EVASION OF HOST INNATE IMMUNITY BY ENTEROCOCCUS FAECALIS: THE ROLES OF CAPSULE AND GELATINASE

by

LANCE ROBERT THURLOW

B.S., Fort Hays State University, 2003
M.S., Fort Hays State University, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the
requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Abstract

Enterococci are gram-positive bacteria typically found as commensals in the gastro-intestinal tracts of most mammals. Enterococci, most notably *Enterococcus faecalis* and *Enterococcus faecium*, have become problematic causative agents of several nosocomially acquired infections including urinary tract infections, bacteremia, surgical sight infections, and endocarditis. These bacteria must first overcome the innate immune response in order to establish infection.

Many bacteria produce capsular polysaccharides that contribute to pathogenesis by helping the microbe evade the host innate immune response. The capsular polysaccharide produced by *E. faecalis* has been shown to play a role in pathogenesis; however the mechanisms of innate immune avoidance were unknown. Moreover, the number of capsule serotypes produced by *E. faecalis* and the genetic differences that contribute to capsule serospecificity were in doubt. In the current study it is made clear that only two capsule serotypes are produced by *E. faecalis* and that both capsule serotypes contribute to evasion of the host innate immune system. This work shows two mechanisms by which the capsule of *E. faecalis* contributes to immune evasion. First, the presence of capsule inhibited complement mediated phagocytosis through limiting the detection of opsonic complement protein C3 on the surface of the bacteria. Secondly, the presence of capsule altered cytokine signaling of macrophages by shielding bacterial components from detection. Many pathogenic strains of *E. faecalis* also produce an extracellular protease known as gelatinase (GelE). This work also shows a novel mechanism involving GelE in innate immune evasion through the degradation of the anaphylatoxin C5a. Degradation of C5a by GelE resulted in decreased neutrophil recruitment in vitro. A rabbit model of endocarditis was employed to assess the effect of GelE production on disease development and progression. Rabbits infected with GelE producing strains had increased bacterial burdens in the heart compared to rabbits infected with strains that were GelE negative. Reduced phagocyte infiltration at primary

and secondary infection sites was also observed in rabbits infected with GelE producing strains compared to GelE negative strains.

The work presented here demonstrates that both the capsular polysaccharide and GelE play roles in *E. faecalis* evasion of innate immune responses. Moreover, these pathogenic determinants would be suitable targets for developing alternative therapeutics used to treat *E. faecalis* infections.

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Approved by:

Major Professor
Dr. Lynn E. Hancock

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Acknowledgements

It has been a long journey, but my time at Kansas State has been an amazing experience. So many people at K-State and in the Division of Biology have helped me along the way that it is impossible to thank them all here. I am very thankful for the support I have received from the division and its members. Everybody that I have had to opportunity to interact with has been willing to help in whatever way they could and for that I am eternally thankful.

One person that I am forever indebted to is my advisor, Dr. Lynn Hancock. I would not be the scientist or person I am today without his the leadership and guidance. From my first day in the lab Dr. Hancock as instilled a work ethic that will always be part of my character. The amount knowledge that I have gained from my experiences in his lab will be the cornerstone of my career. Not only has Dr. Hancock been an outstanding academic and scientific mentor, but he has constantly encouraged me to think on my own and develop my own ideas. His ability to provide encouragement during times of doubt has seen me through some difficult and frustrating situations. It is great feeling knowing that you advisor always has your best interests at heart and is willing to go the extra mile to make sure you succeed. Not only do I consider Dr. Hancock my mentor, but I also consider him a great friend and I am extremely thankful for the opportunities that he has provided me.

I also am very thankful for the support of my committee members both past and present. Dr. Helmut Hirt, Dr. Ludek Zurek, Dr. Larry Takemoto, and Dr. Sherry Fleming have all provided encouragement along the way. The numerous discussions I had with each of them provided me with novel insights into my projects and into the world of science in general. I owe a special thanks to Dr. Fleming. I would not have had the successes I enjoy now if I had not had the great fortune to have someone like Dr. Fleming on my committee. Dr. Fleming was not obligated to provide the level of support that she did, but her generosity with her time and willingness to foster my ideas was above and beyond anything that I could have expected. I also owe a debt of thanks to

many other collaborators including Dr. Sally Olson, Dr. Sanjeev Narayanan, and Dr. Dan Boyle. All three of them contributed significantly to my work and my knowledge of science.

I also acknowledge many of my fellow students. I would not have been successful if it were not for the likes of Bart Bryant, Chris Lehiy, Vamsi Nalem, Joe Coolon, Sara Hoffman, Darin Allen, Alison Fedrow, and Hua Wang providing thoughtful ideas and constant encouragement. I also thank members of the Hancock lab including Vijaya Iyer, Matt Gorman, and Nathan Harms for their contributions to my work and for their friendship. Of all the students I met at Kansas State, Vinai Thomas was by far the most influential. I am very thankful for constant support and unwavering friendship Vinai provided during these past four years.

I am especially grateful for the support of my family. My parents, Larry and Patty Thurlow, have always encouraged me to pursue my goals no matter how difficult they may be. Their support during my 11 years of collegiate studies has been the one constant that I could always depend on. Finally, I thank my loving wife Kathy. To say that I could not have done it without her would be a gross understatement. Her unwavering support and care have been the foundations of all my accomplishments.

CHAPTER 1 - Literature Review: Enterococcus capsule, proteases, and stealth

Introduction

The first descriptions of a bacterial isolates from diseased patients infected by what is now known to be *Enterococcus faecalis* occurred nearly simultaneously in 1899 by Thiercelin (103) and MacCallum and Hastings (54, 103). Early descriptions of these disease causing diplococci from fecal origins resulted in many names including *Micrococcus zymogenes* (54) and *Enterococcus proteiformis* (Proposed in 1903 by Thiercelin and Jouhoud) (17) among several others. It was not until 1906 that the first clear classification of seven distinct groups of streptococci including *Streptococcus faecalis* was conducted by Andrewes and Horder (2). Eventually, in 1984, two of members the gut originating streptococci, *Streptococcus faecalis* and *Streptococcus faecium*, were re-classified as *Enterococcus faecalis* and *Enterococcus faecium* based primarily on comparative 16S rRNA studies (88). Even though *E. faecalis* has been studied as a pathogen for more than a century, it was not until the past couple of decades that members of the genus *Enterococcus* (primarily *E. faecalis* and *E. faecium*) emerged as common and problematic nosocomial pathogens.

E. faecalis and *E. faecium* are common commensal organisms that are found in the intestinal tracts of most mammals. Aside from their generally benign existence as commensals, *E. faecalis* and *E. faecium* are currently some of the most common sources of hospital acquired infections. Most infections caused by enterococcal species are due to either *E. faecalis* or *E. faecium* (45, 68). Historically, 90% percent of enterococcal infectious were caused by *E. faecalis* with close to 8% of infections caused by *E. faecium* (86). Recently, the percentage of infections caused by *E. faecium* compared to *E. faecalis* has increased due to the higher incidence of vancomycin resistant *E. faecium*. Results from a 2005 study conducted in the United Kingdom involving 7066 cases of enterococcal bacterimia revealed that nearly 63% of the infections were caused by *E. faecalis* compared to 28% caused by *E. faecium* (29).

Enterococci as a whole are generally not thought of as community acquired pathogens, but instead are nosocomial pathogens. A nosocomial infection is not present upon admittance to hospital or other clinical setting but is acquired by the patient in the clinical setting. A comprehensive surveillance study carried out from 1992-1998,

comprising intensive care units (ICUs) from 205 hospitals and 498,998 patients revealed that 29,041 (5.8%) patients admitted to the ICU acquired an infection that was not present prior to admittance (84). *Enterococcus* species were responsible for 2468 of the 29,041 infections including 11.8% of ICU acquired bacteremias, 14.3% of ICU acquired urinary tract infections, 17.1% ICU acquired of surgical site infections, and 8.7% ICU acquired of cardiovascular infections (84). Recent estimates indicate that enterococci annually account for 110,000 urinary tract infection, 40,000 wound infections, 25,000 incidents of bacteremia, and 1,110 cases of endocarditis in the United States alone (43). Typically, of all the manifestations of enterococcal disease, endocarditis is the most difficult to treat with mortality rates ranging from 15-20% (60, 69). Enteroccoci cause subacute-chronic endocarditis and are the causative agents of up to 20% of native valve endocarditis and 15% of prosthetic valve endocarditis (25, 63, 69). Enterococcal endocarditis is unique among enterococcal infections in that it is most commonly community acquired. However, more recent studies show that there is a significant risk of nosocomially acquired enterococcal endocarditis (26, 27).

The rapid increase in the rate of enterococcal infections over the past three decades is cause for concern as one of the most problematic trends encountered when treating patients with enterococccal infections is the increase in acquired antibiotic resistances (35). One of the most disturbing resistances seen in enterococci is the acquired resistance to Vancomycin. Vancomycin is often regarded as the antibiotic of last resort for treating various multi-resistant gram positive cocci infections, including enterococcal infections. The first descriptions of vancomycin resistant enterococci were reported in England and France in 1988 and in the US (strain V583) shortly after (50, 87, 107). The incidence of infection with vancomycin resistant strains has rapidly increased since. The most recent (2004) National Nosocomial Infections Surveillance (NNIS) report indicated that nearly 30% of enterococci isolated from clinical settings were resistant to vancomycin. This constituted a 12% rise from the previous five years (73). In some cases, the death risk associated with antibiotic resistant enterococci compared to antibiotic susceptible enterococci is seven fold higher (22).

The emergence of these microbes as nosocomial pathogens coincides with the advent of modern antibiotic therapies. Treatment with antibiotics (primarily 2nd and 3rd

generation cephalosporins) can lead to increased enterococcal burdens in the gut (19, 20, 77). However, new studies indicate that prior treatment with beta-lactams can also increase susceptibility to colonization with VRE in the ICU (77, 98). Most of these studies speculate that the lack of competition from antibiotic susceptible flora allow the enterococci to replicate without inhibition. However, treatment with antibiotics can also cause repression in the expression of a C-type lectin, RegIIIγ, which kills enterococci in the gut (8). Regardless of the conditions required for colonization and eventual infection with *E. faecalis*, the bacteria must also overcome the host barriers to cause infection.

E. faecalis produces several factors that have been shown to contribute to pathogenesis. One of the most studied phenomena associated with *E. faecalis* infections is the formation of biofilms. Strains responsible for infective endocarditis are significantly better biofilm formers than non-endocarditis isolates (66). Furthermore, cells composing biofilms are up to 1000 times more resistant to antibiotics than their planktonic counter parts and biofilms are also considered to be a location conducive to the dissemination of antibiotic resistance genes (10). Adhesion to artificial surfaces such as catheters or in-dwelling medical devices as well as host surfaces is key for the establishment biofilms and infection. *E. faecalis* produces several proteins that aid in adhesion including at least two loci that encode for pili. The endocarditis and biofilm associated pili locus (*ebp*) and the biofilm enhancer in *Enterococcus* locus (*bee*) contain genes encoding for four and five proteins respectively that enhance binding to host cells and aid in biofilm development (71, 90). *E. faecalis* also produces several other cell wall anchored proteins that aid in binding host cells including adhesin to collagen of *E. faecalis* (Ace) which also plays a role in virulence (47, 49, 70, 83). Two other surface proteins involved with *E. faecalis* adhesion are enterococcal surface protein (Esp) and aggregation substance. Esp is involved in colonization of the bladder and biofilm formation (93, 99, 106). Aggregation substance is involve in conjugation, adhesion to eukaryotic cells, and is associated with endocarditis (11, 12, 39, 48, 89).

E. faecalis also produces several proteins with enzymatic properties that contribute to pathogenesis. The proteases gelatinase (GelE) and serine protease (SprE) are co-transcribed through regulation by the *fsr* regulatory system (80, 81). The secreted protease SprE has been implicated in contributing to disease in animal models but the

mechanism of SprE activity is unknown (23, 81, 96, 97). GelE is a zinc-metalloprotease (56) that is related to the *Staphylococcus aureus* protease aureolysin and the *Pseudomonas aeruginosa* protease elastase (79). GelE is known to contribute to biofilm formation (36, 104), but GelE also contributes to virulence through degradation of a broad range of host proteinaceous substrates. There are several known host substrates targeted by GelE including collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and the complement components C3 and C3a (56, 57, 75, 76, 91, 109). Some pathogenic strains also produce a hemolysin known as cytolysin. Cytolysin lyses both prokaryotic and eukaryotic cells and contributes to pathogenicity in rabbit and mouse models of infection (11, 13, 44).

Non-proteinaceous components produced by *E. faecalis* are also known to contribute to pathogenesis. Lipoteichoic acid is the group antigen of enterococci that has been shown to play a role in biofilm formation and resistance to antimicrobial peptides (24). *E. faecalis* also produces two serotypes (C and D) of capsular polysaccharide (105). Studies have shown that both capsule serotypes of *E. faecalis* are anti-phagocytic (34, 42). More recently, a study by McBride et al. indicated that serotype C clinical isolates harbored greater repertoire of antibiotic resistance cassettes, and were more likely to possess multiple virulence factors compared to the other serotypes, suggesting that the presence of the capsule is associated with pathogenic lineages of *E. faecalis* (42, 59). Another cell surface polysaccharide associated with pathogenesis in *E. faecalis* is the rhamnopolymer commonly called Epa. Epa mutants are more susceptible to phagocytic killing (100) although questions remain as to whether the rhamnopolymer is a virulence factor. Unlike the capsular polysaccharides, the Epa polymer and its genetic locus appear to be highly conserved in *E. faecalis* (34, 100). A recent report by Teng et al. (101) demonstrated gross changes in the bacterial cell shape of Epa mutants in the OG1RF background that may partially explain the pleiotropic affects ascribed to the Epa locus in virulence studies (101, 113).

To fully understand the contributions of virulence factors in disease establishment and progression, one must take in to account the factors produced by the host that the pathogen must contend with. All pathogens, whether they are viral, fungal, parasitic, or bacterial in nature, must deal with and eventually overcome the host defense

mechanisms. The first line of host defense is characterized as the innate immune system. The innate immune system is responsible for detection of pathogens, alerting other host cells and systems to infection, and eventual eradication of the invading microbe. The innate immune response is a highly complex interaction of soluble protein elements and cell mediated responses that coordinate a potent rebuttal to all types of pathogenic challenge. Many effectors of the innate immune response are highly specialized and only deal with specific microbial challenges such as viruses, bacteria, or parasites (32). The following section describes key components of innate immune response with a focus on the effectors that are essential for dealing with gram-positive extracelluar pathogens such as *E. faecalis*.

Components of innate immunity contributing to detection and clearance of extracellular bacterial pathogens

Innate immunity is considered the first line of defense against invading pathogens that can react without prior exposure to the invading pathogen. The innate immune system consists of four primary components including physical anatomical and chemical barriers, soluble proteins, pattern recognition receptors, and phagocytes (Table 1.1). The physical and chemical barriers of innate immunity are composed of mucus membranes, epidermis, and chemical barriers such as acidity of the stomach. *Enterococcus faecalis* is an extracellular bacterial pathogen that must find a way past the physical barriers of the host to establish infection. In most cases, the introduction of a catheter or other in-dwelling medical device allows *E. faecalis* to bypass the host physical barriers. Breaks in the skin are also common routes of entry for several bacterial pathogens, including enterococci, which are the most common sources of hospital acquired surgical site infections (43, 84). Once *E. faecalis* has entered the host it must circumvent a myriad of host defense mechanisms. First, invading bacterial pathogens must escape detection by the host. Toll-like receptors found on the surface of several cells in the host detect pathogen associated molecular patterns (PAMPs) which leads to expression of cytokines that alert other cells to infection. Bacterial PAMPs are often components of the cell wall, such as lipopolysaccharide, peptidoglycan, lipoteichoic acids and cell-wall lipoproteins. Invading pathogens must also overcome soluble molecules found in host serum including

antimicrobial peptides and the complement proteins of the host. Complement proteins have several functions in defending against pathogens including opsoniztion, signaling, and the formation of membrane attack complexes. Finally, invading pathogens must escape clearance by host phagocytic cells in order to establish infection. Each component of these host innate immune system (detection, complement, and clearance) works in conjunction with the others to efficiently eradicate most invading microbes.

Table 1.1: Components of the innate immune system

Components	Effectors
Physical and anatomical barriers	Epidermis Stomach acidity Cilia Mucus membranes
Soluble Proteins	Antimicrobial peptides (AMPs) Complement system
Pattern recognition receptors	C-reactive protein (CRP) Mannose binding lectin (MBL) Nucleotide-binding oligomerization domain protiens (NOD) C-type lectins Scavenger receptors Toll-like receptors (TLRs)
Phagocytic cells	Dendritic cells Macrophages Polymorphonuclear neutrophils (PMNs)

Bacterial detection and Toll-like receptors

Bacteria and other potential pathogens produce several pathogen specific molecular patterns that are not commonly found in mammalian hosts. Most complex multi-cellular organisms have evolved numerous mechanisms to detect these pathogen associated molecular patterns (PAMPs). Pattern recognition receptors (PRRs) play an integral role in detection and response to potential pathogens. There are many host receptors associated with detection of invading pathogens, and triggering several PRRs simultaneously can induce diverse innate immune responses. Some PRRs are soluble and recognize specific carbohydrate moieties associated with pathogens. Soluble PRRs including the mannose binding lectin (MBL) and the C-reactive protein (CRP) are

considered acute phase proteins that are only expressed at high levels in response to pathogenic challenge (38, 61). Both CRP and MBL act as opsonins and binding of MBL to terminal mannose residues of bacterial carbohydrates can active the complement cascade (38, 61). However, a strong stimulus is required to stimulate the expression of acute phase proteins. Accute phase response proteins are secreted by hepatocytes in response to the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (61). The production of these pro-inflammatory cytokines is dependent on a separate form of detection and response. Because acute phase PRRs require a stimulus to be expressed in large quantities they are not generally considered the first PRRs to detect and respond to bacterial challenge.

Another class of PRRs are located intracellularly including some Toll-like receptors (TLRs). Most intracellular TLRs are involved in sensing obligate intracellular pathogens or pathogens that have been phagocytosed. Two non-TLR intracellularly located PRRs that recognized bacterial PAMPs are Nod1 and Nod2. Both Nod1 and Nod2 contain a nucleotide-binding oligomerization domain (NOD) and are members of a large family of proteins called the nucleotide-binding domain, luecine rich containing proteins (NLRs) involved in pathogen recognition and recognition of damaged cellular components (46, 61). Nod1 and Nod2 are important detection of bacterial challenge due to their ability to detect specific peptidoglycan moieties (38, 61). However, much like the soluble PRRs, intracellular PRRs are usually not the first responders to challenge with extracellular bacterial pathogens as they require phagocytosis of the microbes prior to detection. The PRRs that are most likely to first come in contact with extracellular pathogens are the membrane spanning or surface associated PRRs.

The three primary groups of the membrane spanning PRRs are scavenger receptors, C-type lectins, and the Toll-like receptors (38). Membrane spanning C-type lectins (CLRs) are a diverse group of proteins that can recognize a variety of PAMPs. The PAMPs recognized by C-type lectins are mostly carbohydrate in nature and include mannose, fucose, and β-1,3 glucan (32). Detection of PAMPs by CLRs leads to changes in cytokine production, and depending on the receptor, to internalization and degradation of the pathogen (32, 108). Membrane spanning CLRs such as Dectin-1 are known to contribute to defense against *Mycobacterium* and fungi but their contributions to

protection against other bacterial pathogens has not been fully elucidated (32, 61). Scavenger receptors are a large and diverse family of transmembrane proteins primarily found on dendiritic cells, macrophages, and endothelial cells (4). The primary role of scavenger receptors is to mediate non-opsonized phagocytosis through recognition of PAMPs, however, scavenger receptors can also act as co-receptors for TLR-2 (4). Scavenger receptor-A (SR-A) and CD36 are two scavenger receptors that are important to host innate immune defense against extracellular bacteria. Recognition of pathogens by SR-A results in non-opsonin mediated phagocytosis of both gram-positive and gram-negative bacteria (4). The scavenger receptor CD36 recognizes LTA and is an essential co-receptor for TLR-2/6 complex response to LTA (4, 21). When expressed on macrophages, CD36 is a phagocytic receptor that has been shown to be important in defense against infection with *Staphylococcus aureus* (4). Scavenger receptors and C-type lectins contribute to defense against infection by extracellular bacteria and are presumably important for protection against infection with *E. faecalis*, but the best characterized and arguably most important PRRs for detection of extracellular and intracellular pathogens are the Toll-like receptors.

The first descriptions of Toll-like receptors (TLRs) and their contributions to immunity was barely a decade ago, but now they are the most studied, best understood, and arguably the most important pattern recognition receptors. The discovery of the importance of TLRs came in 1996 when mutations in *Drosophila* for a receptor called Toll increased susceptibility to fungal infection (53). A human homologue of Toll was subsequently discovered that induced cytokine expression and is now known as TLR-4 (62). TLRs are classified as type 1 transmembrane proteins due to the fact that the N-terminus is outside of the membrane. TLRs are composed of three domains including a luecine rich repeat domain responsible for recognition of PAMPs, a transmembrane domain, and an inracellular domain known as the Toll/IL-1R (TIR) domain (46). TLR signaling is initiated by the dimerization of TLRs following recognition of the cognate PAMP. The dimerization facilitates the recruitment of cytoplasmic adapter molecules that also contain TIR domains.

Table 1.2: The Toll-like receptors

Toll-like receptor	Cellular location	Relevant Ligands	Target organism
TLR-2/TLR-1	Cell surface	Triacyl lipopeptides	Bacteria
TLR-2	Cell surface	Peptidoglycan	Bacteria
		Liparabomannan	Bacteria
TLR-3	Intracellular	dsRNA	Virus
TLR-4	Cell surface	Lipopolysaccharide	Bacteria
TLR-5	Cell surface	Flagellin	Bacteria
TLR-2/TLR-6	Cell surface	Diacyl lipopeptides	Bacteria
		Lipoteichoic acid	Bacteria
		Zymosan	Fungus
TLR-7	Intracellular	ssRNA	Virus
TLR-8	Intracellular	ssRNA	Virus
TLR-9	Intracellular	CpG DNA	Bacteria
TLR-10	Cell surface	Unknown	Unkown

One of a combination of four adapter molecules (MyD88, TIRAP, TRIF, and/or TRAM) are recruited to the receptor complex and elicit a PAMP appropriate response (46). The most commonly used adapter molecule is MyD88 which is used by every TLR with the exception of TLR-3. Recruitment of MyD88 initiates the activation of NF-κB which induces the expression of cytokines. Activation of TLRs generally leads to the production of pro-inflammatory cytokines including TNF-α, interleukin-1β (IL-1β), and IL-6. The cytokines TNF-α and IL-1β induce vasodilation of local endothelium and increase permeability of blood vessels thus allowing leukocytes to be recruited to the infection site (61). Adding to the inflammatory response, IL-6 and IL-1β activate hepatocytes to produce acute phase proteins such as MBL and CRP (38, 61).

Toll-like receptors (TLRs) are expressed on or in a myriad of cells including dendritic cells, macrophages, mast cells, B lymphocytes and intestinal epithelium. The list of TLRs along with the list of TLR ligands is constantly expanding (Table 1.2). The current list of known mammalian TRLs has 12 members. TLRs-1-9 are conserved in both humans and mice but TLRs-11 and 12 are absent in humans (46). The TLRs most associated with recognition of extracellular bacteria, TLR-4 and TLR-2, are surface localized. However, some internally localize TLRs such as TLR-9 are thought to play a

role in responding to bacterial challenge. Currently, TLR-2 is considered the most important TLR for detection of extracellular gram-positive bacteria including *E. faecalis*.

TLR-2 is the crucial PRR for recognition of extracellular gram positives. TLR-2 is unique among TLRs in that it forms heterodimers with TLR-1 or TLR-6 depending on the PAMP. In some cases TLR-2 may also use CD36 as a coreceptor (40). TLR-2 is thought to be able to recognize and respond to a wide variety of PAMPs including lipoteichoic acid (LTA), some lipopolysaccharides (LPS), lipoproteins, lipopeptides, lipoarabinomannans, lipomannans, glycosylphosphatidylinositol, glycoproteins, zymosan and peptidoglycan (PGN) (112). However, recent studies indicate that TLR-2 is not as promiscuous as it may appear and may not be able to respond to such a diverse group of molecules at physiologically relevant concentrations (112).

Critically important to detection of extracellular gram-positive pathogens such as *E. faecalis* is the ability of TLR-2 to detect LTA and PGN. The cell walls of all gram-positive bacteria contain lipoteichoic acid (LTA) with only slight chemical differences between genera. LTA from most bacteria consists of repeating glycerophophate units and D-alanine or *N*-acetylglucosamine substituents anchored by a lipohilic glycolipid (21). The differences in glycosylation of the glycerophosphate backbone on enterococci distinguish them from other streptococci. Peptdioglycan is a heteropolymer consisting of β-1,4 linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) with short oligopeptide stems on the three position of NAM and an interlinking peptide connecting the parallel layers of repeating NAGs and NAMs (112). Currently there is some ambiguity as to which of the two PAMPs is essential for TLR-2 detection with some arguments made for LTA and others for PGN (21, 112). One fact that remains clear is that TLR-2 is important for detection of gram-positive pathogens regardless of which PAMP is critical for detection.

Soluble proteins and the complement system

Soluble molecules such as natural antibodies, antimicrobial peptides, and complement proteins are major contributors to host innate immune defenses. Naturally occurring antibodies are potent opsonins and can also neutralize toxins produced by pathogens. Bound antibodies can also initiate the classical pathway of complement activation.

However, pathogen specific antibody production requires prior exposure to the potential pathogen and is not present in totally naive hosts. Other host proteins are more direct in action and do not require prior exposure to the pathogen to be effective. Soluble proteins with the ability to directly kill or inhibit the growth of microbes are referred to as antimicrobial peptides (AMPs).

Antimicrobial peptides are produced by all forms of life and exhibit a broad spectrum of activity against fungi, viruses, and all types bacteria as is the case with lysozyme, the first described AMP. There are several human proteins that have AMP like properties including enzymes, enzyme inhibitors and some chemokines, but the two primary groups of AMPs are the defensins and cathelicidins (31, 82). Some AMPs have the ability to alter host cell responses to infection, but most AMPs are known for inhibiting microbial growth or killing (31). Most AMPs are cationic and are composed of 20 to 60 amino acid residues. The mechanisms for microbial killing are diverse and depend on the AMP and the nature of the target microbe. The cationic nature of AMPs is thought to allow recognition of the overall negative charge associated with gram-positive and gram-negative cell walls (82). One of the most studied AMPs is a cathelicidin known as LL-37. In its fully processed form LL-37 consists of 37 amino acids with two conserved leucines (31, 82). LL-37 is constitutively present in dermal layers and infection can induce high levels of expression (31). LL-37 has potent broad spectrum antibacterial properties, but it is also a chemotractant involved in neutrophil recruitment and angiogenesis (31, 82). LL-37 is just one example of an AMP, but AMPs in general are formidable components of the innate immune response with their combined abilities of immunoregulation and antimicrobial activity.

Another group of soluble proteins that are important for host defense against infection is the complement system. The complement system is composed of 16 proteins and makes up 10% of the total serum proteins. The complement system plays several roles in defense against pathogens including direct microbial killing through the membrane attack complex (MAC), clearance of pathogens through opsonization, triggering inflammation, and recruitment and activation of phagocytic cells. Activation of the complement system leads to a cascade event where proteins are sequentially modified to produce opsonins, pro-inflammatory anaphylatoxins, and the MAC.

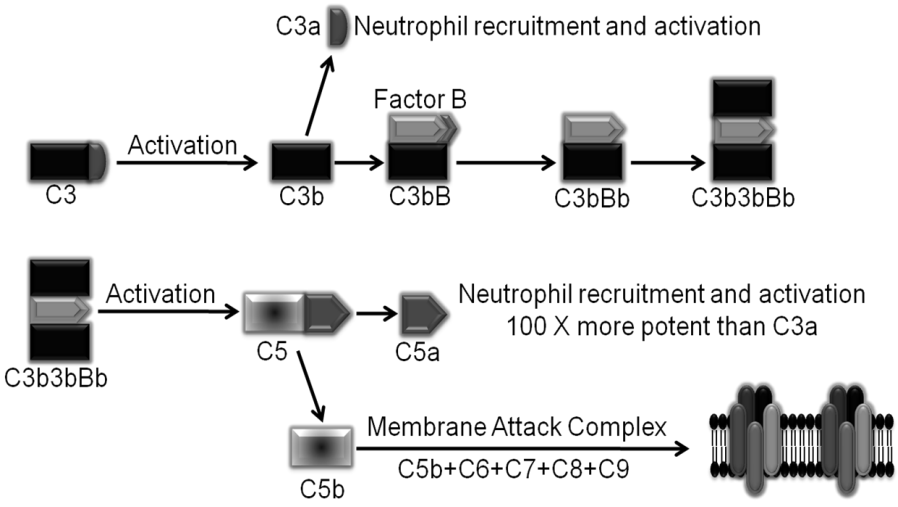

Figure 1.1: Alternative complement activation

Alternative activation of the complement system leads to the production of the opsonin C3b, the anaphylatoxins C3a and C5a, and the membrane attack complex. The anaphylatoxins C5a and C3a are critical for the recruitment of neutrophils. The protein C3b is an effective opsonin of *E. faecalis* in the absence of antibodies. *E. faecalis* is impervious to the membrane attack complex due to the thickness of the peptidoglycan.

The complement system can be activated through one of three ways, the classical, lectin, and alternative pathways. The end result of each pathway is the activation of the C3 convertase which in turn produces the anaphylatoxins C3a and C5a, the opsonin C3b and iC3b, and induces the formation of the MAC (7). The classical pathway of complement activation relies on the recognition of bound antibodies by the complement protein $C1qr_2s_2$ followed by the creation of the C3 convertase utilizing C4 and C2. Activation of the classical pathway relies on prior exposure to the invading microbes and is not utilized in naive hosts. The lectin pathway relies on lectins such as the mannose binding lectin (MBL) for recognition of PAMPs. The microbe bound lectins also induce the production of the C3 convertase through C4 and C2. The alternative pathway (Figure 1.1) relies on the spontaneous hydrolysis of C3 to $C3b(H_2O)$ which can interact with factors B and D to form the C3 convertase, C3bBb. There are several regulatory mechanisms preventing the alternative pathway from getting out of control including degradation of the C3bBb convertase or through degradation by C3b by factors H and I. If not inhibited by factors H and I, C3bBb acts as a C3 convertase and can activate downstream products leading to the MAC.

Arguably the most important components of the complement cascade for defense of extracellular gram-positive pathogens are the opsonins and the anaphylatoxins. The membranes of host cells and pathogens have a net negative charge making it difficult for the two bodies to adhere. Opsonization is the process of coating an object with proteins (antibodies or complement) that enhance phagocytosis. The C3 products C3b and iC3b are two opsonins that are produced during the complement cascade regardless of the method of activation. Inactivated C3b (iC3b) is recognized by host complement receptors 3 and 4 (CR3 and CR4). CR3 and CR4 (also known as CD11b/CD18 and CD11c/CD18) are found primarily on neutrophils and dendritic cells (7). The most efficient opsonin produced during the complement cascade is C3b which is recognized by CR1 (CD35) found on dendritic cells, macrophages, erythrocytes, and neutrophils (7). Coating of an antigen with C3b or iC3b followed by recognition by their cognate receptors on a phagocytic cell induces engulfment of the antigen and can lead to activation of the phagocyte and neighboring cells.

Activation of the complement cascade also leads to the production of anaphylatoxins. Anaphylatoxins are pro-inflammatory and induce mast cell degranulation, increase vascular permeability and can cause anaphylactic shock when injected into animals. Anaphylatoxins also recruit leukocytes including neutrophils, mast cells, and basophils to infection sites. Three anaphylatoxins are produce during the complement cascade including C3a, C4a, and C5a. These three peptides were termed anaphylatoxins at the time of their discovery because of their ability to cause the degranulation of mast cells, basophils and neutrophils. Of the three, C4a is the least active with only some effects on local inflammation. Moreover, C4a is only produced during the classical and lectin binding pathways of complement activation. C3a and C5a are products of all complement pathways and both are key mediators of inflammation and leukocyte recruitment. The C3a anaphylatoxin is involved in the recruitment and activation of eosinophils, but is limited in its ability to activate and recruit neutrophils (16, 18, 28, 57). By comparison, C5a is a least a 100 times more potent than C3a in the activation and recruitment of neutrophils (28, 51). Neutrophil activation and recruitment to infection sites is essential for clearance of extracellular pathogens indicating that the production of C5a is more beneficial to the host than C3a when challenged by these

microbes. Binding of C3a and C5a to their cognate receptors (C3aR and C5aR/CD88 respectively) on macrophages and neutrophils results in increased expression of adhesions essential for extravasion (51). Binding of C5a also activates the phagocytes by inducing the expression of pro-inflammatory cytokines as well as increasing the production of reactive oxygen intermediates that aid in killing phagocytosed microbes.

Complement and other serum proteins along with recognition of extracellular bacterial pathogens by PRRs are essential for alerting the host to infection and starting an effective immune response. However, these components are usually not enough to prevent the spread of the pathogenic bacteria from the original infection site even though antimicrobial peptides and complement have some bactericidal properties. A reoccurring theme associated with microbial detection by PRRs and serum proteins is the production of pro-inflammatory molecules that activate and recruit professional phagocytic cells to infection sites. These professional phagocytes are at the core of the innate immune response through their actions of clearing the infectious agents by phagocytosis.

Phagocytic Cells

Three cells types are primarily responsible for phagocytosis of extracellular bacteria in innate immune responses. Two cell types, neutrophils and macrophages, are responsible for clearance of most infectious agents. Two cell types, dendritic cells and macrophages, are also antigen presenting cells that can stimulate other parts of the immune system. Dendritic cells are responsible for phagocytosis of foreign particles and presentation of processed antigens to T cells. Dendritic cells are found throughout the epithelium and must become activated to fully maximize its potential as an antigen presenting cell. In order for dendritic cells to become active they must first receive a signal. Two primary signal types can activate dendritic cells. The first is activation through chemical signals produced by other cells at the infection. Tumor necrosis factor alpha (TNF-α) and other chemical signals are produced by macrophages and neutrophils when they encounter foreign particles and activate dendritic cells. The second mechanism of activation involves Toll-like receptors on the surface of the cell that recognize pathogen associated molecular patterns (PAMPs) associated with infectious agents. Following activation, dendritic cells phagocytose surrounding particles and migrate to the nearest lymph node.

The phagocytosed antigens are processed, loaded into class I or II MHC molecules, and presented on the dendritic cell surface to T-cells in the lymph nodes (32). Dendritic cells are not considered "professional phagocytes" because of their role in bridging innate immunity and adaptive immunity through antigen presentation and are primarily thought of as professional antigen present cells. Dendritic cells are phagocytic but are more important for their ability to recognize potential pathogens and produce signals that alert other components of the innate immune system including the professional phagocytic cells, macrophages and neutrophils.

Macrophages are a heterogeneous population of leukocytes widely distributed throughout the body that have different properties correlating to their microenvironment such as intestine, lung, or adipose tissue (6). Macrophages have many functions in the host including roles as antigen presenters, sentinels that detect pathogens through PRRs, and clearance of damaged erythrocytes and other cell debris (6, 67). However, once activated, macrophages make important contributions to the clearance of pathogens. Activation of macrophages is rapid and fully reversible indicating that macrophages not only take part in inflammation but also in its resolution (6). Macrophages can be activated by a variety of stimuli including recognition of PAMPs by surface Toll-like receptors, cytokines from neighboring cells, and/or by the anaphylatoxins C3a and C5a. Activated macrophages exhibit greater phagocytic ability and produce high levels of reactive oxygen and nitrogen species that help in killing the ingested pathogens (6). Macrophages also secrete cytokines that trigger other aspects of the immune response including the recruitment of circulating neutrophils and macrophages. Unlike neutrophils, macrophages are distributed throughout all tissues in the host and along with resident dendritic cells are usually the first dedicated immune effector cells that encounter invading pathogens.

Polymorphonuclear neutrophils (PMNs) are the dominant cells in circulation that contribute to the innate immune response. Neutrophils are recruited from the blood to infection sites through a process of extravasion by following the chemical signals from the residential cells or by components of the complement cascade. Neutrophils are professional phagocytes that destroy invading pathogens through phagocytosis. Phagocytosis by neutrophils is mediated by recognition of antibody or complement

opsonins coating the surface of the microbe. Much like in macrophages, the phagocytosed microbes are compartmentalized in an intracellular vesicle referred to as the phagolysosome. Microbes inside the phagolysosome are bombarded with reactive nitrogen and oxygen species along with several enzymatic proteins that have antimicrobial properties. Recruitment of neutrophils to infection sites is essential for the clearance of infections. This evident in the observation that patients with defects in neutrophil extravasion or activation have significantly higher mortality rates than normal individuals due to microbial challenge (72).

Even though dendritic cells have the ability to phagocytose and kill bacterial invaders, their primary duty is as sentinels and professional antigen presenting cells. The bulk of the burden in clearance of invading pathogens falls on activated macrophages and recruited neutrophils. Both cell types can directly recognize and phagocytose extracellular pathogens, but phagocytosis is dramatically increased when foreign substances are coated by opsonins. In non-naive hosts antibodies and complement provide a powerful combination of opsonins. However, a naive host must rely solely on complement opsonization due to the absence of pathogen specific antibodies. Antibodies are generally thought of as part of adaptive immunity even though adaptive immunity and innate immunity are in many ways indistinguishable. However, many bacteria (pathogens) are able to overcome the first line of host defense even as formidable a barrier as innate immune response is to invading microbes. Overcoming this barrier is essential for pathogens to establish infection and disseminate through the host. Over innumerable generations bacterial pathogens have developed several mechanisms for subverting most the innate immune response that will be reviewed here.

Bacterial evasion of the innate immune response

One of the keys to being a successful pathogen is the ability to overcome the host defense mechanisms that try to prevent colonization and dissemination. In order for the potential pathogen to be successful it must find a way to survive in a hostile host environment. Several bacteria are only pathogenic if the host is weakened and cannot mount a full response. Other bacteria are fully pathogenic regardless of the health status of the host. These bacteria have developed many mechanisms for subverting and evading the host

innate immune response. Enterococci fall primarily in the first category as it is rare for a completely healthy individual to acquire enterococcal infections. However, even in a weakened state, the host the innate immune defenses can still be quite substantial, requiring the microbe to employ several mechanisms to subvert the host response.

Bacterial capsular polysaccharides have long been known to enhance pathogenicity. Many pathogenic bacteria produce capsules including the gram-positive cocci *Staphylococcus aureus* (78, 110), *Streptococcus pneumoniae* (1), and group-B streptococci (9). The presence of capsule allows bacteria to escape detection and clearance by the host immune system through several different mechanisms (33, 78, 85, 111). Most capsules convey antiphagocytic properties to the bacteria even in the presence of opsonins including C3b. Several reports have shown that capsule producing species of bacteria are more resistant to opsonophagocytosis by inhibiting the deposition and/or detection of C3b on the surface of the organism (78, 85, 111). Some capsule serotypes of *S. aureus* and *S. pneumoniae* inhibit C3 deposition on the bacteria surface (15, 64). In some cases, such as the capsule of *S. aureus*, the amount C3 deposition is not altered, but C3 is buried beneath the surface of the capsule thus rendering it less accessible to complement receptors on the surfaces of macrophages and neutrophils (110).

Bacterial capsular polysaccharides are also known to aid in the avoidance of innate immune responses including immune surveillance. Immune surveillance relies on pathogen recognition receptors (PRRs), including Toll-like receptors, to sense pathogen associated molecular patterns (PAMPs) as reviewed earlier in the chapter. The primary PAMPs associated with gram-positive bacteria are peptidoglycan and LTA. Bacterial capsules can act as barriers that limit detection of PAMPs by PRRs (1, 33). The encapsulation of *Streptococcus suis* inhibits detection of the bacteria by TLR-2 and alters cytokine production of host cells (33). The capsule of *S. suis* not only alters cytokine production, but can also inhibit signaling pathways involved with phagocytosis (92). A common gut microbe, *Bacteroides fragilis*, produces a capsule polysaccharide that inhibits the production of pro-inflammatory cytokine IL-17 and induces the expression of the anti-inflammatory cytokine IL-10 (58). Capsular polysaccharides are important for

immune evasion, but are not the only mechanisms employed by bacteria to subvert the innate immune response.

Extracellular proteases from pathogenic bacteria assume many roles in manipulation and subversion of host innate immune responses (79). Multiple bacterial species produce extracellular proteases that contribute to pathogenesis through manipulation of the host immune response (79). These proteases target several components of the host innate immune system including complement, antimicrobial peptides (AMPs), cytokines and cytokine receptors (79). *S. aureus* in particular produces many proteins that have roles in inhibiting complement activation, binding antibodies, lyse neutrophils, and neutralize antimicrobial peptides (30).

Enterococcus faecalis and innate immunity

Apart from studies on the roles of gelatinase and cytolysin (14, 65, 75), relatively little is known about the mechanisms employed by *E. faecalis* to circumvent host innate immune responses. Active cytolysin consists of two small peptides that are lytic for a broad range of prokaryotic and eukaryotic cells and increases virulence in animal models (13). Gelatinase is known to contribute to immune evasion through degradation of several host proteins including the immuno-modulatory and antimicrobial peptide LL-37 as well as complement components C3 and C3a, and other host proteins including collagen, fibrinogen, fibrin, endothelin-1, and bradykinin (56, 57, 75, 76, 91, 109). Some studies also implicate capsular polysaccharides of *E. faecalis* in immune evasion. One study involving the known capsular polysaccharides showed that the presence of capsule enhances persistance at infection sites and that capsule inhibited phagocytosis by neutrophils in the presence of complement (35).

Even though not much is known in the method of innate immune evasion by enterococci, several aspects of the innate immune response to enterococci are known. A study employing un-encapsulated strains of *E. faecalis* concluded that *E. faecalis* is not susceptible to the membrane attack complex of the complement system due to the thickness of the peptidoglycan, and that complement C3b is an effective opsonin in the absence of antibodies for effective phagocytic clearance of *E. faecalis* by neutrophils (3). A study involving *Enterococcus faecium*, which produces serologically identical LTA to

E. faecalis, showed that TLR-2 mediated signaling was critical for early immune response and clearance of *E. faecium* (52). Furthermore, TNF-α is thought to play a key role in *E. faecalis*-mediated inflammatory responses even though the exact role remains unclear (5, 74). Based on these studies, recognition of enterococcal LTA and/or peptidoglycan by TLR-2 would appear critical for an efficient host immune response, and the masking of these integral wall components by capsule could result in increased pathogenesis by limiting the host response to the organism.

Scope of thesis

The innate immune response is essential for early detection and clearance of extracellular pathogens including *E. faecalis.* Currently, there is little understanding of the mechanisms employed by *E. faecalis* to escape the host innate immune response. Two virulence factors associated with *E. faecalis* that are known to contribute to *E. faecalis* pathogenesis are GelE and capsular polysaccharide. Several attempts have been made to establish a serotyping system for *E. faecalis* capsular polysaccharides (41, 55, 94, 95). However, these serotyping schemes were flawed in that they included differences in capsular polysaccharide antigens, but were are also based on differences in surface antigens including lipoteichoic acid (41, 102). To date, only one study has linked genetic evidence with capsule production (37). Furthermore, these studies proposed a link to some of their capsular polysaccharide serotypes and innate immune evasion, but could not reconcile the fact that not all proposed capsule serotypes conferred the same advantage (42, 59). Compounding the issue was the lack of a mechanism correlating to inhibition of phagocytosis. As opposed to capsule, several mechanisms for GelE have been reported that contribute to pathogenesis, but none of these has been directly correlated with in-vivo observations.

The effects of capsular polysaccharide and GelE were examined with the broad goal of understanding the mechanisms employed by the virulence factors that contribute to pathogenesis. The first part of this thesis (chapter 2) reconciles the genetics of capsule production with the previously proposed serotyping schemes. Chapter three examines the contributions of capsule to innate immune evasion. This chapter proposes two mechanisms employed by capsule that contribute to innate immune evasion. Chapter

four examines the role of GelE in a rabbit model of endocarditis. This chapter sheds light on a novel mechanism of GelE in immuno-modulation of the host. Finally, chapter five summarizes the work described in the thesis along with prospective future research that may result in the production of novel therapeutics for treating enterococcal infections.

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CHAPTER 2 - Capsular polysaccharide production in *Enterococcus faecalis* and the contribution of *cpsF* to capsule serospecificity

Abstract

Many bacterial species produce capsular polysaccharides that contribute to pathogenesis through evasion of the host innate immune system. The gram positive pathogen *Enterococcus faecalis* was previously reported to produce one of four capsule serotypes (A, B, C, or D). Previous studies describing the four capsule serotypes of *E. faecalis* were based on immuno-detection methods; however the underlying genetics of capsule production did not fully support these findings. Previously, it was shown that capsule production for serotype C (Maekawa type 2) was dependent on the presence of nine open reading frames (*cpsC-cpsK*). Using a novel genetic system, we demonstrated that seven of the nine genes in the *cps* operon are essential for capsule production indicating that serotypes A and B do not make a capsular polysaccharide. In support of this observation, we showed that serotype C and D capsule polysaccharides mask LTA from detection by agglutinating antibodies. Furthermore, we determined that the genetic basis for the difference in antigenicity between serotypes C and D is the presence of *cpsF* in serotype C strains. HPAEC-PAD analysis of serotype C and D capsules indicated that *cpsF* is responsible for glucosylation of serotype C capsular polysaccharide in *E. faecalis*.

Introduction

Enterococcus faecalis is a gram-positive bacterium commonly found as a commensal organism in the gastro-intestinal tracts of most mammals. *E. faecalis* is one of the leading causes of hospital acquired urinary tract infections, bacteremia, and surgical site infections (29). The development of multiple antibiotic resistances, including resistance to vancomycin, makes treatment of enterococcal infections difficult (11). The 2004 National Nosocomial Infections Surveillance (NNIS) report indicated that nearly 30% of enterococci isolated from clinical settings were resistant to vancomycin constituting a 12% rise from the previous five years (26). The development of alternative therapies to treat enterococcal infections has frequently been suggested due to rising percentages of antibiotic resistant enterococcal strains (13-15, 19).

Capsular polysaccharides are major contributors to virulence of many microorganisms. The presence of capsule allows these microbes to escape detection and clearance by the host immune system (9, 27, 30, 41). There have been several publications regarding the role of cell wall polysaccharides in the pathogenesis of enterococcal infections (10, 13, 17, 37, 43). Several attempts have been made to establish a serotyping system for *E. faecalis* capsular polysaccharides (16, 23, 35, 36). These serotyping schemes include differences in capular polysaccharide antigens, but are also based on differences in surface antigens including lipoteichoic acid (16, 38). To date, only one study has linked genetic evidence with capsule production (12). Two loci that have been reported to contain putative genes for capsule production are the *epa* and *cps* operons (10, 42). The polysaccharide produced by the *epa* locus is thought to be the cell wall rhamnopolymer (10), but cannot be detected on the surface of the bacterium (43). Although rhamnopolymer production is reported to be abrogated by mutation (43), the full nature of rhamnopolymer production is yet to be determined for many *E. faecalis* strains. Probing the genomes of serotype A and B strains with a probe specific to the *cps* locus including genes *cpsA* and *cpsB* identified a single ClaI restriction fragment for serotypes A and B (16). However, multiple ClaI restrictions fragments were identified in serotypes C and D (16) suggesting that the genes responsible for capsule production in

serotypes C and D were absent in serotypes A and B. Furthermore, the hybridization pattern between serotype C and D strains indicated a single restriction fragment polymorphism, but the basis for which genes were different between the two serotypes was not fully characterized (16). Studies based on the serotyping scheme proposed by Hufnagel et al. (17) have shown that serotype C and D strains are much more resistant to opsonophagctyosis by neutrophils in the presence of normal human serum. More recently, a study by McBride et al. indicated that serotype C clinical isolates harbored greater repertoire of antibiotic resistance cassettes, and were more likely to possess multiple virulence factors compared to the other serotypes, suggesting that the presence of the capsule is associated with pathogenic lineages of *E. faecalis* (17, 24).

It is essential to understand the underlying mechanisms of capsule production in *E. faecalis* because of ongoing efforts to develop alternative therapies targeting capsule. Here, we use a novel vector system for creating isogenic, in-frame deletion mutants to analyze the genetic basis for capsule production and serotype specificity. Our results show that only serotype C and D strains of *E. faecalis* produce capsular polysaccharides based on the observation that deletions of *cps C D E G* and *I* abolish the production of capsule. In conjunction with these observations we also demonstrate that the presence of capsule prevents detection of lipoteichoic acid on the surface of serotype C and D strains but not on un-encapsulated strains. Our data also show that CpsF is responsible for the difference in serospecificity between serotype C and D strains.

Materials and Methods

Bacterial Strains and Growth conditions

All relevant bacterial strains are listed in Table 2.1. *Escherichia coli* EC-1000 (20) and Electro-10 Blue (Stratagene) were used for plasmid construction. *E. coli* clones were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics when required (32). *E. faecalis* strains were cultivated in Todd-Hewitt broth supplied with the appropriate antibiotics when needed (THB; Becton, Dickinson and Company, Sparks, Maryland). When required for selective growth of *E. coli*, chloramphenicol (Cm) was used at 10 µg/mL and spectinomycin (Sp) was used at 150 µg/mL. When required for

the selective growth of *E. faecalis*, Cm was used at 15 µg/mL and Sp was used at 750 µg/mL. For detection of β-galactosidase activity, X-gal was used at 80 µg/mL for *E. coli* and 120 µg/mL in *E. faecalis.*

Dot Blot Analysis

We performed dot blots with DNA from representative *E. faecalis* strains, including FA2-2, V583, MMH594, Maekawa types 1, 2, 4, 5, 7, 8, 11, 18, and strains OG1RF, 12030, 12107, and E-1 to determine the presence of *cps* operon genes. Purified DNA from each strain was denatured in 0.4 M NaOH to a concentration of 1 µg/ml and spotted onto nylon membranes. The membranes were rinsed several times with TE buffer, pH 8.0. DNA was cross-linked to the membrane using ultraviolet irradiation. Gene specific radiolabeled probes were generated by PCR using primers listed in table 2.3 for each of the *cps*A through K genes, and the downstream gene, *hcp*. Membrane strips were placed in 12 hybridization tubes to be probed independently by each gene-specific probe. Following hybridization, membrane strips were aligned adjacent to one another beginning with the strip probed by the *cps*A-specific probe and continuing through to *hcp*. These membranes were then exposed to X-ray film for autoradiography.

Construction of pLT06

Descriptions of all primers and plasmids are included in tables 2.3 and 2.2 respectively. pLT06 is a combination of pCJK47 (20) pGB354 (3), and pCASPER (6) (Figure 2.1). The *ermC* cassette in pCJK47 was replaced with the chloramphenicol acetyl transferase (*cat*) gene from *Streptococcus agalactiae* plasmid pGB354. Vector pCJK47 was digested with the restriction enzymes *Bgl*II and *Nsi*I resulting in 5.8 Kb and 0.9 Kb fragments. The *cat* gene from pGB354 was amplified by PCR with the primers Cat5' and Cat 3'. The resulting PCR product was cloned as a blunt-end fragment into 5.8 Kb fragment of pCJK47 (T4 DNA polymerase treated). The resulting construct was called pKS05. pKS05 was subsequently digested with SmaI and EcoNI and the 5.7 Kb fragment containing P-*pheS*, *cat*, and *lacZ* was gel extracted (QIAGEN, QUAquick gel extraction kit) followed by klenow treatment (Bioline). pCASPER was digested with EcoRV and PshAI and the 2.15 Kb product containing *orfB, orfC, repA* (Ts), and *orfD* was gel

extracted. The 5.75 Kb pKS05 product and the 2.15 Kb product of pCASPER were blunt end ligated resulting in pLT06.

Construction of markerless exchange vectors

Vector pLT06 was used to create in-frame deletions of *cpsC, cpsD, cpsE, cpsF, cpsG, cpsH,* and *cpsI* in *E. faecalis* strains V583 and FA2-2. Relevant primers are listed in table 2.3. Fragments (1.0 kb) were PCR ampflied upstream and downstream of the gene targeted for mutation. The PCR products were ligated and re-amplified resulting in a 2.0-kb product. The 2.0-kb PCR product was digested by restriction enzymes as described in Table 2.2 and ligated with pLT06. The ligated products were electroporated into *E.coli* Electro 10 Blue (E10B) for propagation and grown on Luria-Bertani (LB) plates containing Cm and X-gal at 30°C. Blue colonies were screened for the presence of the ~2.0 kb inserts using primers OriF and KS05SeqR. Positive clones were grown overnight in liquid LB media containing Cm10 at 30°C. The plasmid was purified using the QIAprep spin miniprep kit (QIAGEN). The ~2.0-kb inserts from each construct were sequenced using primers OriF and KS05SeqR to ensure that no mutations arose during cloning. The resulting deletion constructs pLT08, pLT13, pLT16, pLT18, pLT22, pLT23, and pLT24 were used to generate the *cpsF, cpsH, cpsD, cpsE, cpsC, cpsG,* and *cpsI* deletions respectively.

Generation of deletion mutants

E. faecalis V583 and FA2-2 were used for the generation of isogenic, in-frame *cps* deletion mutants. Both V583 and FA2-2 are classified as serotype C strains and contain *cpsF* (16). Deletion constructs were transformed by electroporation into V583 and FA2-2 as previously described (8). Transformed bacteria were grown on Todd-Hewitt broth (THB) plates containing Cm and X-gal at 30°C. Blue colonies were screened for the presence of the engineered deletion constructs by colony PCR using primers OriF and KS05SeqR. Colonies that were positive for the deletion constructs were inoculated into 5.0 mL of THB containing Cm and grown overnight at 30°C. The cultures were back diluted 1:1000 in fresh THB with 15 μg/mL Cm and grown for 2.5 hours at 30°C followed by shifting to 42°C for 2.5 hours to force single-site integration by homologous

recombination. Following incubation at 42°C, the cells were serially diluted and plated on THB containing Cm and X-gal. Blue colonies growing at 42°C were screened for the targeted integration using PCR with primers flanking the site of integration. Positive integration clones were serially passaged from overnight cultures for two successive days in THB with no selection at 30°C to force the second site recombination event. Following serial passage at 30°C, the cultures were plated by serial dilution on MM9YEG plates containing 10mM *p*-chloro-phenylalanine and X-gal at 37°C. Resulting white colonies were screened for the deletion of the target genes by PCR. Genomic DNA from colonies containing the deletions were purified and sequenced to confirm gene deletions. The resulting deletion mutants are listed in Table 2.1.

Complementation of deletion mutants

The markerless gene deletions were complemented *in trans* by cloning target genes in a pAT28 plasmid background (39). The promoter region for the *cps* operon (*cpsC* promoter) was PCR amplified from the plasmid pCPSC2 using primers Vlac1 and Vlac2 (12, 28). The amplified product was cloned as an *Eco*RI/*Bam*HI fragment into pAT28 generating pLT09 (Table 2.2). PCR amplified gene products were generated for *cpsC*, *D*, *E*, *F*, *G*, *H*, and *I* from purified V583 genomic DNA using primers listed in table 2.3. The amplified products were cloned into pLT09 generating complementation plasmids pLT10 (*cpsF*), pLT14 (*cpsH*), pLT25 (*cpsD*), pLT32 (*cpsE*), pLT33 (*cpsG*), pLT34 (*cpsC*), and pLT35 (*cpsI*) (Table 2.2). The complementation vectors were transformed by electroporation into the corresponding deletion mutants (Table 2.1), resulting in strains LT03, LT04, LT07, LT08, LT25, LT27, LT29, LT31, and LT33. The serotype D strains T-5 and T-18 and the serotype B strain OG1RF were complemented with pLT10 generating strains LT09, LT10, and LT11 respectively (Table 2.1).

Determination of serospecificity by enzyme linked immunosorbent assay (ELISA) and slide agglutination

Serotype C strains, including FA2-2 and V583, can be detected by ELISA or agglutination using the Maekawa Type 2 (MT2) antibody (12, 23). However, serotype D strains such as Maekawa serotypes T-5, T-6, and T-18 cannot be detected by ELISA or

agglutinated by MT2 antibodies (16, 23). We used the MT2 antibodies to compare the serospecificity of V583, FA2-2, LT01, LT03, T-5, LT09, T-18, LT10, OG1RF, and LT11.

Overnight cultures were diluted 1:100 in fresh THB supplemented with the appropriate antibiotics and were allowed to grow to mid-log phase ($O.D._{600}$ of 0.6). Log phase cells were washed three times with and equal volumes of phosphate buffered saline (PBS), aliquoted (50 μL) into wells of a high binding 96 well costar plate (Corning), and allowed to adhere overnight at 4°C. Simultaneously, MT2 antibodies were diluted 1:1000 in PBS and were absorbed against T-5 cells in PBS overnight at 4°C to remove any crossreactivity. Following overnight incubation, the ELISA plates were washed three times in PBS Tween 20 (PBS-T) (0.05%) and blocked with 5.0% skim milk in PBS for two hours. Plates were subsequently washed three times with PBS-T, and the primary MT2 antibodies were added at a dilution of 1:1000 and allowed to bind over night at 4°C. The plates were washed again with PBS-T, and goat anti-rabbit secondary antibodies conjugated to horse radish peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA) were added to the wells. The plates were incubated at room temperature for two hours followed by washing with PBS-T three times followed by washing with PBS three times to remove residual detergent. The ELISA was developed in the presence of o-phenylenediamine dihydrochloride (OPD, Sigma) substrate for 30 minutes in the dark. The ELISA plates were analyzed by a PowerWave XS 96 well plate reader (Bio-Tek instruments) at an optical density of 490 nm.

Slide agglutination assays were performed as previously described (23). Serotype A anti-serum contains antibodies directed towards *E. faecalis* lipoteichoic acid (LTA) (16). Briefly, 5.0 μL of serum was added to 15.0 μL of test cells on a glass slide, and gently rotated for one minute. Agglutination was determined by visual clumping of the cells. Sterile PBS was used in place of antiserum as a negative control.

Preparation and purification of cell wall carbohydrates

Cell wall carbohydrates and capsular polysaccharides were isolated and purified as previously described with slight modifications (10, 14). Briefly, bacteria were grown in

two or four liters of THB supplemented with 1% glucose at 37°C to mid-log phase. Cells were washed in 300 mL of Tris-sucrose solution (10 mM Tris-Cl [pH 8.0]; 25% sucrose), and the resulting cell pellets were re-suspended in Tris-sucrose solution with lysozyme (1mg/mL), mutanolysin (10 U/mL), 0.05% sodium azide, and incubated with gentle rocking at 37° for 16 hours. Following incubation, the samples were centrifuged and the supernatants were treated RNase A (100 μg/mL) and DNase (10U/mL), and incubated for four hours at 37°C with gentle agitation. Pronase (50 μg/mL) was added to the samples and additionally incubated at 37° 16 hours. The supernatants were collected and passed through a 0.2 micron filter followed by extensive dialysis against distilled water. The samples were then lyophilized and re-suspended in minimal volume of gel filtration buffer (50 mM Tris-base/15 0mM NaCl /0.05% sodium azide, pH 7.0), and were run over an S-400 size exclusion column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Collected fractions were analyzed for capsular polysaccharide content using acrylamide gel electrophoresis and the cationic dye Stains-All for detection as previously described (10). Fractions containing capsular polysaccharide were pooled, extensively dialyzed against distilled water, lyophilized, and re-suspended in a minimal volume of 50 mM Tris buffer (pH 8.0). The sample was applied to an anion exchange Q-sepharose column for further purification (GE Healthcare Bio-Sciences, Uppsala, Sweden). Bound capsular polysaccharide was eluted using a step wise gradient starting with 50 mM Tris (pH 8.0) and ending with 50mM Tris/1 M NaCl (pH8.0). Determination of fractions containing capsular polysaccharide was carried out as described above. Capsular polysaccharide containing fractions were pooled, extensively dialyzed against distilled water, lyophilized, and used for downstream applications.

Small scale cell wall carbohydrate preparations for determining production of capsular polysaccharide were performed as stated above with slight modifications. Cells were grown in 25 mL of THB supplemented with 1% glucose until they reached an $O.D._{600}$ of 0.6-0.8. The cells were harvested, washed with 2.0mL of Tris-sucrose solution, and treated with lysozyme and mutanolysin at the same concentrations listed above for 16 hours at 37° C. The cell suspensions were centrifuged and the pellets were discarded. The remaining supernatants were treated with RNase (100 μg/mL) and DNase

(10 U/mL) for 4 hours before final treatment with pronase as described above. Remaining impurities were extracted with 500 μL of chloroform, and the remaining carbohydrates were precipitated with ethanol at a final concentration of 75% at -80°C for 30 minutes. The resulting pellets were air dried, re-suspended in 100 μL of sterile distilled water, and 25 μL was loaded onto an acrylamide gel as previously described. The gels were stained in Stains-All following electrophoresis. Stained gels showed the presence of three distinct staining regions with the highest molecular weight band corresponding to capsular polysaccharide (10).

Carbohydrate Compositional Analysis

Analysis of purified capsular polysaccharide was performed at the Glycotechnology Core Resource at the University of California San Diego using high pH anion exchange chromatography using a Dionex DX 500 HPLC (Dionex, Sunnyvale, CA) with pulsed amperometric detection (ED40; Dionex) (HPAEC-PAD). Samples were hydrolyzed with 2M trifluoroacetic acid at 100° C for five hours, dried, and resuspended in 25 μl distilled water. Sugars were eluted with 120 mM sodium hydroxide at a flow rate of 0.4 ml/min. The carbohydrate composition of each polysaccharide was determined by comparison to known carbohydrate standards that were prepared under identical conditions.

Table 2.1 List of strains used in this study

Strain	Description	Reference
V583	Serotype C	(31)
FA2-2	Serotype C	(7)
MMH594	Serotype C	(18)
OG1RF	Serotype B	(25)
12030	Serotype A	(14)
12107	Serotype B	(14)
E-1	Serotype NT*	(4)
Maekawa Type-1	Serotype B	(23)
Maekawa Type -2	Serotype C	(23)
Maekawa Type -5	Serotype D	(23)
Maekawa Type-7	Serotype A	(23)
Maekawa Type-8	Serotype NT*	(23)
Maekawa Type-11	Serotype NT*	(23)
Maekawa Type-18	Serotype D	(23)
LT01	FA2-2 Δ*cpsF*	This Study
LT02	V583 Δ*cpsF*	This Study
LT03	LT01 + pLT10	This Study
LT04	LT02 + pLT10	This Study
LT05	FA2-2 Δ*cpsC*	This Study
LT06	V583 Δ*cpsC*	This Study
LT07	LT05 + pLT34	This Study
LT08	LT06 + pLT34	This Study
LT09	T-5 + pLT10	This Study
LT10	T-18 + pLT10	This Study
LT11	OG1RF + pLT10	This Study
LT15	V583 Δ*cpsD*	This Study
LT17	V583 Δ*cpsE*	This Study
LT19	V583 Δ*cpsG*	This Study
LT21	V583 Δ*cpsH*	This Study
LT23	V583 Δ*cpsI*	This Study
LT25	LT15 + pLT25	This Study
LT27	LT17 + pLT32	This Study
LT29	LT19 + pLT33	This Study
LT31	LT21 + pLT14	This Study
LT33	LT23 + pLT35	This Study

* These strains were non-typeable by conventional serotyping methods (16).

Table 2.2 Plasmid constructs used in this study

Plasmid	Description	References
pCJK47	Conjugative donor plasmid, , carries $OriT_{pCF10,}$ *lacZ,* and P-*pheS** used in pLT06 construction.	(20)
pGB354	Contains Cat^r used in the construction of pLT06	(3)
pCASPER	Contains *orfB, orfC, RepA* ts, and *orfD* used in pLT06	(6)
pAT28	Broad range shuttle vector, spectinomycin resistant	(39)
pCPSC2	Source of the CpsC promoter used in pLT09	(12)
pKS05	pCJK47 derivative containing Cat^r	This Study
pLT06	Deletion construct used for making mutants	This Study
pLT08	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsF* deletion.	This Study
pLT09	pAT28 containing a 398-bp EcoRI/BamHI fragment containing the native CpsC promoter	This Study
pLT10	pLT09 containing 851-bp SalI/SphI fragment containing *cpsF*.	This Study
pLT13	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsH* deletion	This Study
pLT14	pLT09 containing a 447-bp BamHI/SphI fragment containing *cpsH*	This Study
pLT16	pLT06 containing a 2.0-kb BamHI/SmaI fragment containing engineered *cpsD* deletion	This Study
pLT18	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsE* deletion	This Study
pLT22	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsC* deletion	This Study
pLT23	pLT06 containing a 2.0-kb SmaI/SphI fragment containing engineered *cpsG* deletion	This Study
pLT24	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsI* deletion	This Study
pLT25	pLT09 containing a 1418-bp BamHI/SphI fragment containing *cpsD*	This Study
pLT32	pLT09 containing a 2562-bp BamHI/SalI fragment containing *cpsE*	This Study
pLT33	pLT09 containing a 2555-bp SalI/SphI fragment containing *cpsG*	This Study
pLT34	pLT09 containing a 1319-bp BamHI/SphI fragment containing *cpsC*	This Study
pLT35	pLT09 containing a 1192-bp BamHI/SphI fragment containing *cpsI*	This Study

Table 2.3 Primers used in this study

Primer	Length	Sequence	5' Nucleotide position in source sequence
CpsC1	31mer	5'GAGAGAATTCTATGTCACTGTAATGTTGTTG EcoRI	126 of *cpsB*
CpsC2	31mer	5'CTCTGGATCCGGCTTGATGTATACTATTCTC BamHI	35 of *cpsC* complementary strand
CpsC3	30mer	5'GAGAGGATCCTGCCTTGAAAATCAGGATGC BamHI	1113 of *cpsC*
CpsC4	30mer	5'CTCTCTGCAGCCTTTTACTGAATGGATACC PstI	947 of *cpsD* complementary strand
CpsCseq	20mer	5'GTTTAGGTACCTTGTGAGTT	88 between *cpsB* and *cpsC*
CpsC5'	30mer	5'GAGAGGATCCGATAATCTAATGTAAAGGAT BamHI	445 between *cpsB* and *cpsC*
CpsC3'	30mer	5'CTCTGCATGCCCACGTTTCAGTATCTAACA SphI	68 of *cpsD* complementary strand
CpsD1	30mer	5'GAGAGAATTCTTGATGCCAAGAGCTCAGTA EcoRI	264 of *cpsC*
CpsD2	32mer	5'CTCTGGATCCGGACAGCTTAAATTGACTTAAC BamHI	98 of *cpsD* complementary strand
CpsD3	31mer	5'GAGAGGATCCAGTTATCAGGTAGAGTTGCCA BamHI	1353 of *cpsD*
CpsD4	31mer	5'CTCTCTGCAGGCTGCTGGATCATTTGCAATT PstI	940 of *cpsE* complementary strand
CpsDseq	20mer	5'CAGATTATTCATCGGTTATG	877 of *cpsC*
CpsD5'	31mer	5'GAGAGGATCCTGCCACTAGACAGCTGATTTC BamHI	1130 of *cpsC*
CpsD3'	30mer	5'CTCTGCATGCCAATGACTGACACTTTCACA SphI	27 of *cpsE* complementary strand
CpsE1	32mer	5'GAGAGAATTCAGTAAGCCATATTATGTGGATG EcoRI	519 of *cpsD*
CpsE2 strand	32mer	5'CTCTGAATTCTTCCCTCACTTATCATTTATAG EcoRI	60 between *cpsD* and *cpsE* complementary
CpsE3	31mer	5'GAGAGAATTCGCAAAAGGGTTGTTGAAATAG EcoRI	2490 of *cpsE*

CpsE4	31mer	5’CTCTCTGCAGTTGGTTATACCACCAGCCCAT PstI	38 of *cpsG* complementary strand
CpsEseq	20mer	5’CATGAATGCTTATGCAGAAG	1199 of *cpsD*
CpsE5’	32mer	5’GAGAGGATCCCAGGATATTTTGAGTATACAAC BamHI	18 between *cpsC* and *cpsD*
CpsE3’	31mer	5’CTCTGTCGACCTATTTCAACAACCCTTTTGC SalI	2510 of *cpsE* complementary strand
CpsF1	30mer	5’GAGACCCGGGGCTGACACGAATCGTTAGAC XmaI	1568 of *cpsE*
CpsF1Eco	30mer	5’GAGAGAATTCGCTGACACGAATCGTTAGAC EcoRI	1568 of *cpsE*
CpsF2	33mer	5’CTCTCTGCAGGCCTTTATCAATTCTTTCTCTTC PstI	58 of *cpsF* complementary strand
CpsF3	31mer	5’GAGAATGCATGGTATACACGGCCTTACGATA NsiI	777 of *cpsF*
CpsF4	30mer	5’CTCTGTCGACCGTCTAGTTTCGCCAAGCTC SalI	949 of *cpsG* complementary strand
CpsF4Pst	30mer	5’GAGACTGCAGCGTCTAGTTTCGCCAAGCTC PstI	949 of *cpsG* complementary strand
CpsF5’	31mer	5’GAGAGTCGACAGCAAAAGGGTTGTTGAAATA SalI	2489 of *cpsE*
CpsF3’ strand	32mer	5’CTCTGCATGCCCTACTTTCTCTGTTACTTAAT SphI	17 between *cpsF* and *cpsG* complementary
CpsG1	30mer	5’GAGACCCGGGGATACAATGACAAGTATTGG SmaI/XmaI	2388 of *cpsE*
CpsG2	31mer	5’CTCTCTGCAGCCACCAGCCCATAATTGCTGC PstI	29 of *cpsG* complementary strand
CpsG3	30mer	5’GAGACTGCAGATGTGGGAAGCAAGTTTAAC PstI	2424 of *cpsG*
CpsG4	30mer	5’CTCTGCATGCTAATTCCGTAGCCTTACGTC SphI	509 of *cpsI* complementary strand
CpsGUp	21mer	5’AAGGCTATACCTTACTACAAG	2212 of *cpsE*
CpsGDwn	21mer	5’ATAGCTGTATAACCGTCGACA	610 of *cpsI* complementary strand
CpsG5’	33mer	5’GAGAGTCGACGATTAAGTAACAGAGAAAGTAGG SalI	846 of *cpsF*
CpsG3’	30mer	5’CTCTGCATGCCCACAACCGATACCAATTGC	52 of *cpsH* complementary strand

		SphI	
CpsH1	31mer	5’GAGAGGATCCCATCCGATACAAAAACCTAAG	1410 of *cpsG*
		BamHI	
CpsH2	30mer	5’CTCTGTCGACTTTCTTCATGTCACACACTC	8 of *cpsH* complementary strand
		SalI	
CpsH2B	30mer	5’CTCTGGATCCTTTCTTCATGTCACACACTC	8 of *cpsH* complementary strand
		BamHI	
CpsH3	32mer	5’GAGACTCGAGTAAATAATGCATTTGGTGTTTG	396 of *cpsH*
		XhoI	
CpsH3B	31mer	5’GAGAGGATCCTAAATAATGCATTTGGTGTTT	396 of *cpsH*
		BamHI	
CpsH4	29mer	5’CTCTCTGCAGTCGCCATTCCATTATCCGT	875 of *cpsI* complementary strand
		PstI	
CpsH5’	33mer	5’GAGAGGATCCTAAACAGGGGAGTGTGTGACATG	2451 of *cpsG*
		BamHI	
CpsH3’ strand	32mer	5’GAGAGCATGCCAAACACCAAATGCATTATTTA	20 between *cpsH* and *cpsI* complementary
		SphI	
CpsHSeqF	20mer	5’ATGACATGGTAGGAACTGTC	2134 of *cpsG*
CpsHUp	21mer	5’AGAATCGTCGGTAAATATGTG	1356 of *cpsG*
CpsHDwn	21mer	5’ACTTGGTCCATATCATAGTAT	1084 of *cpsI* complementary strand
CpsI1	30mer	5’GAGAGAATTCATTCCAGGAACCATTGGTGG	1905 of *cpsG*
		EcoRI	
CpsI2 strand	30mer	5’CTCTGGATCCACTAACTTCTCCTAACAAAG	56 between *cpsH* and *cpsI* complementary
		BamHI	
CpsI3	30mer	5’GAGAGGATCCTACTATGATATGGACCAAGT	1065 of *cpsI*
		BamHI	
CpsI4	30mer	5’CTCTCTGCAGTCTTCGTACCAAGAGTCATT	930 of *cpsJ* complementary strand
		PstI	
CpsIUp	21mer	5’ATGACTGCCTTGAATTATCGT	1779 of *cpsG*
CpsIDwn	21mer	5’TTGTATCCTTCCCTGCTACTT	1074 of *cpsJ* complementary strand
CpsI5’	30mer	5’GAGAGGATCCGTAACTTTGTTAGGAGAAGT	33 between *cpsH* and *cpsI*
		BamHI	
CpsI3’	30mer	5’CTCTGCATGCATTTGTTCCTCCGAGTCTAA	2 of *cpsJ* complementary strand

		SphI	
CpsE5	19mer	5’GAACAATTTGTTGGCGAGG	1485 of *cpsE*
CpsE3seq	20mer	5’ATACAAGCAATGCCAGCGGA	2068 of *cpsE*
CpsG5seq	20mer	5’ATCGTCGTCTACCCAACCAT	320 of *cpsG* complementary strand
CpsG3seq	19mer	5’AGCAATCTTTCCAGCGGTC	1040 of *cpsG* complementary strand
CpsB	21mer	5’CATCTTGCCAGGACATGGTGG	584 of *cpsB*
CpsK	21mer	5’GAAGATAAGCCCCAGCTTGTT	705 of *cpsK*
Hcp	22mer	5’CACTTAATGTTGTCACTAACGC	108 of *hcp* complementary strand
Vlac1 pCPSC2	23mer	5’GTTGAATAACACTTATTCCTATC	Amplification of cps promoter from
Vlac2 pCPSC2	21mer	5’CTTCCACAGTAGTTCACCACC	Amplification of cps promoter from
Cat 5’	21mer	5’ AAGCGAACGAAAAACAATTGC	Amplification of Cat^r from pGB354
Cat 3’	22mer	5’ AAAATGTGGTTGTTATACGTTC	Amplification of Cat^r from pGB354
M13F derivatives	19mer	5’ TGTAAAACGACGGCCAGTG	Sequencing and screening pLT09
M13R derivatives	20mer	5’ CAGCTATGACCATGATTACG	Sequencing and screening pLT09
OriF derivatives	21mer	5’ CAATAATCGCATCCGATTGCA	Screening and sequencing pLT06
KS05seqR derivatives	22mer	5’ CCTATTATACCATATTTTGGAC	Screening and sequencing pLT06

Results

Dot blot analysis of the capsule locus from serotype A, B, C, and D strains

Dot blot analysis was performed for representatives of the four *E. faecalis* serotypes. *E. faecalis* serotype A or B strains E-1, OG1RF, Type-1, Type-4, Type-7, 12030, and 12107 as well as serotype C strains FA2-2, V583, MMH594, Type-2, Type-8, and type-11, along with serotype D strains Type-5 and Type-18. Blots were performed to determine the presence or absence of specific capsule operon genes (*cpsC-K*), as well as the conserved flanking genes *cpsA*, *cpsB*, and *hcp* that are known to reside adjacent to the capsule operon. All serotypes contained genes *cpsA*, *cpsB*, and *hcp* (Fig. 2.1). Only serotypes C and D contained genes *cpsC-cpsK* with the only identifiable difference between the two serotypes being that serotype D strains lacked *cpsF* (Fig. 2.1).

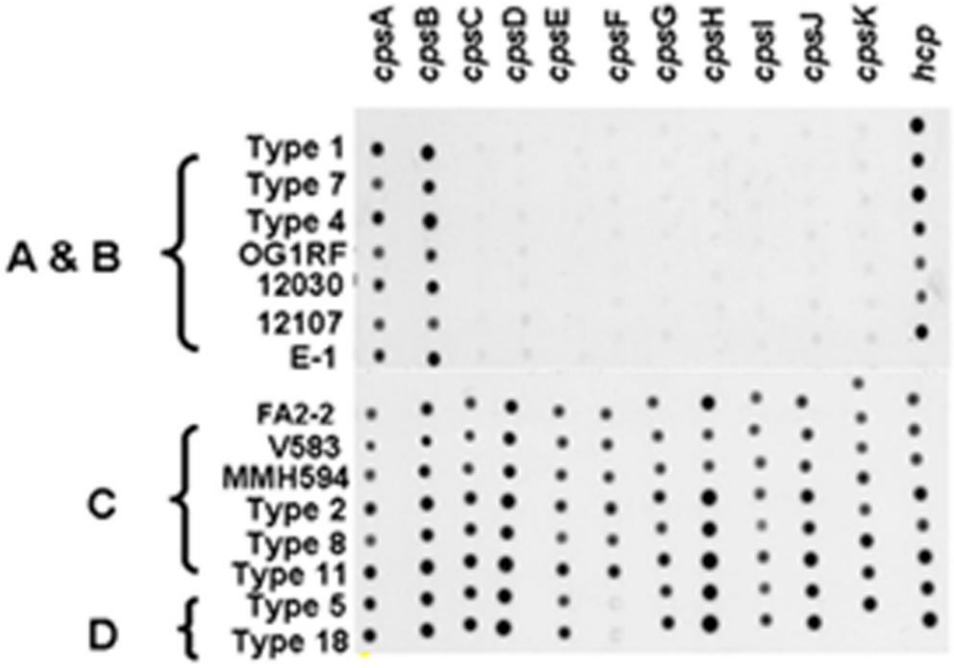


Figure 2.1 Dot blot analysis of the four putative serotypes of *E. faecalis*.
Serotypes A and B (top) only hybridize to *cpsA*, *cpsB*, and the control gene *hcp* that sits outside of the capsule locus. The serotype C strains (middle) hybridize to all the genes in the *cps* locus (*cpsC-cpsK*) as well as *cpsA*, *cpsB*, and *hcp* genes. Serotype D strains (bottom) hybridize to all genes of the *cps* locus except *cpsF*.

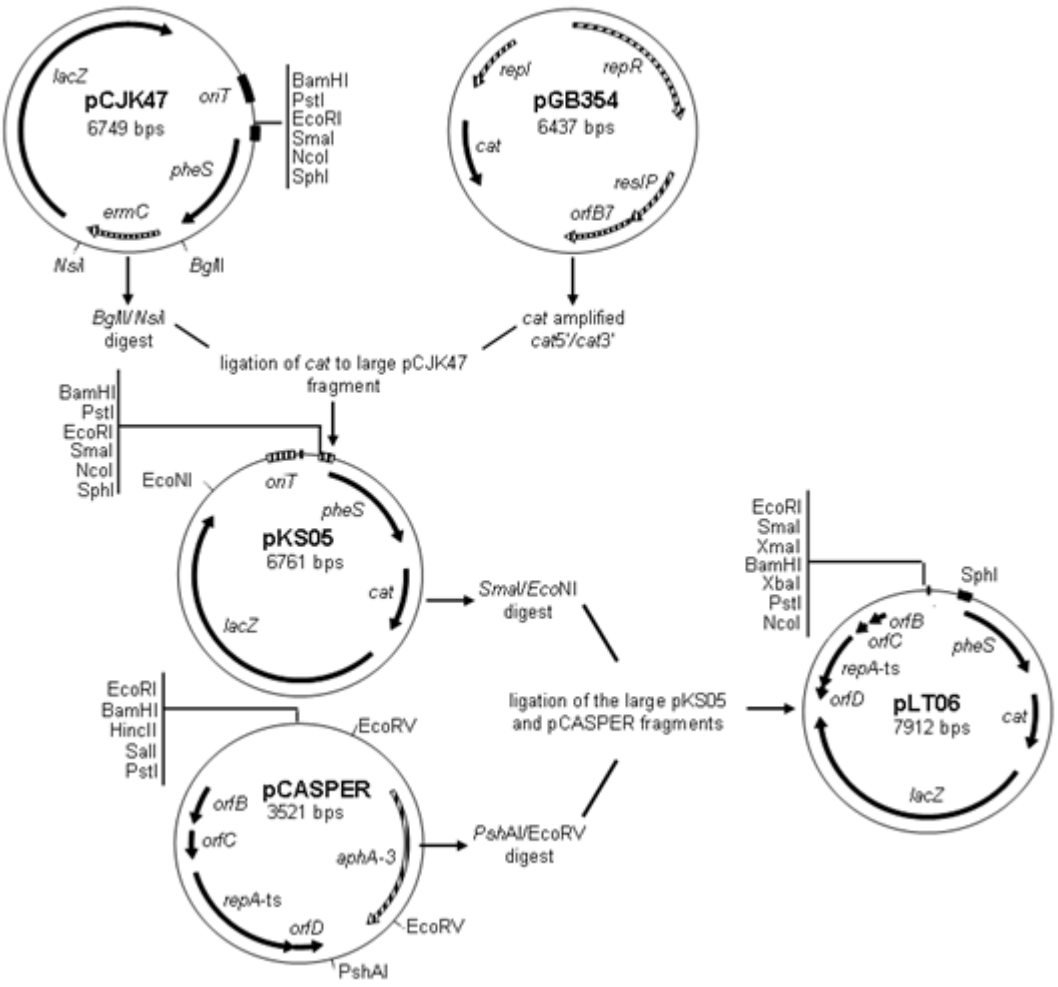
Construction of pLT06 and generation of *cps* operon deletion mutants.

The development of pCJK47 by Kristich et al. was one of the first vector systems for generating gene deletion mutations in *E. faecalis.* (20). Limitations of this system involved the necessity to conjugally mate the plasmid construct from a donor strain (20). This delivery method is inefficient for delivery of cloned DNA into target strains that harbor endogenous plasmids such as the vancomycin resistant strain V583. Another noted obstacle associated with this system is the mobilization and unwanted transfer of genomic DNA from the donor strain into the recipient strain. The *erm* resistance cassette used in pCJK47 for selection was also unsuitable for work with V583 due to inherent resistant to erythromycin.

To counter these limitations we constructed an improved vector system, pLT06, to generate markerless in-frame deletions of *cps* operon genes (Figure 2.2). Insertional inactivation techniques would not have been suitable to assess the contributions of the individual *cps* operon genes to capsule production or serospecificity. The pLT06 vector contains components of pCJK47, including *lacZ* and the counterselectable marker P-*pheS* (Figure 2.2). pLT06 also contains the chloramphenicol acetyl transferase (*cat*) marker from pGB354 for selection purposes, and *orfB, orfC, repA* (Ts), and *orfD* from pCASPER. The combination of genes comprising pLT06 allowed for direct transformation by electroporation of cloned DNA into target *E. faecalis* strains. The plasmid can replicate in *E. faecalis* at permissive temperatures of 30°C, but cannot replicate at the non-permissive temperature of 42°C due to the temperature sensitive nature of the *repA* gene. Flanking regions of the gene targeted for deletion were cloned into pLT06 to serve as templates for targeted recombination. Derivatives of pLT06 designed to delete the targeted genes are forced to integrate into the host genome through single-site homologous recombination when grown at non-permissive temperatures in the presence of chloramphenicol. If recombination does not occur then the subsequent clones of the host cell harboring pLT06 will not survive as they will not carry the *cat* cassette for resistance to chloramphenicol. Clones containing properly integrated pLT06 constructs were serially passaged at the permissive temperature in THB without selection to induce the second site recombination event and subsequent loss of pLT06. Bacteria

harboring integrated or circularized pLT06 constructs should not grow on MM9YEG agar due to the presence of the p-chloro-phenylalanine substrate and the P-*pheS* cassette (20). White colonies from the MM9YEG plates were screened by PCR to confirm deletion of the target gene. Approximately 50% of the screened colonies harbored the desired mutation. PCR amplification from the *cpsE-G* junction in serotype C strains (FA2-2 and V583), the corresponding *cpsF* mutants (LT01 and LT02), and serotype D strains (T-5 and T-18), shows a 2.8 kb amplicon in strains containing *cpsF* and a 2.0 kb amplicon in strains lacking *cpsF*. DNA sequence analysis of LT01 and LT02 and complementation with *cpsF* with pLT10 showed that a non-polar deletion was generated using pLT08.

A.



B.

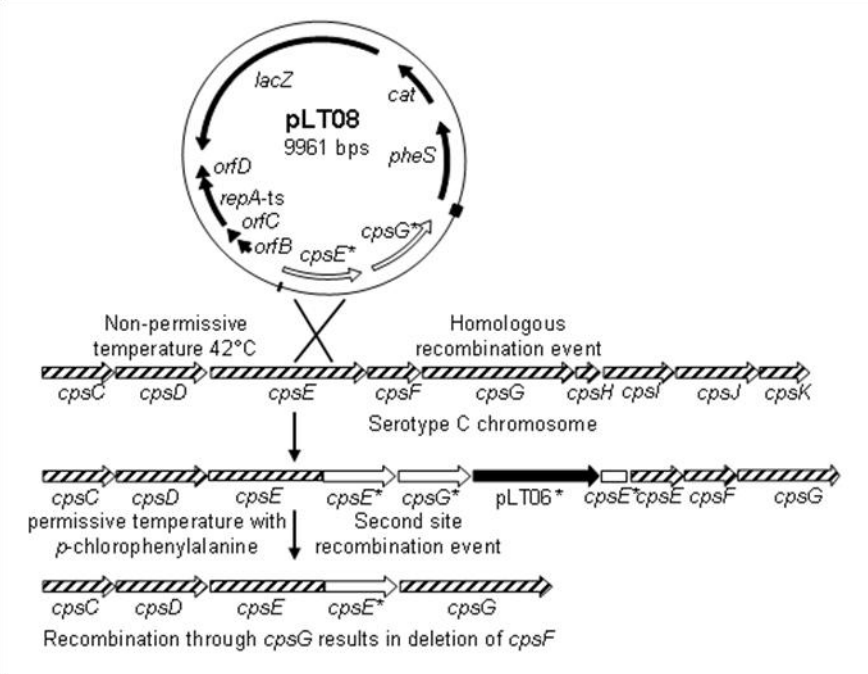


Figure 2.2 Construction of pLT06 and generation of an isogenic, in-frame deletion mutant in *E. faecalis*

A. Strategy for the construction of plasmid pLT06 used in this study for construction of isogenic, in-frame, deletion mutants in *E. faecalis.* See materials and methods for details. The *erm* marker from pCJK47 was replaced with the *cat* marker from pGB354 resulting in pKS05. The *oriT* from pKS05 was replaced with an enterococcal origin of replication and the temperature sensitive *repA* resulting in pLT06. pLT06 was subsequently used to engineer all of the isogenic, in-frame, deletion mutants used in this study. **B.** Diagram of the generation of the in-frame, isogenic *cpsF* mutation using pLT08. Integration through homologous recombination of pLT08 into the *E. faecalis* genome took place at the non-permissive temperature of 42° C. Strains harboring the integrated plasmid were serially passaged at the permissive temperature of 30° C in the absence of the selecting antibiotic chloramphenicol. Serial passaging induced the second site homologous recombination event and the excision of the plasmid. Bacteria were plated on media containing ρ-chlorophenylalanine and X-gal to screen for isolates that lost the plasmid. White colonies were screened by PCR for the deletion event, and isolated DNA was sequenced to confirm that and in-frame deletion had occurred.

Determination of capsular polysaccharide production in serotypes A, B, C, and D

Cell wall polysaccharides were purified from parental and mutant strains to assess capsule production. This method of detection allows for the most direct and solid evidence of the presence of a capsule as opposed to an antibody based method that could

falsely detect other cell wall antigens (38). Small scale cell wall polysaccharide preparations were loaded on polyacrylamide gels, eletrophoresed, and stained with the cationic dye Stains-All. The high molecular weight dark blue band corresponds to capsular polysaccharide, and correlates with previously described high molecular weight *E. faecalis* capsule (10). The light blue band immediately below the capsule corresponds to the rhamnopolymer and the low molecular weight dark blue smear corresponds to teichoic acid as previously described (10). From figure 2.3 it is clear that serotype A and B strains (lanes B and C showing 12030 and OG1RF respectively) do not produce the high molecular weight capsular polyscaccharide. Interestingly, the serotype A strain 12030 did not appear to produce detectable rhamnopolymer, however the basis for this observation is not known at the present time. Consistent with genetic data (Fig.2.1) all serotype C and D strains produced the high molecular weight band corresponding to capsular polysaccharide (Fig. 2.3).

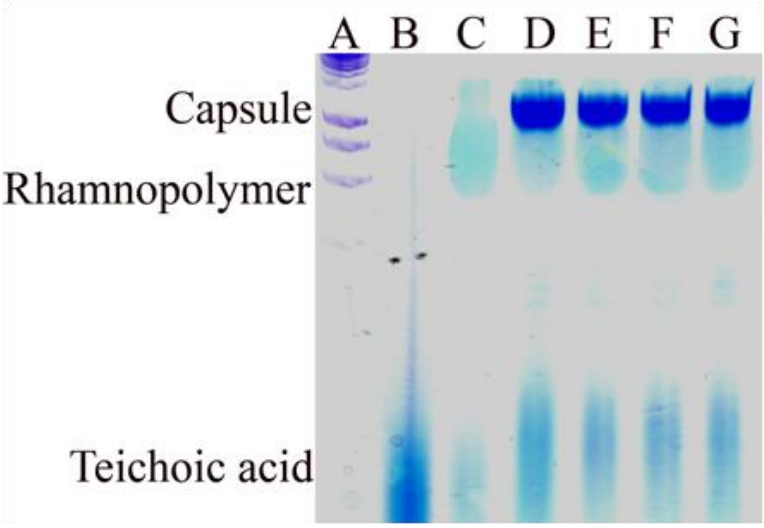


Figure 2.3 Capsule production is serotype A, B, C, and D strains of *E. faecalis*

Acrylamide gel stained with Stains-All showing the presence/absence of capsule production in serotype A-D strains. The high molecular weight bands correspond to capsular polysaccharide as previously described (10). The serotype A strain 12030 (B) as well as the serotype B strain OG1RF (C) do not produce the capsule band. Serotype C strains V583 and FA2-2 (D and E) and the serotype D strains T-5 and T-18 (F and G) produce the high molecular weight capsule band.

Determination of serospecificity between serotype C and D strains

Given that the only genetic difference between serotype C and D strains is the presence of *cpsF* in serotype C strains, we hypothesized that CpsF was the sole contributor to differences in antigenicity between serotype C and D strains. We performed ELISA with Maekawa Type 2 (MT2) antiserum to detect the serotype C antigenic determinant (23). MT2 antisera has been shown to be specific for the serotype C antigen (12). While the serotype C strain FA2-2 was detected by the MT2 antisera, LT01 (FA2-2 Δ*cpsF*) and the serotype D strains T-5 and T-18 were not detected by the MT2 antiserum (Fig. 2.4). Strain LT03 (FA2-2 Δ*cpsF*+pLT10), along with the serotype D strains LT09 (T-5), and LT10 (T-18) containing the complementation vector pLT10 were detected by the MT2 antisera. As expected the serotype B strain OG1RF was not detected by the MT2 antiserum after transformation with pLT10 (LT11) (Fig. 2.4).

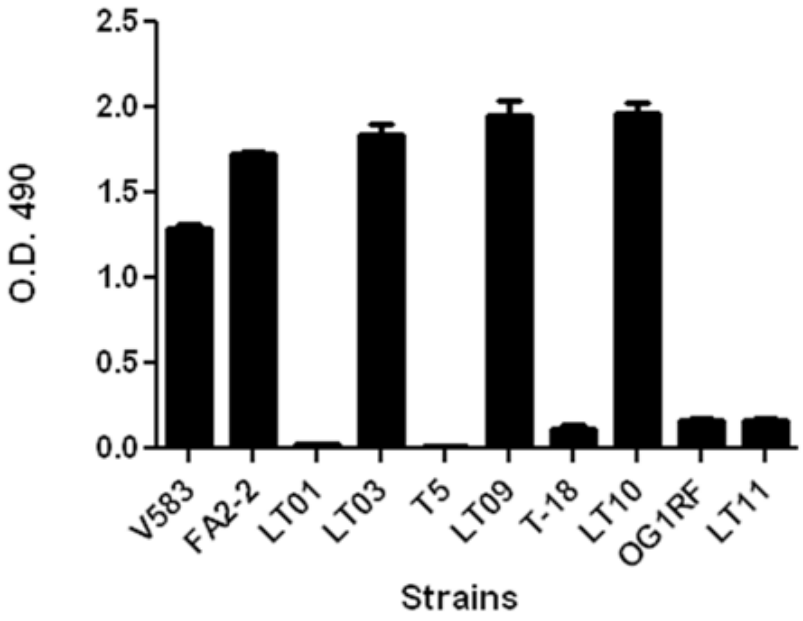


Figure 2.4 Serotype C capsule ELISA

CPS ELISA using MT-2 antibodies to detect serotype C capsule. Serotype C strains V583 and FA2-2 show reactivity with the MT2 antibody. The *cpsF* deletion mutant LT01 is not detected by the antibody, but complementation of LT01 (FA2-2 Δ*cpsF*) with pLT10 (LT03) restores reactivity to the antibody. The serotype D strains T-5 and T-18 are not detected by the serotype C antibody. However, LT09 (T-5 + pLT10) and LT10 (T-18 + pLT10) are seroconverted to serotype C strains when complemented with *cpsF*. The serotype B strain OG1RF is not detected by the MT-2 antibody before or after (LT11) complementation with pLT10 indicating that serotype conversion cannot occur in a strain that does not produce capsular polysaccharide.

Capsule production alters detection of lipoteichoic acid by slide agglutination

Recently, it was discovered that agglutinating antibodies generated to the serotype A strain 12030 were directed towards lipoteichoic acid (LTA), and not towards capsule as previously described (16, 38). This suggests that sera developed for serotyping and detecting serotype A strains should recognize other strains with exposed LTA. We used serotype-A antisera in agglutination assays to determine if our mutant strains could be agglutinated. No agglutination was observed for FA2-2 and LT01 (FA2-2 Δ*cpsF*), but 12030 and LT05 (FA2-2 Δ*cpsC*) agglutinated in the presence of these antibodies (Table 2.4). This suggests that the presence of capsule in serotype C and D strains protects LTA from detection by agglutinating antibodies.

Table 2.4 Slide agglutination using serotype C antiserum

Strain	**Agglutination**
FA2-2	Negative
FA2-2 Δ*cpsF*	Negative
FA2-2 Δ*cpsC*	Positive
12030	Positive

Comparison of serotype C and D capsule polysaccharides by high performance anion exchange chromatography with pulsed amperometric detection

We used purified capsular polysaccharide from FA2-2 (serotype C) and LT01 (FA2-2 Δ*cpsF*/serotype D) to determine the contribution of *cpsF* to the difference in antigenicity between serotype C and D strains. Capsular polysaccharides were purified as described in the materials and methods. Analysis of the FA2-2 capsule compared to the LT01 capsule indicated a difference in the ratio of glucose compared to galactose between the two capsule serotypes (Table 2.5) indicating that CpsF could be a glucosyltransferase.

Contributions of *cps* operon genes to capsule production

We generated in-frame deletions of *cps C*, *D*, *E*, *F*, *G*, *H*, and *I* in the serotype C strain V583 to determine their contribution to capsule production. Figure 2.5 clearly shows that

Table 2.5 HPEAC-PAD analysis

Strain	Galactose	Glucose
FA2-2	1.0	4.4
FA2-2 Δ*cpsF*	1.0	3.0

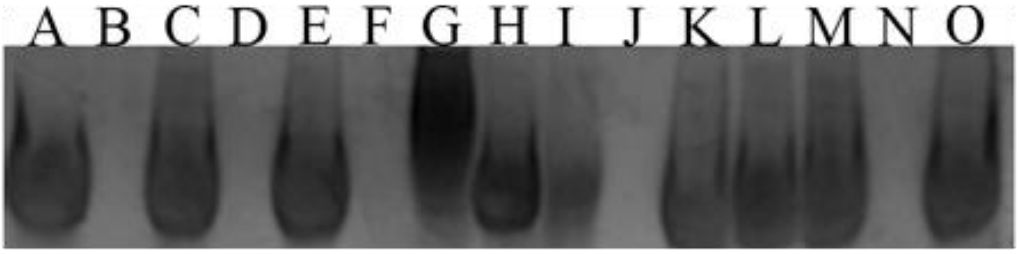


Figure 2.5 Capsule production in *cps* mutant strains

Polyacrylamide gel stained with Stainz-all showing the high molecular weight capsule bands of capsule mutants and complemented mutants. A. V583, B. LT06 (V583 Δ*cpsC*), C. LT08 (V583 Δ*cpsC* + pLT10), D. LT15 (V583 Δ*cpsD*), E. LT25 (V583 Δ*cpsD* + pLT25), F. LT17 (V583 Δ*cpsE*), G. LT27 (V583 Δ*cpsE* + pLT32), H. LT02 (V583 Δ*cpsF*), I. LT04 (V583 Δ*cpsF* + pLT10), J. LT19 (V583 Δ*cpsG*), K. LT29 (V583 Δ*cpsG* + pLT33), L. LT21 (V583 Δ*cpsH*), M. LT31 (V583 Δ*cpsH* + pLT14), N. LT23 (V583 Δ*cpsI*), O. LT33 (V583 Δ*cpsI* + pLT35). Only genes *cpsF* and *cpsH* are not essential for capsule production. Deletion of genes *cpsC, D, E, G,* and *I* completely abrogates capsule production. This observation supports the evidence that serotypes A and B do not produce capsule based on the absence of essential genes for capsule production in these strains. Complementation of these deletions restores capsule production.

genes *cps C, D, E, G,* and *I* are essential for production of the high molecular weight capsular polysaccharide. Further, these phenotypes were not due to polar effects on downstream genes as complementation of each gene *in trans* restores capsule production. The genes *cpsF* and *cpsH* are the only genes in the *cps* operon that are not essential for capsule production (Fig. 2.5).

Discussion

Previous reports of capsule production in *E. faecalis* have focused on differences in antigenicity between cell surface polymers (16, 22). One study divided *E. faecalis* into 21 different serogroups based on differences in agglutination to polyclonal antibodies generated to heat killed cells (23). The antiserum used in this study was possibly detecting capsule as well as other surface antigens (16). A more recent study, grouped strains of *E. faecalis* into four capsular serotypes (A-D) based on serospecificity (16). This study alluded that serotypes A and B shared a locus similar to serotypes C and D that was responsible for capsule production in all four serotypes. Accordingly, the capsular antigen of serotype A was purified, compositionally analyzed, and the structured deduced by NMR (14, 40). However, it was recently reported that the serotyping antibody used to classify serotype A isolates actually recognized LTA, and that the determined structure of the serotype A capsule corresponded to LTA (38). To date, only one genetic locus had been determined to be responsible for capsule production in *E. faecalis* (10). The capsule locus described by Hancock et al. is comprised of nine genes (*cps C-K*) that directly contribute to the expression of a capsular polysaccharide in *E. faecalis* (10). We have shown that serotypes C and D strains contained all genes of the *cps* locus described by Hancock et al. (proceeded by *cps A* and *cps B*) with the variation between serotypes C and D being attributed to the presence (serotype C) or absence of *cpsF* (serotype D).

Previous studies have shown that the genes *cpsA* and *cpsB* are not part of the capsule operon as they are transcribed from a different promoter (12). Attempts to mutate these genes never resulted in the recovery of viable isolates (12). However, reactive capsule antigen could be produced in a heterologous host (*E. coli*) by complementation with the *cpsC-K* operon (12). The absence of capsule production by serotypes A and B (Fig. 2.3) highlights the fact the CpsA and CpsB play no role in capsule production. Therefore, based on sequence homology we propose to rename *cpsA* to *uppS* consistent with its function as undecylprenyl pyrophosphate synthetase. We also propose to rename *cpsB* as *cdsA* as it shares strong sequence similarity with known cytidyl transferase activity. Both UppS and CdsA are known to be essential proteins in

other bacterial systems (1) which explains the inability to recover such mutants in *E. faecalis* (12).

We demonstrate that the production of capsule prevents detection of LTA by agglutinating antibodies (Table 2.4). This observation is consistent with the argument that LTA is shielded from agglutinating antibodies by capsule. Our observations support a role for CpsF in determining serospecificity between serotype C and D strains. Compositional analysis suggests that CpsF is responsible for the altered ratios of glucose to galactose present in the capsules of serotypes C and D (Table 2.5). Additionally, we propose that serotypes C and D are the only *E. faecalis* serotypes that produce a capsular polysaccharide which is supported with the data in figure 2.3 and the underlying genetics known to contribute to capsule production (Figs. 2.1 and 2.5).

CpsF has no known sequence similarity to any characterized protein thus making it difficult to predict a possible contribution to serotype differences. Purified capsular polysaccharide extracts from FA2-2 (serotype C) and LT01 (serotype D) were analyzed by HPAEC-PAD to determine the possible contribution of CpsF. HPAEC-PAD analysis revealed a difference in glucosylation of the polysaccharides with FA2-2 containing an extra glucose relative to galactose when compared to LT01 (FA2-2 Δ*cpsF*) (Table 2.5). The ratio of glucose to galactose for the serotype C strain FA2-2 is identical to previous compositional analysis (10). This result indicates that CpsF is a putative glucosyltransferase, but ongoing studies to reveal the structure of the repeating unit will provide solidifying evidence for the functional role of CpsF.

Opsonophagocytic killing of both serotype C and D strains by healthy human sera is drastically reduced when compared to the unencapsulated serotype A and B strains (17). Additional studies with the serotype B strain, OG1RF, demonstrated the presence of protective antibodies in normal serum leading to clearance of *E. faecalis* (2). This could be due by the presence of opsonizing anti-lipoteichoic acid (LTA) antibodies present in normal human serum (17). Presumably, the presence of capsule in serotype C and D strains masks LTA from detection by the circulating anti-LTA antibodies. Serotype A antibodies that recognize LTA (38) cannot recognize or agglutinate the encapsulated serotype C and D strains (Table 2.4). However, these same antibodies

readily recognize and agglutinate the un-encapsulated strain LT05 (V583 Δ*cpsC*) and the serotype A strain 12030. These observations are consistent with the increased virulence associated with serotype C and D strains (24). Furthermore, LTA is a pathogen associated molecular pattern (PAMP) that is recognized by the pattern recognition receptor (PRR) Toll-like receptor 2 (TLR-2) (33, 34). Recognition of LTA results in increased cytokine production and neutrophil recruitment to the site of infection (5, 21). Presumably, the presence of capsule would attenuate the host innate immune response. Currently, we are conducting studies to determine the effects of capsule on innate immune system evasion.

In summary, the results presented in this study argue that only *E. faecalis* serotype C and D produce a true capsular polysaccharide while serotypes A and B do not. We provide empiric proof that CpsF is the basis for the difference in antigenicity between serotype C and D strains. Finally, the inability to detect LTA on the surface of encapsulated strains indicates that the capsule of *E. faecalis* may play a role in evasion of the host innate immune response. Future studies will aim to address such questions in order to develop targeted therapies to treat infections caused by multi-drug resistant *E. faecalis.*

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CHAPTER 3 - *Enterococcus faecalis* capsular polysaccharide serotypes C and D and their contributions to host innate immune evasion

Abstract

It has become increasingly difficult to treat infections caused by *Enterococcus faecalis* due to the high levels of intrinsic and acquired antibiotic resistances. However, few studies have explored the mechanisms that *E. faecalis* employs to circumvent the host innate immune response and establish infection. Capsule polysaccharides are important virulence factors that are associated with innate immune evasion. We demonstrate that capsule producing *E. faecalis* strains of either serotype C or D are more resistant to complement-mediated opsonophagocytosis compared to un-encapsulated strains using cultured macrophages (RAW 264.7). We show that differences in opsonophagocytosis are not due to variation in C3 deposition, but due to the ability of capsule to mask bound C3 from detection on the surface of *E. faecalis*. Similarly, *E. faecalis* capsule masks detection of lipoteichoic acid which correlates with decreased TNF-α production by cultured macrophages in the presence of encapsulated strains compared to unencapsulated strains. Our studies confirm the important role of the capsule as a virulence factor of *E. faecalis*, and provide several mechanisms by which the presence of the capsule influences evasion of the innate immune response, and suggest that the capsule could be a potential target for developing alternative therapies to treat *E. faecalis* infections.

Introduction

Enterocoocus faecalis is an important nosocomial pathogen associated with many types of infections including surgical site infections, bacteremia, urinary tract infections, and endocarditis (31). Many infections caused by *E. faecalis* are difficult to treat due to increasing resistance to conventional antibiotic therapies including vancomycin (10-12). Apart from studies on the roles of gelatinase and cytolysin (5, 22, 27), relatively little is known about the mechanisms employed by *E. faecalis* to circumvent host innate immune responses.

In other bacterial pathogens, the production of capsular polysaccharide is a known virulence factor as it aids in avoidance of the host innate immune response (25, 30, 36). *E. faecalis* is known to produce two capsular polysaccharide serotypes (C and D)(10, 12, 14, 40) that contribute to pathogenesis and evasion of the host innate immune response (10). Hufnagel *et al*. reported decreased neutrophilic killing of encapsulated serotype C and D strains compared to the un-encapsulated A and B strains (15). In addition, a recent comprehensive analysis of clinical *E. faecalis* isolates indicated that most pathogenic strains of *E. faecalis* belonged to serotype C (19). Despite a link between capsule and virulence, little is known about the specific mechanism(s) of how capsule enhances pathogenesis.

The complement system plays a central role in the activation of the immune system and in the clearance of pathogens. Cleavage of C3 to C3b provides a highly effective opsonin in the absence of antibodies. Several reports have shown that capsule producing species of bacteria are more resistant to opsonophagocytosis by inhibiting the deposition and/or detection of C3b on the surface of the organism (28, 32, 42). Encapsulated bacteria employ numerous mechanisms to resist C3 opsonization and subsequent phagocytosis, including overall reduction in C3 deposition (6). The abundance of C3 deposition is known to differ between capsule producing serotypes of *Streptococcus pneumoniae* (20). In *Staphylococcus aureus,* C3 is buried beneath the surface of the capsule rendering C3 less accessible to complement receptors on the surfaces of macrophages and neutrophils (41).

Bacterial capsular polysaccharides are also known to aid in the avoidance of innate immune responses including immune surveillance. Immune surveillance relies on pathogen recognition receptors (PRRs), including Toll-like receptors, to sense pathogen associated molecular patterns (PAMPs). Two common PAMPs associated with Gram-positive microorganisms are lipoteichoic acid (LTA) and peptidoglycan (PGN). Detection of these PAMPs by Toll-like receptor 2 in conjunction with Toll-like receptors 1 and 6 induces the production of cytokines. In other instances, capsule prevents the detection of PAMPs by PRRs which leads to decreased or altered cytokine production (9). The altered cytokine response to encapsulated pathogens appears to contribute to pathogenicity and virulence.

Our data indicate that the *E. faecalis* capsular polysaccharides from serotypes C and D attenuate C3 opsonized phagocytosis, and that this attenuated response is likely due to decreased recognition of bound C3 on the bacterial surface. Similarly, capsule inhibits detection of *E. faecalis* LTA on the surface and the absence of recognition of this molecule and/or other surface PAMPs in the presence of capsule results in decreased TNF-α production by macrophages.

Materials and Methods

Bacterial Strains, plasmids, and growth conditions

All relevant bacterial strains are listed in table 3.1. *E. faecalis* strains were cultivated in Todd-Hewitt broth supplied with the appropriate antibiotics when needed (THB; Becton, Dickinson and Company, Sparks, Maryland).

Culture of Macrophages

The macrophage like RAW 264.7 (ATCC TIB-71) cells were cultured in DMEM (Invitrogen, Grand Island, N.Y.) supplemented with 100 U penicillin per mL, 100 µg streptomycin per mL, 2 µg L-glutamine per mL, and 5% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA).

Table 3.1 Strains used in this study

Strain	Description	Reference
FA2-2	Capsule + (Serotype C)	(4)
V583	Capsule + (Serotype C)	(33)
OG1RF	Capsule -	(23)
12030	Capsule -	(13)
LT01	FA2-2 Δ*cpsF* Capsule + (Serotype D)	(40)
LT02	V583 Δ*cpsF* Capsule + (Serotype D)	(40)
LT05	FA2-2 Δ*cpsC* Capsule -	(40)
LT06	V583 Δ*cpsC* Capsule -	(40)
LT12	V583 + pMV158gfp	This Study
LT13	LT02 + pMV158gfp	This Study
LT14	LT06 + pMV158gfp	This Study

Complement C3 Deposition

Overnight cultures of *E. faecalis* were diluted 1:100 in fresh media. The cultures were allowed to reach mid-log phase (O.D. 600 of 0.6), and were washed 3X in sterile phosphate buffered saline (PBS) pH 7.4. Approximately $2X10^7$ cells of each strain were re-suspended in 10% normal CD1 mouse serum containing complement (Innovative Research, Southfield, MI) diluted in PBS. Serum for negative controls was heat inactivated prior to the addition of bacteria by incubating at 56ºC for 30 minutes. Bacteria were incubated in 10% serum for 30 minutes at 37ºC with agitation. Complement deposition was stopped by addition of EDTA to a final concentration of 10mM followed by incubation on ice for 5 minutes. The bacteria were pelleted at 4ºC, washed 3 times with sterile PBS to remove unbound complement, and finally re-suspended in 30 µL of 1X SDS-PAGE loading buffer. Whole bacteria were boiled vigorously for five minutes, and the cell debris was removed by centrifugation. The remaining supernatants were loaded on an SDS-PAGE gel and electrophoresed. Proteins in the gel were transferred to nylon membranes, and detection of C3 was carried out by western blot analysis using goat anti-mouse C3 polyclonal antibodies (Bethyl Laboratories, Montgomery, TX) and rabbit anti-goat conjugated with horse radish peroxidase (HRP) as secondary antibody (Bethyl Laboratories, Montgomery, TX)

followed by development with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

ELISA

The concentration of naturally occuring anti-enterococcal antibodies present in the CD1 (Innovative Research, Southfield, MI) mouse serum (used for subsequent phagocytosis assays) was analyzed by ELISA. In addition, ELISA was performed to investigate the serotype specificity conferred by the presence of CpsF among *E. faecalis* isolates using serotype C-specific antibodies. Briefly, log phase *E. faecalis* strains were washed 3 times in PBS and aliquoted (50 μL) into high binding 96 well Costar plates (Corning). The washed cells were allowed to adhere overnight at 4°C. Bound cells were then incubated with either CD1 mouse serum or rabbit anti-serotype C serum (18) followed by incubation with either goat anti-mouse IgG HRP conjugate (Sigma, Saint Louis, MO) or goat anti-rabbit IgG HRP conjugate (Jackson ImmunoResearch, West Grove, PA). ELISAs were developed using o-phenylenediamine dihydrochloride (OPD, Sigma) as the HRP substrate, and the results were read at O.D. 490 on a Bio-Tek PowerWave XS 96 well plate reader.

Opsonophagocytosis assay

E. faecalis strains V583, LT02 (V583 Δ*cpsF*), and LT06 (V583 Δ*cpsC*) were transformed by electroporation with the plasmid pMV158GFP (24) giving rise to LT12, LT13, and LT14 respectively (Table 3.1). Strains LT12, LT13, and LT14 constitutively express GFP allowing fluorescent detection during the opsonophagocytosis assay.

Log phase bacteria were washed three times in PBS prior to re-suspending in HBSS (Invitrogen) media. Harvested RAW 264.7 cells were also re-suspended HBSS media. A concentration of $2X10^6$ CFU/mL bacteria were added to $2X10^5$ RAW 264.7 cells/mL followed by the addition of complement containing CD1 mouse serum to a concentration of 10% to give a final volume of 500 μL and a bacteria to macrophage ratio of 10:1. The samples were incubated at 37ºC for 20 minutes to allow uptake of bacteria by macrophages. Trypsin was then added at 0.25% final concentration and incubated for 10 minutes to remove any bacteria bound to the external surfaces of the RAW 264.7

cells. The free bacteria were removed by three PBS washes with low speed centrifugation (750Xg) (7, 8). The washed cells were fixed to glass slides by cyto-centrifugation. The samples were viewed under 100X oil immersion using a Ziess Axioplan 2 fluorescent microscope to visualize the GFP expressing bacteria inside the RAW 264.7 cells. The intracellular bacteria of at least 100 RAW 264.7 cells were counted for each experimental replicate. The phagocytic index was calculated by dividing the number of phagocytic cells (cells that had consumed bacteria) by the total number of macrophages counted and multiplying that number by the number of bacteria per phagocytic macrophage ($\frac{\#\ phagocytic\ cells}{total\ cells\ counted}$ X $\frac{bacteria}{\#\ phagocytic\ cells}$) as previously described (21, 29). Data are presented as percent phagocytic index with the phagocytic index of LT14 (V583Δ*cpsC*, capsule -) set to 100%. Data were compiled from three separate experiments and the standard error of the mean and statistical significance were calculated with Graphpad Prism software.

Slide Agglutination

Un-encapsulated and encapsulated strains were tested for their reactivity to serotype A antiserum, previously reported to be specific for enterococcal lipoteichoic acid (LTA) (39). Slide agglutination assays were preformed as previously described (18, 40). Briefly, log phase bacteria were washed three times with PBS. Following the PBS washes, 5.0 μL of LTA antiserum or pre-immune serum was added to 15.0 μL of test cells on a glass slide, and gently rotated for one minute. Agglutination was determined by visual clumping of the cells. Sterile PBS and pre-immune serum were used as negative controls.

Flow Cytometry

Flow cytometry was used to determine if C3 or LTA accessibility to antibodies was altered by the presence of capsule. Log phased bacteria were washed three times in PBS, diluted 1:2, and blocked in 5% donkey serum (Jackson ImmunoResearch). Bacteria used for analyzing C3 accessibility were incubated in 50 μL of CD1 mouse serum for 20 minutes at 37ºC to allow for C3 deposition and washed three times in PBS prior to blocking with donkey serum. Blocked cells were incubated for 15 minutes on ice with

2.0% goat anti-C3 antibodies followed by three washes in PBS. Similarly diluted goat serum was used as an isotype control. The bacteria were then incubated with FITC conjugated donkey anti-goat antibody (1:1000) (Jackson ImmunoResearch) for 15 minutes on ice in the dark. The bacteria were again washed three times with PBS and analyzed by flow cytometry. For detection of LTA accessibility, washed and blocked bacterial cells were incubated on ice for 15 minutes with 2.0% anti-LTA rabbit serum (39). Similarly diluted pre-immune rabbit serum was used as an isotype control Cells were then washed three times in PBS and incubated for 15 minutes on ice in the dark with FITC conjugated donkey anti-rabbit antibody (1:100) (Jackson ImmunoResearch). Bacteria were washed three times in PBS and analyzed by flow cytometry. For both the C3 and LTA experiments, flow cytometry analysis of 50,000 bacteria was performed using a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA) at a flow rate of ~2000 cells per second. Data were analyzed using the WinList software program (VerityHouse, Topsham, ME).

TNF-α production

Log phase bacteria were washed three times in PBS and heat killed by incubation at 80ºC for 30 minutes. RAW 264.7 cells were harvested and re-suspended in fresh DMEM culture media to a concentration of $1X10^6$ cells per mL. RAW cells at a concentration of $1X10^6$ cells/mL in a total volume of 2.0 mL were seeded in 24 well plates. The cells were allowed to adhere to the plate surface for two hours prior to induction. Bacteria were added to each well at a concentration of $1X10^7$ Cfu. Lipopolysaccharide (LPS) from *Salmonell enterica* serotype typhimurium (Sigma) was used as a positive control for TNF-α production at a concentration of 10 ng per mL. Clarified supernatants were collected from each well at four hours after the bacterial inoculation. The amount of TNF-α present in the supernatants was determined by ELISA (eBioscience, San Diego, CA) following the manufacturer instructions. One way ANOVA in correlation with a Newman-Kuels post hoc test were used to evaluate statistical significance (GraphPad Prism).

Results

Protective effects of capsule on opsonophagocytosis

The capsular polysaccharides of many bacterial species confer resistance to complement mediated opsonphagocytosis. We examined whether *E. faecalis* capsule conferred resistance to C3 opsonophagocytosis mediated by macrophages. We used ELISA to confirm that our complement source (CD-1 mouse serum) was free of detectable *E. faecalis* antibodies (Data not shown).

Previous studies have shown that *E. faecalis* opsonizing antibodies exist in normal human serum; however, these antibodies are only directed towards the un-encapsulated serotype A and B strains of *E. faecalis* (2, 14). In view of these studies, we determined if *E. faecalis* capsule serotypes C or D conferred resistance to complement mediated opsonophagocytosis compared to an isogenic acapsular mutant. The encapsulated *E. faecalis* strains LT12 (serotype C), LT13, an isogenic *cpsF* deletion mutant which results in the production of a serotype D capsular polysaccharide (40) and LT14, an isogenic *cpsC* deletion mutant which is un-encapsulated (40) were compared. For this assay, we followed the method of Drevets et al. (8, 9) which calls for trypsin treatment and subsequent washes to remove externally bound bacteria as opposed to antibiotic treatment with gentamicin which has been shown to be internalized by macrophages leading to antibiotic killing affects independent of macrophage activity (9). Our data shows a 50% reduction in the opsonophagocytosis of capsule producing strains by macrophages in the presence of complement compared to un-encapsulated strains (Fig. 3.1).

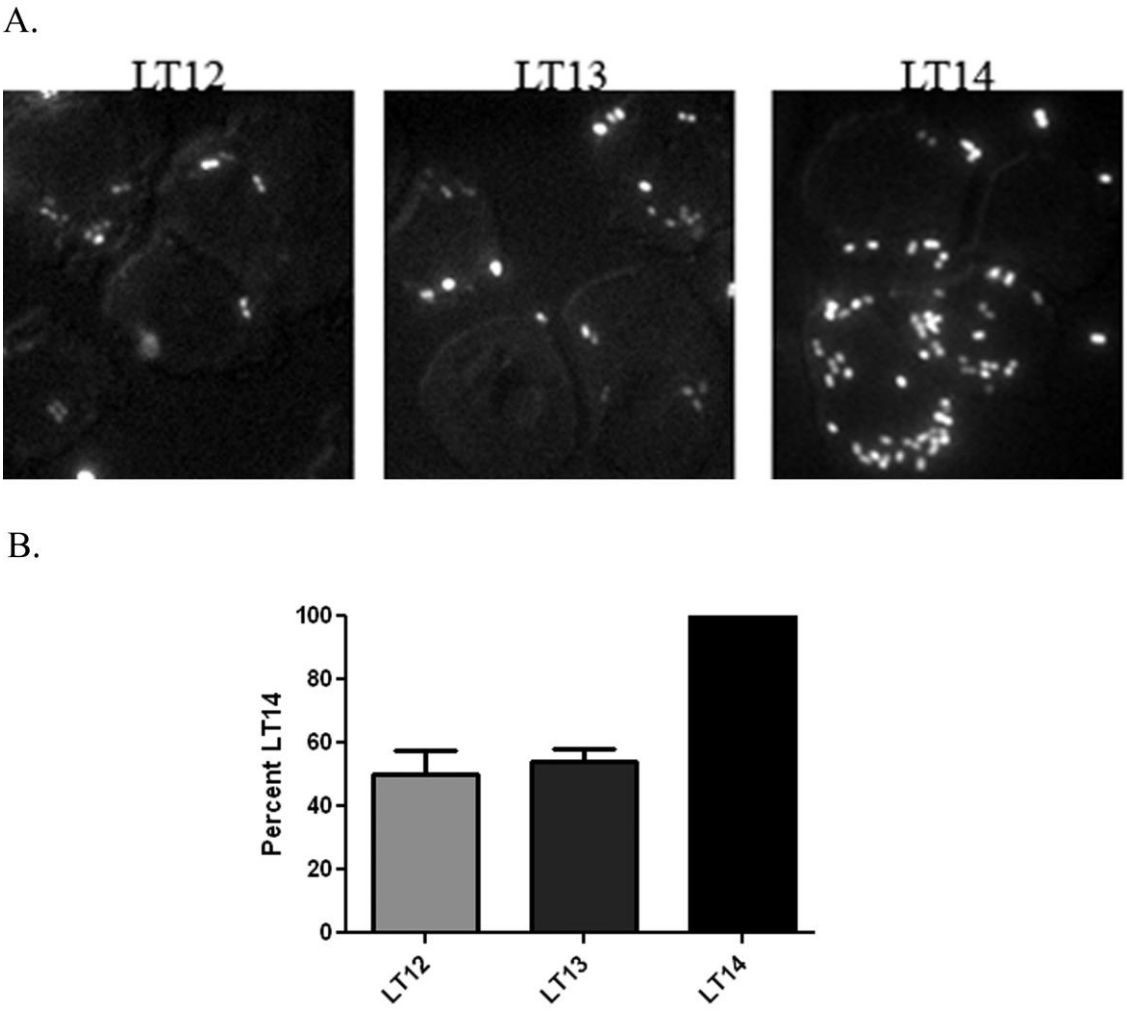


Figure 3.1 Opsonophagocytosis of *E. faecalis*

Capsule serotypes C and D are resistant to opsonophagocytosis in the presence of complement. **A.** Representative micrographs depicting from left to right LT12 (V583 expressing Gfp), LT13 (Δ*cpsF* expressing Gfp), and LT14 (Δ*cpsC* expressing Gfp) incubated with RAW 264.7 macrophage like cells. **B.** Quantification of phagocytic index expressed as the percentage of the un-encapsulated LT14 strain (see Materials and Methods for calculating Phagocytic index). The light gray bar (LT12: serotype C) and the dark gray bar (LT13: serotype D) both show a significant reduction in phagocytic index when compared to LT14 (black bar). Error bars represent SE of three replicate experiments.

These data also show that there is no statistical difference in opsonophagocytosis between isogenic serotype C (LT12) and serotype D (LT13) strains (Fig. 3.1), suggesting that the mere presence of capsule regardless of serotype provides protection against bacterial uptake by macrophages .

Complement C3 deposition and surface accessibility

Bacterial resistance to complement mediated opsonophagocytosis has been attributed to decreased amounts of C3 deposition on the surface of encapsulated strains (6). We used western blot analysis to assess the abundance of complement C3 deposited on both encapsulated and un-encapsulated *E. faecalis* strains.

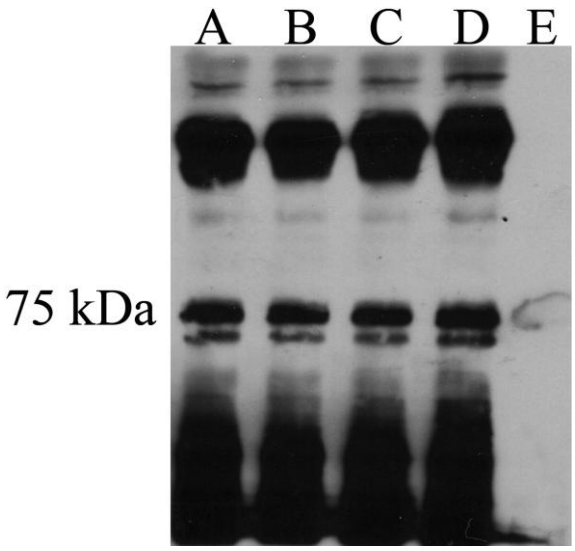


Figure 3.2 Complement deposition on encapsulated and un-encapsulated strains of *E. faecalis.*
The amount of C3 deposition does not differ between strains. Western blot analysis was employed to examine the amount of the C3 deposited on the cell surface of serotype C (FA2-2), serotype D (LT01), and un-encapsulated (LT05 and OG1RF) strains. The blot shows the 75 kDa β chain of C3 for FA2-2 (A**),** LT02 (B), LT05 (C), OG1RF (D) ,and the negative control, FA2-2 incubated with heat inactivated serum (E). The additional bands present on the blot are unprocessed C3, as well as C3 and C3b breakdown products recognized by the polyclonal antibodies to C3.

Two encapsulated strains FA2-2 and LT01(FA2-2Δ*cpsF*) and two un-encapsulated LT05 (FA2-2Δ*cpsC*) and OG1RF strains were used in this experiment. Complement C3 is composed of an α and a β chain (34).

The 75 kDa C3 β chain is left intact through the processing events of C3, and was used to determine differences in overall C3 deposition. Figure 2 shows the deposition of the 75 kDa β chain of C3 on different strains of *E. faecalis*. There is no difference in the amount of C3 deposited on the surfaces of the un-encapsulated strains OG1RF and LT05

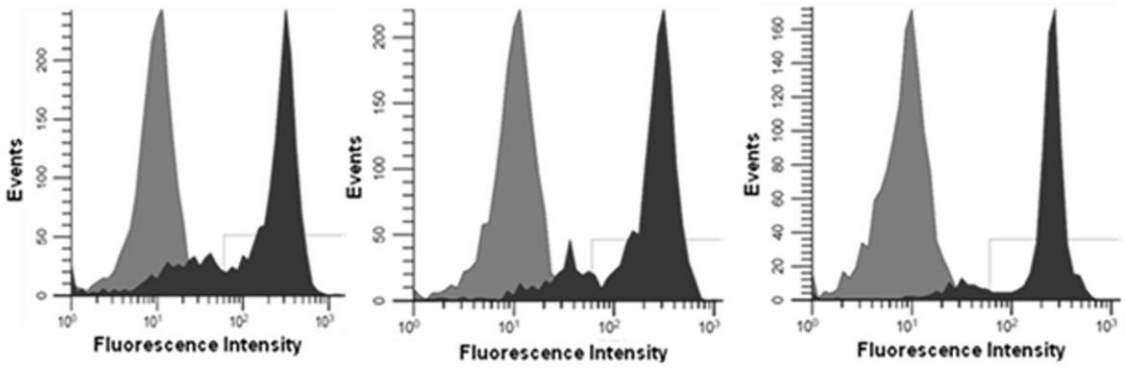
when compared to the encapsulated V583 and LT01 strains (Fig. 3.2). The other detected fragments in this blot are known breakdown products of C3 and C3b.

The amount of complement deposition does not vary between strains, but the presence of complement on the encapsulated strains could be masked from detection by complement receptors leading to decreased phagocytosis. We used complement opsonized strains of V583 (serotype C), LT02(V583Δ*cpsF*, serotype D) and LT06 (V583Δ*cpsC*, capsule -) in conjunction with flow cytometry to determine C3 surface accessibility to antibodies. Our data show that C3 deposited on the surface of LT06 is more detectable than C3 deposited on the surface of encapsulated strains V583 and LT02 (Fig. 3.3). Statistical analysis using one-way ANOVA in conjunction with a Newman-Keuls post hoc test show a significant statistical difference (p-values < 0.05) between V583 and LT06, and also between LT02 and LT06 (Fig. 3.3). There was also a statistically significant difference between V583 and LT02 even though they appear to be equally resistant to complement mediated opsonophagocytosis (Fig. 3.1). The basis for this difference is not known at the present time, but may relate to structural differences in the capsular polysaccharides between these two serotypes.

Lipoteichoic acid and capsule

Lipoteichoic acid (LTA) and peptidoglycan are PAMPs present on *E. faecalis* that are known to stimulate the immune system through pathogen recognition receptors including TLR-2 (35). The capsules produced by other bacteria shield PAMPs resulting in altered cytokine production (30). We examined differences in LTA accessibility between encapsulated and un-encapsulated strains by slide agglutination assays. *E. faecalis* serotype A anti-serum is directed against enterococcal LTA (39). We tested the ability of these antibodies to agglutinate either encapsulated or un-encapsulated *E. faecalis* strains. The encapsulated strains V583 (serotype C) and LT02(Δ*cpsF*, serotype D) were not agglutinated by the anti-serum, whereas the un-encapsulated strains LT06(Δ*cpsC*) and 12030 (serotype A reference strain) were both agglutinated (data not shown).

A.



B.

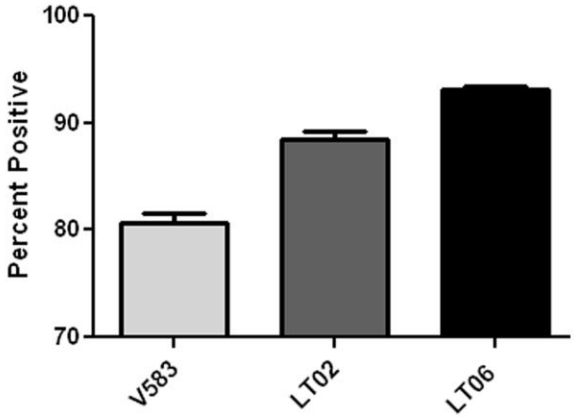
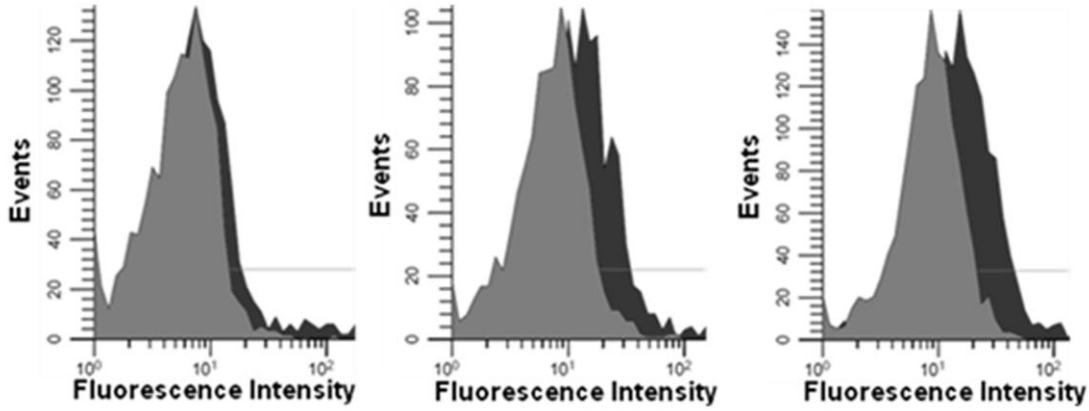


Figure 3.3 Complement C3 accessibility

Complement C3 is masked from detection by capsule. Flow cytometry was used in conjunction with anti-C3 antibodies and FITC conjugated secondary antibodies to evaluate the availability of C3 to detection. **A.** Representative histograms depicting (from left to right) flow cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated (LT06) *E. faecalis* strains. The isotype controls are light gray and the C3 antibody treated cells are dark gray. **B.** Quantification of the C3 positive cells. Using one-way ANOVA in conjunction with a Newman-Keuls post test, statistical analysis for three replicates showed statistically significant differences (p-value < 0.05) in the amount of positively labeled bacteria when V583 (light gray bar) and LT06 (black bar) were compared, and when LT02 (dark gray bar) and LT06 were compared. Statistical analysis also revealed a significant difference in C3 detection between V583 and LT02 ($P < 0.05$). Error bars represent SE for three replicate. Approximately 50,000 bacteria were analyzed for each replicate.

A.



B.

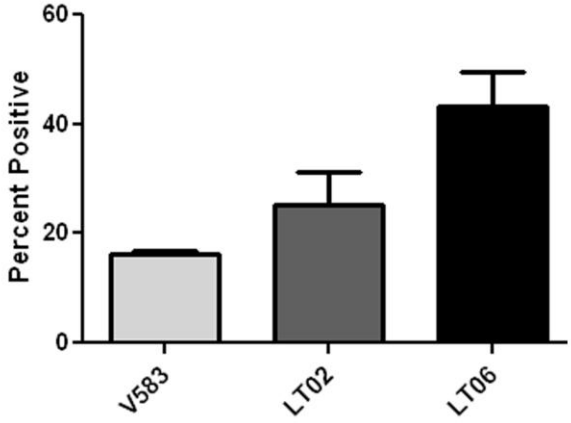


Figure 3.4 Accessibility of LTA to antibodies

The presence of capsule masks LTA from detection by antibodies. Flow cytometry was used in conjunction with LTA antiserum and FITC conjugated secondary antibodies to evaluate the levels of LTA accessibility. **A.** Representative histograms depicting (from left to right) flow cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated (LT06) *E. faecalis* strains. The isotype controls are in light gray and the anti-LTA antibody treated cells are dark gray. **B.** Quantification of LTA detection by flow cytometry. Statistical analysis for three replicates using a one-way ANOVA in conjunction with a Newman-Keuls post test showed significant differences ($P < 0.05$) in the amount of LTA detected between V583 (light gray bar) and LT06 (black bar), and between LT02 (dark gray bar) and LT06 with p-values less than 0.05. However, there is no statistical difference in LTA detection when LT02 is compared V583. Error bars represent SE for three replicate. Approximately 50,000 bacteria were analyzed for each replicate.

As agglutinating antibodies are generally of the IgM class, we also used flow cytometry to quantify the differences of LTA availability to the IgG class. Strains V583, LT02, and LT06 were incubated with Serotype A antiserum followed by a FITC conjugated secondary antibody. Figure 3.4 shows the percentage of the cells that were positive for FITC labeling. One-way ANOVA followed by a Newman-Keuls post hoc test showed significant statistical differences (p-values < 0.05) in the amount LTA detected between V583 (serotype C) and LT06 (capsule -), and also between LT02 (serotype D) and LT06. However, there was no significant statistical difference when the encapsulated strains V583 and LT02 were compared.

TNF-α production in response to capsule

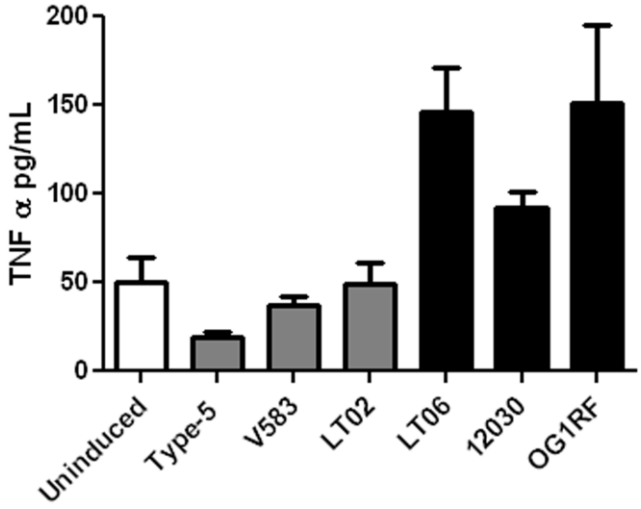


Figure 3.5 The production TNF-α by RAW 264.7 cells increases when exposed to un-encapsulated strains of *E. faecalis.*
E. faecalis capsule reduces TNF-α production by RAW 264.7 cells. Macrophage like RAW 264.7 cells were incubated with serotype C (V583), serotype D (T-5 and LT02), and un-encapsulated (LT06, 12030, and OG1RF) *E. faecalis* strains. Supernatants were collected and analyzed by ELISA for TNF-α content. Results show pg/mL of TNF-α production by RAW 264.7 cells in the presence of each strain. Statistical analysis of three replicates using one way ANOVA and a Newman-Kuels post hoc test shows significant differences between the amount of TNF-α produced in response to T-5, V583 and LT02 when compared to LT06, 12030, and OG1RF. Interestingly, there is no statistically significant difference between the amount of TNF-α produced by un-induced RAW cells when compared to the three encapsulated strains. Error bars represent SE for three replicate experiments.

These data indicate that capsule produced by either serotype C or D strains masks LTA from antibody detection.

The presence of a capsule is known to alter the macrophage cytokine response in other microorganisms (10). To examine this possibility in *E. faecalis*, we used ELISA to assess the ability of capsule producing and non-producing strains to induce TNF-α production by RAW 264.7 cells. We predicted that the ability of capsule to inhibit detection of LTA (Fig. 3.4) would translate to less TNF-α production by RAW 264.7 cells. The capsule producing strains T-5 (serotype D), V583 (serotype C), LT02 (serotype D) along with the un-encapsulated strains (LT06, 12030, OG1RF) were heat-killed and incubated with RAW 264.7 cells. Clarified supernatants were collected at 4 hours post inoculation, and were analyzed for TNF-α production. The TNF-α produced in response to the un-encapsulated strains is significantly higher than that produced in response to encapsulated strains with p-values < 0.05 using one-way ANOVA and a Newman-Keuls post hoc test analysis (Fig. 3.5). However, there is no statistically significant difference when comparing the encapsulated strains with each other or when comparing the un-encapsulated strains with each other. Strikingly, there is no statistically significant difference in the amount of TNF-α produced by RAW cells when comparing the strains T-5, V583, and LT02 to the un-induced RAW control cells.

Discussion

Capsular polysaccharides contribute to the virulence of microorganisms through multiple mechanisms including resistance to opsonophagocytosis, and masking bacterial surface antigens from detection by the host immune system (1, 9). Several Gram-positive cocci including *S. aureus* (26), *S. pneumoniae* (1), and group-B streptococci (4) produce capsular polysaccharides that are known to contribute to virulence. Previous reports have indicated that *E. faecalis* strains can be classified by the presence or absence of capsular polysaccharide (11, 15, 16, 40). Hancock and Gilmore (11) showed that the presence of capsule enhances persistence at infectious sites using a murine infection model, and subsequently showed that encapsulation protects the bacteria from killing by neutrophils, whereas an unencapsulated isogenic mutant was readily killed by neutrophils. The

killing of the unencapsulated mutant by neutrophils was dependent on the opsonic activity of complement.

Here, we demonstrate that *E. faecalis* capsular polysaccharide serotypes C and D provide resistance to complement opsonized phagocytosis by macrophages. In good agreement with previously reported work on the role of the *E. faecalis* capsule in affecting resistance to opsonic killing by neutrophils (11, 16), we observed a 50% reduction in phagocytic killing in encapsulated strains compared to the isogenic acapsular mutant. An additional cell wall polysaccharide in *E. faecalis* termed Epa has also been shown to contribute to resistance to phagocytic killing (37), and may account for why the protective effect of the capsule is not more substantial in *E. faecalis*. Unlike the capsule, the Epa polymer and its genetic locus appear to be highly conserved in *E. faecalis* (10, 37). However, a direct comparison on the relative contribution of Cps and Epa in the same strain background has not possible to date, because the OG1RF strain in which Epa mutants were created lacks the capsule locus (14, 40), and in our hands we have been unable to generate Epa mutants in encapsulated strain backgrounds (L.T., unpublished data). A recent report by Teng et al. (38) demonstrated gross changes in the bacterial cell shape of Epa mutants in the OG1RF background and this may account for our inability to generate such mutants in our encapsulated strains and may partially explain the pleiotropic affects ascribed to the Epa locus in virulence studies (38, 43).

An additional benefit of the macrophage system is the use of cultured cells that are less likely to vary from experiment to experiment compared to the neutrophil assay, which requires fresh isolation of neutrophils from human blood donors. Furthermore, because the strains used in this comparative study were isogenic derivatives we can make a direct assessment on the role of capsule and serotype differences in host immune evasion as has been observed in other microbial pathogens (28, 32, 42, 43). Our findings show that *E. faecalis* capsular polysaccharides alter the detection of C3 and LTA by antibodies (Figs. 3.3-3.4). Paralleling these findings, we also demonstrate that the presence of capsule also abrogates TNF-α production by macrophages (Fig. 3.5). Together these data provide a mechanism by which the presence of capsule alters complement-mediated opsonophagocytosis by altering accessibility of the bound C3b opsonin, and by altering the production of TNF-α in response to encapsulated *E. faecalis*.

It is noteworthy that capsule serotype differences in an isogenic background did not result in significant changes in resistance to opsonin-mediated phagocytosis, or in altered TNF-α response. McBride et al. (19) recently showed that clinical isolates of *E. faecalis* possessing multiple virulence factors, as well as multi-drug resistance were more likely to be identified as capsule serotype C. Our findings suggest that either of the encapsulated serotypes (C or D) benefit the bacterium in evasion of the host innate response. We did however observe a significant difference in the amount of bound C3 detectable on the surface of isogenic serotype C compared with serotype D capsule, but this difference did not correlate with changes in the phagocytic index of these strains, leaving open the question as to why the more pathogenic and drug-resistant clinical isolates are more frequently identified as serotype C as opposed to D. In *S. aureus*, comparison of the contribution of type 5 and type 8 capsule in the same strain background revealed that the presence of N-acetylation on the type 5 capsule structure conferred a fitness advantage *in vivo* (41). Whether a similar affect will also be observed in the comparison of *E. faecalis* serotype C and D strains *in vivo* will be the focus of future studies.

Aside from anti-phagocytic properties, bacterial capsules also act as barriers that limit detection of PAMPs by PRRs (1, 9). A common PAMP shared by all strains of enterococci is LTA. The LTA of *E. faecalis* is known to stimulate TNF-α production via TLR-2. Although not fully understood, TNF-α is thought to play a key role in *E. faecalis*-mediated inflammatory responses (3, 26). A study involving *Enterococcus faecium*, which produces serologically identical LTA to *E. faecalis*, showed that TLR-2 mediated signaling was critical for early immune response and clearance of *E. faecium* (17). Based on these studies, recognition of enterococcal LTA and/or peptidoglycan by TLR-2 would appear critical for an efficient host immune response, and the masking of these integral wall components by capsule could result in increased pathogenesis by limiting the host response to the organism. Interestingly, a study by Kau et al. (16) demonstrated that the response to *E. faecalis* in a urinary tract infection model is not TLR-2 dependent. The capsule phenotype of the clinical isolate used in this study is not known, and based on our finding that the presence of the capsule alters recognition of an

important PAMP (LTA) known to be recognized by TLR-2, suggest that TLR-2 signaling might only be of benefit against *E. faecalis* strains that lack capsule.

Our goal was to understand the mechanism of how encapsulation enhances the resistance of *E. faecalis* to innate immunity. Taken together, our results show that two capsule serotypes produced by *E. faecalis* can subvert host innate immune responses by conferring resistance to complement-mediated phagocytosis, as well as altering the innate response to the pathogen. This study provides mechanistic evidence demonstrating that the *E. faecalis* capsule alters the accessibility of bound C3 supporting the observation that the most pathogenic lineages of *E. faecalis* are encapsulated (19, 39). By masking PAMPs on the surface of *E. faecalis*, the capsule also alters the host response to infection by encapsulated strains. It is our contention that the capsule produced by *E. faecalis* serotypes C and D is an important virulence determinant that plays multi-faceted roles in evasion of host innate immune responses. Because of this, the *E. faecalis* capsule could serve as a target for developing future therapeutics.

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CHAPTER 4 - *Enterococcus faecalis* proteases degrade complement C5a and contribute to evasion of innate immunity

Abstract

The gram-positive pathogen *Enterococcus faecalis* is a leading agent of nosocomial infections including urinary tract infections, surgical site infections, and bacteremia. Among the infections caused by *E. faecalis*, endocarditis is the most serious clinical manifestation and unique as it is commonly acquired in a community setting. Enterococcal endocarditis is a complex disease with many host and microbial components contributing to disease outcome. Using a rabbit model of endocarditis and isogenic protease [gelatinase (GelE) and serine protease (SprE)] mutants, we examined the contributions of the *E. faecalis* extracellular proteases in the development and progression of endocarditis. Both of these proteases have been shown to contribute to the virulence of *E. faecalis* in other animal models, and here we show a novel mechanism by which the proteases contribute to host innate immune evasion. Our work shows that GelE and SprE can proteolyze the anaphylatoxin complement C5a and that this proteolysis leads to decreased neutrophil migration *in vitro*. However, using protease mutants in the rabbit endocarditis model we only observed a significant decrease in bacterial burden in rabbits infected with $GelE^-$ strains, suggesting that GelE is the principal protease involved in disease progression. Correlating with these observations was the decrease of heterophil (neutrophil-like cells) infiltrate at tissue sites infected with GelE producing strains. Taken together, these observations provide a novel mechanism employed by *E. faecalis* to avoid host immune responses by limiting the recruitment of host phagocytes (neutrophils) to the site of infection.

Introduction

Enterococci are leading causes of hospital acquired infections including bacteremia, surgical site infections, and urinary tract infections (29). However, the most frightful manifestation of enterococcal infection is endocarditis with mortality rates ranging from 15-20% (22). Enterococci, most commonly *E. faecalis*, are the third leading cause of infective endocarditis (20). Enteroccoci cause subacute-chronic endocarditis and are the causative agents of up to 20% of native valve endocarditis and 15% of prosthetic valve endocarditis (20, 22). Unlike other enterococcal infections, endocarditis is most commonly community acquired, although recent studies indicate that there is a significant risk of acquiring enterococcal endocarditis in a clinical environment (6, 7).

Infectious endocarditis is responsible for 1 in 1000 hospital admissions, and is relatively uncommon with an incidence of 1.7-6.2 cases per 100,000 persons each year (5, 22). Despite improvements in diagnosis and treatment, infectious endocarditis continues to cause significant morbidity and mortality with mortality rates ranging between 4-50% depending on the causative agent (22). Infective endocarditis is caused by colonization of damaged heart endothelium followed by encasement in fibrin and platelets resulting in a lesion known as a vegetation (15, 22), and complications from infective endocarditis can arise following embolization to secondary sites (2, 22).

Enterococci form vegetations on the valves of the heart, and emboli that dislodge from these vegetations can spread to other body sites (15). In experimental endocarditis in rabbits, mortality is often associated with embolization to secondary infectious sites including blood vessels of the heart, brain, and kidneys (10). Occasionally the emboli occlude blood vessels in the secondary infection sites leading to tissue damage. Of these secondary infection sites, *E. faecalis* displays affinity for the kidneys (10). Previous studies indicated that the presence of extracellular proteases (GelE and SprE) significantly increased mortality in animal infection models, but the relative contribution of each protease in experimental endocarditis has not been examined to date (10, 33).

Multiple bacterial species produce extracellular proteases that contribute to pathogenesis through manipulation of the host immune response (26). These proteases target several components of the host innate immune system including complement, antimicrobial peptides (AMPs), cytokines and cytokine receptors (26). The *E. faecalis* proteases GelE and SprE are co-transcribed through regulation by the *fsr* regulatory system (27, 28). SprE has been shown to contribute to disease in animal models (4, 28, 32, 34), but mechanistically how it contributes is not known at the present time. Gelatinase is a zinc-metalloprotease (17) that is related to aureolysin from *Staphylococcus aureus* and elastase from *Pseudomonas aeruginosa* (26). Gelatinase is known for its contribution to biofilm formation (12, 36), and is also thought to contribute to virulence through degradation of a broad range of host substrates including collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a (17, 18, 24, 25, 31, 37).

Complement C3a is an anaphylatoxin involved in activation and recruitment of eosinophils, but is limited in its ability to activate and recruit neutrophils (1, 3, 8, 18). Comparatively, complement C5a is at least 100 times more potent in activation and recruitment of neutrophils than C3a (8). Determination of the effects of *E. faecalis* proteases on C5a is of particular importance because of the relevance of neutrophil recruitment for bacterial clearance.

The broad substrate specificity of GelE probably contributes significantly to the complexity of endocarditis pathology, but specific mechanistic contributions to endocarditis have not been elucidated. We sought to elucidate the specific contributions of each protease to endocarditis as well as assess direct mechanisms that are associated with increased pathogenesis. Here we show that GelE, and to a lesser extent SprE, degrade human complement component C5a. Incubation of C5a with GelE or SprE curtailed chemotaxis of neutrophil like cells across a membrane *in-vitro*. Using a rabbit model of endocarditis and isogenic protease mutants we demonstrated that the production of GelE by *E. faecalis* correlates with increased bacterial burdens at the primary infection site and decreased heterophil recruitment to primary and secondary infection sites. Our results show a new mechanism employed by *E. faecalis* to circumvent host innate

immune responses, and adds to the growing list of roles of GelE and SprE as important virulence factors in *E. faecalis* infections.

Materials and Methods

GelE and SprE purification

GelE was purified as previously described with some minor differences (12). Two liters of Todd Hewitt Broth (THB) were inoculated with 20 mL of an overnight culture of the GelE over expressing *E. faecalis* strain FA2-2 harboring the pML29 plasmid (12). The 2.0 L culture was incubated at 37º C for 24 h. Bacteria were removed by centrifugation for 30 min at 15,000 × g. The recovered supernatants were filter-sterilized and incubated at 37º C for 24 h with 10 μg/mL RNase A and 1.0 U/mL DNase. The GelE was precipitated from the supernatant upon addition of ammonium sulfate to 60% saturation followed by incubation overnight at 4º C. The mixture was centrifuged for 30 min at 27,500 × g, and the pellets were recovered by dissolving in 150 mL of GelE buffer (50mM Tris and 1 mM $CaCl_2$, pH 7.8). The 150 mL sample was applied to a CL-4B column (2.5 × 17 cm) at a flow rate of 5.0 mL/min using a Bio-Rad BioLogic LP. The column was washed with six column volumes of GelE buffer. Five millimeter fractions were collected as GelE was eluted from the column by washing with three column volumes of 50% ethylene glycol (vol/vol) in GelE buffer. Ten microliters from each fraction was spotted on a THB agar plate containing 1.5% skim milk. Fractions showing proteolytic activity on the THB 1.5% skim milk plates were pooled and dialyzed extensively against GelE buffer or 5.0 mM sodium phosphate (pH 7.0) using dialysis tubing (M_r cutoff of 12,000 – 14,000). After dialysis, the protease purity was checked by SDS-PAGE and silver stained. Purified GelE was aliquoted and stored at 20º C. Each aliquot was tested for activity on a THB 1.5% skim milk plate prior to use. SprE was purified as previously described (35).

C5a Degradation

Recombinant human complement C5a and recombinant human complement C5a (His-Tag) were commercially obtained from BioVision (Mountain View, CA). Human

Table 4.1 Strains used in this study

Strain	Relevant Genotype	Relevant Phenotype	Reference
V583	Parental	$GelE^+$ $SprE^+$	(30)
VT01	V583 Δ*gelE*	$GelE^-$ $SprE^+$	(36)
VT02	V583 Δ*sprE*	$GelE^+$ $SprE^-$	(36)
VT03	V583 Δ*gelE* Δ*sprE*	$GelE^-$ $SprE^-$	(36)

complement protein C5a (His-Tag) (M.W. 12 kDa) was incubated with purified GelE or SprE to determine the ability of the proteases to hydrolyze C5a. Equal amounts (0.5 µg) of GelE or SprE and C5a were incubated in total volume of 25.0 µL in GelE buffer (50mM Tris pH 7.8, 1 mM $CaCl_2$) or SprE buffer (50 mM Tris pH 7.4, 5 mM $CaCl_2$) for 20 minutes at 37º C. A 15.0 µL aliquot from each sample was analyzed on a Tris-Tricine 10-20% gradient gel (Invitrogen) by silver staining as previously described (23). The remaining 10 µL were prepared for MALDI-TOF analysis using a ZipTip (Millipore, Bedford, MA) following the manufacturer instructions. Samples were eluted in a solution of 50% acetonitrile containing 0.1% trifluoroacetic acid, mixed with 2,5 dihydroxy benzoic acid (Sigma, Saint Louis, MO), and spotted on a Bruker aluminum plate for MALD-TOF analysis. Samples were analyzed using a Bluker Ultraflex II mass spectrometer.

HL-60 growth and differentiation

The human promyelocytic leukemia HL-60 cells (ATCC CCL-240) were grown in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37º C with 5% CO_2.

It is known that HL-60 cells can be differentiated into neutrophil like cells upon the addition of dimethylsulfoxide (DMSO)(13), and that differentiated HL-60 (dHL-60) cells are a reliable substitute for isolated neutrophils in chemotaxis and migration studies (14, 38). HL-60 cells for use in downstream applications were differentiated as previously described (14). Briefly, HL-60 cells were incubated for five days in Iscove's

modified Dulbecco's media supplemented with 1.2% DMSO at a concentration of $5X10^5$ cells/mL. Cell differentiation was evaluated by analyzing CD11b expression on the surface of HL-60 and dHL-60 cells by flow cytometry. Briefly, HL-60 and dHL-60 cells were harvested and resuspended in culture media to a concentration of $1X10^6$ cells/mL. The cells were washed three times in 200 µL of stain media containing PBS (pH 7.0), 10% fetal bovine serum, and 0.2% sodium azide. The Fc receptors were blocked with FcR block (BD Biosciences, San Jose, CA), followed by incubation on ice for 15 minutes with anti-CD11b APC conjugated antibodies (BioLegend, San Diego, CA) or anti-F4/80 FITC conjugated antibodies (eBiosciences, San Diego, CA) as a negative control. Cells were washed three times in stain media and resuspended to a final volume of 500 µL and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA) at a flow rate of ~200 cells per second. Data were analyzed using the WinList software program (VerityHouse, Topsham, ME).

dHL-60 Transmigration Assay

Differentiated HL-60 (dHL-60) cells were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) prior to the migration assay. Briefly, dHL-60 cells were pelleted and resuspended in three milliliters of PBS containing 0.1% BSA at a concentration of $1X10^6$ cells/mL followed by the addition of an equal of volume CFDA-SE in PBS at a concentration of 20 µM. The cells were incubated with CFDA-SE for 10 minutes at 37º C and subsequently washed three times with DMEM supplemented with human serum albumin (HSA) (5.0 mg/mL) and HEPES (15 mM). Washed cells were resuspended in DMEM HSA/HEPES at a concentration of $1X10^6$ cells/mL and 100 µL of cells were aliquoted into the upper chamber (3.0 µM polyester membrane) of a 24 well Transwell (Corning) plate. A volume of 600 µL of DMEM HSA/HEPES containing either C5a (10^{-9} M) alone, C5a incubated with GelE or C5a incubated with SprE was added to the lower wells prior to the addition of upper chambers. Culture media containing either GelE or SprE was used in the lower wells as a negative control. The dHL-60 cells were allowed to migrate towards the bottom chamber for 70 minutes at 37º C. Cells that had migrated to the bottom well were collected, washed three times in PBS, and lysed with 0.2 M NaOH. The amount of CFDA-SE present from the cell lysates was

measured spectrofluorometrically with excitation at 492 nm and emission at 571 nm on a Perkin Elmer Victor 3 fluorescent plate reader. Fluorescence values for the negative controls were subtracted from the samples and data were analyzed as percent fluorescence with C5a alone set to 100 percent. Statistical analysis was performed using GraphPad Prism software.

Experimental endocarditis

New Zealand White rabbits weighing approximately 2 kg were anesthetized by intramuscular injection with ketamine (25 mg/kg) and xylazine (20 mg/kg). The right carotid artery was exposed for catheterization by surgical incision and a polyethylene catheter with an internal diameter of 0.86 mm (Becton Dickinson, MD) was introduced in the right carotid artery and advanced until it traversed the aortic valve into the left ventricle. Proper catheter placement was determined by feeling the resistance and noting the pulsation of the catheter line. Wound clips were used to close the incision, and all rabbits recovered without complications. Groups of 6-8 catheterized rabbits were injected with 1 ml of diluted cultures (1 X 10^7 cfu) of *E. faecalis* strains V583, VT01 (ΔgelE), VT02 (ΔsprE), or VT03 (ΔgelEsprE) (Table 4.1) via the marginal ear vein 24 hours after catheter insertion. Two negative control rabbits received sterile saline. To prepare the bacteria for injection, enterococci (V583, VT01, VT02 and VT03) were grown to stationary phase, washed twice and diluted to a final cell density of ~10^7 cfu/ ml in sterile saline. The rabbits were euthanized 48 hours after the bacterial challenge by intraperitoneal administration of sodium pentobarbital. Research was conducted in compliance with the Animal Welfare act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory animals, NRC publication, 1996 edition.

Determination of bacterial burden

Animals with macroscopic valvular vegetations and proper catheter placement were analyzed for data in this study. Blood was drawn just prior to euthanasia to determine bacterial CFU in blood at the time of sacrifice. At the time of sacrifice, aortic valves and left ventricular vegetations were removed, weighed, and homogenized in 1.0

ml of sterile PBS, pH 7.4 and quantitatively cultured by plating serial dilutions on THB agar plates (referred to collectively as heart tissue). To determine the extent to which emboli formed from cardiac vegetations, enterococci present in the spleen, liver, and kidney were also assessed by plate count. Harvested organs were introduced into 3 mL of sterile PBS, pH 7.4 and thoroughly homogenized with a tissue homogenizer. Tissue homogenates were serially diluted and plated on THB agar and colonies counted after overnight incubation at 37ºC. Bacterial loads were expressed as $\log_{10}$CFU per gram of tissue.

Histology

The left kidneys, walls of the aorta, and aortic valves exhibiting vegetations from representative rabbits infected with V583, VT01, VT02 and VT03 were fixed in 10% buffered formalin for histopathology. For general histology, tissues (kidneys and regions of the aorta including the aortic valve) were embedded in paraffin and serial sections (5µm thick) were stained with either HE (hematoxylin and eosin) or Gram-stain.

Image analysis and statistical analysis

Images were obtained at a final magnification of 400X and analyzed using imageJ software. For quantitative analysis of heterophils surrounding emboli in the kidneys or in the matrix layer (ML, generally thought to be composed of host fibrin, fibronectin, plasma proteins and platelets, (21)) around the vegetations in the aorta, images were initially converted to 8-bit and a threshold was applied to contrast heterophils from the background. Heterophils were counted from images using dimensions obtained from a training dataset. In cases where heterophils overlapped, the watershed algorithm was applied to delineate heterophil boundaries before counting particles. The total number of heterophils from each bacterial treatment was normalized to the area of the surrounding kidney emboli or ML layer and reported as the number of heterophils per 10 mm^2 (10000 μm^2).

Statistical analysis of heterophil counts and bacterial tissue burdens was carried out with GraphPad software. One way analysis of variance followed by Neuman-Keuls

post hoc test was carried out to determine statistical significance. A P< 0.05 was considered to be statistically significant.

Results

Rabbit Endocarditis Bacterial Burden

We used a GelE (VT01), a SprE (VT02), and a double GelE/SprE (VT03) mutant in an isogenic background (V583) (Table 4.1) to determine the pathogenic effects of the proteases in a rabbit endocarditis model. Bacterial burdens were determined from the hearts, kidneys, blood, livers, and spleens from each rabbit. The kidneys were of special interest because *E. faecalis* is known to have a tropism for kidneys (10, 16). Figure 4.1 shows the log10 CFU per gram of tissue from the hearts (Fig. 4.1A) and both kidneys (Fig. 4.1B). A one way ANOVA with a Neuman-Keuls post hoc test indicates a

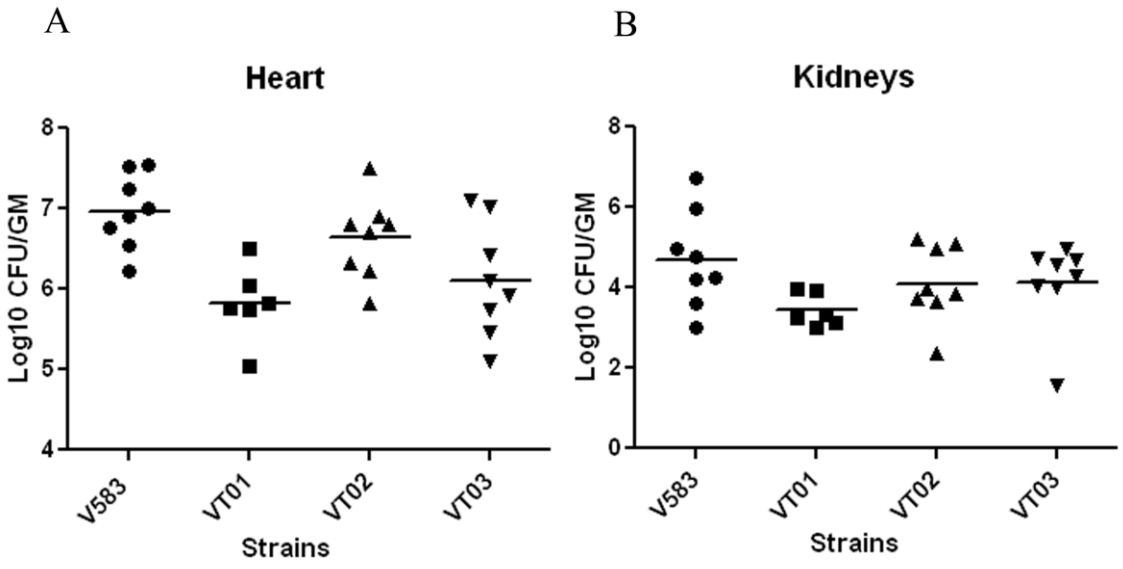

Figure 4.1 Bacterial burdens from the hearts and kidneys of infected rabbits

A rabbit endocarditis model was used to assess the pathogenesis of isogenic GelE, SprE and double GelE/SprE mutants compared to a wild type (V583) strain. Horizontal bars represent the mean **A.** Rabbits infected with GelE producing strains (V583 and VT02) have higher bacterial burdens in the heart than rabbits infected with strains lacking GelE (VT01 and VT03). (N=6-8) **B.** Bacterial burdens in the kidneys do not differ significantly. (N=6-8)

statistically significant difference ($P<0.05$) in the bacterial burdens in the hearts of rabbits infected with the wild type strainV583 and compared to VT01 ($GelE^{-}SprE^{+}$) and VT03 ($GelE^{-}SprE^{-}$) (Fig 4.1A). Conversely, there is no significant difference in bacterial burdens in the heart between rabbits infected with V583 compared to VT02 ($GelE^{+}SprE^{-}$), nor is there a significant difference in rabbits infected with VT01 ($GelE^{-}SprE^{+}$) compared to VT03 ($GelE^{-}SprE^{-}$). Bacterial burdens in the hearts from rabbits infected with VT02 ($GelE^{+}SprE^{-}$) were significantly different when compared to rabbits infected with VT01 ($GelE^{-}SprE^{+}$). Even though we did not observe a statistically significant difference in the bacterial burdens in the hearts of rabbits infected with VT02 ($GelE^{+}SprE^{-}$) when compared with VT03 ($GelE^{-}SprE^{-}$), the overall trend of increased bacterial burden in the hearts of rabbits infected with $GelE^{+}$ strains remained consistent. Other tissues harvested from the rabbits including the kidneys (spleen, liver, blood, and kidneys) did not display significant difference in bacterial burden for any of the *E. faecalis* strains (Fig 4.1B and data not shown).

Heterophil Recruitment

Rabbit heterophils are the equivalent of human neutrophils. Based on the observations that GelE, and to a lesser extent SprE (Fig. 4.4) degrade C5a and other pro-inflammatory peptides, we predicted that rabbits infected with *E. faecalis* strains producing one or both proteases should have decreased heterophil recruitment to infection sites. The aortas and half sections of the left kidney were collected from rabbits infected with V583, VT01 ($GelE^{-}$), VT02 ($SprE^{-}$), and VT03 ($GelE^{-}$ $SprE^{-}$). The number of heterophils/ 10 mm^2 of matrix layer (ML) was determined from four aorta sections containing vegetations for each strain. Figure 4.3 shows that rabbits infected with strains lacking GelE (VT01 and VT03) had significant ($P<0.05$) higher numbers of heterophils/ 10 mm^2 in ML than rabbits infected with strains producing GelE (V583 and VT02).

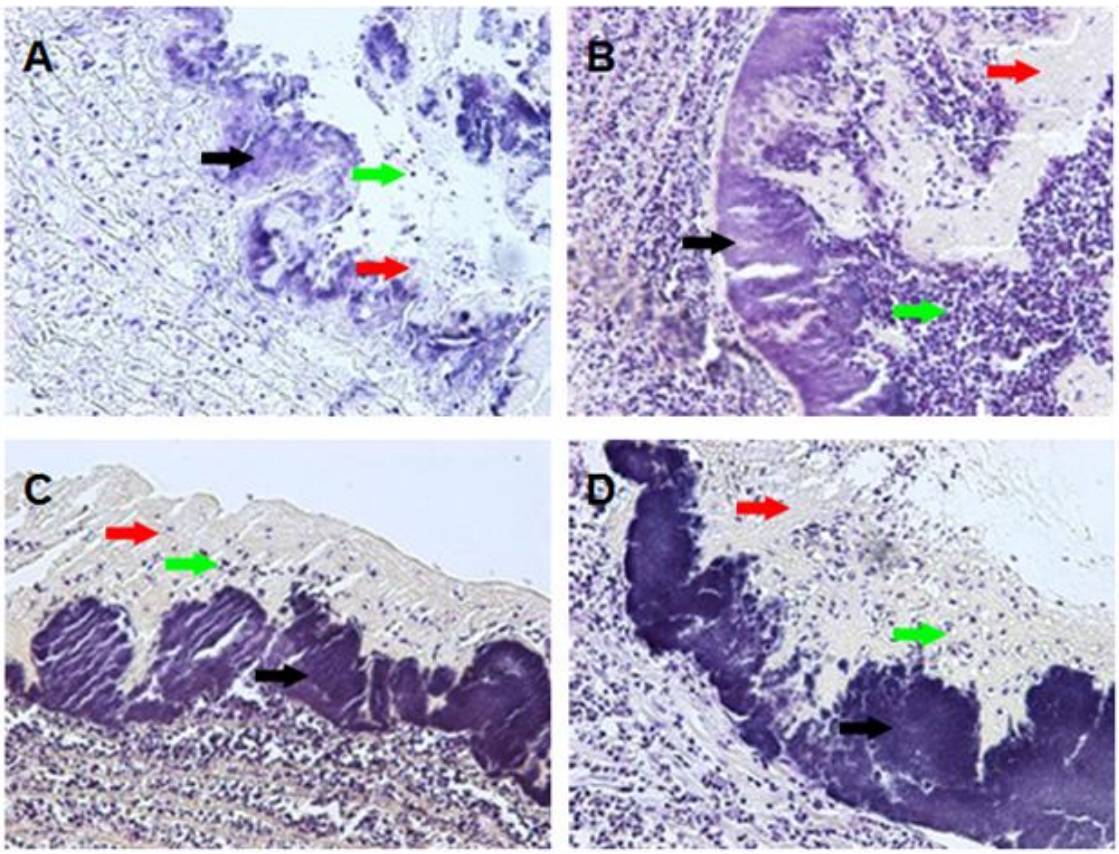

Figure 4.2 Histopathology of rabbit aortic vegetations

Histology of aortic vegetations. Panels A, B, C and D are representative images of gram-stained cross-sections (5µm) of vegetations formed on the ascending aorta of rabbits infected with V583, VT01, VT02 and VT03 respectively (magnification, x 200). Black arrows point to *E. faecalis* biomass on the surface of the endothelium. Red arrows point to deposited matrix layer (ML) composed mostly of platelets and fibrin. Green arrows point to influx of heterophils and other immune cell infiltrates.

There was no significant difference in the amount of heterophils/ 10 mm^2 of ML between rabbits infected with VT01 ($GelE^-$) or VT03 ($GelE^-$ $SprE^-$) or between rabbits infected with V583 or VT02 ($SprE^-$).

The number of heterophils/ 10 mm^2 surrounding the emboli in the kidneys were also assessed from rabbits infected with V583 or the three protease mutant strains. Similar to results seen from the heart vegetations, GelE producing strains (V583 and VT02) had significantly ($P<0.05$) less heterophils/ 10 mm^2 surrounding the emboli than did strains

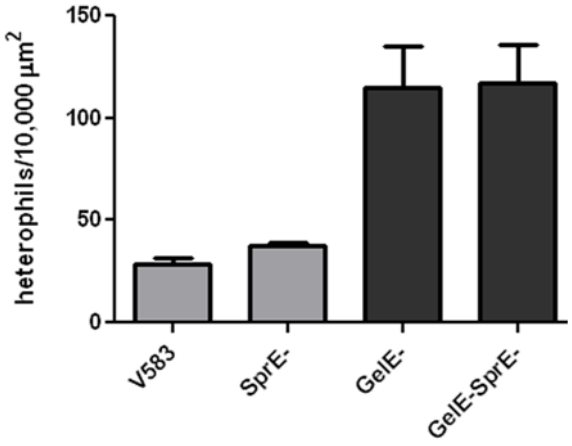

Figure 4.3 Quantification of heterophil infiltration in vegetation matrix layers

Quantification of heterophil chemotaxis in the hearts or rabbits infected with *E. faecalis*. Differences in the number of heterophils that have migrated to the bacterial vegetations were determined from histological images (magnification, x 400) and were normalized to the area of ML surrounding them. Heterophils were counted using Image J software from 4 random images of vegetations from each strain and reported as the total number of heterophils trapped per 10 mm^2 of ML (Mean ± SEM). (N=4)

lacking GelE (VT01 and VT03) (Figs. 4.3 and 4.5). There was no statistically significant difference in the number of heterophils/ 10 mm^2 around the kidney emboli in rabbits infected with the GelE producing strains (V583 and VT02) which is also similar to the results from the heart vegetations. However, unlike the observations from the heart vegetations, rabbits infected with VT01 ($GelE^-$) had significantly ($P<0.05$) fewer heterophils surround kidney emboli than did rabbits infected with VT03 ($GelE^-$ $SprE^-$) suggesting that SprE has some effect on heterophil recruitment in this tissue.

GelE and SprE Degradation of C5a

Previous studies showed that GelE degrades C3a (24), however, C5a is 100 times more potent in neutrophil activation and recruitment (8). We incubated purified human C5a with purified GelE and SprE to determine if either protease possessed proteolytic activity targeting C5a. We used Tris-Tricine gel analysis and MALDI-TOF analysis to determine activity of the enterococcal proteases towards C5a. Our results show that GelE completely degrades C5a as shown in Figures 4.6A and 4.6B. These results are similar to

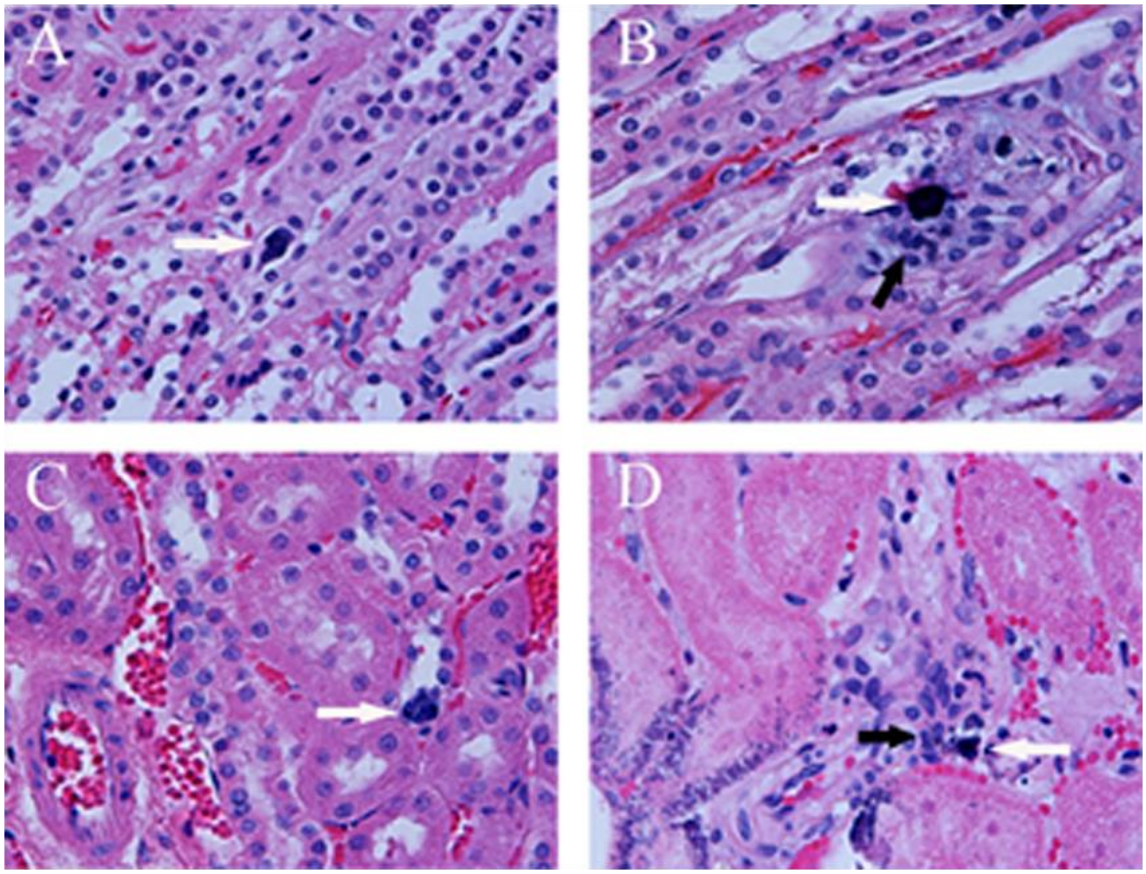

Figure 4.4 Histopathology of rabbit kidneys

Representative histopathology slides of rabbit kidneys stained with hematoxylin and eosin. White arrows indicate emboli and black arrows indicate heterophils. Panels A and C show emboli from rabbits infected with GelE producing strains (V583 and VT02 respectively). Panels B and D show emboli from rabbits infected with GelE$^-$ strains (VT01 and VT03 respectively). Emboli consisting of V583 (A) and VT02 (C) do not have any significant heterophil influx compared to emboli consisting of VT01 (B) and VT03 (D).

the reported GelE activity towards C3a (24). Conversely, SprE had limited proteolytic activity towards C5a, with some degradation observed on the Tricine gel compared to C5a alone (Fig. 4.6B), which was confirmed by MALDI-TOF analysis (Fig. 4.6A).

In-Vitro Neutrophil Chemotaxis in Response to C5a incubated with GelE and SprE

GelE and to a lesser extent, SprE, can hydrolyze the complement protein C5a (Fig. 4.6). Because C5a is a powerful neutrophil chemotractant, we determined if incubation of C5a

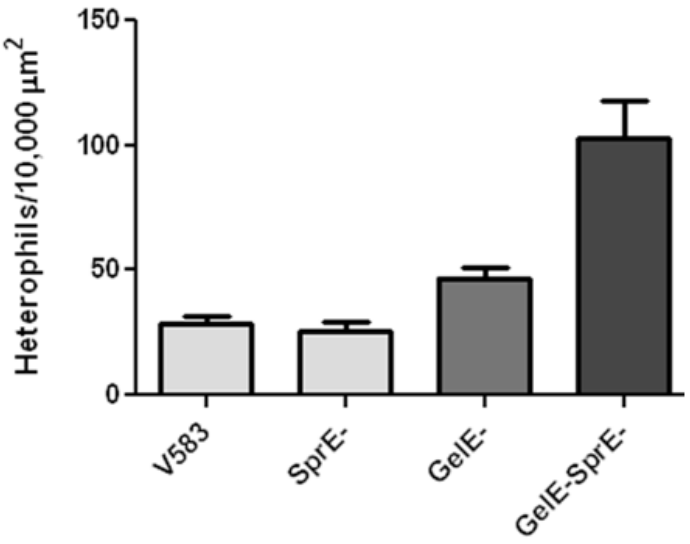

Figure 4.5 Quantification of heterophil infiltration surrounding emboli in the kidneys

Quantification of heterophils/10 mm^2 of tissue surrounding the emboli in the kidney shows a significant decrease ($P<0.05$) in influx in GelE producing strains (V583 and VT02) compared to $GelE^-$ strains (VT01 and VT03). There is also a significant difference in heterophil density per 10 mm^2 between VT01 ($GelE^-SprE^+$) and VT03 ($GelE^-SprE^-$) suggesting a limited role for SprE in limiting heterophil recruitment. (N=8-11)

with GelE and SprE decreased neutrophil chemotaxis *in-vitro*. We used dHL-60 cells in conjunction with Transwell migration assays to determine the effect of dHL-60 movement across a membrane in response to C5a or C5a incubated with GelE or SprE. Flow cytometry in conjunction with CD11b antibodies was employed to ensure that HL-60 cells incubated with DMSO had differentiated into neutrophil like cells (Fig. 4.7). As previously described (14, 38), HL-60 cells displayed increased levels of CD11b on their surface following five days of incubation with DMSO indicating differentiation into neutrophil like cells.

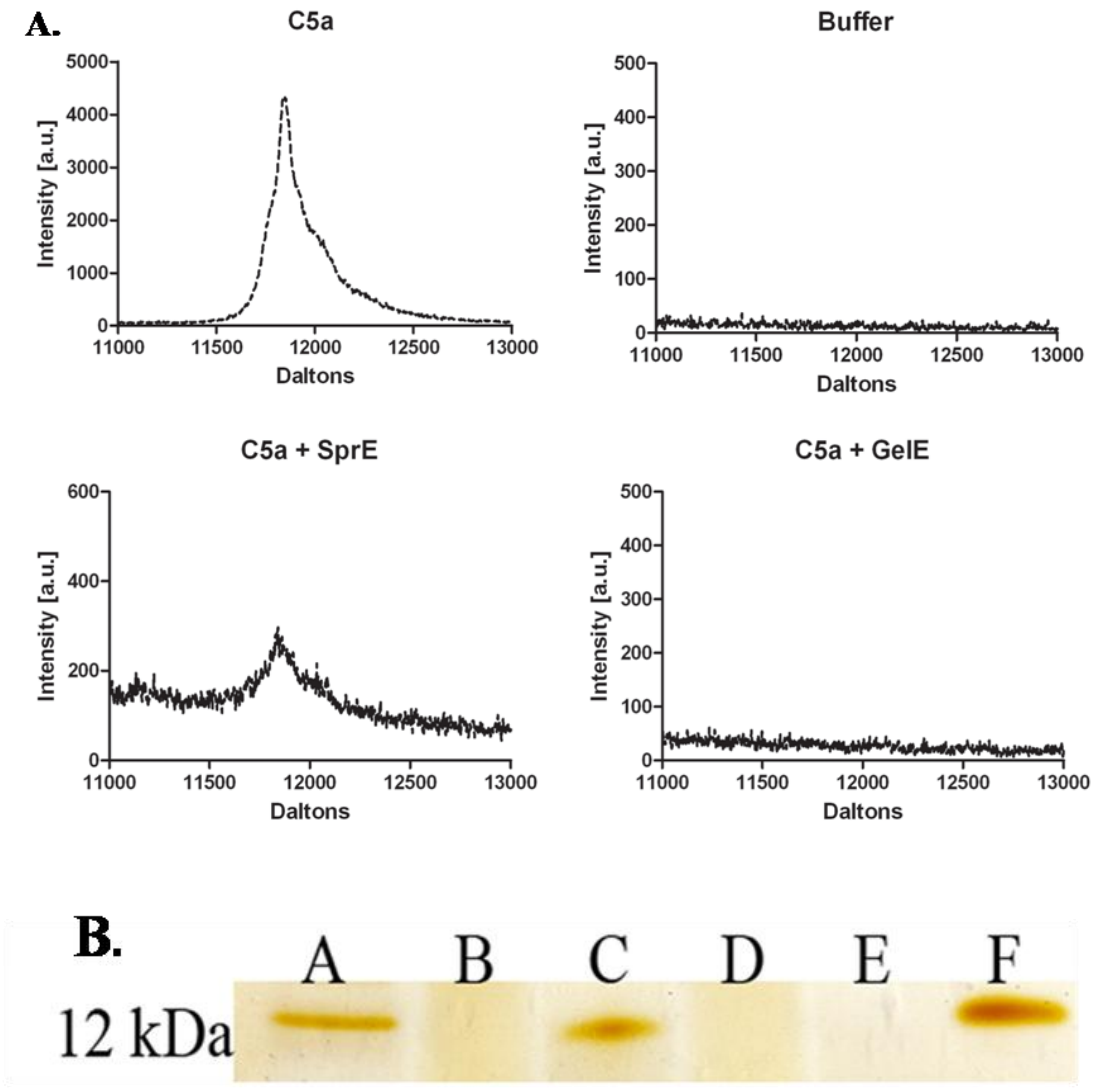

Figure 4.6 Degradation of C5a by GelE and SprE

GelE and SprE degrade C5a. **A.** MALDI-TOF spectra of C5a (~12 kDa) alone, Buffer alone, C5a incubated with SprE, and C5a incubated with GelE. Incubation of C5a with GelE results in complete hydrolysis of C5a where as incubation of C5a with SprE results in partial degradation. **B.** Silver stained Tris-Tricine showing the molecular weight marker ~12 kDa (A), C5a incubated with GelE (B), C5a incubated with SprE (C), GelE buffer (D), SprE buffer (E), and C5a (F). GelE completely degrades C5a.

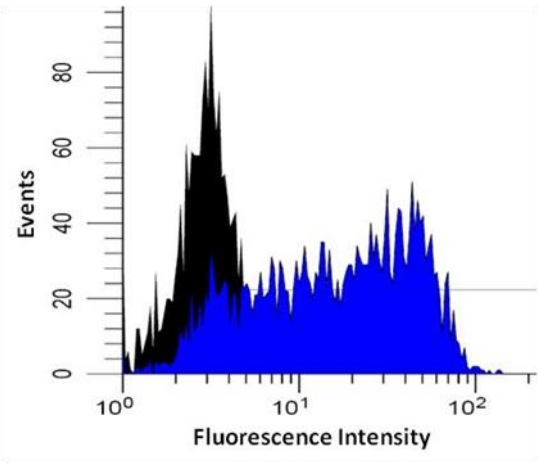

Figure 4.7 Differentiation of HL-60 cells

Flow cytometry analysis of CD11b expression on dHL-60 cells. Black shows CD11b expression on HL-60 cells prior to incubation with DMSO. Blue shows CD11b expression on HL-60 cells following incubation with DMSO. Increased expression of CD11b on HL-60 cells following incubation with DMSO indicates differentiation into neutrophil-like cells. Differentiated HL-60 cells were used to study chemotaxis in response to C5a incubated with *E. faecalis* proteases.

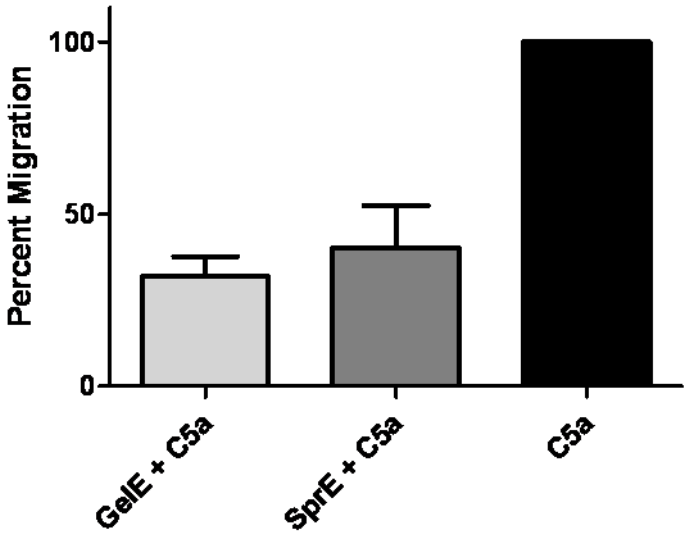

Figure 4.8 Transwell transmigration assay

Incubation of C5a with GelE or SprE inhibits dHL-60 migration through Transwell membranes. Neutrophil like dHL-60 cells were labeled with fluorogenic CFDA-SE and allowed to migrate through a 3.0 µM membrane in response to C5a or C5a previously incubated with GelE or SprE. Incubation of C5a with GelE or SprE significantly ($P<0.05$) reduces dHL-60 chemotaxis compared to C5a alone.

The dHL-60 cells (labeled with CFDA-SE) were allowed to migrate towards C5a or C5a incubated with GelE or SprE for 70 minutes. As expected, incubation of C5a with GelE resulted in a significant reduction in neutrophil movement across the Transwell membrane compared to C5a alone (Fig. 4.8). Interestingly, incubation of C5a with SprE also led to a significant decrease in neutrophil chemotaxis compare to C5a (Fig. 4.8) despite the observation that hydrolysis of C5a by SprE is limited (Fig. 4.6). There is no statistically significant difference in the amount of neutrophil migration across the Transwell membranes when C5a is incubated with GelE or SprE, even though GelE is more efficient in hydrolyzing C5a.

Discussion

Extracellular proteases from pathogenic bacteria assume many roles in manipulation and subversion of host innate immune responses (26). The *E. faecalis* extracellular proteases GelE and SprE are known to contribute to pathogenesis through contributions to biofilm production as well as degradation of important immune peptides (12, 24, 31, 36). Here we show a new mechanism of innate immune evasion for GelE and SprE through degradation of C5a. The complement protein C5a is a potent inflammatory peptide with a broad spectrum of functions including the modulation of cytokine production, induction of oxidative bursts, and also serves as powerful chemoattractant for neutrophils and monocytes (9, 11). GelE thoroughly hydrolyzes C5a which leads to decreased neutrophil migration *in vitro*. SprE has limited proteolytic activity towards C5a, which is sufficient to limit neutrophil chemotaxis *in vitro.* However, our data indicate that SprE is not as effective as GelE in limiting rabbit heterophil chemotaxis in the heart suggesting that the limited proteolytic activity of SprE alone is not sufficient to hinder heterophil recruitment *in vivo*. The matrix layer covering the vegetations in the heart from rabbits infected with VT01 ($GelE^-$ $SprE^+$) contain as many heterophils/10 mm^2 as similar sites from rabbits infected with VT03 ($GelE^-$ $SprE^-$). In contrast, rabbits infected with GelE producing strains of *E. faecalis* (V583 and VT02) had significantly decreased heterophil density in the matrix layer compared to rabbits infected with VT01 ($GelE^-SprE^+$) or VT03 ($GelE^-$ $SprE^-$). Interestingly, matrix layers from rabbits infected with V583 and VT02

(GelE$^+$SprE$^-$) show no difference in heterophil density, implying that GelE alone is sufficient for limiting heterophil recruitment to the matrix layer and that SprE does not augment this effect in the heart. Correlating with this data is the observation that bacterial burdens from rabbit hearts infected with GelE producing strains (V583 and VT02) are significantly higher than those from rabbits infected with the GelE$^-$ strains (VT01 and VT03).

Previous investigations into the role of *E. faecalis* proteases in endocarditis have been conducted by others (10, 33). Gutschick et al.(10) compared proteolytic isolates of *E. faecalis* (*Streptococcus faecalis* subspecies *liquefaciens*) to non-proteolytic isolates (*S. faecalis*) and found that rabbits infected with proteolytic strains had shorter mean survival times (3.9 days compared to 7.1 days) and significantly more emboli in the kidneys than those infected with non-proteolytic strains. Rabbits that succumbed to the infection also had significantly higher bacterial burden in the heart when infected with proteolytic strains compared to non-proteolytic isolates, which is consistent with our observations for GelE expressing strains. For humane reasons, we did not use death as our endpoint, but still found that vegetations were smaller in rabbits infected with GelE producing strains. This is principally due to the fibrinolytic activity observed in histology (Fig. 4.2) and consistent with the ability of GelE to cleave fibrin (37). Gutschick et al. (10) used proteolytic isolates but could not distinguish between a role for GelE or SprE, as isogenic mutants were not available at that time. More recently, Singh et al. (33) compared an isogenic *fsrB* deletion mutant and a *gelE* insertion mutant in an OG1RF strain background in the rat model of experimental endocarditis. These authors also reported a significant role for protease production in the early stages of endocarditis. As *gelE* and *sprE* are cotranscribed, an insertion in *gelE* is known to exert a polar affect on *sprE* transcription (28). Therefore, the study by Singh et al. was unable to discern a role for either GelE or SprE in the endocarditis model. Here we used isogenic deletion mutants in both GelE and SprE, as well as a double deletion mutant, and have shown that GelE is the principle protease involved in mediating bacterial burden in the heart.

Despite *in vitro* cleavage of C5a by SprE, there appeared to be little effect on heterophil recruitment or bacterial burden when comparing strains with (VT01) or without (VT03) SprE expression in the absence of GelE. One possible explanation for

the lack of correlation between *in vivo* and *in vitro* SprE activity could be that serum protease inhibitors are selectively targeted to serine proteases as compared to metalloproteases. Additionally, the molar ratio of C5a to either protease in vivo is not known and this may also explain the absence of correlation. At a 1:3 molar ratio of GelE to C5a, we observed complete proteolysis of C5a in 10 minutes. Under similar conditions, C5a was only partially degraded by SprE, yet this partial degradation was sufficient to interfere with C5a-dependent neutrophil (dHL-60) chemotaxis *in vitro*. However, we did observe some affect of SprE expressed in the emboli lodged in the kidney, in that fewer heterophils were recruited to this site when infected with strain VT01 ($GelE^-$,$SprE^+$) compared to VT03 ($GelE^-$,$SprE^-$).

Infective endocarditis begins with bacterial colonization of damaged heart endothelium followed by encasement in fibrin in platelets resulting in a characteristic lesion called a vegetation (15). Vegetations are prone to embolization often leading to leading to secondary infections within the kidney, spleen, and brain (15). Because emboli resulting from *E. faecalis* endocarditis have a tropism for the kidney (10) we explored kidney pathology of rabbits infected with our protease mutants. We observed a greater influx of rabbit heterophils around the emboli localized to the kidney of rabbits infected with VT01 ($GelE^-SprE^+$) and VT03 ($GelE^-SprE^-$) compared to rabbits infected with V583 or VT02 ($GelE^+SprE^-$) despite the absence of difference in overall bacterial burdens from this tissue. These results indicated that GelE is important for innate immune evasion following colonization of secondary sites of infection as well as the primary site of infection. We also observed a lesser extent of heterophil recruitment around the emboli in rabbits infected with VT01 ($GelE^-SprE^+$) compared to rabbits infected with VT03 ($GelE^-SprE^-$). This result was surprising based on the observation that the density of heterophils recruited to the site of infection in the heart is the same in the rabbits infected with either strain. We speculate that the limited proteolytic effect of SprE on C5a may be sufficient early in infection to partially delay heterophil recruitment, but may not be sufficient to continually repress heterophil recruitment over an extended period of time. However, one can not discount the timing of emboli release from the vegetation as being a factor. Perplexingly, we did not see a decrease in the bacterial burden in rabbits infected with VT01 ($GelE^-$ $SprE^+$) or VT03 ($GelE^-$ $SprE^-$) compared to

V583 or VT02 ($GelE^+$ $SprE^-$), even though we see significant differences in heterophil recruitment in the kidneys. The similarity in bacterial burden in the kidneys may be due to the short duration of infection in the rabbits. During the 48 hour period post inoculation, the bacteria must colonize the damaged heart valve, form vegetations, and expel emboli that then lodge in other organs in the rabbit. Even though 48 hours was enough time to see a difference in bacterial burden in the original infection site (heart) it may not have been enough time to allow for efficient bacterial clearance in other organs. However, we do see an increased influx of rabbit heterophils around the emboli in the kidneys of rabbits infected with VT01 ($GelE^-$ $SprE^+$) and VT03 ($GelE^-$ $SprE^-$) suggesting that given sufficient time these tissues would have decreased bacterial loads compared to the kidneys of rabbits infected with the GelE producing strains V583 and VT02.

Infective endocarditis is a complex disease with many bacterial and host factors contributing to diverse pathologies. Most virulence factors studied in relation to enterococcal endocarditis have focused on adherence (19). The extracellular proteases GelE and SprE are two known virulence factors that contribute to *E. faecalis* pathogenesis in other disease models. Elevated bacterial burden in the hearts of rabbits infected with the GelE producing strains V583 and VT02 is consistent with a crucial role for GelE in pathogenesis. Additionally, reduced heterophil recruitment to infections sites in animals infected with GelE producing strains is consistent with the observation of C5a degradation. The role of SprE is more ambiguous than that of GelE. The presence of SprE does not significantly increase bacterial burden in the heart as does GelE, nor does SprE inhibit heterophil recruitment in the matrix layer. However, SprE may play a limited role in early stages of infection by limiting phagocyte recruitment. Despite the indistinct role for SprE, it remains clear that GelE is important for innate immune evasion in endocarditis, thus adding to the ever growing list of GelE contributions to pathogenesis.

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CHAPTER 5 - Summary and Discussion

General Summary

Over the past several decades enterococci have emerged as one of the most common sources of hospital acquired infections. Enterococci have been known to cause disease for nearly a century, but modern medical practices using antibiotic therapeutics combined with innate and acquired antibiotic resistances in the bacteria have resulted in a rapid increase in the incidence of enterococcal diseases (7, 8, 13, 28). Enterococci are resistant to a broad range of antibiotics with the resistance to vancomycin being the most disturbing from a clinical standpoint. Glycopeptide antibiotics including vancomycin are often turned to as the last resort to treat many infections. A 2005 NNIS report revealed that 30% of enterococci isolated from a clinical setting were vancomycin resistant (25). Predictably, mortality rates for individuals infected with vancomycin resistant enterococci are significantly higher than for individuals infected with susceptible strains (9). Multiple antibiotic resistant enterococci are not only more difficult to treat, but antibiotic resistance also provides enterococci with a selective advantage over competing microbes in the gastrointestinal (GI) tract. Treatment of intensive care unit (ICU) patients with 2^{nd} and 3^{rd} generation cephalosporins or with other beta-lactams results in increased GI colonization with enterococci due to the lack of competition from susceptible bacterial species (7, 8, 28, 40).

Regardless of antibiotic resistance profiles and increased colonization due to antibiotic treatment, enterococci must still overcome a myriad of host defense mechanisms to establish infection at extraintestinal sites. Host innate immune barriers include physical and chemical barriers, soluble proteins, pattern recognition receptors, and phagocytic cells. These innate immune effectors are reviewed in **Chapter 1** with special attention paid to those effectors that are key in defense against extracellular gram-positive pathogens. Like most pathogens, *E. faecalis* produces several virulence factors (reviewed in **Chapter 1**) that aid in establishing infections. Most of the studies involving *E. faecalis* virulence factors have focused on adherence or biofilm formation, while only a handful have focused on mechanisms employed to circumvent host immune responses. Studies from this work have shown two virulence factors (capsule and GelE) produced by *E. faecalis* that contribute to host innate immune evasion. Furthermore, these studies not

only demonstrate that these factors contribute to immune evasion but also yield mechanisms for subversion of the host response. Adding to these studies is the elucidation of the genetics required for capsule production and how the underlying genetics correlate with differences in capsule serotypes.

Bacterial capsular polysaccharides are known to contribute to the pathogenicity of numerous microbes including several gram-positives (1, 5, 29, 45). The influence of *E. faecalis* surface polymers thought to be capsule have been studied in relation to pathogenesis in the past (12, 16, 18, 41, 48). Most of these studies have divided *E. faecalis* into groups based on one of several serotyping schemes (17, 20, 36, 37). However, these studies are not reliable due to the fact that these serotyping schemes were based on many surface antigens including lipoteichoic acid (17, 42). As discussed in C**hapters 1 and 2**, only two loci (*epa* and *cps*) had been reported to contain putative genes for capsule production (12, 47), but only one study had directly linked the genetic evidence with physical proof of capsule production (15). Prior to the studies reported in this thesis, the capsule serotyping scheme divided *E. faecalis* into four groups (A, B, C, and D).

In **Chapter 2**, evidence presented demonstrates that only two serotypes (C and D) of *E. faecalis* produce a capsular polysaccharide. Previous studies had shown that the genes *cpsA* and *cpsB* are not part of the capsule operon as they are transcribed from a different promoter (15). This indicated that serotypes A and B did have the necessary set of genes to produce a capsular polysaccharide. This was clearly demonstrated to be the case in **Chapter 2** by the absence of capsule production in isogenic mutants lacking the genes *cps C, D, E, G,* or *I*. Furthermore, data in this chapter shows that differences in serospecificity between serotypes C and D is due to the presence of *cpsF*. Data from this work indicates that *cpsF* may be a glucosyltransferase. Data in **Chapter 2** also indicated that the group antigen (LTA) is hidden from detection by agglutinating antibodies thus providing some insight into how capsule may be limiting immune detection of encapsulated *E. faecalis* strains. Consistent with this finding is the observation that serotype C and D strains tended to be more pathogenic than their A and B counterparts (18, 24).

Several studies have shown that bacteria employ capsules as a means to defend against components of the host innate immune system. The inhibition of phagocytosis and evasion of detection by the host are two primary mechanisms attributed to capsule in the evasion of the innate immune response. In **Chapter 3** the role of capsule with regard to immune evasion by *E. faecalis* was explored. Hufnagel et al. had previously reported that opsonophagocytic killing of both the serotype C and D strains by healthy human sera was drastically reduced when compared to the un-encapsulated serotype A and B strains (18). At the time of the Hufnagel et al. manuscript (18), it was thought that all four serotypes were encapsulated. However, in light of the discoveries in **chapter 2,** we hypothesized that only strains producing capsule (serotypes C and D) were protected from opsonophagocytosis. This hypothesis was tested in **chapter 3** using isogenic mutants that were defective in capsule production that had been incubated in serum containing opsonic C3. As suspected, the absence of capsule increased the rate of opsonophagocytosis by macrophages when compared to encapsulated strains. The contribution of capsule to the reduction of opsonophagocytosis can be attributed to either an inhibition of complement deposition or to the ability of capsule to mask the detection of bound complement on the surface of the microbe (29, 31, 46). The results presented in **Chapter 3** showed that the decrease in opsonophagocytosis of encapsulated *E. faecalis* strains was due to the ability of capsule to hide C3 from detection. Similarly, the capsule also hid surface LTA from detection by specific antibodies.

It is known that LTA is recognized by the pattern recognition receptor (PRR) Toll-like receptor 2 (TLR-2), and that recognition of LTA results in increased cytokine production and neutrophil recruitment to the site of infection (4, 19, 33, 35). Presumably, the presence of capsule would attenuate the host innate immune response by limiting cytokine production. Encapsulated strains along with naturally un-capsulated and isogenic capsule negative mutants were incubated with macrophages to test this hypothesis.

Results presented in **chapter 3** showed that the presence of capsule significantly reduces the production of the pro-inflammatory cytokine, TNF-α. These results indicate that the capsule of *E. faecalis* provides protection from the host innate immune response

through inhibition of phagocytosis, and by inhibiting the detection of pathogen associated molecular patterns which limits cytokine production by the host (Figure 5.1).

Even though capsular polysaccharide production is associated with the most pathogenic lineages of *E. faecalis*, several un-encapsulated strains also cause disease. Two factors that were known to contribute to *E. faecalis* pathogenesis were the secreted proteases GelE and SprE. The secreted protease SprE had been implicated in contributing to disease in a number of animal models but the mechanism of SprE activity is unknown (10, 30, 38, 39). GelE was also known to contribute to virulence presumably through degradation of a broad range of host proteinaceous substrates including LL-37 and complement components C3 and C3a (21, 22, 26, 27, 32, 44). In **Chapter 4** a new mechanism is described for both SprE and GelE with regards to innate immune subversion. Both proteases were able to degrade the anaphylatoxin C5a with SprE having slightly reduced activity compared to GelE. However, incubation of C5a with either purified GelE or SprE resulted in decreased neutrophil migration in-vivo when compared to whole C5a. The relative contributions of GelE and SprE were tested in a

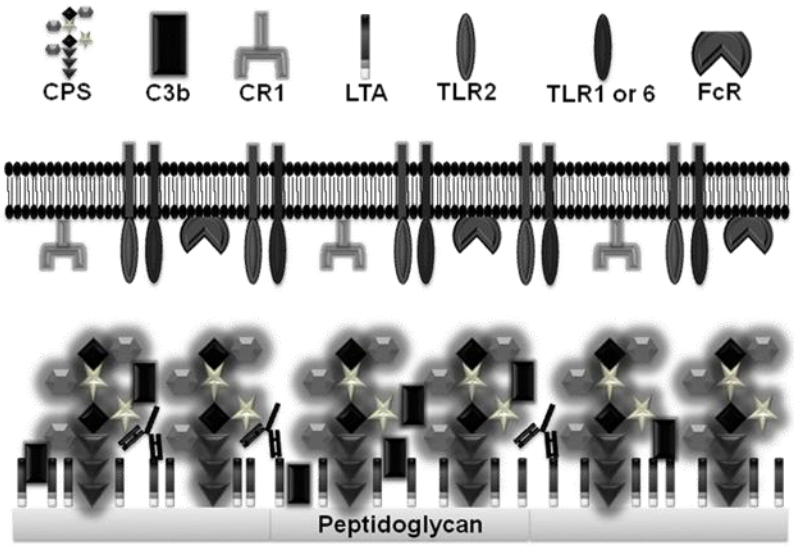

Figure 5.1: The capsule of *E. faecalis* contributes to innate immune evasion.
The capsular polysaccharide produced by *E. faecalis* inhibits the detection of LTA from detection by Toll-like receptors. Capsule also prevents detection of opsonic antibodies and complement by their cognate receptors.

rabbit model of endocarditis. Hearts and Kidneys from rabbits infected with either a wild type strain, a GelE$^-$ strain, an SprE$^-$ strain, or a GelE$^-$/SprE$^-$ strain (all in an isogenic background) were compared in terms of bacterial burden and heterophil influx. The data showed that in the primary infection site (heart) the bacterial burden decreased if the bacteria lacked the ability to produce GelE. Corresponding to this data was the observation that the matrix layers of the vegetations of rabbits infected with GelE producing strains had significantly less heterophil density than the matrix layers from GelE$^-$ infected rabbits. Surprisingly, all rabbits had similar bacterial burdens in the kidney regardless of which strain they were infected with. However, there was significant difference in the amount of heterophil infiltrate around the emboli. Rabbits infected with the double protease mutant strain had significantly higher levels of heterophil infiltrate compared to rabbits infected with any of the other three strains. Interestingly, rabbits infected with the GelE$^-$ strain had significantly higher heterophil influx than did the rabbits infected with either of the GelE producing strains. This would indicate that early in infection, the activity of SprE may be enough to suppress the initial levels of C5a signaling. However, as infection progresses the amount of C5a generated during the continual activation of the complement cascade may eventually overwhelm the limited proteolytic activity of SprE. This is evident in the heart where the effect of SprE by itself was null in terms of limiting heterophil influx. Regardless of the effect of SprE, it was evident that GelE contributed significantly to pathology presumably through the degradation of C5a as well as other immune effectors it is known to target.

Future Directions

The data in **Chapter 4** shows a novel mechanism the contribution of GelE to pathogensis through the degradation of C5a, and also indicates a limited role for SprE in immune evasion. The data in **Chapters 2 and 3** provide insight into the relevance of capsule production, and how the production of capsule contributes to host innate immune evasion. Even though discovery of these novel mechanisms is relevant, there is much more to understand about the interaction of the microbe and the host. The studies in **Chapter 4** show one mechanism of GelE in pathogenesis and it is well known that GelE also contributes to biofilm formation, especially on artificial surfaces (14, 43). The

subject that requires more study is the contribution of GelE to biofilm formation in-vivo. The size of the vegetations in the hearts of the rabbits infected with GelE producing and GelE mutant strains did not differ, which would indicate that GelE may not be directly involved with biofilm formation on heart valves. Because of the complex nature of endocarditis, the relative conditions of factors known to contribute to in-vitro biofilms may not extend to this disease model. Enterococci are one of the leading causes of urinary tract infections. Urinary tract infections (UTI) are thought to be caused by bacterial biofilm formation in the catheter which then serves as point of dissemination for the microbes. Enterococci are also known to form biofilms on in-dwelling medical devices and on artificial heart valves. However, the contribution of GelE to biofilm formation in these conditions has yet to be elucidated.

The work presented here explored the effects of capsular polysaccharide on resistance to opsonophagocytosis and cytokine production. From a bacterial standpoint, the mechanisms of capsule for immune evasion are quite simple: limit the detection of opsonins and mask the detection of surface PAMPs. However, enterococci exist primarily as gut commensals and are only seen as pathogens when the natural balance of the host has been disrupted. Disruption in this case could be trauma which temporarily weakens host defenses, or treatment with antibiotics which kill other gut commensals, thereby allowing enterococci to outgrow their natural niche. In some cases it seems to be a combination of these events that leads to enterococci becoming pathogens. Regardless of the conditions required for enterococci to be pathogens, one question remains: why do normally non-pathogenic microbes such as enterococci produce a capsular polysaccharide? The production of a capsule by a commensal organism would not seem to be beneficial from an evolutionary standpoint. There should be no selective pressure for keeping a genetic locus that leads to the production of an energy demanding surface polymer which only seems to aid the bacteria during a pathogenic lifestyle. The answer to this question may have been revealed during the study of other gut commensals. The capsular polysaccharides produced by other organisms of gut origin trigger an anti-inflammatory response by the residential host cells (23). In this scenario the presence of capsule is not beneficial in terms of pathogenesis, but exists to provide an advantage for a commensal lifestyle. The capsule of *E. faecalis* could be providing this same advantage.

As previously stated, prior treatment with various types of antibiotics can result in the explosion of enterococcal populations in the gut which predisposes patients to infection. It is also known that encapsulated strains of *E. faecalis* contain higher levels of antibiotic resistance and are more pathogenic (24). The presence of a capsule may provide a selective advantage that allows the serotype C and D strains to outcompete un-encapsulated counterparts in a gut environment that is devoid of other competition. This could be tested in a competition animal model in which the animals are treated with high levels of antibiotics and are subsequently inoculated with equal numbers of encapsulated and un-encapsulated *E. faecalis* strains.

Along with possible contributions of capsule to commensalism, capsule is almost certainly playing a role in pathogenesis. Some of the work presented here indicates this. Nevertheless, studies exploring the full effect of encapsulation on the host response have barely scratched the surface. As described earlier, the production of capsule by other gut microbes can have an anti-inflammatory effect. Furthermore, the presence of capsule on *Streptococcus suis* inhibits pro-inflammatory cytokine production, and alters cytokine production in a MyD88 independent manner suggesting that the encapsulated strains are no longer recognized by TLR-2 (11, 34). This study has shown that the presence of *E. faecalis* capsule decreases the amount of TNF-α produced by macrophages, but the effect on the production of other cytokines is not known. A comprehensive analysis of pro- and anti-inflammatory cytokine production in response to un-encapsulated and encapsulated strains of *E. faecalis* has never been done, but could provide many relevant insights into the contribution of capsule to pathogenesis. Additional studies on the effect of capsule in in-vivo models (including bacterial burdens, cytokine production, and immune cell recruitment) should also be investigated to provide more comprehensive view into the world of host pathogen interactions.

Determining the mechanisms employed by bacteria to establish infection and subvert the host innate immune response is critical for developing new therapeutics. However, this is only the first step in a long process of developing novel treatments. The high level of antibiotic resistance in enterococci is well documented and illustrates the need for development of new treatment strategies. One relatively novel approach for treating multi-drug resistant bacteria is the development of humanized monoclonal

antibodies. Antibody based serum therapies have been around since the 1890s but were abandoned due to the discovery of antibiotics (6). Today, serum based therapies have too many negative drawbacks to be considered a safe alternative to traditional therapeutics. However, the ability to produce fully humanized monoclonal antibodies in mice has brought back the idea of highly specific immuno-therapies for treating bacterial infections. Several clinical trials have been conducted for such therapies in treating multi-resistant *Staphylococcus aureus* strains, yet most of these trials have failed. The natural antibody response to a pathogen consists of multiple antibodies targeting multiple antigens produced by the microbe (polyclonal), but the antibodies in these studies targeted one highly specific antigen (3). As reviewed in **chapter 1**, it is obvious that even a relative weak pathogen such as *E. faecalis* produces several factors that contribute to pathogenesis. It has now become clear that multiple monoclonal antibodies targeting several antigens may be more relevant.

The number of virulence factors produced by *E. faecalis* is relatively modest when compared to other pathogens like *S. aureus.* If nothing else, *E. faecalis* could serve as a microbial model organism for determining proof of principle for the development of humanized immuno-therapies targeting several antigenic determinants. Several virulence factors expressed by *E. faecalis* contribute to adhesion which leads to the development of biofilms. Bacteria in biofilms are not only more resistant to antibiotics, but are impervious to penetration by antibodies. Again, most enterococcal infections are acquired in a clinical setting, such as surgical site infections, endocarditis due to valve replacement, and UTIs due to catheterization, which could be prevented by inhibiting the bacteria from establishing infection. This could be done in a prophylactic manner by treating patients with antibodies targeting the adhesion proteins Esp and Ace prior to these invasive procedures.

Most healthy humans have circulating antibodies that efficiently opsonize *E. faecalis* (2, 18). However, these antibodies are only known to target un-encapsulated *E. faecalis* strains and are not effective in clearing capsule producing strains (18). Targeting of surface antigens with antibodies directed towards capsule could allow for clearance of encapsulated strains. Indeed, preliminary evidence from unpublished data (L. R. Thurlow) shows that polyclonal antibodies produced to encapsulated strains of *E.*

faecalis dramatically increases the rate of opsonophagocytosis in a macrophage assay. Additionally, this work and the work of others has shown that GelE makes significant contributions to pathogenesis and biofilm formation. The production of highly specific polyclonal antibodies targeting GelE has provided some interesting preliminary data. The antibodies are able to inhibit GelE activity in-vitro and the incubation of antibodies with *E. faecalis* inhibits biofilm formation on the surface of plastic plates (data not shown). These preliminary results should be studied in greater detail to determine the potential of these antibodies as prospective therapeutics. Furthermore, these and other antibodies targeting *E. faecalis* virulence factors should be tested for both the prophylactic and therapeutic potential in animal models of infection. Hopefully, the continuation of the work presented here will lead to the discovery of important aspects of host pathogen interactions, and potentially to the development of novel therapeutics for preventing and treating *E. faecalis* infections.

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In

CHAPTER 2

Capsular polysaccharide production in *Enterococcus faecalis* and contributions of CpsF to capsule serospecificity

Thurlow L. R., Thomas, V. C., and Hancock L. E. (2009)

Journal of Bacteriology 191, 6203-6210.

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October 30, 2009

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License Number	2298840332615
License date	Oct 30, 2009
Licensed content publisher	American Society for Microbiology
Licensed content publication	Journal of Bacteriology
Licensed Content title	Capsular Polysaccharide Production in Enterococcus faecalis and Contributions of CpsF to Capsule Serospecificity
Licensed Content Author	Lance R. Thurlow, Lynn E. Hancock
Volume	191
Issue	20
Start page	6203
End page	6210
Type of Use	Dissertation/Thesis
Format	Print and electronic
Portion	Full article
Order reference number	
Title of your thesis/dissertation	EVASION OF INNATE IMMUNITY BY ENTEROCOCCUS FAECALIS: THE ROLES OF CAPSULE AND GELATINASE
Expected complete date	Dec 2009
Estimated size (pages)	150
Total	0.00 USD