (Yother, 2011) or slime layer (Ayers et al., 1979). It was not known whether the reference SMG strains studied for this project express EPS. The genomes of six strains from the Surette laboratory SMG strain collection have been sequenced and well annotated. These strains are: B196 and C270 (*S. intermedius)*, C232 and C1050 (*S. constellatus*), and C238 and C1051 (*S. anginosus*). Searches were done on these genomes to look for EPS biosynthesis and capsular polysaccharide synthesis (cps) genes; the presence of these genes indicates that these strains potentially express EPS. The capsule biosynthesis gene locus of *S. anginosus* ATCC 33397 (Tsunashima et al., 2012) was used as a reference. The results of this search can be found in Table 3.2. In the B196 genome, the following genes were found: *cps A* (cps operon transcriptional regulator), *cps B* (cps biosynthesis protein), *wzx* (flippase – required to export carbohydrate polymers), *cps 3U* (UDP-glucose pyrophosphorylase), *gbpB* (glucan binding protein), and *cps M* and *N* (glycoslytransferases) (Table 3.2). A number of these genes were also found in the other five SMG strain genomes (Table 3.2).

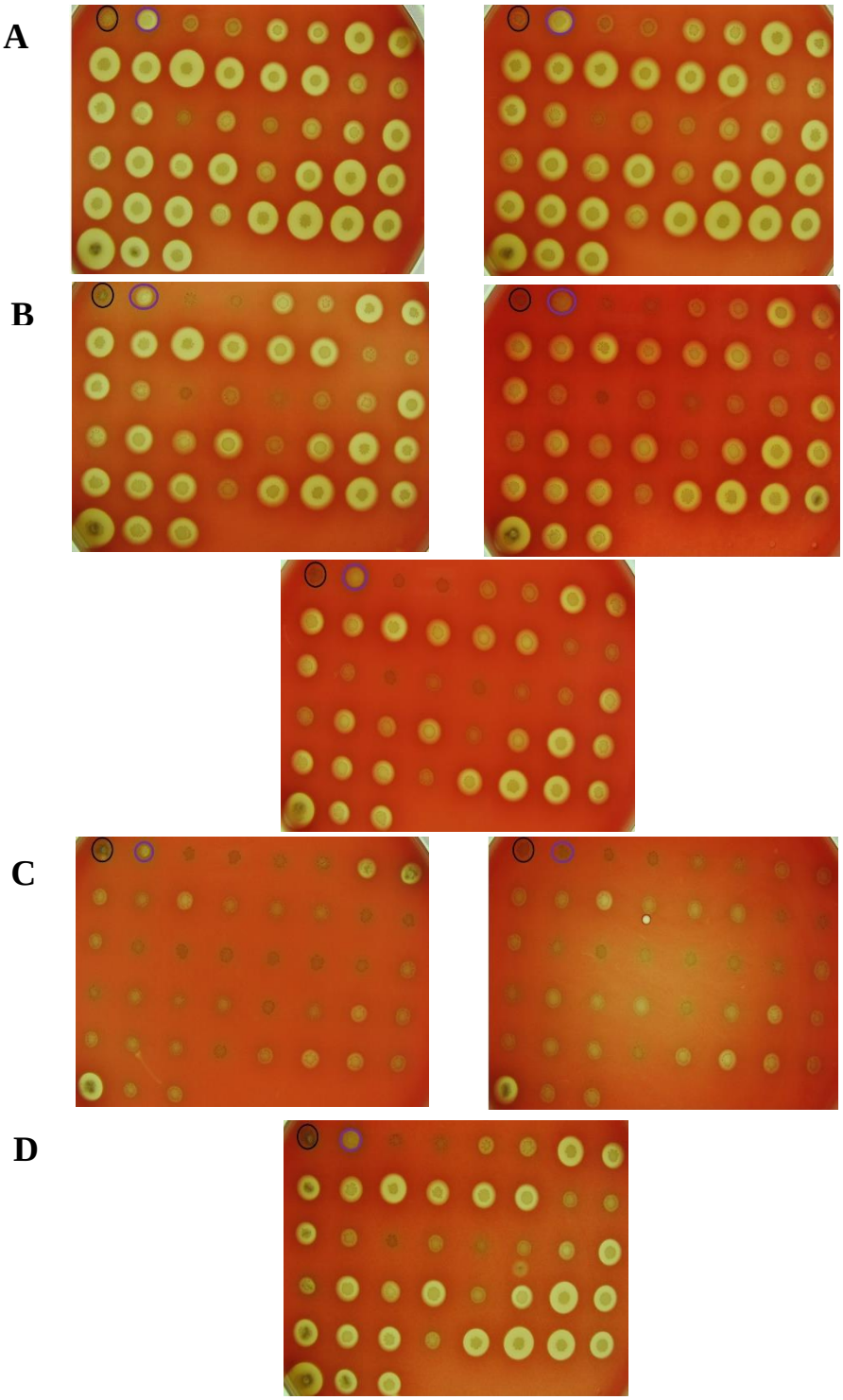

Figure 3.8: 7 human blood donors were placed into one of three groups based on their hemolytic profile.

WT B196 (circled in black), M60R (circled in purple), and 41 β-hemolytic transposon library mutants were stamped on individual and pooled hCBA plates which were incubated for 48 hrs in 5% CO_2 in 37ºC. Response groups: A) sensitive, B) intermediate resistance, or C) resistant to hemolysis. D) is pooled blood from all 7 donors. For the strain or mutant at each position on the stamped plates, refer to Table A.2.

Table 3.2: EPS biosynthesis and capsular polysaccharide synthesis (cps) genes were found in the annotated genomes of six SMG strains from the Surette laboratory SMG strain collection.

Strain	EPS Biosynthesis Genes[a]
B196 (*S. intermedius*)	*cps A*[b], *cps B, cps C, cps D, cps F, cps M, cps N, cps 3U, gbpB,*
C270 (*S. intermedius*)	*cps A, cps B, cps C, cps D, cps F, cps M, cps N, cps 3U, gbpB,*
C232 (*S. constellatus*)	*cps F, cps M, cps N, cps 3U, gbpB*
C1050 (*S. constellatus*)	*cps F, cps M, cps N, cps 3U, gbpB*
C238 (*S. anginosus*)	*cps F, cps M,* c*ps N, cps 3U, gbpB,*
C1051 (*S. anginosus*)	*cps A, cps B, cps C, cps D, cps E, cps F, cps G, cps I, cps K, cps M, cps N, wzx, cps 3U, gbpB,*

[a]The presence of these genes indicates that these strains potentially express EPS. The capsule biosynthesis gene locus of *S. anginosus* ATCC 33397 (Tsunashima et al., 2012) was used as a reference.

[b]The functions of each identified gene are as follows: *cps A* = putative transcriptional regulatory protein, *cps B* = putative phosphotyrosine-protein phosphatase, *cps C* = putative regulatory protein, *cps D* = putative protein-tyrosine kinase, *cps E* = undecaprenylphosphate glucosyl transferase, *cps F* = β-1,4-rhamnosyltransferase, *cps G* = N-acetylglucosamine transferase, *cps I* = glucosyl transferase, *cps K* = putative Galf transferase, *cps M* = putative glycosyltransferase, *cps N* = putative glycoslytransferase, *wzx* = flippase, *cps 3U* = UDP-glucose pyrophosphorylase, and *gbpB* = glucan binding protein B (Tsunashima *et al.,* 2012)

3.2.7 Transmission Electron Microscopy Revealed that C1392, B196, and C984 Express Extracellular Polysaccharide

Figures 3.1 and 3.2 demonstrate the heterogeneity in the ability of the SMG reference strains to trigger hTLR2 activation and cytokine release, respectively. It was hypothesized that SMG EPS enables evasion of innate immune cell responses. Therefore, to test whether the results of the hTLR2 NF-κB:SEAP reporter assay and TNFα ELISA were due to differential expression of EPS, transmission electron microscopy was done to look for extracellular structures in three SMG strains. C1392, B196, and C984 were chosen to be analyzed by transmission electron microscopy because they gave a high, medium, and low response, respectively, in assays. Figure 3.9 shows that each of these strains express a layer of EPS. However, no strain appears to express significantly more or less EPS than the other.

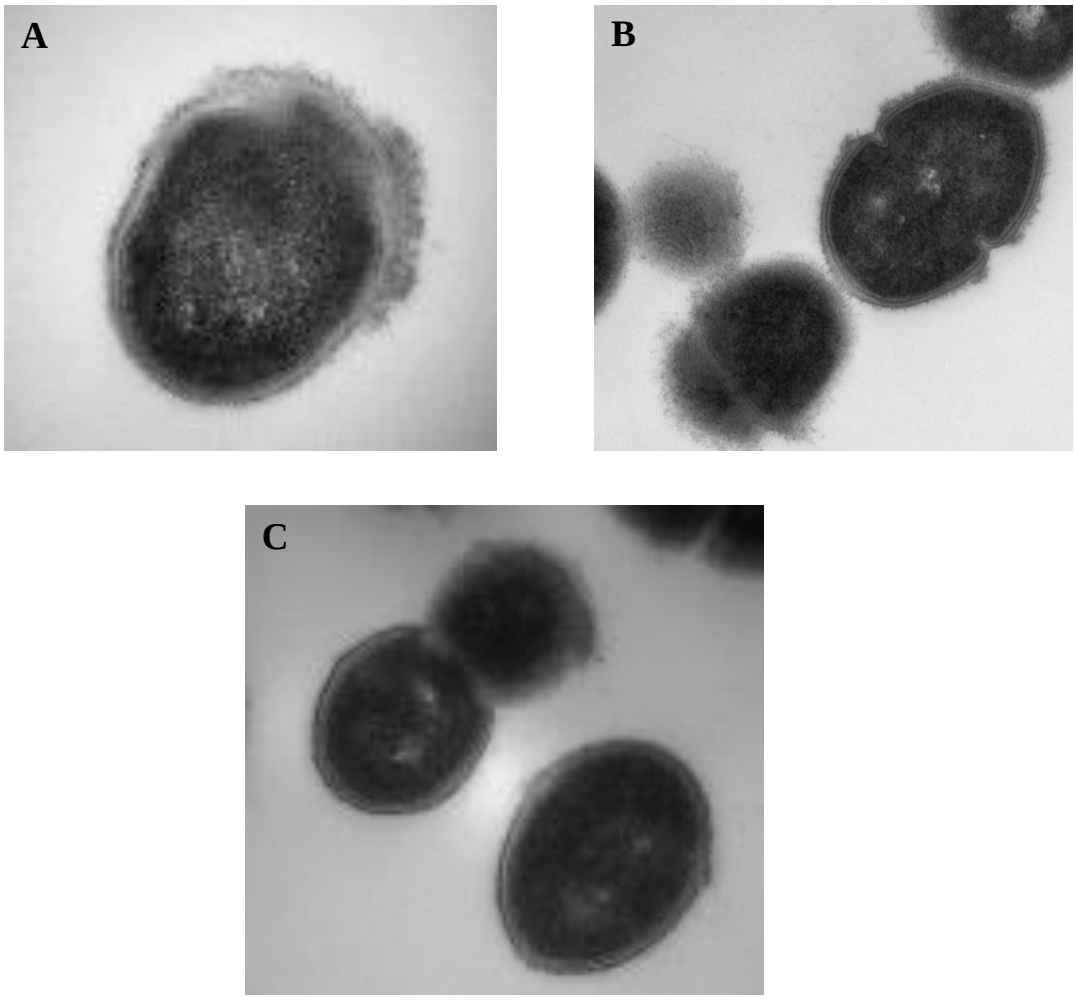

Figure 3.9: C1392, B196, and C984 express extracellular polysaccharides.

C1392, B196, and C984 were grown in liquid broth for 24 hrs prior to chemical fixation and analysis by transmission electron microscopy at 100 000x magnification. The appearance of each of these strains reveals the expression of extracellular polysaccharides. A) C1392, B) B196, C) C984

Chapter 4

Discussion

4.1 The Host Response to the SMG

When investigating host-microbe relationships, both sides of the relationship must be considered. During the course of this investigation, discoveries were made about how human cells respond to members of the SMG as well as how the SMG interact with human cells. The findings on host cell responses to the SMG will be discussed first.

4.1.1 hTLR2 Activation Heterogeneity may be Due to Variation in Receptor-Ligand Affinity

The results from the hTLR NF-κB:SEAP reporter assay (Figure 3.1) show that hTLR2 was activated by each of the reference SMG strains to some extent. This demonstrates that these strains express cell wall components that bind hTLR2. However, there was heterogeneity in the extent to which different SMG strains activated hTLR2. This difference between strains might be explained by higher expression of hTLR2 ligand in the cell wall of some strains and lower expression in others. Or it could be due to expression of cell wall components that have a greater or weaker affinity for hTLR2. Jiménez-Dalmaroni *et al* found that the ectodomain of hTLR2 can bind cell wall ligands (ex: lipoproteins and lipoteichoic acid) with acyl chains composed of between 16 and 19 carbon atoms (Jiménez-Dalmaroni *et al.,* 2015). Research on TLR4 activation has revealed that hexa- and hepta-acylated LPS triggers stronger TLR4 activation than tetra- and penta-acylated LPS molecules (Bäckhed *et al*., 2003; Miller *et al*., 2005). Perhaps a similar phenomenon occurs with hTLR2 activation. Most likely, different SMG strains express different

proportions of ligands with varying affinities for hTLR2. The discrepancies in activation between live and dead *S. intermedius* strains will be discussed in section 4.2.1.

Larsen *et al* hypothesize that a key difference between Chronic Obstructive Pulmonary Disease- and asthma-causing Proteobacteria and commensal *Prevotella* spp. is in their ability to trigger a pro-inflammatory immune response via TLR4 (Larsen *et al*., 2015). They attribute this difference to variation in LPS structure and the degrees to which different structures activate TLR4 (Larsen *et al.*, 2015). The hypothesis that more pathogenic SMG strains trigger higher levels hTLR2 activation than commensal strains was not tested in this study. It was found, however, that C1392, isolated from a brain abscess, activated hTLR2 to the greatest extent and that C984, a CF airway isolate, activated hTLR2 to the least extent. There are, of course, a multitude of factors that coalesce to form a host-microbe relationship. However, in future work on human-SMG relationships, it may be advantageous to investigate whether variation in hTLR2 activation is an important contributing factor to their formation.

4.1.2 hTLR2 Activation is Appears to Play a Key Role in Triggering a TNFα Response to SMG Strains

Figure 3.2 shows that blocking hTLR2 before stimulation with C984, B196, and C1392 decreases the concentration of TNFα released by differentiated THP-1s by 2- or 3- fold. This is preliminary data. Only the TNFα response was measured and more replicates of this experiment are required to generate statistics to determine the significance of the cytokine decrease following hTLR2 inhibition. Additionally, differentiated THP-1s are macrophage-like cells. Different cell types express varying proportions of TLRs (Stewart *et al.*, 2015; Trivedi and

Greidinger, 2009), so the observations made with this cell line can only be used to make inferences about how macrophages launch a TNFα response to the SMG.

The data collected suggests that hTLR2 activation plays a key role in triggering the release of TNFα from macrophages in response to the SMG. This is not a surprising result, as it is known that Gram positive microbes express high proportions of TLR2-binding ligands (Elson *et al*., 2007; Yoshimura *et al*., 1999). The conclusions drawn from work with cell lines can be used to guide experiments with primary cells. The implication from work with the HB2 and THP-1 cell lines that hTLR2 is an important player in human-SMG interactions can now be tested with primary human cells. It can also be investigated whether different host responses to the SMG (Kaiser *et al*., 2014) are due to hTLR2 polymorphisms. Certain hTLR2 polymorphisms have been linked to infection and particular diseases (Cheng *et al*., 2015; Proença *et al*., 2015; Wu and Yang, 2015). Again, as stated in the previous section, it is not logical to assume that the nature of human-SMG relationships hinges solely on SMG interactions with hTLR2. However, the work of Madrenas and colleagues has demonstrated how heavily initial TLR-ligand interactions impact the formation of host-microbe relationships (Mele and Madrenas, 2010; Peres *et al.*, 2015). Therefore, it is worthwhile to investigate SMG-hTLR2 interactions further in the future.

It should be noted that C984, along with triggering low levels of hTLR2 activation in the HB2 cell line, also triggers low levels of TNFα release from THP-1s. In addition, the concentration of cytokine released by THP-1s was decreased with pre-treatment with anti-hTLR2 antibody. The decrease in TNFα release following anti-hTLR2 antibody treatment suggests that the data from the hTLR2 NFκB:SEAP reporter assay was not the result of active hTLR2 inhibition. It also shows that C984 does not trigger a cytokine response from an alternate

TLR besides hTLR2. These results indicate that C984 stimulation causes low activation of human PRRs in general – not just hTLR2.

4.1.3 Sensitivity to Intermedilysin Varies Between Hosts

Unexpectedly, it was found that the blood of seven different donors displayed different hemolytic profiles when stamped with WT B196, M60R, and 41 B196 transposon library mutants. Each of the donors fell into one of three groups: sensitive, intermediate resistance, and resistant. Since hemolysis varied with WT B196, the B196 transposon mutants, and M60R, it can be inferred that the differences in hemolysis are due to varying susceptibilities to ILY. B196 and M60R are both *S. intermedius* strains, which are known to produce ILY, and the selected B196 transposon mutants over-produce ILY (discussed in section 4.2.2).

Work with *Caenorhabditis elegans* and various human cells lines has shown that mammalian cells do have defense systems in place against pore-forming toxins. Mammalian cells are able to protect themselves via activation of: p38 mitogen-activated protein kinase (Huffman *et al.*, 2004; Kao *et al.*, 2011), the unfolded protein response (Bischof *et al.*, 2008; Statt *et al.*, 2015), vesicle trafficking (Los *et al.*, 2011), and autophagy (Kloft *et al.*, 2010). However, it is unlikely that any of these mechanisms are responsible for the donor-to-donor variation observed. Firstly, the aforementioned mechanisms only occur in nucleated cells (Kloft *et al.*, 2010); these pathways would not be activated in red blood cells (RBC). Furthermore, these are general, conserved defense mechanisms that were detected in entire nematode populations and multiple cell lines – they weren't observed in only individual worms or particular cells lines. The donor blood cells must be protecting themselves from ILY by a different mechanism.

It is known that ILY is able to insert itself into human plasma membranes by binding CD59 and cholesterol (Johnson *et al*., 2013). CD59 deficiency leads to serious autoimmune disease (Asimakopoulos *et al*., 2014; Haliloglu *et al*., 2015; Höchsmann and Schrezenmeier, 2015), but each of the blood donors is healthy, so variation in levels of CD59 expression is not a viable explanation of the data. Though CD59 polymorphisms in the ILY binding site have not been screened for in the donors, this is not one of the more likely explanations. The ILY and Membrane Attack Complex (MAC) binding sites on CD59 overlap with one another (Johnson *et al*., 2013). If the resistant donors had polymorphisms at this location which resulted in lower ILY binding, they would be expected to display symptoms similar to those displayed by those with CD59 deficiencies. The CD59 of these donors would have a lower affinity for the MAC and would be less able to protect their own cells from lysis by the complement system. In addition, the affinity of ILY for cholesterol has been found to be very low (Tabata *et al*., 2014), so variation in cholesterol composition of donor blood cells would not be expected to effect ILY binding affinity. Whole, unclotted blood was used to make the hCBA plates used in experiments (ie: there were not just washed RBCs in the media). When the blood of the seven donors was mixed, the hemolytic profile resembled that of the donors in the intermediate resistance group. This suggests that resistance to ILY is conferred by some factor that is diluted by mixing.

Further work is required to discover what this protective factor is. It is most likely that host resistance is conferred by a soluble factor that binds ILY, preventing it from acting on cells. However, differential expression of a human factor that interacts with bacterial cells, preventing ILY release, is also a possible explanation. Differential production of an antibody that targets ILY seems like a plausible explanation for the varying β-hemolysis phenotype. Since adaptive immunity is acquired, the concentration of antibody would vary from host to host. Differences in

antibody levels could explain why some donors are more or less susceptible to the activity of ILY. It is known that the SMG is widespread in the human population; perhaps more resistant donors are carriers of *S. intermedius* strains. These findings are significant because studies on host-to-host variation focus on different responses to a bacterial species (Broderick *et al*., 2010) or groups of strains (Kaiser *et al*., 2014), but not to specific virulence factors. Studies must be done to test the discussed hypotheses.

4.2 The Contribution of SMG Factors to Virulence

Both microbial and host factors contribute to the formation of relationships between the two. In this study, the perspectives of both human immune cells and the SMG were considered. In order to understand how members of the SMG cause disease, two virulence factors commonly expressed by streptococcal pathogens which enable their evasion innate immunity, cytolysins (Littmann *et al*., 2009) and EPS (Lakkitjaroen *et al*., 2014), were investigated in the SMG. The ways by which these factors affect human innate immune cell responses to the SMG were probed.

4.2.1 Intermedilysin Expression Affects hTLR2 Activation

As seen in Figure 3.1, B196, C1365, and M60R - the *S. intermedius* reference strains – activate hTLR2 to a greater extent when dead versus alive. To tease out the cause of this difference in activation, a cell viability assay was conducted on HB2 cells following stimulation with the various control and experimental groups used for the hTLR2 NFκB:SEAP reporter

assay. It was found that the HB2 cell viability was 100% or greater following stimulation with all groups except for the live *S. intermedius* strains (Figure 3.3). (HB2 cell viability could be higher than 100% because the cells are not differentiated when seeded, so they can proliferate between the time they are seeded and the time the viability assay is conducted.) As mentioned previously, *S. intermedius* strains express a secreted cytolysin called intermedilysin (ILY), which specifically targets human cells thru recognition of the ubiquitously expressed membrane protein CD59 (Wickham *et al*., 2011).

The *S. intermedius* strains studied triggered low levels of hTLR2 activation and caused decreases in HB2 cell viability only when alive. Since this phenomenon was only observed with the *S. intermedius* reference strains and because it is well-documented that this species produces a human-specific cytolysin (Johnson *et al.*, 2013; Nagamune *et al*., 2000; Sukeno *et al*., 2005), it can be assumed that it was the action of ILY that decreased both HB2 cell viability and hTLR2 activation. However, it can be seen in Figure 3.2 that live B196 cells triggered the same level of TNFα release from differentiated THP-1s as live C1392 cells – even though these strains had opposite effects on HB2 cell viability. Kinetic experiments were not done on the HB2 and THP-1 cell lines to determine the time it takes ILY to cause cell death. It could be that in the hTLR2 NFκB:SEAP reporter assay, the HB2 cells were killed before they were able to produce enough SEAP to cause a colour change in the detection media. In the THP-1 stimulations, the cells may have had sufficient time to release detectable amounts of TNFα before dying.

Even though TNFα release was not affected by ILY production by B196, the data do suggest that ILY production is an important mechanism of innate immune evasion by *S. intermedius*. ILY is a potent cytolysin against human cells. In a physiological context, the release of ILY by *S. intermedius* strains would be expected to affect the response of innate immune cells

they encounter, as ILY could kill these cells before they had a chance to launch a response, or before they had fully performed their function (Bernatoniene *et al*., 2008; Littmann *et al*., 2009). However, testing this hypothesis *in vivo* with an animal model is problematic because ILY only acts on human cells. To study the expression of *ily* as a mechanism of evasion further, primary human cells or transgenic mice that express human CD59 could be used.

4.2.2 Transposon Mutants Express Varying Levels of Intermedilysin

During the phenotype screen of the B196 transposon mutant library on mixed human blood, it was found that WT B196 was α-hemolytic and 41 transposon mutants were β-hemolytic to various extents (Figure 3.4). The cytotoxicity assays conducted after this screen support the conclusion that the blood plate results were due to varying expression of ILY (Figure 3.6). When measuring the cytotoxicity of WT B196 and the selected B196 transposon mutants against the HB2 cell line, the cytotoxicity of WT B196 was measured at 25%. The cytotoxicity of the 41 transposon mutants varied from approximately 10% to 100%. When this collection of bacteria was stamped on sCBA, it was found that only 9-D10 and 18-C14 (the H_2O_2-producers) were β-hemolytic (Figure A.1). Since the *S. intermedius* B196 transposon mutants displayed varying degrees of β-hemolysis on human blood, varying cytotoxicity against HB2 cells, but no hemolysis against sheep blood, it can be concluded that the isolated transposon mutants express various levels of ILY. Though WT B196 was α-hemolytic on pooled human blood, it is β-hemolytic on the blood of sensitive human donors, and it is cytotoxic against HB2 cells, demonstrating that the WT does produce ILY – just not as much as many of the isolated transposon mutants.

As discussed in the introduction, *ily* expression is known to be affected by *ccpA* (Tomoyasu *et al.*, 2010b), *lacR* (Tomoyasu *et al*., 2013), and *luxS* (Pecharki *et al*., 2008). The sites of transposon insertion in the transposon mutants that were β-hemolytic on hCBA have been determined (Table 3.1). Though none of the insertions were in the three mentioned genes, the work published of *ily* regulation provides enough information to allow hypotheses to be formed for why the various insertions resulted in increased production of ILY. Several mutants had an insertion in or near to *adcA*, a gene encoding a zinc-binding lipoprotein required for zinc ion import (Bayle *et al*., 2011). It has been found in *Streptococcus pneumonia* that knocking out *adcA* significantly impairs growth in zinc-deficient medium. Growth is restored by supplementing media with zinc (Bayle *et al*., 2011). It could be that in the *adcA* B196 transposon mutants, *ily* expression is increased in order to lyse human cells, releasing the zinc they are otherwise unable to acquire. In fact, it has been hypothesized that ILY evolved for the purpose of nutrient acquisition (Tomoyasu *et al*., 2010b).

In a number of mutants, transposon insertions were found in genes involved with carbohydrate metabolism. Insertions were found in: the putative lactose phosphotransferase system transcriptional repressor, *lacA* (putative galactose-6-phosphate isomerase subunit LacA – required for lactose catabolism), and *pulA2* (putative alkaline ampylopullulanase – required for breakdown of carbohydrate storage molecules into glucose monomers). The first two mutations would lead to the buildup of lactose in the cell and the last mutation would prevent the accumulation of glucose monomers. It has been published that lactose (Tomoyasu *et al*., 2013) activates *ily* transcription, while glucose (Tomoyasu *et al*., 2010b) represses it. That could explain why the mutants with insertions in the mentioned locations over-produce ILY.

24 transposon mutants had insertions in one of three areas upstream of the *ily* gene (Figure 3.7). In four of the mutants, the site of transposon insertion was roughly 40 bps upstream of *ily*. The region going from 116 765 -> 116 748 (39 bps upstream of *ily*) has been identified as a catabolite repressible element (*cre*) (Tomoyasu *et al*., 2010b). When glucose is present, it binds to ccpA, which will then bind the *cre*, blocking transcription. The insertions 2 and 4 bps upstream of the *cre* may have been close enough to this element to relieve transcriptional repression of *ily*, leading to greater expression of ILY by those transposon library mutants (Figure 3.7). Multiple independent insertions were also found approximately 200 and 300 bps upstream of *ily*. These are likely genetic elements required for repression of *ily*; an insertion in these locations would relieve repression, increasing ILY production. I hypothesize that one of these regions is the sequence that is recognized by LacR. Tomoyasu *et al* published that LacR binds the *ily* promoter, but they were unable to find a sequence in this region that resembles the conserved LacR binding sequence (Tomoyasu *et al*., 2013). Perhaps the binding sequence they were looking for is in this region of transposon insertion. It is intriguing that so many transposon insertions occurred in the *ily* promoter; the possibility that this location is a transposon insertion hotspot could be probed in future investigations.

Each of these hypotheses is thought-provoking and remain to be tested.

4.2.3 B196 Expresses a Hemolysin that is Active against Sheep Blood

During the B196 transposon library screen, a potential novel hemolysin was identified. Shown in Figure 3.5, the two transposon mutants that produce H_2O_2 (9-D10 and 18-C14) also cause β-hemolysis on sCBA. On mixed human blood and on the blood of resistant and

intermediately resistant donors, these two mutants were α-hemolytic. In addition, in the cytotoxicity assay, these mutants caused less than 10% cytotoxicity against HB2 cells. None of the transposon mutants that were β-hemolytic on pooled hCBA were β-hemolytic on sCBA (Figure A.1). All of these data demonstrate that B196 produces a factor that is specifically hemolytic and that factor targets sheep, but not human, blood. This could be a hemolysin that targets various non-human animal RBCs, but sheep's blood was the only non-human blood tested. When looking at the hemolysis caused by 9-D10 and 18-C14, it appears to be contact-dependent hemolysis (ie: the hemolysis occurs only around the growth of the mutants). H_2O_2 can alter the gene expression of microbes (Li *et al*., 2015; Wang *et al*., 2013a). It could be that H_2O_2 production up-regulates the expression of a B196 contact-dependent hemolysin. However, this explanation has yet to be explored.

4.2.4 EPS Thickness does not Affect hTLR2 Activation or Cytokine Response

Given that the level of hTLR2 activation in the HB2 cell line and the concentration of TNFα release by THP-1s triggered by C984 was consistently low, it was hypothesized that this strain expresses a thick layer of EPS. This would physically block cell wall ligands from being bound and triggering a cytokine response. Conversely, it was hypothesized that C1392 did not express EPS – or expressed a thin layer of EPS – because it triggered high levels of hTLR2 activation and TNFα release. Transmission electron microscopy was done at the electron microscopy facilities at McMaster University on C984, B196, and C1392. These strains gave the lowest, an intermediate, and the highest response in the hTLR2 NFκB:SEAP reporter assay, respectively. As seen in Figure 3.9, each of these strains expresses a layer of EPS. However,

C984 does not express an EPS layer that is thicker than that of the other two strains and C1392 does not express a layer of EPS that is thinner. Therefore, the differences between SMG strains in the hTLR2 NFκB:SEAP reporter assay and the THP-1 stimulation experiment are not due to varying EPS thickness.

If an investigation were to be done on the SMG ligand recognized by hTLR2, this is an avenue that could be further explored. Since each of the strains analyzed do express EPS, the heterogeneity in hTLR2 activation may be due to varying receptor affinity for the specific carbohydrates that make up the EPS layer. EPS could be isolated from each SMG reference strain and used in stimulations of the HB2 cell line to test whether the EPS from different strains could activate hTLR2 to different extents. The composition of EPS of each strain could be probed from there. However, for the purposes of this study, experiments such as these were not necessary.

4.3 This Study has Implications for the Future Study of the SMG and other Pathobionts

The results from this study demonstrate that, in the investigation of host-microbe relationships, one side of the coin cannot be separated from the other. In the investigation of hTLR2 recognition of the SMG, it was found that hTLR2 does bind the SMG – but different strains activate hTLR2 to different extents. On the other hand, while delving into mechanisms of regulation of production of the *S. intermedius* cytolysin ILY, it was found that different humans vary in their susceptibility to its hemolytic activity. This brings up an important point about research on pathobionts: oftentimes, the approaches taken in the investigation of commensal *vs.*

pathogen lifestyles of pathobionts are one-sided. Most studies are conducted from a microbial perspective: searching for the presence of virulence genes in a pathobiont genome or phenotype screening for virulence factors (Calder *et al*., 2015; Newell *et al*., 2014; Wei *et al*., 2006). In recent years, studying host-microbe relationships from the host perspective has become more popular. Investigators have demonstrated how host genetics (Emonts *et al*., 2008; Ruimy *et al*., 2010; Xue *et al.*, 2015) and host lifestyle choices (Jaspers, 2014; Wang *et al.*, 2013b) can correlate with microbial carriage and incidence of infection.

However, studying pathobionts and hosts in isolation is problematic because it is not known how closely (or how distantly) results from these studies resemble the host-microbe interactions occurring *in vivo* (Bermudez-Brito *et al*., 2013; Meijerink *et al*., 2013; Yan *et al*., 2013). *In vivo*, host and microbe are constantly responding to one another. Host factors alter microbial gene expression and microbial factors alter host gene expression. The resulting phenotypes of the two interact, contributing to the formation of a relationship. Since the gene expression, and therefore phenotype, of the two are dynamic, this results in the dynamic nature of host-microbe relationships. In order to obtain a deeper understanding of host-microbe dynamics, researchers of this topic need to move towards integration of microbe and host perspectives. A number of research groups have begun to show that virulence is the result of how bacterial and host factors come together (Caspi-Fluger *et al*., 2011; Hava and Camilli, 2002; Song *et al*., 2012). Studying this topic from the microbial and host perspectives simultaneously can be a challenging endeavor, but it is a vital undertaking for the purpose of gaining a profound understanding of the causes of infectious disease in a physiological context.

4.4 Future Directions to Investigate the SMG Transition from Commensalism to Pathogenicity

There are many avenues yet to explore in the investigation of human-SMG relationships. The heterogeneity between SMG reference strains in the extents to which they activate hTLR2 could be probed. Preparations of EPS and cell wall components (ex: peptidoglycan, lipoteichoic acid, and lipoproteins) from each SMG strain could be used to determine which class of molecule is recognized by hTLR2. This experiment would also elucidate why some strains cause greater or less activation than others (ie: more or less ligand or differences in receptor affinity). In addition, the preliminary results that suggest that hTLR2 activation is important for the launch of a cytokine response to the SMG. These results should be followed up on to determine whether the decrease in cytokine release cause by blocking hTLR2 is significant and whether this decrease is observed in SMG strains in general. If hTLR2 turns out to play a vital role in the THP-1 cytokine response to the SMG, this investigation could be extended to primary cells to determine if innate immune cells from a human host rely on hTLR2 to respond to the SMG. Correlations between hTLR2 polymorphisms and magnitude of host cytokine response could also be investigated.

The results yielded from sequencing the sites of insertion in the transposon mutants that were β-hemolytic on pooled human blood have left multiple hypotheses to be tested. It should be tested whether zinc-supplementation will decrease the β-hemolysis of the *adcA* mutants. The hypothesis that four of the mutants have insertions in the *cre* upstream of *ily* could be tested with supplementation of media with glucose. The production of ILY should not decrease even in the presence of glucose if this element is mutated. In addition, the frequency of transposon insertion

in the region approximately 300 bps upstream of *ily* is an interesting find. It could be that this is the sequence bound by LacR. This could be determined with an immunoprecipitation assay.

The finding that there is heterogeneity in the susceptibility of human blood donors to ILY deserves to be extensively explored. An *in vitro* hemolysis assay could be done with WT B196 and either the isolated RBCs of individual donors or mixtures of RBCs and serum. This would elucidate whether resistance is conferred by some difference in RBCs or by a factor in the serum. If it is found that some component in the serum is responsible for protecting against the action of ILY, Western blotting could be done to detect binding of this factor to ILY. The discovery of this factor would be a fascinating and important find. This information would be valuable to research groups working on developing ILY as an anti-tumour therapeutic agent (Cai *et al*., 2014; Ge *et al*., 2011; You *et al.*, 2011). Findings could also be used by physicians to stratify patients that are being treated for SMG infection. Patients that express low levels of protective factors against the SMG may require additional, longer, or alternative treatment to be cured. It is possible that the concentration of these factors in populations at higher risk to infection (ex: those with autoimmune diseases, hospitalized patients, and the elderly) could be used to predict susceptibility to and severity of SMG disease. This find could also contribute to the explanation of why some individuals form pathogenic relationships with the SMG while others do not.

Chapter 5

Conclusion

In conclusion, this study provided insight into how human immune cells and members of the SMG interact with one another. This work contributes to the field of study on SMG-human relationships by identifying variance in the immunogenicity of different SMG strains and by corroborating recent findings that human responses to the SMG cannot be generalized across hosts (Kaiser et al., 2014). The results of this study illustrate that a number of microbial as well as host factors coalesce to catalyze the shift from commensalism to pathogenicity. The genomes of members of the SMG contain various virulence genes. However, the expression of these genes may vary depending on environmental conditions, such as nutrient availability. In addition, some hosts may be more capable of defending themselves from virulence gene products than others. In the future, this knowledge may be used to predict host susceptibility to SMG disease. This project provides a foundation for the future study of how and under what conditions the SMG transition from commensalism to pathogenicity.

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