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Evaluating the impact of post-translational modifications by the secreted zinc metalloprotease, GelE, on the major autolysin of E. faecalis, AtlA, and a stress-induced protein, SalB

Emily K. Stinemetz

EVALUATING THE IMPACT OF POST-TRANSLATIONAL MODIFICATIONS BY THE SECRETED ZINC METALLOPROTEASE, GELE, ON THE MAJOR AUTOLYSIN OF *E. FAECALIS,* ATLA, AND A STRESS-INDUCED PROTEIN, SALB

by

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DISSERTATION

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by

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Houston, Texas

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Dedication

To my parents, Carol Stinemetz and the late Doug Stinemetz, words cannot even begin to describe the gratitude I owe you. You provided me with the love, encouragement, and strength to further my education.

To my father:

"Once upon a midnight dreary, while I pondered, weak and weary,
Over many a quaint and curious volume of forgotten lore,
While I nodded, nearly napping, suddenly there came a tapping,
As of some one gently rapping, rapping at my chamber door.
Tis some visitor," I muttered, "tapping at my chamber door —
Only this, and nothing more."

-Edgar Allen Poe

To my mother:

"Listen my children and you shall hear
Of the midnight ride of Paul Revere,
On the eighteenth of April, in Seventy-five;
Hardly a man is now alive
Who remembers that famous day and year."

-Henry Wadsworth Longfellow

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Evaluating the impact of post-translational modifications by the secreted zinc metalloprotease, GelE, on the major autolysin of *E. faecalis,* AtlA, and a stress-induced protein, SalB

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Advisory Professor: Barrett R. Harvey, Ph.D.

AtlA is the major peptidoglycan hydrolase of *E. faecalis* involved in cell separation of dividing cells. SalB is a secreted stress-induced protein regulated by the CroRS system. In addition, these two proteins also appear to be affected by the virulence factor, gelatinase (GelE). GelE is a secreted zinc metalloprotease known to impact various cellular functions by post-translational modification of protein substrates. The overall objective of this work was to understand how GelE cleavage of secreted proteins, specifically AtlA and SalB, changes their function. Herein, I discovered that GelE modifies both AtlA and SalB. As visualized by Western blot analysis and flow cytometry, when GelE is expressed, AtlA exists in a N-terminally truncated form. Furthermore, N-terminal-sequencing analysis identified the GelE-cleavage site within AtlA to occur near the catalytic region, Domain II. Thus, cleavage removes the majority of the N-terminal T/E rich region, Domain I. Truncation of AtlA at this site caused no significant difference in the peptidoglycan hydrolysis activity compared to the full-length protein. Nevertheless, the modification of AtlA was shown to be required for cell separation and the completion of cell division. Additionally, GelE-modified AtlA was shown to localize to the cell septum. Taken together, these results demonstrate that post-translational modification of AtlA by GelE regulates AtlA septum localization and successful cell separation. Similarly, in the presence of GelE, SalB was found in multiple fragments. Western blot and flow cytometry analysis demonstrated that SalB was found in the media supernatant, but not associated with the

cell surface. Overall, this dissertation demonstrates that GelE post-translationally modifies these two secreted proteins, AtlA and SalB, impacting the function of AtlA in cell division. Future experiments will strengthen our knowledge of how these modifications impact *E. faecalis* virulence.

Table of Contents

Table of Figures

List of Tables

Chapter 1: Background and significance

Significance

Enterococci are important bacterial pathogens that cause hospital-acquired infections (Murray, 1990). These bacteria develop resistance to various antibiotics, in particular vancomycin, which is compounded by their ability to form biofilms on both biotic and abiotic surfaces (Moellering, 1991, Lewis, 2001). Due to their propensity for developing antibiotic resistance, alternate approaches should be explored to target bacterial virulence. One such strategy is the use of antibodies targeting virulence-associated surface proteins.

Bacteria produce many virulence factors that allow them to evade host immune defense mechanisms, thus permitting their colonization. Virulence factors are diverse and can exhibit specific environment-dependent functions. For example, virulence factors that have a crucial role in wound infections might not necessarily be functionally associated with other types of infections, such as those of the urinary tract. In order to improve our understanding on how to better treat bacterial infections, the following basic research questions need to be answered: What is the role of these virulence factors *in vivo*? Are virulence factors impacted by post-translational modifications? Do these factors have specific targets? It is my belief that addressing these research questions could ultimately lead to the development of novel agents against enterococci.

The work presented in this dissertation focuses on the interaction of a virulence factor, GelE, with two extracellular *Enterococcus faecalis* secreted proteins: AtlA and SalB. GelE is a secreted zinc metalloprotease recognized as an important regulator of cellular functions because of its ability to modify proteins (Makinen *et al.*, 1989, Waters *et al.*, 2003). AtlA is the major peptidoglycan hydrolase of *E. faecalis* and is important in separating daughter cells during cell division (Qin *et al.*, 1998, Eckert *et al.*, 2006). SalB is a stress-induced protein whose function is not fully understood, yet is known to play a role in both cell morphology and biofilm

development (Shankar *et al.*, 2012, Mohamed *et al.*, 2006). Specifically, uncovering the functional role of these exoproteins in different phases of *E. faecalis* cell division and the nature of their relation with each other will significantly improve our knowledge of the mechanisms impacting cell separation during cell division.

Background

1.1 *Enterococcus* species

Enterococci are Gram-positive cocci that were first identified as intestinal organisms at the end of the 19th century (Evans & Chinn, 1947, Lebreton *et al.*, 2014). Based on the ability of these species to form cellular chains, enterococci became grouped as part of the *Streptococcus* genus in 1906 (Schleifer *et al.*, 1984). The *Enterococcus* genus was not characterized until the mid-1980s (Schleifer *et al.*, 1984). Based on 16S rRNA and DNA-rRNA hybridization studies, more than 30 bacterial species, such as *E. faecalis* and *E. faecium,* are found in the enterococcal classification (Arias & Murray, 2008, Klein, 2003, Fisher & Phillips, 2009).

The *Enterococcus* genus is composed of facultative anaerobes with low G + C content (Fisher & Phillips, 2009, Willems & van Schaik, 2009). Enterococci can survive a broad range of temperatures from 6.5°C to 47.8°C, and up to 60°C for short periods (approximately 30 minutes), and can also survive high salinity conditions and pH ranges from 4.6 to 9.9 (Fisher & Phillips, 2009, Van den Berghe *et al.*, 2006). Due to their survival versatility and adaptability in various conditions, enterococci are found in the environment, in fermented foods such as cheeses and sausages, as well as in the human, animal, insect, and nematode gastrointestinal (GI) tracts (Lebreton *et al.*, 2014, Foulquie Moreno *et al.*, 2006). Enterococci primarily colonize the GI tract and the genital tract in humans (Cetinkaya *et al.*, 2000).

In the mid-1970s, probably due to the introduction of 3rd-generation cephalosporins, there was an emergence of hospital-acquired infections (HAIs) from enterococci (Arias & Murray, 2012, Murray, 1990). Since then, enterococci have become the second most prevalent cause of HAIs in the United States (Murray, 1990, Nallapareddy *et al.*, 2011a), responsible for wound infections, urinary tract infections (UTIs), bacteremia, and endocarditis (Nallapareddy *et al.*, 2011b). *E. faecalis* and *E. faecium* are the two primary *Enterococcus* species known to cause

infections (Fisher & Phillips, 2009). Historically, *E. faecalis* accounted for 80-90% of all clinical isolates, but recently the number of *E. faecium* infections has risen (Hidron *et al.*, 2008, Murray, 1997).

Enterococci have either intrinsic resistance to certain antibiotics or have evolved specific resistance to many different antibiotics such as vancomycin or penicillin (Moellering, 1991). Since the late 1980s, there has been a rise in the incidence of vancomycin-resistant enterococci (VRE) in Europe and the United States (Cetinkaya *et al.*, 2000) and, as a consequence, enterococcal infections have become difficult to treat. Thus, it is imperative to pursue alternative approaches to traditional antibiotic therapies.

1.2 Bacterial Peptidoglycan

Gram-positive bacteria, like enterococci, have a cell wall composed of a thick layer of peptidoglycan that allows the cell to hold its shape, withstand osmotic pressure, enable cell-cell communication, and protect the cell from antimicrobial drugs (Silhavy *et al.*, 2010). Peptidoglycan is a polymer comprising of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) disaccharides linked via a tetrapeptide cross-bridge that gives the cell wall both form and flexibility (van Heijenoort, 2001, Ton-That *et al.*, 1997). In order for growth to occur, autolysins, such as AtlA in *E. faecalis,* turn over bacterial peptidoglycan through cleavage of the old peptidoglycan and insertion of the newer peptidoglycan (Holtje, 1995).

Eukaryotic cells do not produce peptidoglycan. During infection, hosts have receptors, such as peptidoglycan recognition proteins (PGRPs) in insects and mammals (Kang *et al.*, 1998, Dziarski & Gupta, 2006), that recognize bacterial peptidoglycan and mount an appropriate host response in order to remove the invading bacteria (Atilano *et al.*, 2014). Peptidoglycan-PGRP complexes increase the immune response by increasing activation markers CD80/86 and CD14.

In addition, the peptidoglycan-PGRP complex encourages inflammation by increasing pro-inflammatory IL-8, IL-12, and TNF-α (De Marzi *et al.*, 2015). One way that bacteria have responded to this peptidoglycan recognition is through the use of autolysins to inhibit peptidoglycan-PGRP complexes. These autolysins are responsible for peptidoglycan cleavage. They digest and modify peptidoglycan in order to avoid host peptidoglycan recognition (Humann & Lenz, 2009). Recently it was discovered that in *S. aureus,* the major autolysin Atl, is necessary for the cleavage of small fragments from the end of peptidoglycan strands (Atilano *et al.*, 2014). Without these ends peptidoglycan receptors do not recognize the bacterial peptidoglycan, consequently making the bacteria camouflaged from the host immune response.

The peptidoglycan layer contains cellular surface proteins and glycopolymers such as teichoic acids. The function of these teichoic acids is to protect the cellular envelope, to defend the bacterial cell from host immune defenses, and to promote bacterial adhesion/colonization (Baddiley, 1972, Brown *et al.*, 2013). They can be either peptidoglycan-anchored wall teichoic acids (WTAs), or membrane-anchored, lipoteichoic acids (LTAs) (Weidenmaier & Peschel, 2008).

1.2.1 Teichoic acids and their interaction with autolysins

In *Staphylococcus aureus,* WTAs inhibit the major autolysin, Atl, by preventing the binding of Atl to the cell wall (Schlag *et al.*, 2010). Thus, Atl binds to areas of the cell wall where WTA concentration is low, such as the cellular septum, allowing the cell to carry out efficient separation of the two daughter cells. The deletion of *tagO*, a gene required in the biosynthesis of WTA, allows for the random binding of Atl to the cell surface, resulting in increased cellular lysis (Biswas *et al.*, 2012, Schlag *et al.*, 2010).

Alternatively, transmembrane LTA polymers that extend through the cell wall to the cell

surface can interact with autolysins. Using crystallography, one study demonstrated that LTA binds to the repeat regions of Atl and further confirmed that Atl does not bind to WTA (Zoll *et al.*, 2012). At areas where new cell wall synthesis is occurring, there is considerably more LTA exposed and a lower concentration of WTA. Thus, the LTA-Atl interaction allows Atl to bind to the cell septum. It was hypothesized that Atl changes its localization by modifying LTA partners. This allows Atl-mediated enzymatic activity against peptidoglycan to occur at different points, resulting in successful cell separation (Zoll *et al.*, 2012).

Unlike in *S. aureus,* the mechanism for AtlA localization to the cell septum is not fully understood in *E. faecalis*. In this dissertation, I will provide strong evidence that post-translational processing of AtlA, the major autolysin of *E. faecalis,* by a metalloprotease, GelE, can alter AtlA localization to the cell septum. It is possible that GelE-mediated cleavage of AtlA might cause AtlA to interact with teichoic acids as described above; however, the question of how cleaved AtlA localizes to the cell septum has yet to be resolved.

1.3 Biofilms

A bacterial biofilm is a group of cells that are attached to each other and to either a biotic or an abiotic surface (Donlan, 2002, Flemming & Wingender, 2010). The development of a biofilm occurs in three major stages: attachment, maturation, and dispersal (Kostakioti *et al.*, 2013, O'Toole *et al.*, 2000). Often times, bacterial cells have appendages such as flagella, pili, or fimbriae that help the bacterial cells during initial contact of the surface (Donlan, 2001). During the attachment stage, an extracellular matrix (ECM) surrounds the community providing protection, stability, and structure. The ECM is also composed of proteins, polysaccharides, and extracellular DNA (eDNA) that is released from the bacteria (Flemming & Wingender, 2010, Vilain *et al.*, 2009). Through channels, nutrients, oxygen, and small communication molecules are allowed to pass from cell to cell in the ECM.

This thick, dense matrix allows bacteria to become more resistant to antibiotics, environmental stresses, and host immune responses compared to free-moving, planktonic cells (Mandsberg *et al.*, 2009, Bjarnsholt *et al.*, 2013). The ECM prevents antimicrobials from affecting bacterial cells by blocking their passage to the cells. At the same time, bacterial cells in the ECM are growing at a decreased rate causing them to have a decreased susceptibility to antibiotics (Donlan, 2001, Duguid *et al.*, 1992, Hoyle *et al.*, 1992, Abee *et al.*, 2011). Due to their high antibiotic resistance, biofilms are difficult to eliminate and can ultimately lead to chronic infections (Lewis, 2001).

In the clinical setting, areas on which biofilms can form include teeth, lungs, within wounds, or on medical devices, such as urinary catheters and heart valves (Mandlik *et al.*, 2008, Mandsberg *et al.*, 2009, Costerton, 2002). Not only do these bacterial cells grow adjacent to one another, they are also able to communicate via small diffusible molecules, a process known as quorum sensing (Miller & Bassler, 2001, Waters & Bassler, 2005, Li & Tian, 2012). Quorum sensing allows the bacteria to sense when a certain cell density is reached and appropriately respond. For example, quorum sensing can allow bacterial cells to escape from the host immune response by coordinating the expression of certain virulence factors that can impact the host immune response. Thus, quorum sensing impacts host removal of bacterial cells and helps establish a bacterial infection (Costerton, 1999, Costerton *et al.*, 1999, Davey & O'Toole G, 2000).

In a study examining the biofilm formation capacity of 128 enterococcal isolates (83 *E. faecalis,* and 45 *E. faecium),* 95% of the time *E. faecalis* was a biofilm producer compared to only 28% for *E. faecium* (Di Rosa *et al.*, 2006). However, the reason *E. faecalis* is more likely to form biofilms is not yet understood. Environmental signals such as glucose, serum, or temperature are known to regulate biofilm formation (Mohamed & Huang, 2007). In addition, different enterococcal virulence factors may also play a role in biofilm development. For

example, *fsr, gelE*, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and autolysins have all been shown to influence biofilm formation (Nallapareddy *et al.*, 2000, Nallapareddy *et al.*, 2011b, Thomas *et al.*, 2009). Taken together there are many factors in the enterococcal species that can impact biofilm formation. These factors range from the growth conditions to the bacterial secreted proteins.

1.4 Proteases

Proteases, also known as peptidases, are enzymes that carry out proteolysis, the hydrolysis of peptide bonds in proteins or peptides (Hase & Finkelstein, 1993, Lebrun *et al.*, 2009). They exists as either endoproteases, which cleave within internal peptide bonds, or exoproteases, which cleave N- or C-terminal peptide bonds (Hase & Finkelstein, 1993). Proteases also demonstrate limited or unlimited proteolytic activity. In limited proteolytic activity, proteases cleave specific peptide bonds while in unlimited proteolytic activity, proteases are able to completely break down peptides to their amino acids.

In addition, proteases are classified into one of six groups. This is based on the functional group located at their catalytic site. These include the aspartic proteases, metalloproteases, serine proteases, cysteine proteases, threonine proteases, and glutamic acid proteases (Lebrun *et al.*, 2009). Of these, the aspartic, metallo and serine proteases are the most common (Hoge *et al.*, 2010). Bacterial proteases only include metalloproteases, serine proteases, cysteine proteases, and aspartic proteases (Hase & Finkelstein, 1993). Gelatinase, a metalloprotease, and serine protease, SprE, are two proteases secreted by *E. faecalis.*

1.4.1 Gelatinase

Originally discovered in 1955 for its ability to clot milk and hydrolyze casein, gelatinase (GelE) is an extracellular zinc metalloprotease produced by *E. faecalis* (Grutter & Zimmerman,

1955, Bleiweis & Zimmerman, 1964, Makinen *et al.*, 1989). GelE is a member of the M4 family of zinc metalloproteases (Del Papa *et al.*, 2007). The majority of zinc metalloproteases, including GelE, contain HEXXH as the primary sequence motif for zinc association (Jongeneel *et al.*, 1989, Hase & Finkelstein, 1993). In addition to binding to zinc, three to four calcium atoms are found bound to GelE, potentially stabilizing the structure (Del Papa *et al.*, 2007).

Like other proteases, GelE is synthesized as an inactive precursor (Del Papa *et al.*, 2007, Hase & Finkelstein, 1993). This precursor is composed of a signal peptide, a propeptide and mature GelE. GelE is not activated until it is secreted outside the cell. The signal peptide functions to guide the GelE precursor to the secretion system (Del Papa *et al.*, 2007). Activation of GelE occurs by an autocatalytic process and the association of the propeptide with the active form (Marie-Claire *et al.*, 1998, O'Donohue & Beaumont, 1996, Del Papa *et al.*, 2007). Similar to the zinc metalloprotease of *Pseudomonas aeruginosa,* elastase*,* the propetide of GelE acts as an intramolecular chaperone, allowing the active GelE to properly fold (McIver *et al.*, 1995, Marie-Claire *et al.*, 1999, Del Papa *et al.*, 2007). In addition, Papa *et al.* demonstrated that C-terminal processing of GelE is required for full protease activation, which is unique for M4 zinc metalloproteases (Del Papa *et al.*, 2007). Although the function of this C-terminal region is unknown, the authors of this work suggested that this C-terminal region either impacts processing of the major autolysin of *E. faecalis,* serves as a secondary signal, or impacts the gelatinase activity (Del Papa *et al.*, 2007).

In addition, the expression of GelE is regulated by the fsr quorum-sensing system, which is encoded by the *fsrABDC* operon (Figure 1.1) (Qin *et al.*, 2000). Gelatinase biosynthesis-activating pheromone (GBAP) is an 11-amino acid peptide lactone encoded by *fsrD.* Once expressed, GBAP is processed and exported outside the cell by the transporter FsrB. As GBAP accumulates outside the cell, a histidine kinase, FsrC, phosphorylates FsrA. This activation causes FsrA to induce the *fsrABDC* operon, as well as induces a second operon containing

gelEsprE (Nakayama *et al.*, 2001, Nakayama *et al.*, 2006, Teixeira *et al.*, 2013). In *fsrA, fsrB,* and *fsrC* mutants, expression of *gelE* and *sprE* does not occur (Qin *et al.*, 2000). *gelE* encodes the metalloprotease, gelatinase, while *sprE* encodes a serine protease (Qin *et al.*, 2000, Kawalec *et al.*, 2005).

GelE has broad substrate activity, cleaving both host and bacterial substrates as well as proteins. In addition, GelE preferentially cleaves at hydrophobic amino acids. Specifically, purified GelE cleavage occurs between serine and leucine-isoleucine residues (Makinen *et al.*, 1989). Below, I will discuss the numerous substrates that GelE can cleave and examine the impact this has on *E. faecalis* pathogenesis.

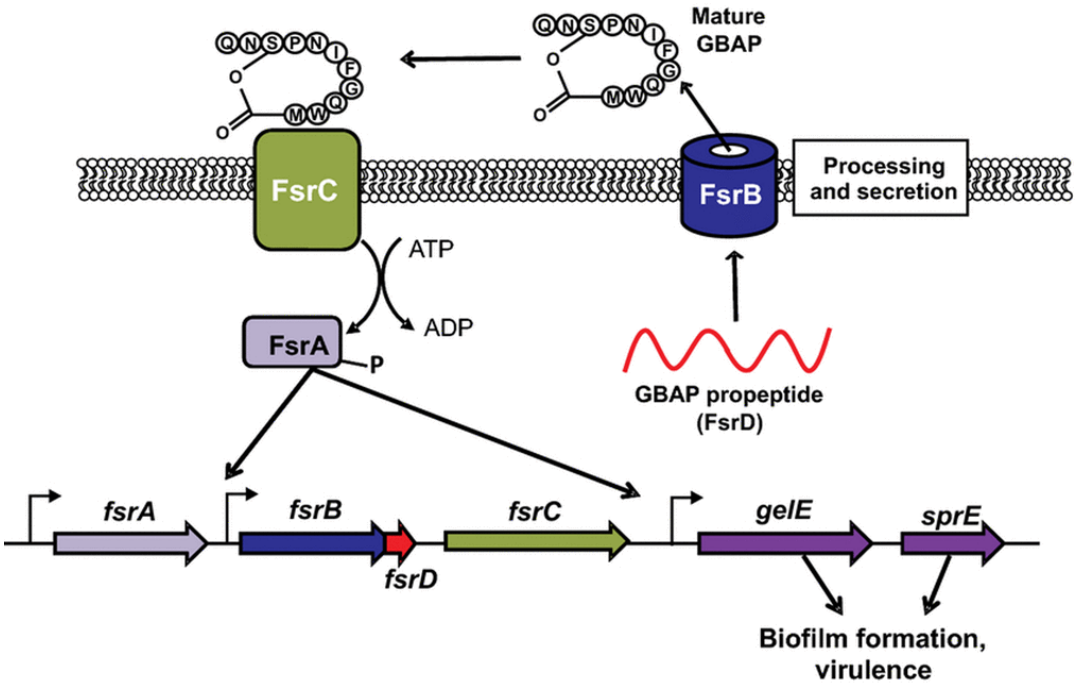


Figure 1.1: Fsr-quorum sensing pathway. *fsrD* encodes the GBAP precursor, which is processed and secreted outside the cell by FsrB. As GBAP accumulates, it binds to the histidine sensor kinase, FsrC. This interaction causes phosphorylation of FsrA, which binds to *fsrB* and *gelE/sprE* promoters. *gelE* produces gelatinase while *sprE* produces serine protease. This figure was reproduced and adapted from: Cook LC, Federle MJ. Peptide pheromone signaling in Streptococcus and Enterococcus. FEMS microbiology reviews. 2014;38(3):473-492. Permission to use this figure was granted by Copyright Clearance Center (License number: 4032660717362).

1.4.1.1 Functions of GelE

1.4.1.1a The impact of GelE on pathogenesis

The impact of *E. faecalis* proteases on pathogenesis has been confirmed in many disease models. Although, it is difficult to separate GelE and SprE activity, GelE has displayed significant impact to pathogenesis. As described below, GelE impacts the host immune response by either directly or indirectly inhibiting AMP activity, through cleavage of complement components, through cleavage of the host extracellular matrix, or through cleavage of bacterial exoproteins. This helps in the establishment of *E. faecalis* infections.

In a study examining 50 *Enterococcus* isolates (30 *E. faecalis* and 20 *E. faecium*), only 60% of the isolates contained the *gelE* gene, among which, only 66.6% displayed GelE activity (Comerlato *et al.*, 2013). Overall, *E. faecalis* maintains *gelE* and GelE activity more frequently than *E. faecium* (Qin *et al.*, 2000, Roberts *et al.*, 2004, Comerlato *et al.*, 2013). In enterococcal strains lacking *gelE,* other factors may compensate for the absence of GelE, with specific activity related to certain infection locations.

In a mouse peritonitis model, an insertion *gelE* mutant resulted in a significant delay in time to death (Singh *et al.*, 1998). In a *G. mellonella* infection model, both *ΔgelE* and *ΔfsrB* (GelE-negative) were less virulent than the wild-type strains, although *ΔfsrB* showed a greater impact (Gaspar *et al.*, 2009). Through observations of a rabbit endocarditis model, GelE non-expressing strains (*ΔgelE* and *ΔgelEΔsprE*) demonstrated a significant decrease in bacterial burden at disseminated sites compared to strains that expressed GelE (V582 and *ΔsprE*) (Thurlow *et al.*, 2010). Together these results confirmed that GelE is an important factor in pathogenesis in multiple animal infection models.

Viable bacteria spread from the gastrointestinal (GI) tract to the extraintestinal sites by a process called translocation, which results in systemic infection and death (Berg, 1999). In a

mouse model, *E. faecalis* was translocated across the intestinal tract, which resulted in systemic infection and death (Wells *et al.*, 1990). *ΔgelE* demonstrated a decreased in bacterial translocation across human enterocyte-like T84 cells compared to the wild-type strain OG1RF (Zeng *et al.*, 2005). Complementation of *gelE* in *ΔgelE* resulted in a restoration of the ability of *E. faecalis* to translocate (Zeng *et al.*, 2005). A peptide antagonist (ZBzl-YAA5911) against GBAP decreased the translocation of *E. faecalis* from the aqueous humor into the vitreous cavity (Nakayama *et al.*, 2013). Together, these results suggest that GelE impacts the ability of *E. faecalis* to translocate. The mechanism for this translocation remains in question.

ΔgelE showed a decrease in biofilm production compared to the wild-type OG1RF (Hancock & Perego, 2004, Mohamed *et al.*, 2004). In addition, a GelE-negative strain, JH2-2, does not readily form biofilms (Kristich *et al.*, 2004). Expression of *gelE* in JH2-2 resulted in biofilm formation (Kristich *et al.*, 2004). Based on these results, Kristich *et al.* concluded that GelE is required for biofilm formation (Kristich *et al.*, 2004). The addition of purified GelE to strains that do not form biofilms caused the strain to produce biofilms (Hancock & Perego, 2004). Thus, expression of GelE has been demonstrated to impact biofilm formation in *in vitro* studies.

Although GelE was found to be necessary for biofilm formation in genetic analysis, the presence of GelE was not found to impact biofilm formation in clinical isolates. There was no difference found in biofilm production between isolates that expressed GelE and isolates that lacked GelE expression (Mohamed & Murray, 2005). In agreement with this study, the examination of a larger group of *E. faecalis* isolates demonstrated no significant difference in the biofilm formation ability of GelE expressing and non-expressing isolates (Seno *et al.*, 2005). In clinical isolates from UTIs, the presence of *gelE* demonstrated no significant impact on biofilm formation (Kafil *et al.*, 2016). In opposition, in clinical isolates of clinical root canal

retreatment, expression of *gelE* was found to be higher in strains that produce biofilm than in strains that do not (Wang *et al.*, 2011). Since biofilm production did not correlate in clinical isolates that differed in GelE status for most of these clinical studies, this indicates that other factors could play a role in biofilm production, compensating for a lack of GelE.

1.4.1.1b GelE and host immune response system

In order to effectively establish an infection, bacteria must first combat the host immune response. One part of the host innate immune response is antimicrobial peptides (AMPs). These are small proteins which kill a broad range of microbes, fungi, and viruses. AMPs act as the first line of defense, responding to the presence of microbes prior to inflammation (Izadpanah & Gallo, 2005). One way bacteria combat AMPs is through extracellular proteases. In *E. faecalis*, GelE impacts the activity of various AMPs. For example, Schmidtchen *et al*. demonstrated that GelE degrades the AMP, LL-37 (Schmidtchen *et al.*, 2001). This cleavage resulted in decreased LL-37 binding to *E. faecalis* and increased bacterial survival (Schmidtchen *et al.*, 2001). LL-37 functions as both an antimicrobial peptide as well as a regulator of immune responses (Reinholz *et al.*, 2012). Therefore, GelE cleavage helps the bacteria to establish an infection by removing one of the hosts' first line of defense.

Not only does GelE directly cleave AMPs and inhibit AMP activity, but GelE indirectly inhibits AMP activity. Schmidtchen *et al.* also determined that GelE cleavage of proteoglycans releases dermatan sulphate, which binds to α-defensin and eliminates its antimicrobial activity (Schmidtchen *et al.*, 2001). However, not all α-defensins demonstrate inhibition by GelE cleavage. For example, incubation of two human α-defensins, the human neutrophil peptide-3 and α-defensin-5, with purified GelE did not impact their bactericidal activity, suggesting that GelE does not impact either human neutrophil peptide-3 or α-defensin-5 (Miyoshi *et al.*, 2010).

The greater wax moth, *Galleria mellonella,* is a model organism used for the study of bacterial infections (Tsai *et al.*, 2016). In *E. faecalis* infections with this model organism, GelE was identified as a major virulence factor (Park *et al.*, 2007). Injection of purified GelE into *G. mellonella* resulted in *G. mellonella* death (Park *et al.*, 2007). After further analysis, Park *et al.* demonstrated that purified GelE degrades *G. mellonella* cecropin, an inducible AMP important for host defense upon bacterial infection (Park *et al.*, 2007). This result further demonstrates GelE cleavage of a host AMP in order to eliminate the first line of host defense. All together these results establish that GelE is capable of evading AMPs directly or indirectly through their degradation in both insects and mammals.

Another important aspect of the host innate immune response is the complement system. Three pathways activate the complement system: the alternative, the classical, and the mannose-binding lectin (MBL) pathway (Mathern & Heeger, 2015). The complement system functions to produce complement proteins that bind to pathogens for phagocytosis, to recruit more phagocytes, and to damage bacteria by producing pores in the bacterial cell membrane. The early events in these three different pathogens generate C3 and C5 convertase, a protease responsible for activating further downstream complement components. Many bacterial proteases target either the C3 or the C5 to deactivate and evade the complement system (Potempa & Potempa, 2012).

GelE inhibits the human complement system through multiple complement components. Park *et al.* demonstrated that GelE cleaves C3a (Park *et al.*, 2007). In addition to its role in the complement system, C3a demonstrated antimicrobial activity and is important for immune regulation (Kohl, 2001). In the complement system, C3 is activated to C3b by the C3 convertase assembly. *E. faecalis* modifies this change slightly through GelE cleavage. Purified GelE cleaves C3 to C3b near the original site that C3 convertase acts upon (Park *et al.*, 2007). This GelE-activated C3b reacts with water and loses its ability to bind to the bacterial surface,

essentially inactivating the complement system (Park *et al.*, 2008). Furthermore, GelE impacts polymorphonucelar cell family (PMN) bacterial targeting and killing of bacterial cells by cleaving and removing iC3b from the surface of *E. faecalis* cells (Park *et al.*, 2008). In addition, GelE cleaves C5a as well (Thurlow *et al.*, 2010). Together, these results indicate that GelE impacts multiple complement components, which encourages *E. faecalis* infections.

1.4.1.1c GelE cleavage of host substrates

Another aspect of host colonization is bacterial cell adhesion, followed ultimately by dissemination. In order to adhere, bacteria produce adhesins that bind to components of the host extracellular matrix, such as collagen or fibronectin (Ribet & Cossart, 2015). Proteases, like GelE, facilitate bacterial dissemination by cleavage of these same known host substrates that bacterial adhesins bind to. For example, GelE has shown collagen I cleavage (Makinen *et al.*, 1989, Shogan *et al.*, 2015). Inhibition of GelE activity by 1,10-phenanthroline did not completely inhibit collagen I degradation, suggesting that a non-metalloprotease is also necessary for collagen degradation (Shogan *et al.*, 2015).

GelE also indirectly effects the degradation of collagen through matrix metalloprotease-9 (MMP9). MMP9 is a host enzyme that is produced in response to tissue injury in order to assist in the remodeling of tissues. Shogun *et al.* observed that GelE is necessary for recombinant MMP9 cleavage *in vitro* (Shogan *et al.*, 2015). However, upon analysis of MMP9 cleavage *ex vivo,* GelE was not required for MMP9 cleavage, whereas the *E. faecalis* serine protease, SprE, was necessary for *ex vivo* MMP9 cleavage (Shogan *et al.*, 2015). The differences observed between GelE cleavage of recombinant versus naturally occurring MMP9 were hypothesized to be due to the folding of MMP9 (Shogan *et al.*, 2015). Cleavage of MMP9 results in the activation of MMP9. Further analysis demonstrated that the complementation of

gelE with a Δ*gelE* strain increased MMP9 activation (Shogan *et al.*, 2015). Together, these results suggested that GelE and SprE impact MMP9 cleavage and activation, but did not separate the major player in MMP9 cleavage and activation. Ultimately, the activation of MMP9 by GelE and SprE impacts the degradation of collagen IV (Gioia *et al.*, 2009).

GelE is also able to degrade fibrin. Strains that do not express GelE lack fibrin degradation while strains that do express GelE demonstrate fibrin degradation (Waters *et al.*, 2003). In established vegetations, the matrix layer surrounding the vegetation includes fibrin (McCormick *et al.*, 2002). Strains that express GelE resulted in a significantly smaller matrix layer surrounding the bacteria compared to strains that failed to express GelE (Thurlow *et al.*, 2010). The impact of GelE cleavage on fibrin, which impacts the surrounding matrix layer, allows the bacterial cells to disseminate from the area and spread to other parts of the body.

1.4.1.1d GelE cleavage of bacterial exoproteins

Aggregation substance, Asc10, is an *E. faecalis* surface protein encoded by plasmid pCF10. The presence of GelE is important for the removal of misfolded Asc10 from the cell surface (Waters *et al.*, 2003). Waters *et al* hypothesized that GelE removes all misfolded proteins from the surface in a similar fashion to Asc10.

In addition, GelE activates certain bacterial antimicrobial peptides by cleaving bacterial exproteins. These protease active bacterial AMPs are used against other bacteria while competing for limited resources (Faye *et al.*, 2002). In *E. faecalis,* Dundar *et al.* found that GelE is responsible for the extracellular processing of EF_1097 into the bacterial AMP enterocin O16 (Dundar *et al.*, 2015). *ef1097* expression is controlled by the fsr-regulatory system (Dundar *et al.*, 2015). *E. faecalis* is resistant to enterocin O16, which primarily inhibits lactobacilli (Dundar *et al.*, 2015). Since lactobacilli are the prominent species found in the female genital tract

(Larsen & Monif, 2001), one could speculate that the primary function of this *E. faecalis* AMP is to help encourage *E. faecalis* infection in the genital tract. In a *Drosophila* infection model, deletion of *ef1097* resulted in decreased virulence (Teixeira *et al.*, 2013).

The Harvey laboratory has previously worked on the interaction between GelE and a secreted microbial surface component recognizing adhesive matrix molecules (MSCRAMM) known as Ace (Pinkston *et al.*, 2011). Ace is cleaved by GelE, which removes the collagen adhesion domain, domain A, of Ace from the cell surface. The maintenance of domain A on the cell surface is important for *E. faecalis* adherence to collagen. In wild-type OG1RF, at an early growth phase when GelE is not present, Domain A is cleaved from the cell surface and binding to collagen is high. At a later growth phase when Domain A was removed from the cell surface, binding to collagen was significantly decreased. An OG1RFΔ*gelE* strain, which has Domain A on the cell surface, maintains high collagen binding (Pinkston *et al.*, 2011). The cleavage of Ace by GelE could function to release cells from host collagen encouraging dissemination.

To study the interaction of GelE and the major autolysin of *E. faecalis,* AtlA, the Hancock laboratory examined the surface autolysin profiles of *E. faecalis* OG1RF, OG1RFΔ*gelE,* V583, and V583Δ*gelE.* Consistent with previous reports (Eckert *et al.*, 2006), cell wall extracts from the OG1RFΔ*gelE* and V583Δ*gelE* strains displayed a full-length version of AtlA of approximately 72 kDa while the cell wall extracts from the wild-type strains displayed a processed form of AtlA of approximately 62 kDa (Thomas *et al.*, 2009). Together these results suggest that GelE is responsible for the processing of AtlA to the 62 kDa product. To further examine the GelE-AtlA interaction, Thomas *et al.* incubated recombinant AtlA with purified GelE and AtlA was processed into many minor fragments (Thomas *et al.*, 2009). As opposed to the autolytic profile studies, the results from the recombinant protein incubation suggest that purified GelE cleaves AtlA at multiple cleavage sites resulting in the minor

fragments of AtlA. Based on MALDI-TOF MS/MS peptide mass mapping, GelE initially cleaves within the N-terminal region of AtlA and then further cleaves within the C-terminal region of AtlA (Thomas *et al.*, 2009). The Hancock laboratory postulated that GelE cleavage of AtlA would localize AtlA to other parts of the cell besides the cell septum (Thomas *et al.*, 2009).

In addition, upon *gelE* inactivation, higher levels of a *E. faecalis* secreted protein, SalB, were found in the supernatant, suggesting a relationship between GelE and SalB (Shankar *et al.*, 2012). A double *gelE* and *salB* deletion strain demonstrated an increase in septum misplacement compared to a *salB* deletion alone (Shankar *et al.*, 2012). Similarly, a double *gelE* and *salB* deletion strain displayed increased clumping under fluorescence laser scanning microscopy compared to the *salB* deletion alone (Shankar *et al.*, 2012). Based on these experiments, there appears to be a relationship between SalB and GelE, which has yet to be determined.

Overall, these results suggest that GelE is responsible for the cleavage of various secreted *E. faecalis* proteins. The cleavage of these proteins by GelE could either impact the function of the protein or remove the protein from the extracellular environment or cell surface when no longer needed.

1.4.1.2 SprE

The *sprE* gene is located downstream of the *gelE* gene. Expression of SprE is regulated by the fsr-quorum sensing system (Figure 1.1) and *sprE* is co-transcribed with *gelE.* Due to polar effects on downstream transcription, an insertion mutation in *gelE* impacts the expression of *sprE* (Qin *et al.*, 2000).

SprE is a serine protease that is classified within the gluatmyl endopeptidsase I staphylococcal group (Stennicke *et al.*, 1998, Kawalec *et al.*, 2005). SprE shares homology with the V8 protease in *S. aureus* (Qin *et al.*, 2000). SprE has a molecular mass of 25 kDa. Kawalec

et al. demonstrated that GelE is responsible for processing of SprE and that in the absence of GelE, SprE is in a "superactive" form (Kawalec *et al.*, 2005).

1.4.1.2a Functions of SprE

Of the two proteases discussed, GelE has been more frequently studied. Due to the lack of SprE expression in a *gelE* insertion mutant, it was difficult to distinguish the impact of each protease on pathogenesis. For example, work by Singh *et al.* demonstrated that a *gelE* insertion mutant (GelE-, SprE-) had a significant decrease in endocarditis induction rate compared to wild-type OG1RF (Singh *et al.*, 2005). Since the *gelE* insertion mutant did not express SprE, the role of each protease in pathogenesis was unconfirmed. In order to understand the role of each protease, in-frame deletion mutants of *gelE* and *sprE* were analyzed (Thurlow *et al.*, 2010, Thomas *et al.*, 2008). Upon analysis of these mutants, it was determined that GelE and not SprE is necessary for endocarditis pathogenesis (Thurlow *et al.*, 2010). In addition, Thurlow *et al.* demonstrated that SprE cleaves C5a at a molar ratio of 2:1 (C5a to SprE), but does not cleave C5a at any lower concentrations, suggesting that SprE is not involved in cleavage of host immune response components (Thurlow *et al.*, 2010).

Thomas *et al.* observed that a Δ*sprE* strain had a higher autolytic rate compared to wild-type OG1RF (Thomas *et al.*, 2008). Furthermore, they observed a ~2-fold higher amount of released eDNA in the Δ*sprE* strain compared to OG1RF (Thomas *et al.*, 2008). These data suggest that SprE is a negative regulator of autolysis. Upon analysis of biofilm formation, Thomas *et al.* saw that the Δ*sprE* strain developed a denser biofilm layer compared to the wild-type strain and looked consistent with quick biofilm growth (Thomas *et al.*, 2008). Based on these results, Thomas *et al.* postulated that SprE negatively regulates GelE activity, which would prevent early biofilm maturation (Thomas *et al.*, 2008).

Due to the impact of SprE on autolysis, the impact of SprE on AtlA was examined. Upon purified SprE incubation with recombinant AtlA, AtlA was processed into a major band of approximately 62 kDa (Thomas *et al.*, 2009). In opposition to these results and as previously discussed, analysis of the autolytic profile of OG1RF and Δ*gelE* strains demonstrated that GelE was responsible for cleavage of AtlA to the 62 kDa fragment (Thomas *et al.*, 2009). Based on MALDI-TOF MS/MS peptide mass mapping, SprE cleaves AtlA within the N-terminal region (Thomas *et al.*, 2009). Thomas *et al.* postulated that SprE cleavage of AtlA could allow AtlA to localize to the cell septum (Thomas *et al.*, 2009). Ultimately, they proposed a model where SprE modifies the surface associated AtlA, preventing further GelE-AtlA modification and subsequent cell lysis and death (Thomas *et al.*, 2009).

1.5 Exoproteins and their impact on the virulence of *E. faecalis*

Virulence of *E. faecalis* is complex and, as such, is associated with a multitude of factors (Marra *et al.*, 2006). Some of the *E. faecalis* virulence factors that are classically studied include: gelatinase, *Enterococcus* surface protein (Esp), aggregation substance (AS), adhesins, Ace, Ebp pili, and sex pheromones (Rich *et al.*, 1999, Kayaoglu & Orstavik, 2004, Comerlato *et al.*, 2013, Nallapareddy *et al.*, 2006). Frequently, specific infection locations involve different virulence determinants (Baldassarri *et al.*, 2004). There are a broad range of virulence factors that influence the ability of *E. faecalis* to grow and invade the host. This dissertation focuses on two extracellular proteins of *E. faecalis*: AtlA and SalB, and further examines how their interaction with GelE influences the virulence of *E. faecalis*.

1.5.1 AtlA

E. faecalis secretes three autolysins, AtlA, AtlB, and AtlC, among which, AtlA is the

major peptidoglycan hydrolase. AtlB and AtlC are not present in all enterococcal strains because *atlb* and *atlc* genes are located on putative prophages (Mesnage *et al.*, 2008). The wild-type *E. faecalis* strain, OG1RF, used in this work, only contains AtlA. In a study by Mesnage *et al.,* the authors proposed that AtlB has similar functions to AtlA, and AtlB can carry out compensatory cellular functions upon inactivation of AtlA. Deletion of AtlC caused no change in cell morphology, suggesting that AtlC does not carry out similar functions to AtlA (Mesnage *et al.*, 2008). This dissertation will primarily concentrate on AtlA.

1.5.1.1 Functions of AtlA

While AtlA carries out multiple cellular functions, one of its primary roles is in cellular division. As a bacterial cell grows and divides, the cellular septum is formed to separate the two new cells (Weiss, 2004). At the last stage of cell division, hydrolases such as AtlA cleave the newly formed peptidoglycan separating the cell into two new daughter cells (Yamada *et al.*, 1996, Ohnishi *et al.*, 1999). Specifically, AtlA is a *N*-acetylglucosaminidase, cleaving the glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) in glycan strands (Eckert *et al.*, 2006). An *atlA* deletion grows in long chains, where the dividing cells remain connected at their cell septa (Qin *et al.*, 1998, Mesnage *et al.*, 2008).

In addition, AtlA has been shown to be important for biofilm formation and autolysis activity. Autolysis is the ability of the cell to lyse itself with its own enzymes. Upon deletion of *atlA,* no autolysis activity can be detected, suggesting that AtlA is the primary *E. faecalis* autolysin (Qin *et al.*, 1998). Deletion of *atlA* resulted in decreased biofilm development (Kristich *et al.*, 2008, Mohamed *et al.*, 2004, Qin *et al.*, 1998). As AtlA lyses the *E. faecalis* cell, eDNA is released and begins to form the biofilm extracellular matrix (Thomas *et al.*, 2009). Thus, AtlA is important for cell separation, biofilm formation, and autolysis activity. However, it is not currently known how AtlA is regulated.

1.5.1.2 Domain organization of AtlA

Based on sequence comparisons, AtlA is composed of a signal peptide and three domains: Domain I, Domain II, and Domain III (Figure 1.2) (Eckert *et al.*, 2006). The function of Domain I remains largely uncharacterized, but is a threonine and glutamic acid (T/E) rich region. Domain I does not appear to have an effect on enzymatic activity since truncation of Domain I does not change the enzymatic activity level compared to full-length AtlA (Eckert *et al.*, 2006). These observations suggest that Domain I does not serve as a propeptide. Domain II is the only domain of AtlA that has demonstrated enzymatic activity (Eckert *et al.*, 2006). The catalytic activity of Domain II is lower than the catalytic activity of full-length AtlA, suggesting that the presence of either the N- or C-terminal region is necessary for full enzymatic activity (Eckert *et al.*, 2006). Domain III contains six lysine motif (LysM) modules of approximately 50 amino acids in length. The LysM domain is conserved across most bacteria and is necessary for binding to peptidoglycan. Domain III anchors AtlA to the cell wall (Buist *et al.*, 2008, Eckert *et al.*, 2006). Eckert *et al.* tested different domain truncations of AtlA for their enzymatic activity. Truncation of Domain III from AtlA caused a marked reduction in enzymatic activity, suggesting that the ability to bind to the cell is necessary for protein function (Eckert *et al.*, 2006). Domain III is also thought to anchor AtlA to specific locations both at the cell septum and at cell poles (Buist *et al.*, 2008). A recent study demonstrated that the presence of multiple LysM modules increase peptidoglycan binding affinity (Mesnage *et al.*, 2014). In this dissertation, I have determined that the GelE cleavage site on AtlA occurs between Ala 173 and Leu 174 within Domain I, 7 amino acids from the start of Domain II. GelE-cleavage of AtlA was found to impact AtlA localization to the cell septum and to encourage cell separation. The possibility exists that Domain I might be necessary for localization of AtlA to other regions of the cell or for prevention of AtlA binding to the cell septum until the cell is ready for division.

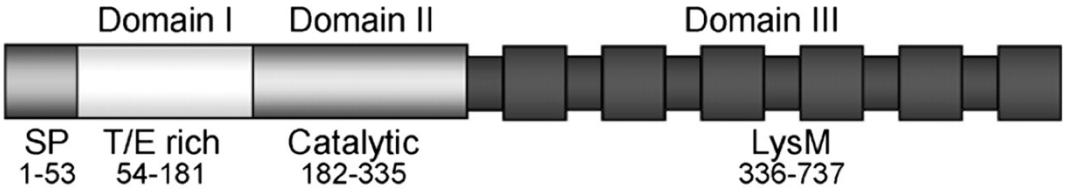


Figure 1.2: Domain organization of AtlA. AtlA is composed of three domains: Domain I, Domain II, and Domain III. The function of Domain I is poorly understood. Domain II contains catalytic activity. Domain III contains six LysM modules that allow AtlA to bind to peptidoglycan. This figure was reproduced and adapted from: Eckert, C., M. Lecerf, L. Dubost, M. Arthur and S. Mesnage (2006). Functional analysis of AtlA, the major N-acetylglucosaminidase of *Enterococcus faecalis*. J Bacteriol 188(24): 8513-8519. Permission to use this figure was granted by Copyright Clearance Center (License number: 11618320).

1.5.1.3 Interaction of AtlA with GelE and SprE

Much evidence points to GelE interacting with AtlA. Analysis of the lytic activity of OG1RF against *M. lysodeikticus* cells showed AtlA as having two major bands: one at 62 kDa and one at 72 kDa (Eckert *et al.*, 2006). Both bands were absent when analyzing the lytic activity of OG1RFΔ*atlA* (Eckert *et al.*, 2006). As discussed, both *gelE* and *atlA* deletions display a long chain phenotype, though an *atlA* deletion exhibits longer cellular chains (Qin *et al.*, 1998, Waters *et al.*, 2003). Interestingly, upon the complementation of *gelE* into a *gelE* deletion strain, the cells appeared either singly or in pairs, further supporting a role for GelE in cell division (Waters *et al.*, 2003). Prior studies have examined the presence of GelE in clinical isolates and how this affected chain length. Arias *et al.* reported that neither the presence nor the absence of GelE impacted chain length (Arias *et al.*, 2007). One possible explanation for this discrepancy could be that the clinical isolates examined might lack AtlA expression. This is consistent with the hypothesis that the interaction between GelE and AtlA impacts cellular chaining.

As previously discussed, the Hancock laboratory demonstrated that purified GelE cleaved recombinant AtlA into multiple fragments while purified SprE processed recombinant AtlA into one single fragment (Thomas *et al.*, 2009). These results seemed to be in contradiction to analysis of the autolytic profiles of the OG1RF and Δ*gelE* strains, which demonstrated that the presence of GelE resulted in a single fragment (Thomas *et al.*, 2009). Regardless of these conflicting results, the Hancock lab concluded that GelE processes AtlA.

Not only does GelE directly interact with AtlA, but it also appears that GelE impacts the function of AtlA. In addition to cell separation, one of the other major functions of AtlA is that of autolysis. Deletion of *atlA* resulted in no autolytic activity while deletion of *gelE* resulted in reduced autolytic activity compared to the wild-type OG1RF autolytic activity (Qin *et al.*, 1998,

Waters *et al.*, 2003). These results suggest that interaction between GelE and AtlA could also affect the autolytic function of AtlA.

The Hancock laboratory suggested that GelE-mediated cleavage of AtlA releases AtlA from peptidoglycan allowing AtlA to localize to non-septal areas. The authors propose that SprE, not GelE, is important for AtlA localization to the cell septum (Thomas *et al.*, 2009). As part of this thesis project, the primary GelE cleavage site of AtlA within Domain I between Ala 173 and Leu 174 was determined via N-terminal sequencing. Not only have I been able to identify the GelE cleavage site, but I will show that cleavage of AtlA by GelE is important for AtlA localization to the cell septum, which can be tracked with in house generated anti-AtlA monoclonal antibodies that are able to delineate cleaved and uncleaved AtlA. In addition, GelE-mediated cleavage of AtlA will be shown to regulate cell separation by cleaving long chaining cells into single or paired cells.

1.5.2 SalB

SalB is secreted in response to stress (Le Breton *et al.*, 2003). The activity of SalB allows the cell to be resistant to different stressors such as ethanol, peroxide, heat, and low pH (Rince *et al.*, 2003). SalB expression is regulated by the CroRS two-component system (Muller *et al.*, 2006). The histidine-kinase, CroS, and the response regulator, CroR, constitute the CroRS system (Muller *et al.*, 2006). As shown via an EMSA, purified CroR bound to the *salB* promoter. Specifically, only the phosphorylated form of CroR bound to this promoter region (Muller *et al.*, 2006).

The CroRS system is involved in β-lactam resistance (Comenge *et al.*, 2003). To determine if SalB was involved in CroRS β-lactam resistance, the sensitivity of a *salB* deletion strain against several cell-wall antimicrobials was examined. The *salB* deletion strain displayed

resistance levels close to that of a wild-type strain (Muller *et al.*, 2006). Overall, this suggests that SalB is not a major factor in CroRS-mediated β-lactam resistance.

SalB is composed of a signal peptide region followed by an N-terminal region that shares sequence homology with SagA from *E. faecium* and the PcsB-like protein from *Streptococcus* species. The central domain of SalB is predominantly composed of the amino acids serine, threonine, and proline. Specifically, 46 out of 81 amino acids are one of those three amino acids, which does not represent any known motif (Muller *et al.*, 2006). The C-terminal region shows homology with proteins that contain a Lys7 domain, necessary for binding to peptidoglycan (Muller *et al.*, 2006, Birkeland, 1994). SagA has been shown to be associated with the cell wall (Teng *et al.*, 2003) and both SagA and PcsB are thought to be peptidoglycan hydrolases (Rangan *et al.*, 2016, Yang *et al.*, 2011, Bartual *et al.*, 2014). PcsB has been shown to interact with a cell division protein FtsX and this interaction might be necessary for peptidoglycan hydrolase activity (Sham *et al.*, 2011).

A *salB* deletion strain shows a decrease in biofilm formation compared to the wild-type strain, OG1RF. It was also noted that SalB appears to have an effect on biofilm formation at the primary adhesion stage (Mohamed *et al.*, 2006). In addition, a *salB* deletion displays morphological defects. For example, the *salB* deletion strains grows in cell clumps and has misaligned cell septa (Le Breton *et al.*, 2003, Shankar *et al.*, 2012). Since the homolog PcsB is a peptidoglycan hydrolase, it was proposed that SalB might also be a peptidoglycan hydrolase, but it was determined that SalB had no significant peptidoglycan hydrolase activity. In addition, Shankar *et al*. examined the autolysis activity of a *salB* deletion strain and observed an increase in the autolysis rate compared to the wild-type strain (Shankar *et al.*, 2012). Overall, it appears that SalB has a profound impact on cell division via cell septum placement and cell morphology.

Upon *gelE* inactivation, higher levels of SalB were found in the supernatant, suggesting a relationship between GelE and SalB (Shankar *et al.*, 2012). A double *gelE* and *salB* deletion strain demonstrated an increase in septum misplacement compared to a *salB* deletion alone (Shankar *et al.*, 2012). Similarly, a double *gelE* and *salB* deletion strain displayed increased clumping under fluorescence laser scanning microscopy compared to the *salB* deletion alone (Shankar *et al.*, 2012). Based on these experiments, there appears to be a relationship between SalB and GelE, which has yet to be determined. Herein, I hypothesize that GelE cleaves SalB, which subsequently affects the function of SalB. Initially, due to the impact of SalB on autolysis and cell division, I believed that SalB could have an effect on either the autolysis activity or the localization of AtlA to the cell septum.

1.6 Motivation, rationale, and organization of this dissertation

The overall objective of my dissertation project has been to determine how GelE cleavage of two secreted proteins, AtlA and SalB, alters their function. I hypothesized that GelE regulates cell division by processing both AtlA and SalB, affecting their cellular function in regards to division. The methodology used in this project is explained in Chapter 2. Discussed in Chapter 3 is the impact of GelE cleavage on AtlA function, particularly in cell division and localization to the cell septum. The relationship between GelE and SalB and accumulation of SalB in the supernatant in an *E. faecalis* Δ*gelE* strain is explored in Chapter 4. Proposed experiments and the future direction of the project will be examined in Chapter 5. I anticipate that in elucidating the role GelE processing plays on AtlA and SalB, this work will provide further insight into the regulation of cell division.

Chapter 2: Materials and Methods

Chemicals, strains and growth conditions

All chemicals were purchased from Sigma (St. Louis, MO). All culture media were purchased from Difco Laboratories. Strains used in this study are listed in Table 2.1. Brain heart infusion (BHI) and Luria broth (LB) were prepared based on manufacturer instructions (Becton, Dickinson). For semisolid media, Bactor agar was used. Lipoteichoic acid (LTA) was purchased from Sigma Aldrich and is isolated from *Bacillus subtilis*.

Enterococcus strains were grown in BHI broth or BHI agar plates and incubated at 37°C. If needed, growth medium was supplemented with the following antibiotics: 100 μg/mL ampicillin, 200 μg/mL gentamicin, 100 μg/mL rifampicin, 25 μg/mL fusidic acid, and 2000 μg/mL kanamycin.

E. coli strains used for protein purification were grown in LB medium at 37°C and supplemented with 50 μg/mL kanamycin and 100 μg/mL ampicillin as needed. Cells were induced with 0.5 M isopropyl- β - D-galactopyranoside (IPTG).

DNA Manipulation and analysis

Standard methods or manufacturers' instructions were used to isolate DNA from and for transformations. Plasmid DNA was purified with a QIAprep Spin miniprep kit. DNA fragments were purified with either a QIAquick Gel Extraction kit or a QIAquick PCR purification kit. Lone Star Labs (Houston, TX) carried out genetic sequencing. Oligonucleotides were obtained from Sigma-Aldrich (St. Louis, MO) and are listed in Table 2.2. Restriction enzymes were purchased from New England BioLabs, Inc (NEB). Phusion high-fidelity DNA polymerase and

Taq DNA polymerase were used for PCR. In order to verify correct construction, all final versions were subjected to sequencing.

Construction of OG1RFΔ*atlA*Δ*gelE* occurred with the assistance of Maria Camila Montealegre Ortiz from the Murray Laboratory. To construct OG1RFΔ*atlA*Δ*gelE,* the primers, AtlAUpFor/AtlAUpRev with a BamHI site and AtlADownFor/AtlADownRev with an EcoRI site, were used to amplify sequences upstream and downstream of *atlA*. Using crossover PCR, the upstream and downstream sequences were joined and a 5' BamHI site and 3' EcoRI site was made. Using those sites, the resulting sequence was inserted into the pHOU1 vector (Panesso *et al.*, 2011). This plasmid was then electroporated into *E. faecalis* CK111, which was then filter mated with *E. faecalis* OG1RF*ΔgelE*. Colonies were cultured on MM9YEG plates supplemented with 10 mM p-chloro-phenylalanine to select for the OG1RF*ΔatlAΔgelE* double deletion mutant. Verification of double deletion was carried out with two sets of primers outside the AtlA deleted region and by using pulsed field gel electrophoresis (PFGE).

Protein construction, expression, and purification

His_6-AtlA and His_6-SalB and derivatives thereof were expressed from pQE30 in *E. coli* strain M15 pREP4. Briefly, primers corresponding to the gene of interest (listed in Table 2.2) with BamH1 and Kpn1 sites were used to amplify the region from OG1RF by PCR and ligated into pQE30 (Qiagen, Inc.). This plasmid was then transformed into M15 *Esherichia coli* harboring pREP4 (Qiagen, Inc.). For expression and purification, cells were grown to an optical density (OD_{600}) of 0.5 and then induced with 0.5 M IPTG for 2.5 hours at 37°C. Cells were harvested by centrifugation (10,000g x 10 min) and cell pellets were stored at -20°C. Cell pellets were then resuspended in BugBuster reagent (EMD Millipore) and Benzonase. The cell suspension was then incubated for 15 minutes at room temperature while mixing. Lysates were

centrifuged at 16,000 X g for 20 minutes at 4°C. Lysates were then poured into gravity-flow nickel columns and allowed to flow through. The column was washed with phosphate buffered saline (PBS) with 20 mM imidazole. His-tagged proteins were eluted in 1 mL fractions with PBS with 0.1 M EDTA. Similar protein concentrations were pooled together and were dialyzed three times at 4°C for a total of 20 hours in PBS. Recombinant protein concentrations were determined using a NanoDrop spectrophotometer ND-1000 (Thermo-Fisher Scientific) and purity was determined by immunoblot. In order to prevent cleavage of recombinant proteins, 50 μL aliquots of purified proteins were stored in the -20°C freezer, used once and disregarded.

Monoclonal antibodies generation

Monoclonal antibodies (mAbs) were generated as previously described (Pinkston *et al.*, 2011, Gao *et al.*, 2010). Recombinant proteins of interest (either AtlA or SalB) were used to immunize BALB/c mice according to standard protocols (Murray *et al.*, 1993, Kohler & Milstein, 1975). Then, the spleen cells from the mouse were collected and fused with SP2/0 mouse myeloma cells to make hyrbridomas (Kohler & Milstein, 1975). MAbs were selected for binding specificity to the recombinant proteins of interest via ELISAs and harvested. Surface plasmon resonance carried out on a Biacore T100 (GE Healthcare) was used to screen for the highest affinity clones (Canziani *et al.*, 2004). Briefly, a CM5 chip coated with goat anti-mouse IgG was used to capture anti-AtlA or anti-SalB mAbs from hybridoma culture supernatants. Then 10 μg/mL recombinant protein of interest (either AtlA or SalB) was evaluated for binding to the mAbs. The kinetic rate constants and affinity can be calculated from this result.

Protein Analysis

Samples (purified proteins, supernatants, cell pellets, etc) were resuspended in SDS loading buffer (0.5% bromophenol blue, 10% SDS, 25% glycerol, 0.5 M Tris-HCl - pH 6.8) and heated to 95°C for 10 minutes. Then samples were separated by SDS-PAGE in a Mini-PROTEAN 3 cell (Bio-Rad) with 10% or 4% to 12% Novex Bis-Tris (Invitrogen) polyacrylamide gels under reducing conditions.

In order to visualize protein, gels were stained with Coomassie protein strain (0.2% Coomassie, 40% methanol, and 10% acetic acid) for one hour followed by destaining solution (40% methanol and 10% acetic acid) until proteins were visible.

For Western Blot analysis, after separation of proteins on polyacrylamide gels, proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Milipore) using a XCell II Blot Module (Invitrogen). Prior to blocking, protein levels on the PVDF membranes were viewed by staining with Ponceau S (Amresco) in 5% acetic acid. Blocking was carried out with 5% Blotto (milk-PBST). 3 wash steps were carried out with PBST between each step. Monoclonal primary antibodies were purified as described above. Primary antibody of interest at 10 μg/mL in 5% Blotto were used to probe the membrane. Horseradish peroxidase (HRP) conjugated goat-derived anti-mouse IgG secondary antibody (Jackson Immunoresearch) was used to probe the membrane at a 1:5,000 dilution in 5% Blotto. TMB membrane peroxidase substrate system kit (KPL) was used to detect horseradish peroxidase (HRP) bound secondary antibody.

For ELISA analysis, as previously described (Gao *et al.*, 2010), recombinant proteins of interest (10 μg/mL) were coated overnight in 50 mM carbonate buffer, pH 9.6, at 4°C onto a 96-well Microlon 600 ELISA microtitier plate (Greiner Bio-one, NC). Following 3 washes with PBS-0.2% Tween (PBST), wells were blocked for nonspecific binding with 5% milk in PBST

for 1 hour at room temperature. Following blocking, wells were washed three times with PBST. For primary antibody binding, 100 μL of 10 μg/mL of the mAb of interest in 5% milk-PBST was added to each well and incubated at room temperature for one hour. Wells were washed three times with PBST followed by incubation of 100 μL per well of a 1: 3,000 dilution of goat-derived anti-mouse IgG conjugated with HRP (Jackson Immunoresearch) in 5% milk-PBST for one hour at room temperature. Following a final wash of three times with PBST, tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for 10-15 minutes. TMB substrate was stopped by the addition of 2 M H_2SO_4. An optical density reading at 450nm (OD_{450}) was used to measure the absorbance. All ELISA data was analyzed with Microsoft Excel.

To precipitate proteins, trichloracetic acid (TCA) precipitation was used. One fourth the volume of sample of TCA was added to bacterial supernatant and incubated for 10 minutes at 4°C. Following, incubation the sample was centrifuged for 5 minutes at 14,000 rpm. The resulting pellet was washed with 200 μL cold acetone and the centrifuged for 5 minutes at 14,000 rpm. The acetone wash step was repeated for a total of 2 times. Any excess acetone was removed by heating the pellet at 95°C for 5 minutes. Then the pellet was subjected to SDS-PAGE analysis.

All protein concentrations (for recombinant proteins, antibody concentrations, etc.) were measured with a Nanodrop spectrophotometer ND-1000 (Thermo-Fisher Scientific).

Biacore analysis

Biacore anlaysis was carried out as previously described (Hall *et al.*, 2012). To determine if SalB and AtlA interact, association was monitored via a Biacore T100 instrument (GE Healthcare). First, a CM5 chip was coated with goat anti-mouse IgG (Fc-specific) (Jackson

ImmunoResearch) with an NHS/EDC Amine Coupling Kit (BR-1000-50; GE Healthcare). The running buffer used was HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 0.05% v/v polysorbate 20, pH 7.4). 10 μg/mL anti-AtlA mAb 44 or 88 was diluted in HBS-EP+ and applied to the coated chip surface. Then, 10 μg/mL recombinant AtlA or D2D3 was diluted into HBS-EP+ and bound to the anti-AtlA mAbs. Both recombinant AtlA and D2D3 showed binding to the anti-AtlA mAbs. Then, 10 μg/mL recombinant SalB was diluted in HBS-EP+ and association to either recombinant AtlA or D2D3 was determined. Flow cells were regenerated with 100 mM phosphoric acid. All samples were applied in duplicate.

Gelatinase-cleavage assays

All cleavage assays used purified GelE. Purified GelE was obtained from *E. faecalis* OG1RF*ΔsprE* as described previously (Hancock & Perego, 2004, Makinen *et al.*, 1989, Pinkston *et al.*, 2011). Briefly, cells were grown to stationary phase and the culture medium was filtered with a 0.45 and then a 0.22 μm pore-size filter. Proteins were precipitated overnight at 4°C by the addition of ammonium sulfate crystals to a saturation of 60%. After centrifugation, the protein pellet was dialyzed overnight at 4°C in 50 mM Tris-HCl-1 mM $CaCl_2$, pH 7.8. Another concentration step was carried out with a Millipore stirred-cell concentrator with a 10,000-molecular weight cutoff membrane. This was then applied to a HiPRep 16/60 Sephacryl S-200 high-resolution column (GE Healthcare) and test for gelatinase activity via gelatin agar palates (Singh *et al.*, 2005).

Cleavage reactions were carried out by incubating 3 μg of recombinant protein of interest (either SalB or AtlA) and various amounts of purified GelE (range, from 0 to 1.5 μg) in 0.1 ml of PBS at 37°C for 30 minutes. In order to stop GelE protease activity, SDS-PAGE

sample buffer with 2-mercaptoethanol was added to the samples and heated to 95°C for 5 minutes. As controls, purified GelE (1.5 μg) and recombinant proteins of interest (3 μg) in 0.1 mL PBS were incubated in a similar manner. Samples were then separated by SDS-PAGE with 4 to 20% gels (NuSep, Inc.) and stained with Coomassie Brilliant blue protein staining (Sigma).

N-terminal sequencing

To cleave recombinant AtlA, 10 μg/mL purified GelE was incubated in 0.1 mL PBS with 10 μg recombinant AtlA for 30 minutes at 37°C. In order to stop protease activity, SDS-PAGE sample buffer with 2-mercaptoethanol was added to the GelE-AtlA mix and boiled at 95°C for 5 minutes. Samples were separated by SDS-PAGE with 4-20% gradient gels (NuSep, Inc). Following manufacturer's protocols, the sample was transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Milipore). The samples were viewed with Ponceau stain and the band representing the cleaved AtlA was cut out and sent to the Protein Chemistry Laboratory at Texas A&M University (College Station, TX), where N-terminal sequencing was carried out.

Microscopy

To examine cellular chain length, measurements were determined as previously described with slight modifications (Arias *et al.*, 2007). Briefly, OG1RF, OG1RF*ΔatlA,* OG1RF*ΔatlAgelE,* or OG1RF*ΔsalB* cells were grown overnight in BHI + appropriate antibiotics and in the morning diluted to an OD_{600} of 0.1. The resulting cells were grown for 4 hours at 37°C while shaking at 240 RPM. At 3.5 hours, recombinant proteins of interest (full-length AtlA, AtlA', DII, DIII, or SalB) were incubated with either OG1RF*ΔatlA,* OG1RF*ΔatlAgelE,* or OG1RF*ΔsalB* cells at 5 μg/mL for 0.5 hour while shaking at 240 RPM. OG1RF, OG1RF*ΔatlA,*

OG1RF*ΔatlAgelE*, or OG1RF*ΔsalB* cells alone without the addition of recombinant proteins were incubated in parallel as controls. Cells were centrifuged and resuspended in 8 mL 0.9% NaCl. 10μL was spotted onto a glass slide and visualized under a light microscope at 1000x magnification. 50 randomly chosen chains were counted for number of cells per chain length. Based on that number, cells were categorized either as a short chainer (80% or more had 4 or less cells per chain length), a medium chainer (41 to 79% of cells had 4 or less cells per chain length), a long chainer (less than 40% of cells had less than 4 cells per chain length), or an extra-long chainer (more than 70% of cells had more than 10 cells per chain length) (Table 2.3).

Transmission electron microscopy (TEM) experiments were carried out as previously descried (Chang *et al.*, 2013, Pinkston *et al.*, 2014). *E. faecalis* cells (OG1RF, OG1RF*ΔgelE*, or OG1RF*ΔatlAΔgelE*) were grown to an OD_{600} of 1.0 and centrifuged. If recombinant proteins were added to *E. faecalis* cells, then prior to washing the cell pellets, 5 μg/mL of recombinant proteins were added for to the cell culture for 0.5 hour while shaking. Cells were then pelleted and washed with 0.1 M NaCl and resuspended in PBS. 5 μL of bacteria cells in PBS was pipetted on a nickel-coated carbon grid for 1 minute. The grid was washed 3 times with wash buffer (1% BSA in PBS). Then, the grid was blocked with 0.1% gelatin buffer for 1 hour at room temperature. After blocking, the sample was washed once with 1% BSA-PBS. For primary antibody labeling, the grid was then labeled with 10 μg/mL of mAb of interest in 1% BSA-PBS for 1 hour at room temperature. Following 3 wash steps with 1% BSA-PBS, the grid was blocked with 0.1% gelatins in PBS for 1 hour at room temperature. For secondary antibody binding, a 1:20 dilution of gold-conjugated anti-mouse IgG (Jackson ImmunoResearch) in 1% BSA-PBS was used to label the samples for 1 hour at room temperature. The samples were stained with 1% uranyl acetate in H_2O and imaged on a JEOL JEM-1400 electron microscope.

Flow Cytometry

As previously described (Gao *et al.*, 2010), *E. faecalis* cells in BHI + antibiotics were grown to an optical density (OD_{600}) of 0.4 and harvested by centrifugation at 10,000 g X 2 minutes. Cells were washed twice with 2% bovine serum albumin (BSA) phosphate buffered saline (PBS) (PBS-2%BSA). Following the wash step, the cells were labeled for 0.5 hour at room temperature with 10 μg/mL of the mAb against the protein of interest in PBS-2%BSA. After, primary antibody incubation, another wash step was carried out. The cells were then labeled with 1:100 R-phycoerythrin (PE) conjugated goat $F(ab')_2$ anti-mouse IgG (Jackson Immunoresearch) in PBS-2% BSA for 0.5 hour at room temperature in the dark. Following a wash step, the cells were fixed in 1% paraformaldehyde and examined with a Becton Dickenson FACSCalibur (BD Bioscience, CA). Data were analyzed with either WinMIDI or FlowJo software.

Peptidoglycan Hydrolysis Assay

Peptidoglycan purification and hydrolysis assays were carried out as previously described (Atrih & Foster, 1999, Eckert *et al.*, 2006)(Atrih 1999)(Eckert 2006). Briefly, peptidoglycan was purified from 500 mL of OG1RF Δ*atlA* grown to an OD_{600} of 0.7. In order to extract peptidoglycan, cells were harvested and the resulting pellet was treated with 14 mL 4% SDS for 30 minutes at 100°C. Following the SDS-treatment, peptidoglycan was washed five times with 20 mL of H_2O. Peptidoglycan was incubated overnight at 37°C with Pronase (200 μg/mL) in 1mL Tris-HCl (10 mM, pH 7.4) and with trypsin (200 μg/mL) in 1mL of phosphate buffer (20 mM, pH 7.8) In the morning, peptidoglycan was washed twice with 20 mL of H_2O and subsequently used in a peptidoglycan hydrolysis assay.

Peptidoglycan hydrolysis was determined by measuring the turbidity over 6 hours at an optical density of 450 nm on a Genesys 20 Spectrophotometer (Thermo Fisher). In order to digest peptidoglycan, peptidoglycan was resuspended in a 25 mM Tris-HCl, 100 mM NaCl buffer and 10 μg/mL recombinant proteins (AtlA or AtlA'), recombinant AtlA + purified GelE, or purified GelE alone were added. The optical density was measured every 30 minutes and the peptidoglycan mixture was incubated at 37°C while shaking. Microsoft Excel was used to analyze the data.

Strain Name	Genotype	Reference
OG1RF	Laboratory strain Fus^r Rif^r, p-Cl-Phe^r	(Murray *et al.*, 1993)
TX5264	OG1RF Δ*gelE*	(Sifri *et al.*, 2002)
JH2-2	Laboratory strain	(Jacob & Hobbs, 1974)
TX5127	OG1RF Δ*atlA*	(Qin *et al.*, 1998)
TX5243	OG1RF *sprE* insertion mutant [▽*sprE*]	(Qin *et al.*, 2000)
TX5471	OG1RF Δ*gelE*Δ*sprE*	(Pinkston *et al.*, 2011)
TX5266	OG1RF Δ*fsrB*	(Qin *et al.*, 2001)
	OG1RF Δ*atlA*Δ*gelE*	This study
TX5123	OG1RF Δ*salB*	(Mohamed *et al.*, 2006)
CK111	*E. faecalis* electroporation strain used for cloning	(Kristich *et al.*, 2007)
M15	*E. coli* host strain used for cloning	Qiagen, Inc.
EC1000	*E. coli* host strain used for cloning	(Leenhouts *et al.*, 1996)

Table 2.1: Strains used in this study.

Primer Name	Sequence
AtlA Up For	CGGGATCCTGACTGATTTTTCCGCTTG
AtlA Up Rev	CATTGCCACGTGACATTGATTCTTTCTTC
AtlA Down For	CAATGTCACGTGGCAATGCTTCTTCAAC
AtlA Down Rev	CGGAATTCGCTCCTTCAGAACGTCCA
AtlA Bam H1 For	GCAGGATCCACAGAAGAGCAGCCAACAAATGC
AtlA Kpn1 Rev	CTCCGGTACCTCATTAACCAACTTTTAAAGTTTGACC
AtlA D2 Bam H1 For	GCAGGATCCGAATTTATTGCCGAGTTAGCTCG
AtlA D3 BamH1 For	GCAGGATCCCCATCTTCTGGT
AtlA' BamH1 For	GCAGGATCCTTATCACCGACGCAAAGTCC
SalB BamH1 F	GGCCGGATCCGACAATGTTGATAAAAAAATTGAAG
SalB Kpn1 R	GCGCGGTACCCTATTAGGCTGAGTGTCCTACGATTG

Table 2.2: Primers used in this study.

Short chainer	≥80% of chains have ≤4 cells
Medium Chainer	41-79% of chains have ≤4 cells
Long chainer	≤40% of chains have ≤4 cells
Extra-long chainer	>70% of chains have ≥10 cells

Table 2.3: Qualification parameters for chain length experiments. This table is based upon the parameters described in Arias, C. A., L. Cortes and B. E. Murray (2007). Chaining in enterococci revisited: correlation between chain length and gelatinase phenotype, and gelE and fsrB genes among clinical isolates of *Enterococcus faecalis*. J Med Microbiol, 56(Pt 2): 286-288.

Chapter 3: GelE processing of AtlA impacts AtlA cell surface localization, altering the cellular chain length

NOTE: Kenneth Pinkston was responsible for the generation of the anti-AtlA mAb panel, which was used to examine surface expression of AtlA by Peng Gao, Ph.D. Dr. Gao is responsible for the discovery that cleaved AtlA localized to the cell septum and any electron microscopy was carried out with his help. OG1RF*ΔgelEΔsprE* was constructed with the help of Maria Camila Montealegre Ortiz, Ph.D.

Introduction

AtlA, the major peptidoglycan hydrolase of *E. faecalis,* plays a pivotal role in both cell separation during cell division and in cellular autolysis (Tomasz, 1974, Shockman *et al.*, 1996, Thomas *et al.*, 2009). OG1RFΔ*atlA* cells grow as long chains linked together at their septa because the dividing cells are unable to complete septum cleavage. In addition, OG1RFΔ*atlA* cells lack autolysis activity, and display a decreased ability to form biofilms (Qin *et al.*, 1998).

Gelatinase (GelE), a secreted zinc metalloprotease regulated by the Fsr quorum-sensing system, is a major virulence factor of *E. faecalis* known to be important in both cellular adherence and biofilm formation (Engelbert *et al.*, 2004, Mohamed *et al.*, 2004, Nakayama *et al.*, 2001, Teixeira *et al.*, 2013, Qin *et al.*, 2000). Additionally, GelE has been shown to play a role in the regulation of AtlA. OG1RFΔ*gelE* cells grow in chains similar to OG1RFΔ*atlA* cells, although chain lengths of OG1RFΔ*gelE* cells are not as long compared to those of OG1RFΔ*atlA* cells (Arias *et al.*, 2007). Not only does GelE impact chain length, but OG1RFΔ*gelE* cells also have a decreased autolytic ability (Waters *et al.*, 2003). Based on these observations, it is likely that GelE and AtlA interact. To that end, Thomas *et al.* proposed a model where GelE processes AtlA, allowing for the lysis of bacterial cells and consequently the formation of the biofilm by release of extracellular DNA (Thomas *et al.*, 2009, Thomas *et al.*, 2008). The purpose of this dissertation is to improve our understanding of the interaction between GelE and AtlA.

E. faecalis is known to have three autolysins: AtlA, AtlB and AtlC (Mesnage *et al.*, 2008, Beliveau *et al.*, 1991). AtlA is the major autolysin and this dissertation will mainly concentrate on it. The wild-type strain used in this work, OG1RF, only expresses AtlA (Bourgogne *et al.*, 2008). Through sequence comparison to other autolysins, it was determined that AtlA is composed of three domains. Domain I has no known function and is T/E rich, Domain II provides AtlA with its peptidoglycan hydrolysis activity, and Domain III contains six

LysM modules that allow for peptidoglycan recognition and binding (Eckert *et al.*, 2006, Mesnage *et al.*, 2014). Based on studies that examined the enzymatic activity of different domain truncations of AtlA, Domain III was deemed necessary for enzymatic activity, while Domain I did not confer any enzymatic activity. Based on these findings, Eckert *et al.* concluded that Domain III is necessary for bringing Domain II within close proximity to the peptidoglycan in order to carry out peptidoglycan cleavage (Eckert *et al.*, 2006).

This chapter presents a study of AtlA and GelE, in an effort to understand their interaction and the impact GelE has on the function of AtlA. I hypothesized that GelE-mediated cleavage of AtlA regulates the function of AtlA in cell division. An anti-AtlA monoclonal antibody panel was generated as a tool set to improve our understanding of the impact GelE-cleavage has on AtlA function. As the mAb panel was undergoing evaluation, it became apparent that anti-AtlA mAb 44 recognized both an N-terminally cleaved and full-length form of AtlA while anti-AtlA mAb 88 only recognized full-length AtlA. Using N-terminal sequencing, GelE was found to cleave AtlA at a single site, between Ala 173 and Leu 174 towards the terminus of Domain I. Using mAbs generated in the laboratory, I was able to determine that this cleavage event changed the localization of AtlA and impacted the chain length phenotype.

Specifically, I will show that GelE-cleaved AtlA localizes to the cell septum while full-length AtlA localizes randomly across the cell surface. In an effort to determine the effect of localization on cell division, the chain length was monitored in an OG1RFΔ*atlA*Δ*gelE* strain with the addition of either full-length or GelE-cleaved AtlA. The addition of recombinant GelE-cleaved AtlA to OG1RFΔ*atlA*Δ*gelE* caused the cells to display a short chain phenotype while the addition of recombinant full-length AtlA to OG1RFΔ*atlA*Δ*gelE* caused cells to maintain a chaining phenotype. This finding suggests that GelE cleavage of AtlA encouraged separation of

the dividing cells. Although I have determined that GelE cleavage is important for AtlA septal localization, further study of GelE-cleaved AtlA localization needs to be conducted to completely understand the mechanism mediating these events.

Results

Anti-AtlA mAb panel shows unique binding epitopes

As previously discussed, the Hancock laboratory has shown that GelE cleaves AtlA at an unknown site (Thomas *et al.*, 2009). In an effort to determine the impact of GelE-cleavage on AtlA function, an anti-AtlA monoclonal antibody panel was developed. While assessing the ability of the anti-AtlA mAb panel to bind to surface associated AtlA on both OG1RF and OG1RF*ΔgelE* strains via flow cytometry, two distinct binding profiles became apparent. As shown in Figure 3.1, when exponentially grown cells were analyzed for surface AtlA expression via flow cytometry, anti-AtlA mAb 44 recognized AtlA on the surface of both the wild-type OG1RF and the OG1RF*ΔgelE* strains. Contrary, anti-AtlA mAb 88 only recognized AtlA on the surface of the OG1RF*ΔgelE* strain. As a negative control, OG1RF*ΔatlA* showed less anti-AtlA mAb binding to the surface, which was at a similar level as a secondary antibody only control.

To determine which domain within AtlA was recognized by each antibody, recombinant full-length AtlA, recombinant AtlA domain II, III (D2D3) and recombinant AtlA domain III (D3) were constructed, expressed, and purified (Figure 3.2A). Then, using ELISA, anti-AtlA mAb 44 and mAb 88 were evaluated for their ability to bind to these three different recombinant protein versions of AtlA (Figure 3.2B). Anti-AtlA mAb 88 bound to full-length recombinant AtlA, but not recombinant D2D3 or recombinant D3, suggesting that mAb 88 binds within Domain I of AtlA. Anti-AtlA mAb 44 bound to both recombinant full-length AtlA as well as to recombinant D2D3, but not recombinant D3, suggesting that mAb 44 binds within Domain II of AtlA. As controls, recombinant Ace, another *E. faecalis* surface protein, and an anti-Ace mAb (mAb 70) were used. Recombinant Ace shows no binding to anti-AtlA mAbs while anti-Ace mAb 70 shows no specific binding to recombinant versions of AtlA.

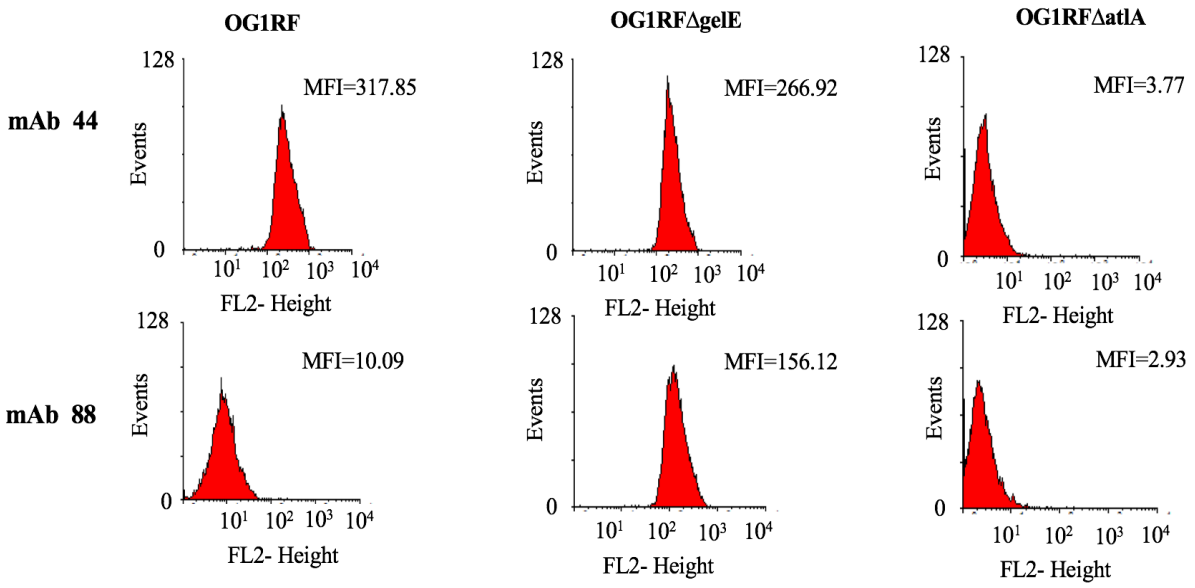


Figure 3.1: AtlA surface binding analysis of OG1RF, OG1RF△*gelE*, and OG1RF△*atlA* strains with anti-AtlA mAbs 44 and 88. Exponentially grown wild-type OG1RF, OG1RF△*gelE*, and OG1RF△*atlA* strains were analyzed for AtlA surface expression via flow cytometry with either anti-AtlA mAb 44 or mAb 88 as the primary antibody. In the wild-type OG1RF strain (GelE+), only mAb 44 was able to recognize AtlA on the surface. In the OG1RF△*gelE* strain (GelE-), both mAb 44 and mAb 88 were able to recognize AtlA on the cell surface. The OG1RF△*atlA* strain was used as a negative control to show no anti-AtlA mAb binding. Kenneth Pinkston was responsible for the generation of anti-AtlA mAbs, Peng Gao, Ph.D., carried out the first AtlA surface analysis with those antibodies, and I carried out the repetitions of the AtlA surface analysis. Results shown are representative of three independent experiments.

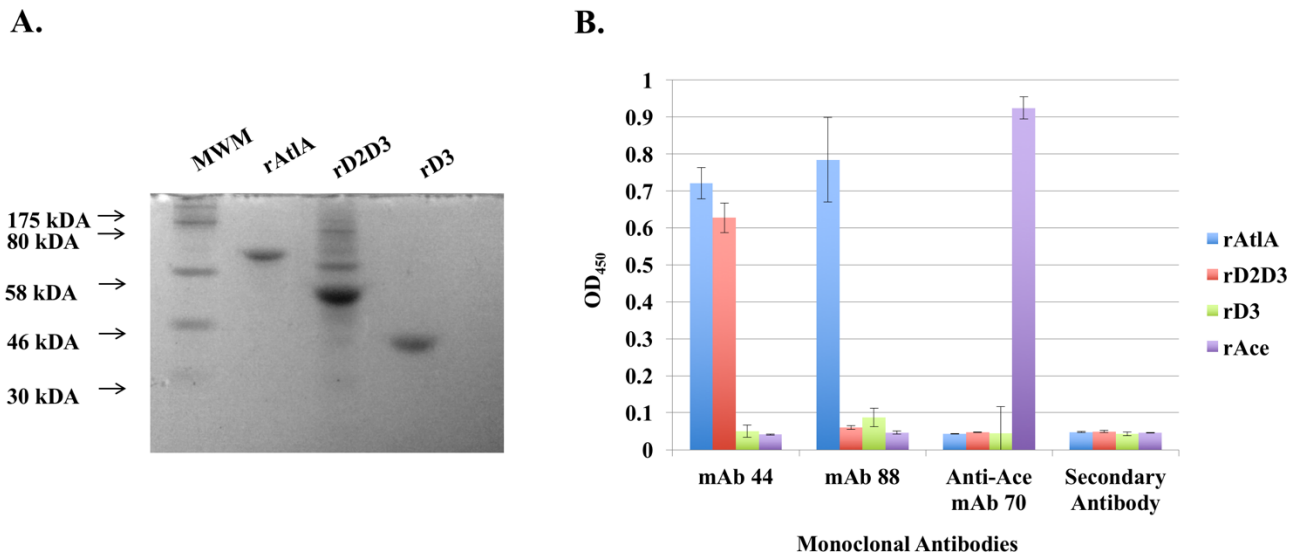


Figure 3.2: Anti-AtlA mAb 88 binds within Domain I while anti-AtlA mAb 44 binds within Domain II. (A) Recombinant AtlA, D2D3 and D3 were expressed and purified. Lanes represent 10 μg/mL of each recombinant protein. Proteins were visualized by Coomassie Brilliant Blue staining. Molecular mass standards are indicated to the left in kilodaltons. (B) Anti-AtlA mAb 44 and 88 were evaluated for their ability to bind to recombinant full-length, D2D3, and D3 via ELISA analysis. Each recombinant protein was labeled with each anti-AtlA mAb, followed by goat anti-mouse IgG-HRP conjugate and developed with TMC substrate. Recombinant Ace, anti-Ace mAb 70 and secondary antibody alone were used as either positive or negative controls. Results shown are averages of three independent experiments, each performed in triplicate. Error bars represent one standard deviation of the mean.

Anti-AtlA mAbs differentiate between the GelE-dependent truncated form of AtlA and the full-length AtlA form

To evaluate protease processing of AtlA, AtlA was extracted from exponentially grown OG1RF, OG1RF*ΔgelE*, OG1RF ▽*sprE*, and OG1RF*ΔgelEΔsprE* strains and evaluated with anti-AtlA mAb 44 and anti-AtlA mAb 88 using Western blot analysis. The OG1RF ▽*sprE* and OG1RF*ΔgelEΔsprE* (SprE-) strains were included because SprE has previously been shown to process AtlA (Thomas *et al.*, 2009). In the evaluation, OG1RFΔ*atlA* was included as a negative control to demonstrate lack of anti-AtlA mAb binding in the absence of AtlA. Examining AtlA expression in the OG1RF and OG1RF ▽*sprE* strains (GelE+), anti-AtlA mAb 44 recognized a major band that was smaller than recombinant full-length AtlA and similar in size to a recombinant truncated form of AtlA (molecular weight is 59 kDa) (Figure 3.3A). Contrary, in the absence of GelE (OG1RF*ΔgelE* and OG1RF*ΔgelEΔsprE*), anti-AtlA mAb 44 recognized a major band consistent with recombinant full-length AtlA (molecular weight is 72 kDa) (Figure 3.3A). Upon examination of the OG1RF*ΔgelE* and OG1RF*ΔgelEΔsprE* strains with anti-AtlA mAb 88 as the primary antibody, a band equal in size to recombinant full-length AtlA was present (Figure 3.3B). OG1RF and OG1RF*ΔsprE* (GelE +) also displayed this same band, but at a lower intensity compared to the band seen in the OG1RF*ΔgelE* and OG1RF*ΔgelEΔsprE* strains (Figure 3.3B). Consistent with ELISA (Figure 3.2B) and flow cytometry (Figure 3.1) results, these results suggest that mAb 88 was only capable of binding full-length AtlA via Domain I, while mAb 44, via binding Domain II, was capable of binding the truncated version of AtlA, as identified only in the presence of GelE.

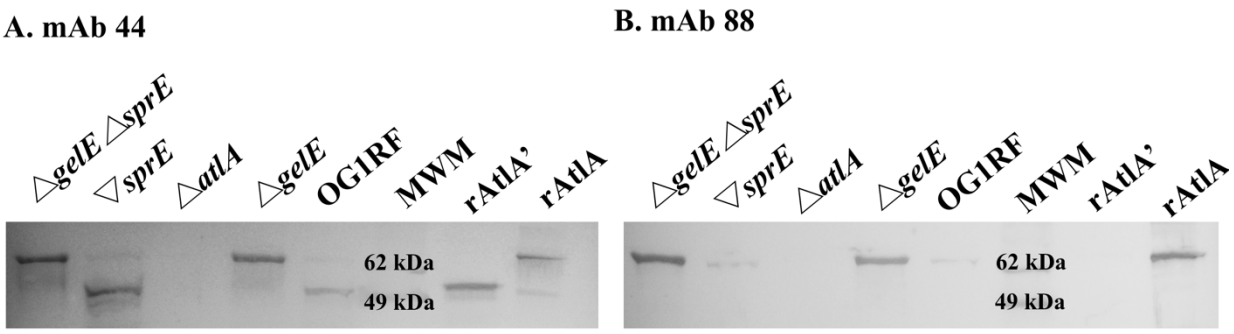


Figure 3.3: Western blot analysis of different *E. faecalis* strains with anti-AtlA mAb 44 and mAb 88. Exponential phase wild-type OG1RF, OG1RF△*gelE*, OG1RF△*atlA*, OG1RF▽*sprE*, and OG1RF△*sprE*△*gelE* strains were collected and centrifuged. In order to disassociate the surface associated AtlA, the pelleted cells were then boiled at 95ºC for 10 minutes in SDS-PAGE sample buffer. Either mAb 44 or mAb 88 were used as the primary antibody in Western blot analysis. As controls, recombinant AtlA and AtlA' are found in the right lanes. (A) Anti-AtlA mAb 44 shows binding to two separate bands, one representing the full-length AtlA and one representing a truncated form of AtlA. (B) Anti-AtlA mAb 88 shows binding to one band, representing the full-length AtlA. This result represents three independent experiments.

The presence of SprE does not impact AtlA surface association

To determine if SprE has an impact on AtlA processing and surface association, surface-associated AtlA in wild-type OG1RF, OG1RF*ΔgelE*, OG1RF*ΔgelEΔsprE*, and OG1RF ▽*sprE* strains was monitored by flow cytometry with anti-AtlA mAbs 44 and 88. As shown in Figure 3.4A, anti-AtlA mAb 44 displayed robust signal for all four strains. Conversely, anti-AtlA mAb 88 only showed robust signal for strains that do not express GelE (OG1RF*ΔgelE* and OG1RF*ΔgelEΔsprE*). OG1RF and OG1RF ▽*sprE* (GelE+) showed a decrease in mAb 88 signal (Figure 3.4A). These results suggest that there is no difference in the AtlA surface association pattern in the absence of SprE (OG1RF ▽*sprE* and OG1RF*ΔgelEΔsprE*).

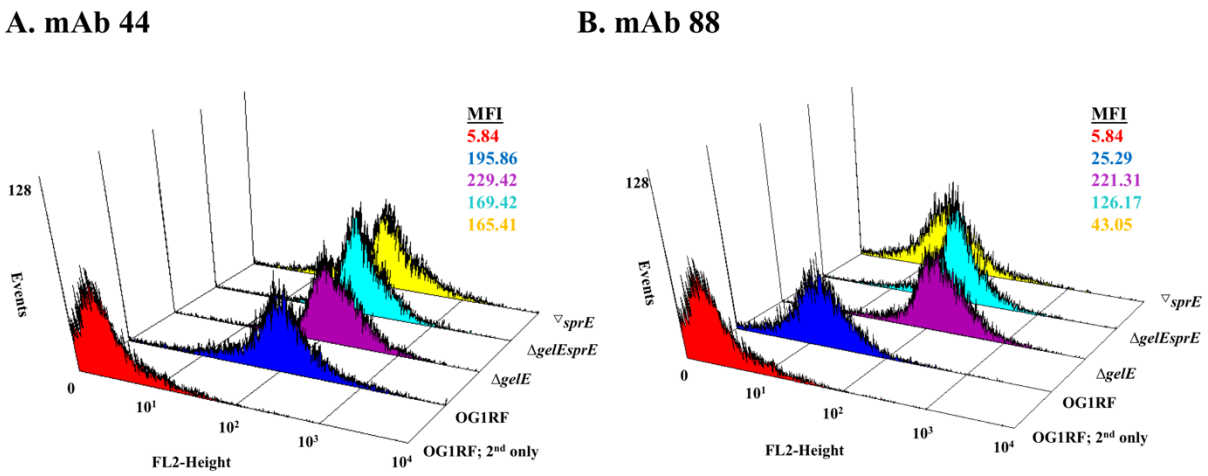


Figure 3.4: Strains that express GelE (OG1RF and OG1RF▽*sprE*) show a decrease in the mAb 88 signal of full-length AtlA. Flow cytometry analysis of exponentially grown wild-type OG1RF, OG1RF△*gelE*, OG1RF ▽*sprE*, and OG1RF△*gelE*△*sprE* strains. As a negative control, wild-type OG1RF cells were labeled with only secondary antibody. (A) Anti-AtlA mAb 44 was used as the primary antibody at 10 µg/mL. (B) Anti-AtlA mAb 88 was used as the primary antibody at 10 µg/mL. Results shown are representative of three independent experiments.

GelE cleavage of AtlA occurs between Ala 173 and Leu 174 within Domain I via N-terminal sequencing

Together Western blot (Figure 3.3) and flow cytometry analysis (Figure 3.1) of different *E. faecalis* strains strongly suggested that GelE cleavage of AtlA results in a N-terminally truncated version of AtlA that remains surface associated and that the mAb panel was capable of detecting the full-length and the GelE-truncated versions of AtlA. In an effort to determine at what concentration GelE cleavage of AtlA occurs, recombinant AtlA was incubated with different concentrations of GelE (ranging from 0 to 15 μg/mL) at 37°C for 30 minutes. Figure 3.5A shows that full-length AtlA begins to get cleaved by purified GelE at 0.15 μg/mL and is completely cleaved by 4.5 μg/mL.

To determine where within AtlA GelE cleavage occurs which generates a band at approximately 60 kDa, N-terminal sequencing analysis was employed. Recombinant AtlA was cleaved by 10 μg/mL purified GelE from *E. faecalis* OG1RF*ΔsprE* for 30 minutes at 37°C in order to isolate a clean cleavage product. The isolated approximately 60 kDa band of recombinant AtlA was subjected to N-terminal sequencing analysis. Based on the analysis, the GelE cleavage site occurs 7 amino acids from the previously defined start of Domain II, between Ala 173 and Leu 174 within Domain I (Figure 3.5B). Using the cleavage site information, we constructed, expressed, and purified recombinant cleaved AtlA (recombinant AtlA') for further functional analysis.

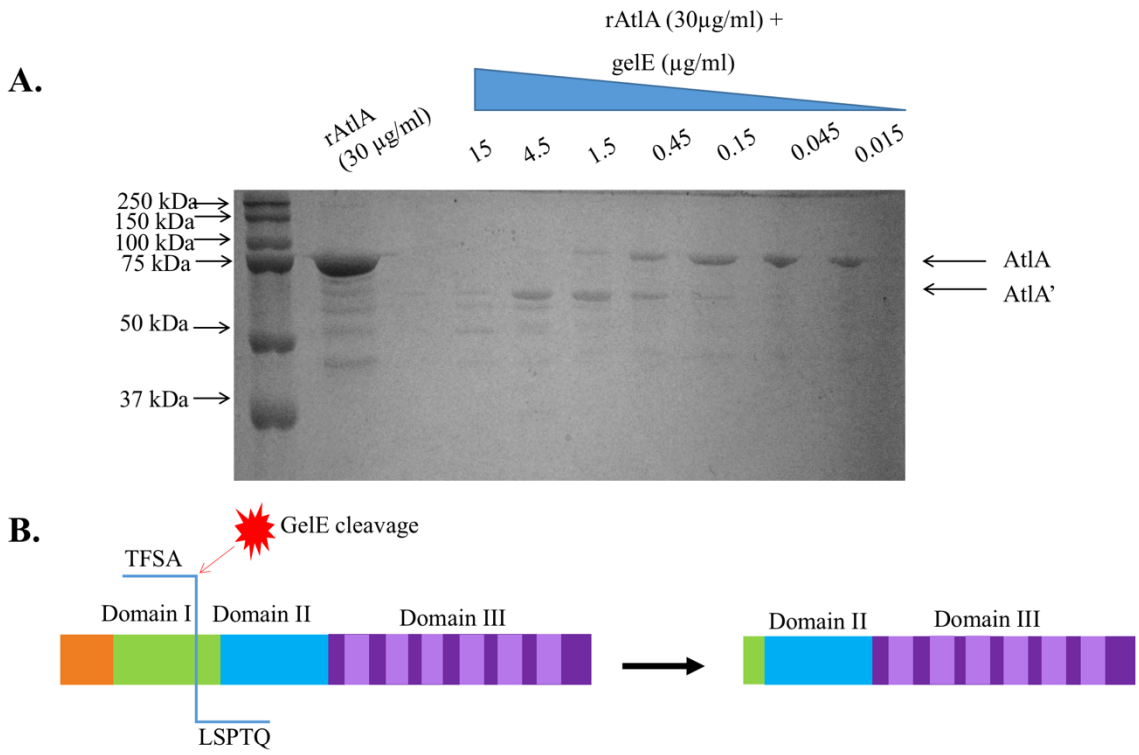


Figure 3.5: GelE cleavage of AtlA occurs between Ala 173 and Leu 174 within Domain I. (A) Cleavage of recombinant AtlA by purified GelE. Sample lanes from right to left represent reaction mixtures containing different amounts of purified GelE (0.015, 0.045, 0.15, 0.45. 1.5, 4.5, and 15 µg/ml) were incubated with 30 µg/mL of recombinant AtlA. Recombinant AtlA (30 µg/ml) incubated alone is shown as a control. (B) Recombinant AtlA was cleaved by purified GelE. Then the truncated approximately 60 kDa band was N-terminally sequenced. The reported N-terminal sequence was LSPTQXP, suggesting that GelE-cleavage to produce this primary approximately 60 kDa product occurs between Ala 173 and Leu 174. Results presented in Figure A are representative of three independent experiments.

GelE-mediated cleavage of AtlA does not significantly impact the enzymatic activity of AtlA in peptidoglycan hydrolysis

One of the major functions of AtlA is that of peptidoglycan hydrolysis. Specifically AtlA is a *N*-acetylglucosaminidase (Eckert *et al.*, 2006). To determine whether GelE-cleavage of AtlA impacted the peptidoglycan hydrolase activity of AtlA, the effect of the addition of recombinant AtlA and recombinant AtlA' to peptidoglycan was monitored in a peptidoglycan hydrolase activity assay (Figure 3.6). The results demonstrated no significant difference in the ability of recombinant AtlA compared to recombinant AtlA' to hydrolyze peptidoglycan ($p>0.05$). As a control, peptidoglycan alone showed no peptidoglycan hydrolysis. The addition of either recombinant AtlA or AtlA' showed a significant ability to hydrolyze peptidoglycan compared to the peptidoglycan only control ($p<0.05$). This result demonstrated that GelE cleavage of AtlA does not impact the peptidoglycan hydrolase activity of AtlA.

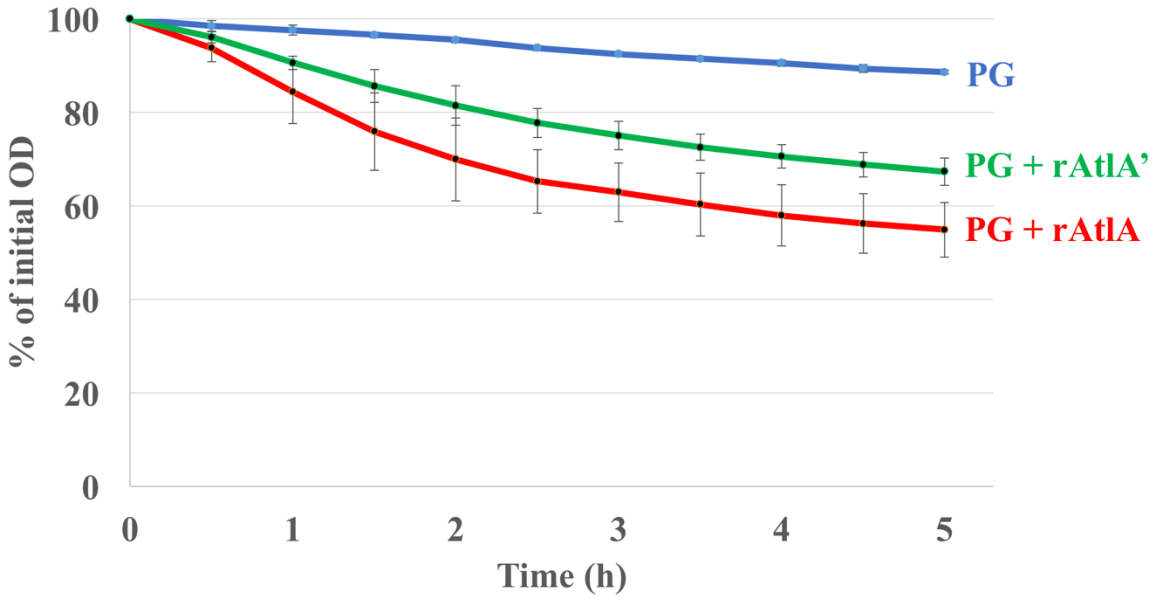


Figure 3.6: Peptidoglycan hydrolysis analysis demonstrates no significant difference in activity of recombinant AtlA and AtlA' on the peptidoglycan substrate. Equal molar concentrations (65 nM) of either recombinant full-length AtlA or AtlA' were added to purified peptidoglycan and the peptidoglycan hydrolysis rate was measured by examining the OD_{675} every 30 minutes over a 5-hour time period. As a control, the hydrolysis rate for peptidoglycan alone was measured. Results are averages of three independent experiments. Error bars represent one standard deviation of the mean. Statistical significant differences ($P < 0.05$) in peptidoglycan hydrolysis were determined for PG + rAtlA and PG + rAtlA' relative to PG only, using Student's *t* test. No significant difference ($P > 0.05$) was determined for PG + rAtlA relative to PG + rAtlA', using Student's *t* test**.**

GelE-mediated cleavage of AtlA impacts cell separation during cell division

AtlA plays an important role in cell division by cleaving the peptidoglycan at the cell septum between the two daughter cells, allowing for cell separation (Emirian *et al.*, 2009). In an effort to determine the impact of GelE-cleavage of AtlA on cell division, the chain length of OG1RF*ΔatlA* and OG1RFΔ*atlAΔgelE* cells was monitored when incubated with either recombinant AtlA or AtlA'. As previously defined (Arias *et al.*, 2007), the number of cells per chain were counted in 50 randomly chosen chains and defined as having either short, medium, long, or extra-long chains (for evaluation criteria see Table 2.3). As controls, OG1RF, OG1RF*ΔatlA*, and OG1RFΔ*atlAΔgelE* strains were examined under light microscopy. As anticipated, wild-type OG1RF appeared as pairs or as single cells (100% ± 0 ≤ 4 cells per chain) (Figure 3.8A). Consistent with previous studies, OG1RF*ΔatlA* appeared as long chains, with cells attached at septum cleavage sites, and with multiple cells per chain (40% ± 5.3 ≤ 4 cells per chain) (Figure 3.8B) (Qin *et al.*, 1998). OG1RF*ΔatlAΔgelE*, a strain developed for this study, also displayed a long chain phenotype (28% ± 2 ≤ 4 cells per chain), with chains longer than those seen in OG1RF*ΔatlA* (Figure 3.8C).

The addition of recombinant full-length AtlA and recombinant AtlA' were then examined for their ability to restore the wild-type short chain phenotype. Addition of recombinant full-length AtlA to the OG1RF*ΔatlA* cells appeared as individual cells with short chains observed (98.7% ± 2.3 ≤ 4 cells per chain) (Figure 3.8D). In contrast, the addition of recombinant full-length AtlA to the OG1RF*ΔatlAΔgelE* strain displayed a medium chaining phenotype (52.7% ± 3.8 ≤ 4 cells per chain) (Figure 3.8E) as opposed to the long chain phenotype observed in the OG1RF*ΔatlAΔgelE* strain (Figure 3.8C). Furthermore, the addition of the recombinant AtlA' to the OG1RF*ΔatlAΔgelE* strain displayed a short chain phenotype (93.3% ± 3.1 ≤ 4 cells per chain) (Figure 3.8F) like that seen in wild-type OG1RF cells. Since

recombinant AtlA' was able to restore the wild-type single or paired cell phenotype, I concluded that GelE-cleavage of AtlA regulates the function of AtlA in the completion of cell separation during cell division.

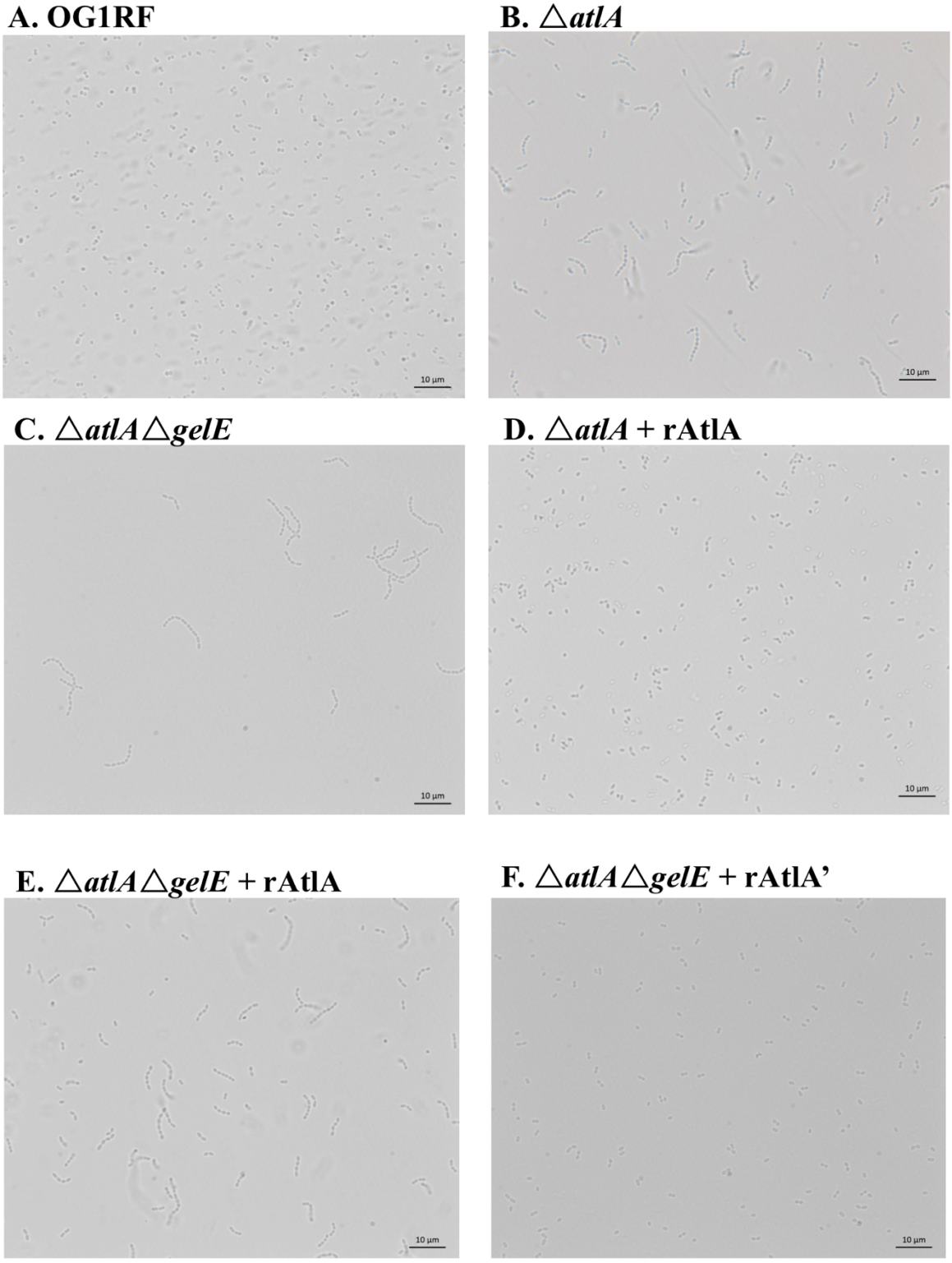

A. OG1RF
B. △atlA
C. △atlA△gelE
D. △atlA + rAtlA
E. △atlA△gelE + rAtlA
F. △atlA△gelE + rAtlA'
10 μm

Figure 3.7: GelE cleavage of AtlA impacts cellular chain length. OG1RF, OG1RF△*atlA*, and OG1RF△*atlA*△*gelE* were cultured overnight. Overnight cultures were diluted to an OD_{600} of 0.1 and incubated for an additional 3.5 hours. Where indicated, equal molar concentrations (13nM) of either recombinant AtlA or AtlA' were incubated with the cultures for another 0.5 hours. Cells were harvested and resuspended in 0.9% saline and imaged on a light microscope at 1000X magnification: (A) OG1RF (B) OG1RF△*atlA* (C) OG1RF△*atlA*△*gelE* (D) OG1RF△*atlA* + recombinant AtlA (E) OG1RF△*atlA*△*gelE* + recombinant AtlA (F) OG1RF△*atlA*△*gelE* + recombinant AtlA'. 50 randomly chosen chain per strain were counted and the percentage of cells with less than or equal to 4 cells was determined, which is shown in Table 3.1. These results represent three independent experiments. The scale bar in the lower right is 10 μm.

	No treatment	+ rAtlA	+ rAtlA'
OG1RF	100% ± 0	N.D.	N.D.
Δ*atlA*	40% ± 5.3	98.7% ± 2.3	N.D.
Δ*atlA*Δ*gelE*	28% ± 2	52.7% ±3.8	93.3% ± 3.1

Table 3.1: Percentage of cells that had less than or 4 cells per chain. Averages of three independent experiments are shown in this table. ± indicates one standard deviations from the mean. All groups were compared to their no treatment control. $P > 0.05$ in all cases using Student's *t* test.

GelE–cleaved AtlA localizes to the cell septum and the cell poles

Based on the observation that GelE-mediated cleavage of AtlA impacted cell separation (Figure 3.8), I hypothesized that GelE-cleaved AtlA would localize to the cell septum. To investigate whether GelE-mediated cleavage impacted AtlA localization on daughter cells prior to separation, anti-AtlA mAb 44 and anti-AtlA mAb 88 were used to monitor AtlA septal localization in both the wild-type OG1RF and the OG1RF*ΔgelE* strains via transmission electron microscopy. Recall that anti-AtlA mAb 44 binds within Domain II of AtlA and is able to recognize both the full-length and GelE-dependent truncated from of AtlA while anti-AtlA mAb 88 binds within Domain I and is unable to recognize the GelE-dependent truncated form of AtlA (Figures 3.2 and 3.3). Wild-type OG1RF labeled with anti-AtlA mAb 44 showed AtlA localization primarily to either the cell septum or cell pole (Figure 3.9A). Conversely, in an OG1RF*ΔgelE* strain, anti-AtlA mAb 44 showed binding to AtlA that was dispersed across the surface of the strain rather than localizing to either the cell septum or cell pole (Figure 3.9B). Alternatively, examination of AtlA surface binding with anti-AtlA mAb 88 on an OG1RF strain identified little full-length AtlA binding (Figure 3.10A). Detecting AtlA with anti-AtlA mAb 88 in a OG1RF*ΔgelE* strain showed a dispersal of AtlA across the surface of the cell similarly to anti-AtlA mAb 44 detection, albeit less robust than anti-AtlA mAb 44 binding (Figure 3.10b). This is consistent with the data shown in Figure 3.1, where GelE cleavage of the mAb 88-detected epitope within AtlA Domain I prevented AtlA detection on the cell surface. Altogether, our results demonstrate that GelE-dependent AtlA cleavage plays a pertinent role in AtlA localization to the cell septum.

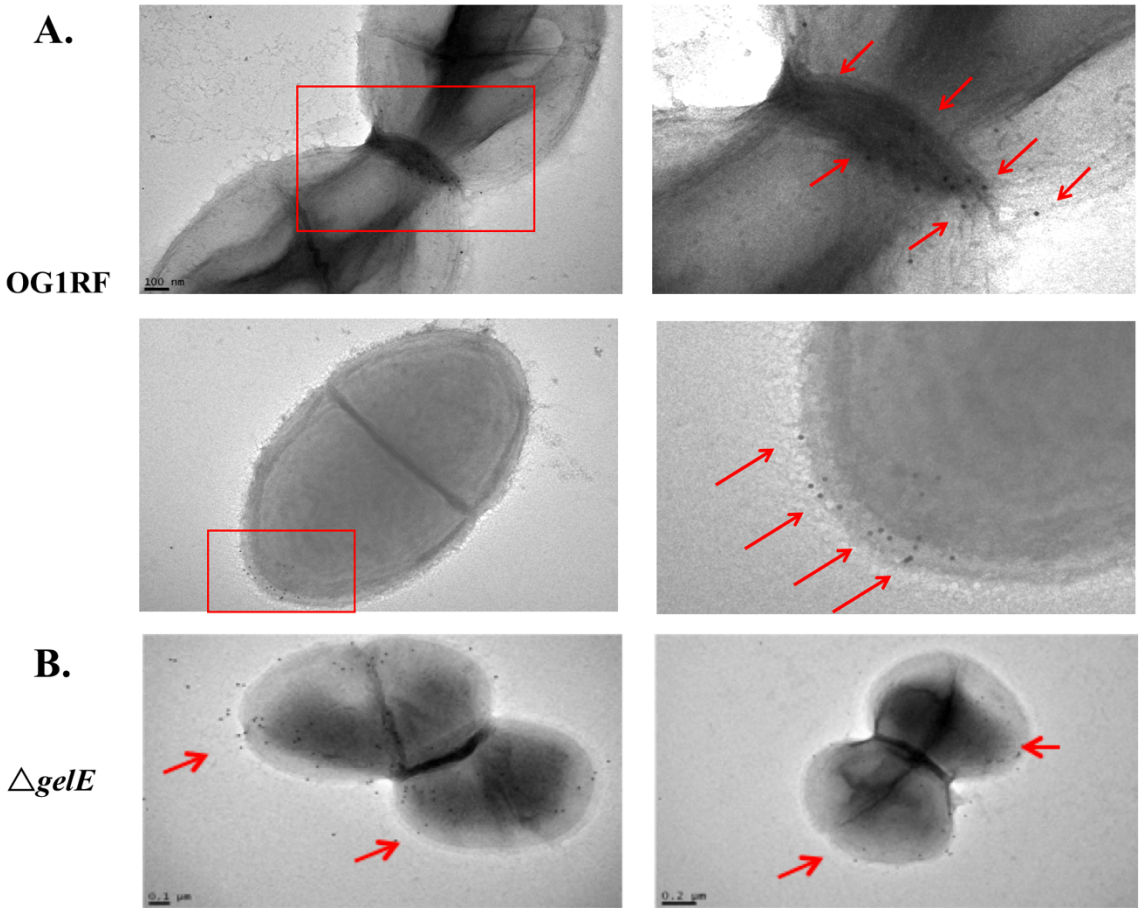


Figure 3.8: AtlA surface localization is impacted by the presence of GelE. OG1RF and OG1RF△*gelE* were cultured overnight in BHI and placed on nickel-coated carbon grid. Anti-AtlA mAb 44 was used to label AtlA on the surface of cells. Samples were then labeled with gold-conjugated anti-mouse IgG and then imaged with a JEOL JEM-1400 electron microscope. (A) OG1RF (B) OG1RF△*gelE*. Initial electron microscopy experiments were carried out by Peng Gao, Ph.D. Results shown are representative of three independent experiment.

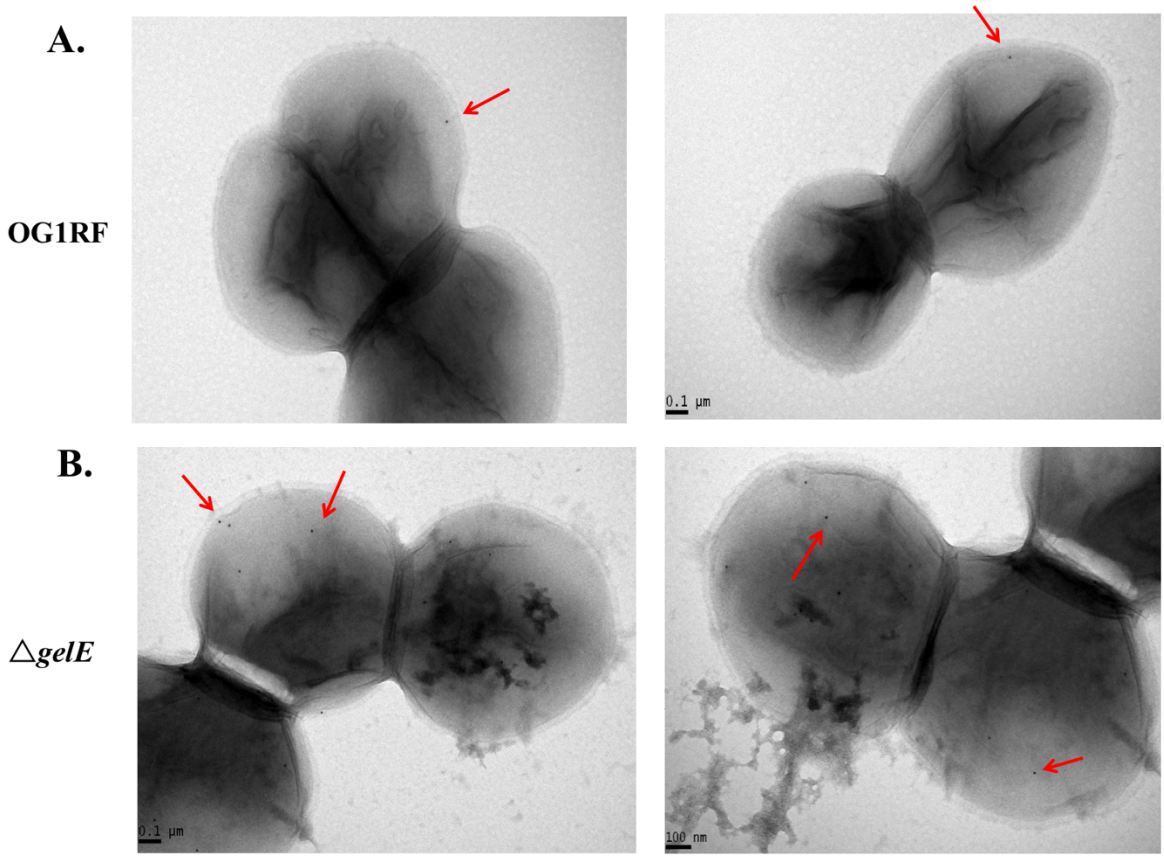


Figure 3.9: AtlA surface localization with anti-AtlA mAb 88. OG1RF and OG1RF△*gelE* cells were cultured overnight in BHI and placed on nickel-coated carbon grid. Anti-AtlA mAb 88 was used to label AtlA on the surface of cells. Samples were then labeled with gold-conjugated anti-mouse IgG and then imaged with a JEOL JEM-1400 electron microscope. (A) OG1RF (B) OG1RF△*gelE*. Initial electron microscopy experiments were carried out by Peng Gao, Ph.D. Results shown are representative of three independent experiment.

The addition of either recombinant full-length AtlA or AtlA' to the OG1RF*ΔatlAΔgelE* strain displays random cell surface localization

In the OG1RF strain, GelE-cleaved AtlA was found to primarily localize to either the cell septum or to the cell poles while, in the OG1RF*ΔgelE* strain, AtlA randomly localized to the cell surface (Figure 3.9). To determine if recombinant AtlA or AtlA' localizes to either the cell septum or to the cell surface, the exogenous addition of recombinant AtlA or AtlA' to the OG1RF*ΔatlAΔgelE* strain was monitored for localization with anti-AtlA mAb 44 by transmission electron microscopy. Upon the addition of recombinant AtlA to the OG1RF*ΔatlA* strain (GelE+), anti-AtlA mAb 44 detected AtlA randomly dispersed across the cell surface (Figure 3.11A). The addition of recombinant AtlA to the OG1RF*ΔatlAΔgelE* strain (GelE-) displayed random AtlA cell surface binding (Figure 3.11B). The addition of recombinant AtlA' to the OG1RF*ΔatlAΔgelE* strain (GelE-) also displayed random cell surface binding (Figure 3.11C). These findings demonstrate that exogenous addition of both recombinant AtlA and AtlA' localizes randomly across the cell surface, suggesting that exogenous AtlA interacts differently with the cell wall than endogenous AtlA.

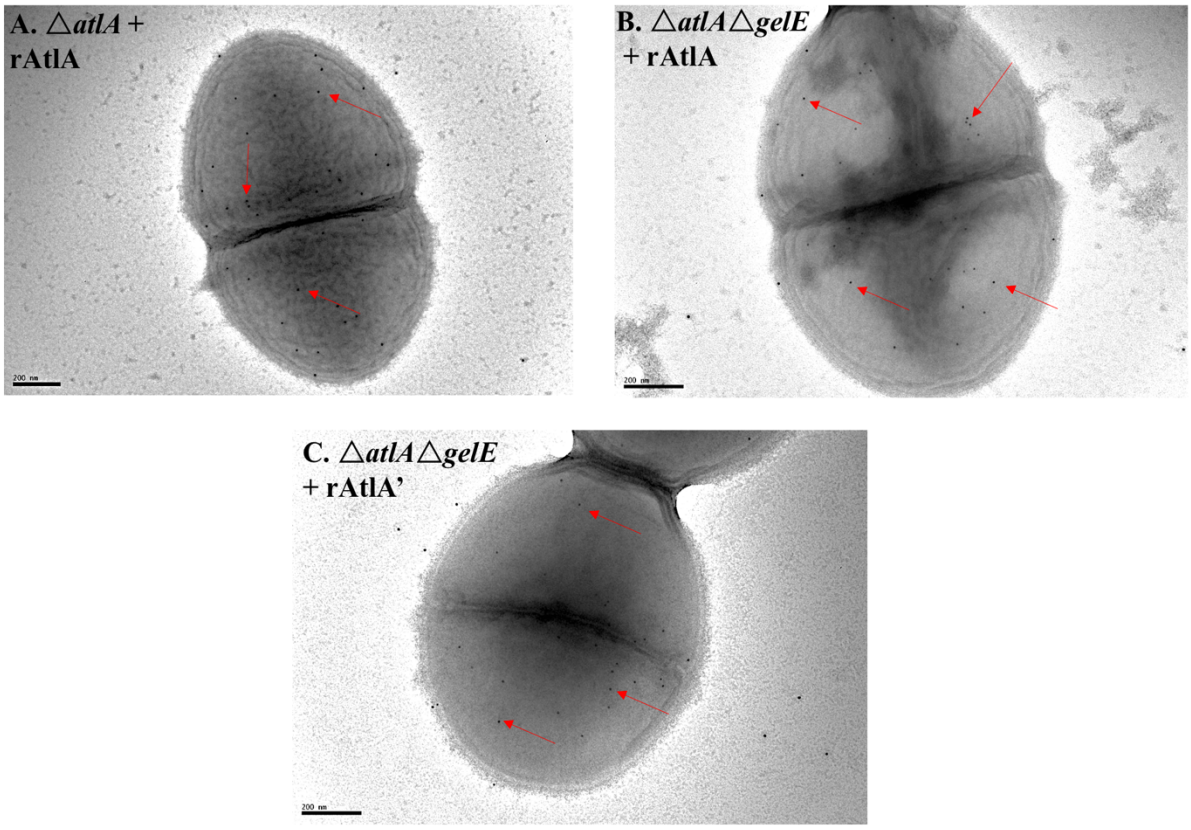


Figure 3.10: The addition of recombinant AtlA and AtlA' to OG1RF△*atlA*△*gelE* show random localization across the cell surface. Overnight cultures of OG1RF△*atlA* and OG1RF△*atlA* △*gelE* cells incubated with either recombinant AtlA or AtlA' for 30 minutes prior to being placed on nickel-coated carbon grid. Anti-AtlA mAb 44 was used as the primary AtlA antibody. A secondary antibody, gold-conjugated anti-mouse IgG, was then used to label cells. The cells were imaged on a JEOL JEM-1400 electron microscope. (A) OG1RF△*atlA* + rAtlA (B) OG1RF△*atlA*△*gelE* + rAtlA (C) OG1RF△*atlA*△*gelE* + rAtlA'. Results shown are representative of three independent experiment.

The exogenous addition of either recombinant Domain II or Domain III to an OG1RF*ΔatlA* strain demonstrated limited surface association

As shown by electron microscopy, GelE-mediated cleavage impacted AtlA septal localization (Figure 3.9). Domain III of AtlA contains six LysM modules that provide AtlA with the ability to bind to peptidoglycan (Eckert *et al.*, 2006). In theory, due to the presence of the LysM modules, AtlA is able to bind anywhere there is peptidoglycan. Yet, although there is peptidoglycan across the whole cell surface, GelE-cleaved AtlA localizes to the cell septum. Currently, it is not understood how only cleaved AtlA localizes to the cell septum. Initially, I hypothesized that GelE-mediated cleavage impacted AtlA localization by encouraging Domain II to bind at the cell septum instead of Domain III.

In an effort to determine which domain of AtlA is necessary for localization to the cell septum, recombinant AtlA Domain II (rD2) was additionally constructed and purified. Similar to Figure 3.2B, the anti-AtlA mAbs were evaluated for their ability to bind each domain by ELISA analysis. In this analysis, another anti-AtlA mAb, mAb 65, was also evaluated. Anti-AtlA mAb 44 bound recombinant AtlA, AtlA', and D2, but not recombinant D3, further verifying that mAb 44 binds within Domain II (Figure 3.12). Anti-AtlA mAb 65 was found to bind recombinant AtlA, AtlA', and D3, but not recombinant D2 (Figure 3.12). These observations further demonstrated that anti-AtlA mAb 44 bound within AtlA Domain II, while anti-AtlA mAb 65 was found to bind within AtlA Domain III.

To determine if Domain II is able to associate with the cell surface, the exogenous addition of either recombinant AtlA, AtlA', D2, or D3 to OG1RF*ΔatlA* cells was evaluated for the ability to associate with the surface by flow cytometry. As shown in Figure 3.13, the addition of recombinant AtlA to OG1RF*ΔatlA* cells displayed surface association when detected with both mAb 44 and 65. Similarly, the addition of recombinant AtlA' to OG1RF*ΔatlA* cells

displayed robust signal when detected with both mAb 44 and 65, though recombinant AtlA' surface association was higher than that of recombinant AtlA. The addition of either recombinant D2 or D3 displayed limited surface association, suggesting that neither Domain II nor Domain III alone is able to associate with the surface. Together, these findings suggest that Domain II and Domain III must be connected together in order for cleaved AtlA to bind to the cell.

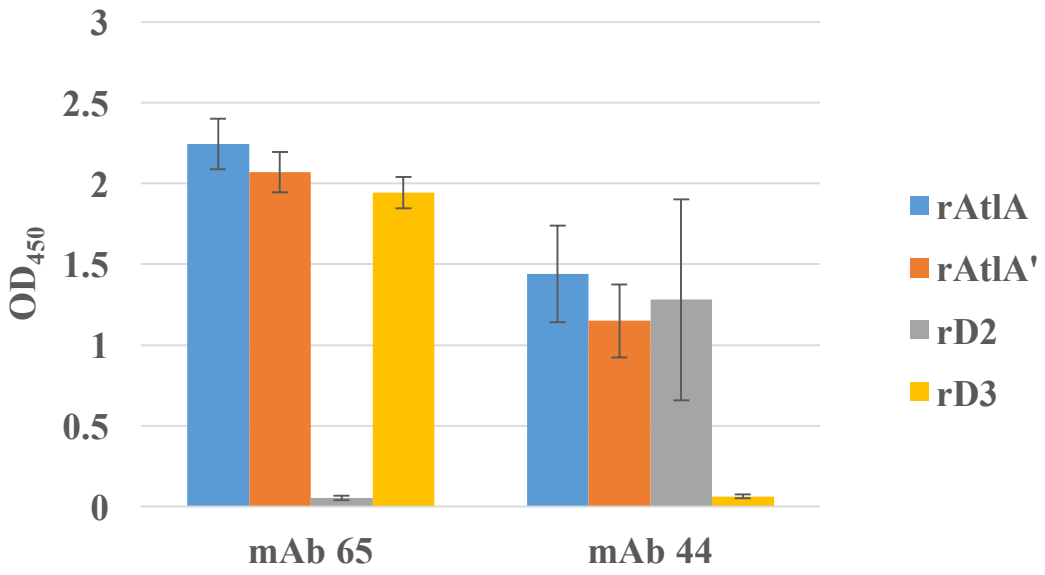


Figure 3.11: ELISA analysis of anti-AtlA mAb 44 and 65 binding to recombinant proteins further confirms that mAb 44 binds within Domain II while mAb 65 binds within Domain III. Anti-AtlA mAb 44 and 65 were evaluated for their ability to bind to recombinant full-length, AtlA', D2 and D3 via ELISA analysis. Each recombinant protein was labeled with each anti-AtlA mAb, followed by goat anti-mouse IgG-HRP conjugate and developed with TMC substrate. Results shown are an average of three independent experiments and error bars are 1 standard deviation from the mean.

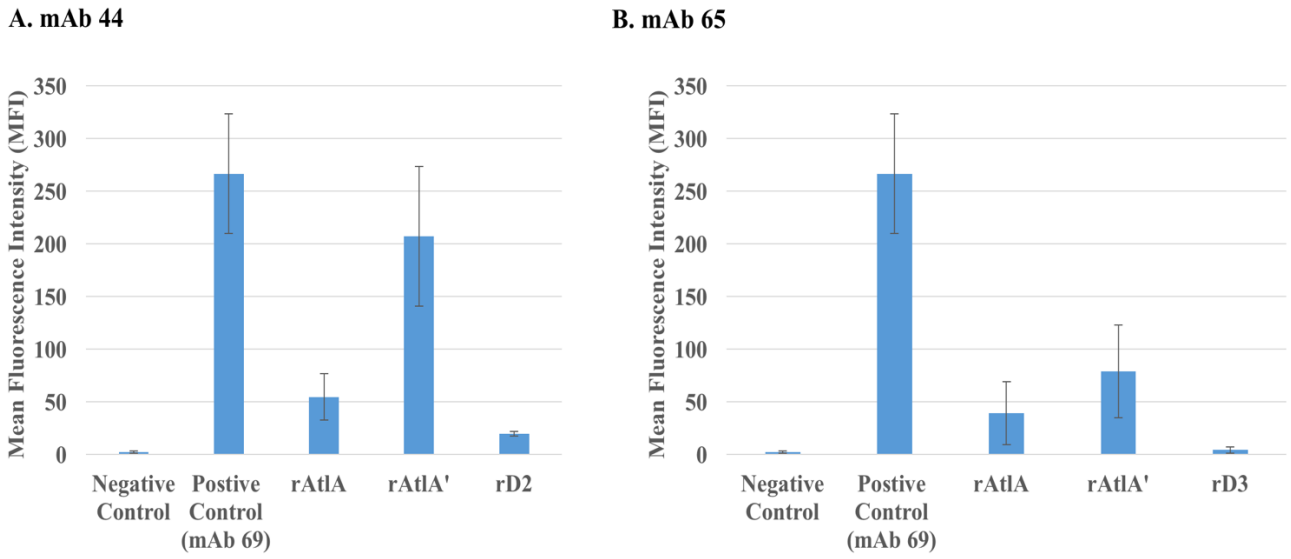


Figure 3.12: **Flow cytometry analysis displays limited surface association when recombinant D2 or D3 are exogenously added to OG1RF△*atlA* cells.** Flow cytometry analysis of the OG1RF△*atlA* strain with the addition of different recombinant proteins (full-length AtlA, AtlA', DII and DIII) analyzed with either anti-AtlA mAb 44 or mAb 65. As a negative control, wild-type OG1RF△*atlA* cells were labeled with only secondary antibody. As a positive control, OG1RF△*atlA* cells were labeled with an irrelevant antibody (anti-EbpC mAb 69). A) mAb 44 and B) mAb 65. Results shown are an average of three independent experiments and error are 1 standard deviation from the mean.

Limited LTA-AtlA interaction is detected by ELISA

In staphylococcus strains, the major autolysin, Atl, has also been shown to localize to the cell septum (Baba & Schneewind, 1998, Heilmann *et al.*, 1997). In addition, by crystallography studies, Atl has been shown to bind to lipoteichoic acid (LTA), one of the primary molecules that makes up the cell wall and is anchored to the cell membrane. At the cell septum more LTA is exposed than other areas of the cell surface, thus the localization of Atl to the cell septum was reasoned to be caused by the interaction between Atl and LTA (Zoll *et al.*, 2012). Based on this observation, I hypothesized that AtlA may also bind to LTA.

In an attempt to resolve the interaction of AtlA and LTA, ELISA analysis was employed. In this assay, different concentrations of purified *Bacillus subtilis* LTA was first bound to an ELISA plate. Then, either recombinant AtlA, D2D3, or AtlA + GelE was incubated with the bound LTA. Anti-AtlA mAb 44 was used to detect bound AtlA. As shown in Figure 3.14, regardless of GelE cleavage, limited AtlA detection occurred. Based on this result, our experiment cannot confirm an interaction between AtlA and LTA.

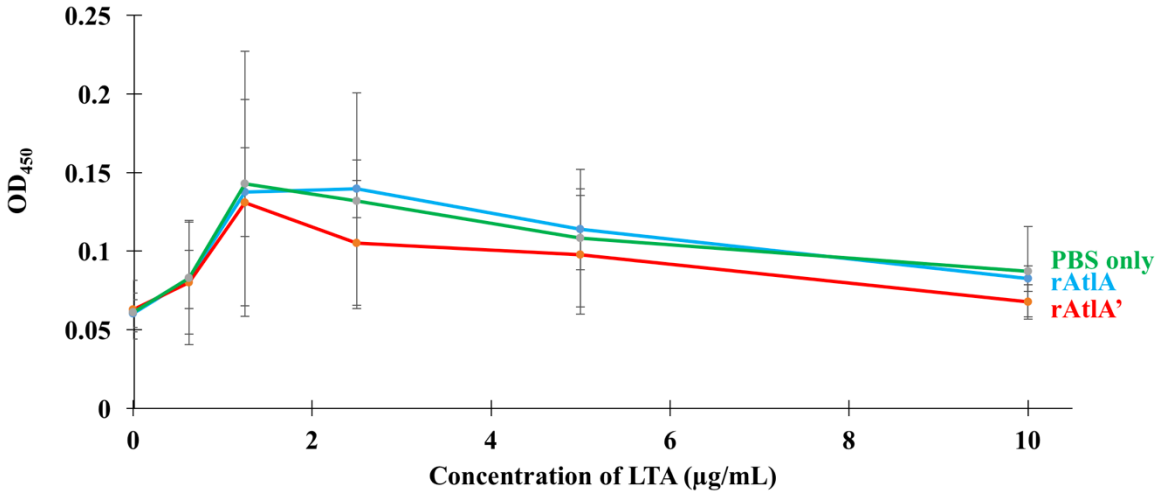


Figure 3.13: **ELISA analysis of LTA-AtlA binding exhibits limited AtlA detection.** Different concentrations of LTA (ranging from 0 µg/mL to 10 µg/mL) were coated onto the ELISA plate. 10 µg/mL recombinant AtlA, AtlA + GelE, or D2D3 were incubated overtop of the bound LTA. Finally, binding of AtlA was detected anti-AtlA mAb 44 followed by goat anti-mouse IgG-HRP conjugate and development with TMC substrate. Results shown are an average of three independent experiments and error bars are 1 standard deviation from the mean.

Discussion

In *E. faecalis,* AtlA is the major peptidoglycan hydrolase. The ability to cleave peptidoglycan makes AtlA pivotal in separating dividing cells (Qin *et al.*, 1998). As such, AtlA requires strict modulation in order to regulate its function in cell division. The results presented in this dissertation provide strong evidence that post-translational modification of AtlA by GelE impacts the function of AtlA during cell division. This conclusion is supported by the evidence that cleaved AtlA localized to the cell septum (Figure 3.9) and the addition of recombinant AtlA to a OG1RF*ΔatlAΔgelE* strain maintained a chaining phenotype (Figure 3.8).

While examining the lytic activity of OG1RF against *M. lysodeikticus* cells, Eckert *et al.* demonstrated that AtlA displayed two protein bands that were present in the wild-type strain, but lacking in the OG1RF*ΔatlA* strain: one at 72 kDa (the approximate molecular weight of AtlA) and the other at 62 kDa (Eckert *et al.*, 2006). Thomas *et al.* examined the impact that proteases induced by the Fsr quorum-sensing system of *E. faecalis* had on the autolytic profiles of wild type OG1RF and OG1RF*ΔgelE* strains (Thomas *et al.*, 2009). A processed AtlA band of approximately 62 kDa was observed only in cells producing GelE, whereas those lacking GelE expressed AtlA of approximately 72 kDa (Thomas *et al.*, 2009). Consistent with these results, analysis of AtlA expression in different *E. faecalis* strains by Western blot showed that strains that expressed GelE (OG1RF and OG1RF ▽*sprE*) displayed a major band at the same size as recombinant AtlA' while those strains that were incapable of producing GelE (OG1RF*ΔgelE* and OG1RF*ΔgelEΔsprE*) displayed a major band consistent in size with recombinant full-length AtlA (Figure 3.3). Together these results suggested that the presence of GelE is necessary for the processing of AtlA to the cleaved fragment. After further analysis, the GelE-cleavage site on AtlA was identified within Domain I. This cleavage site occurs between Ala 173 and Leu 174,

near the start of the pre-defined Domain II (Figure 3.5). AtlA lacking this N-terminal region would have a molecular weight of 58.9 kDa, consistent with the 62 kDa observed.

While work by Thomas *et al.* supports a role for SprE mediated processing of AtlA in regulating autolysis activity (Thomas *et al.*, 2009), I was unable to find evidence supporting a major role of SprE processing of AtlA in mediating cell division, as GelE processing AtlA alone could alleviate the chaining phenotype when incubated with our OG1RF*ΔatlAΔgelE* strain *in vitro* (Figure 3.8). In addition, analysis of different *E. faecalis* strains lacking SprE expression (OG1RF ▽*sprE* and OG1RF*ΔgelEΔsprE*) had no detectable impact on AtlA bands detected by Western blot analysis, producing similar results to that of OG1RF and OG1RF*ΔgelE* respectively (Figure 3.3). I also addressed the role of SprE on AtlA surface association by monitoring wild-type OG1RF, OG1RF*ΔgelE,* OG1RF*ΔgelEΔsprE,* and OG1RF ▽*sprE* cells for AtlA surface association by flow cytometry. In the absence of SprE (OG1RF ▽*sprE* and OG1RF*ΔgelEΔsprE*), no difference was observed in the AtlA surface association pattern compared to those of OG1RF and OG1RF*ΔgelE* respectively (Figure 3.4). Taken together, these findings suggest no appreciable role for a SprE-AtlA interaction in the regulation of cell division or AtlA surface association.

Once the GelE cleavage site on AtlA was discovered, I sought to determine its impact on AtlA function. Eckert *et al.* examined the enzymatic activity of different domain truncations of AtlA (Eckert *et al.*, 2006). Truncation of Domain I did not significantly alter the enzymatic activity. In contrast, truncation of Domain III from Domain I and II led to a significant decrease in enzymatic activity, suggesting that Domain III is required for enzymatic activity to occur (Eckert *et al.*, 2006). Domain III contains six LysM modules that confer AtlA with the ability to bind to peptidoglycan. Based on these findings, Eckert *et al.* proposed that Domain III is required to bring Domain II within the vicinity of its peptidoglycan substrate (Eckert *et al.*,

2006). Consistent with the work presented here, cleavage of the N-terminal domain by GelE did not significantly impact the ability of AtlA to hydrolyze peptidoglycan (Figures 3.6 and 3.7).

OG1RF*ΔatlA* cells grow as long chains linked together at their cell septa, unable to properly separate (Qin *et al.*, 1998). Similarly, OG1RF*ΔgelE* cells display a chaining phenotype, suggesting that GelE is required for cell separation. Upon the complementation of *gelE* into an OG1RF*ΔgelE* strain, the cells appeared either in pairs or as single cells, further suggesting that the presence of GelE impacts cell division (Waters *et al.*, 2003). During early growth stages, long chains of cells are produced (Lominski *et al.*, 1958). In *Streptococcus pneumoniae*, a streptococcus that has both short and long chain phenotypes similar to *E. faecalis,* the long chain phenotype is associated with an advantage in human epithelial cell adherence due to an increase in the number of adhesive events per particle (Rodriguez *et al.*, 2012). Moreover, it was hypothesized that a short chain length in *S. pneumoniae* allows the bacterium to evade host immune defense cells, such as phagocytes, promoting invasive disease (Weiser, 2010). Based on these findings, I speculate that human cell adherence would be most important at early stages of growth, potentially prior to Fsr signaling, when GelE cleavage of AtlA has not yet occurred and long chains are present (Lominski *et al.*, 1958). As GelE reaches maximum activity levels at higher cell density, after colonization is established, AtlA would be cleaved and short cell chains would result. These short chains could potentially aid in bacterial dissemination or help *E. faecalis* evade host immune defense. In fact, GelE has already been shown to be associated with an increase in bacterial burden at dissemination sites (Thurlow *et al.*, 2010). Thus, GelE could aid in bacterial cell dissemination by cleaving AtlA at a later growth stage, resulting in short chains.

Using our anti-AtlA mAbs (44 and 88) to track both GelE-cleaved and full-length AtlA, localization of AtlA in wild-type OG1RF and OG1RF*ΔgelE* cells was monitored via electron

microscopy. Wild-type OG1RF cells labeled with anti-AtlA mAb 44 showed localization of AtlA to the cell septum (Figure 3.9A) while OG1RF*ΔgelE* cells labeled with anti-AtlA mAb 44 showed random localization of AtlA across the cell surface (Figure 3.9B). These findings indicate that GelE-mediated AtlA cleavage is responsible for the localization of AtlA to the cell septum.

While evidence described in this dissertation independently demonstrates that GelE mediated cleavage of AtlA is sufficient to encourage cell separation and cleaved AtlA localization to the cell septum, my efforts to date have not yet provided a complete understanding of the mechanism that mediates these events. In staphylococcus strains, the major autolysin, Atl, was found to localize to the cell septum due to an interaction with one of the major peptidoglycan constituents, LTA (Zoll *et al.*, 2012). Based on these results, I proposed that LTA might regulate cleaved AtlA localization. Using ELISA analysis, I was unable to confirm a direct interaction between LTA and AtlA (Figure 3.14). Alternatively, in staphylococcus strains, the other major peptidoglycan constituent, wall teichoic acid (WTA), prevents Atl binding. At the cell septum, WTA is found at a lower concentration, which would cause Atl to bind to the septum (Schlag *et al.*, 2010). Based on these findings, I propose that the presence of WTA could impact the localization of AtlA. Continued study will provide a deeper understanding of cell separation during the final stage of cell division and the potential importance of timing cell separation with cellular density through Fsr-mediated GelE expression.

Chapter 4: SalB, a secreted stress-induced protein, is detected as a GelE cleavage product in the cellular supernatant, but not on the cell surface in *in vitro* studies

NOTE: Experiments using the sandwich ELISA to detect soluble SalB in different strains were carried out by Ken Pinkston.

Introduction

SalB is an extracellular *E. faecalis* protein controlled by the CroRS two-component system (Muller *et al.*, 2006). *salB* is induced by extracellular stress (Muller *et al.*, 2006). Particularly, *salB* can be induced by different environmental stressors such as low pH, heat, peroxide, and detergents (Rince *et al.*, 2003, Le Breton *et al.*, 2003). In addition, SalB is proposed to be a peptidoglycan hydrolase due to its similarities to the orthologs, SagA in *E. faecium* and PcsB in streptococci, which are classified as such (Sham *et al.*, 2011, Yang *et al.*, 2011, Bartual *et al.*, 2014, Rangan *et al.*, 2016). Shankar *et al.* tested the ability of purified SalB to cleave purified peptidoglycan and saw no significant peptidoglycan hydrolase activity (Shankar *et al.*, 2012). Interestingly, the SalB ortholog, PcsB, did not initially display peptidoglycan hydrolase activity either, yet it was found that, upon binding to an essential cell division protein, FtsX, PcsB could indeed cleave peptidoglycan (Sham *et al.*, 2011). Thus, although SalB has not been shown to have peptidoglycan hydrolase activity, the possibility exists that similarly to PcsB, SalB might require an initial binding to another protein to be activated.

SalB shares sequence homology with *E. faecium* SagA and streptococcus PcsB. The C-terminal region of SalB shares homology with proteins from *L. mesenteroides*, which contain a Lys7 domain, important for the binding of proteins to cellular peptidoglycan (Muller *et al.*, 2006). Both SagA and PcsB are associated with the cell wall (Teng *et al.*, 2003, Sham *et al.*, 2011). Based on these C-terminal domain similarities and homology to SagA and PcsB, it is possible that once secreted, SalB is able to bind to cellular peptidoglycan.

Although SalB is a stress-induced protein, which suggests a role for SalB in stress response, other major functions of SalB remain mysterious. It appears that SalB might also be important in the regulation of different cellular processes such as cell division, adhesion, biofilm

formation, and autolysis (Shankar *et al.*, 2012, Mohamed *et al.*, 2006). Upon *salB* deletion, biofilm formation decreases, binding to fibronectin and collagen type I increases, autolysis increases, and the cells display more morphological defects, such as misplaced cell septa and cell clumps (Mohamed *et al.*, 2006, Shankar *et al.*, 2012).

As discussed in Chapter 3, GelE and AtlA impact similar cellular functions to SalB. For example, OG1RFΔ*gelE* and OG1RFΔ*atlA* grow in long chains where the individual cells are connected at their cell septa (Qin *et al.*, 1998, Waters *et al.*, 2003), suggesting that both GelE and AtlA impact cell separation. OG1RFΔ*salB* grow in cell clumps with misplaced cell septa, suggesting a role in septum formation (Shankar *et al.*, 2012). In addition, the OG1RFΔ*gelE* strain displayed a decrease in autolysis, while the OG1RFΔ*atlA* strain displayed no autolytic activity (Qin *et al.*, 1998, Waters *et al.*, 2003). Contrary, OG1RFΔ*salB* displayed an increase in autolytic (Shankar *et al.*, 2012). Based on the similarities between SalB, GelE, and AtlA deletions, I proposed that the functions of these three proteins are linked.

It has also been shown that higher levels of SalB are found in an OG1RF*ΔgelE* culture supernatant (Shankar *et al.*, 2012), suggesting a possible link between GelE and SalB. Due to these results, Shankar *et al.* constructed an OG1RFΔ*gelE*Δ*salB* strain to study the relationship between SalB and GelE. The OG1RFΔ*gelE*Δ*salB* strain grew with more clumps and more misplaced septa compared to the OG1RFΔ*salB* strain (Shankar *et al.*, 2012). In contrast, the OG1RFΔ*gelE*Δ*salB* strain showed a significant increase in autolysis compared to the OG1RFΔ*gelE* strain and decreased autolysis compared to the OG1RFΔ*salB* strain. Shankar *et al.* concluded that an epistatic relationship between *gelE* and *salB* did not occur (Shankar *et al.*, 2012). In this dissertation, I hypothesized that GelE directly interacts with SalB affecting its function, which ultimately impacts the function of AtlA in cell separation during cell division.

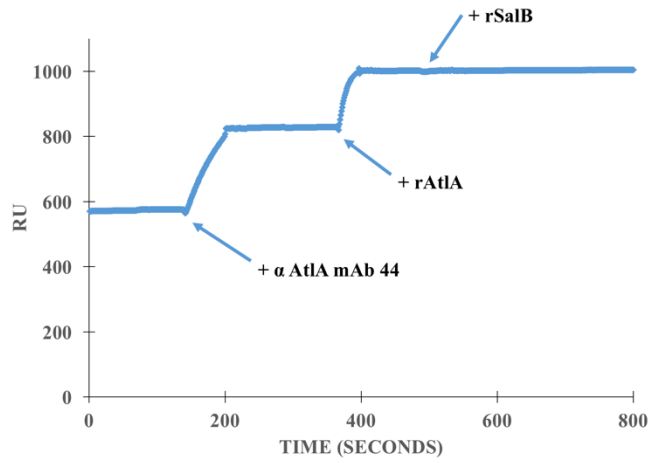
In this dissertation, an anti-SalB monoclonal antibody panel was generated as a tool to help understand the localization and function of SalB. Specifically, the findings presented here indicated that SalB did not localize to the cell surface and instead was found as a soluble form in the supernatant as a cleaved product of GelE. Overall, there are two ways that GelE could impact the function of SalB either through the destruction of SalB or by modifying SalB such that an alternative function occurs. To determine the impact of GelE cleavage on SalB function, I examined the impact of GelE cleavage on SalB in cell division. The addition of either recombinant SalB or native GelE-cleaved SalB to OG1RFΔ*salB* cells did not restore the wild-type phenotype. Further study of SalB and GelE may give us a greater understanding of the impact GelE cleavage has on SalB function.

Results

Recombinant SalB displays no detectable binding to either recombinant AtlA or D2D3

OG1RF*ΔsalB* displayed an increase in autolysis compared to the wild-type OG1RF strain, suggesting that the presence of SalB decreases the autolysis activity (Shankar *et al.*, 2012). Since AtlA is the major autolysin of *E. faecalis*, I considered the hypothesis that SalB directly binds to AtlA, impacting the role of AtlA in autolysis. In an effort to determine whether a direct interaction between SalB and AtlA occurs, the interaction between recombinant SalB and either recombinant AtlA or D2D3 was monitored via surface plasmon resonance (SPR) analysis. Briefly, anti-AtlA mAb was captured on a CM5 sensor chip coupled with goat anti-mouse IgG. Then, the bound anti-AtlA mAb was used to capture either recombinant full-length AtlA or recombinant D2D3 (representing cleaved AtlA). Finally, recombinant SalB was tested for the ability to bind to either the recombinant AtlA or recombinant D2D3. As shown in Figure 4.1, upon the addition of recombinant SalB to either full-length AtlA or D2D3, no binding could be detected.

A.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+ rAtlA
+ α AtlA mAb 44
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400
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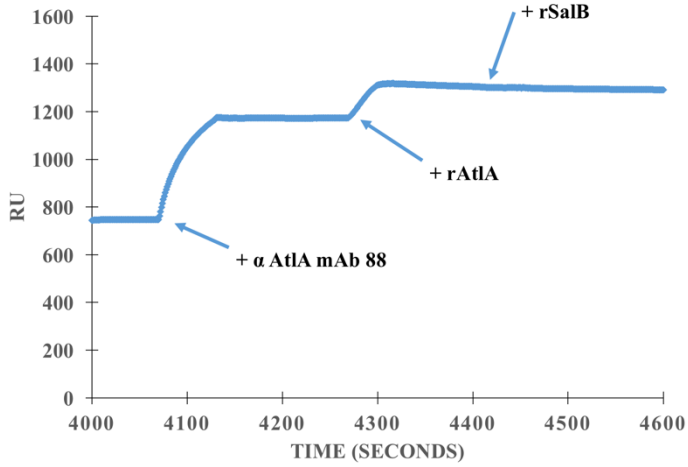
1000

B.


+ rSalB
+ rAtlA
+ α AtlA mAb 88
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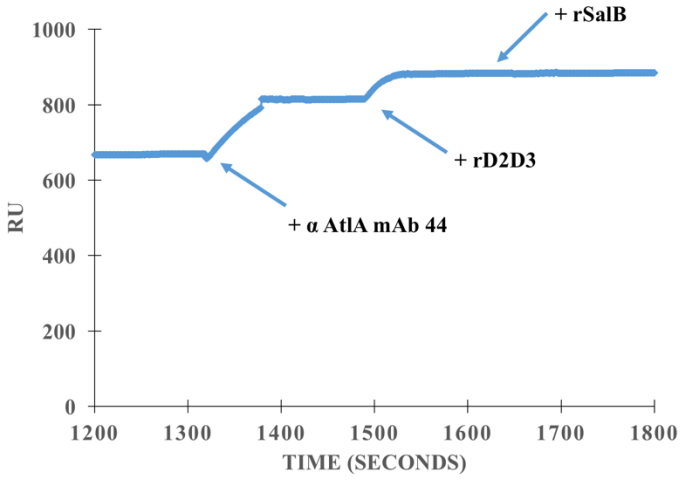
C.


+ rSalB
+ rD2D3
+ α AtlA mAb 44
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TIME (SECONDS)
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800
1000
1200
1300
1400
1500
1600
1700
1800

Figure 4.1: Recombinant SalB demonstrates no detectible binding to either recombinant AtlA or recombinant D2D3 by SPR analysis. The interaction between SalB and AtlA was monitored by SPR analysis. (A) Anti-AtlA mAb 44 was captured on a CM5 sensor chip, followed by the addition of recombinant full-length AtlA. The addition of recombinant SalB is shown by the arrow. (B) Anti-AtlA mAb 88 captured on a CM5 sensor chip, followed by the addition of recombinant full-length AtlA. The addition of recombinant SalB is shown by the arrow. (C) Anti-AtlA mAb 44 captured on a CM5 sensor chip, followed by the addition of recombinant AtlA D2D3. The addition of recombinant SalB is shown by the arrow.

SalB is detected in media supernatant and minimal SalB is detected on the cell surface in *in vitro* culture

Bacterial secreted proteins are most often associated with helping bacterial cells adhere to host cells, helping bacterial cells fight against host immune cells, damaging target cells, or discovering available resources in the environment (Lee & Schneewind, 2001, Green & Mecsas, 2016). Secreted proteins can associate with the cell surface through covalent or non-covalent interactions (Schneewind & Missiakas, 2012). SalB is a known secreted protein with a C-terminal domain that suggests that SalB is able to bind to cellular peptidoglycan (Rince *et al.*, 2003, Muller *et al.*, 2006). To investigate whether SalB is associated with the cell surface, an anti-SalB mAb panel was generated. The anti-SalB mAb panel was then evaluated for its ability to recognize surface associated SalB on wild-type OG1RF strain using flow cytometry (Figure 4.2). All four anti-SalB mAbs (mAb 20, mAb 25, mAb 32, and mAb 42) detected minimal SalB on the cell surface in either the wild-type OG1RF or the OG1RFΔ*gelE* strain. An OG1RFΔ*salB* strain was used as a negative control to demonstrate minimal mAb binding in the absence of SalB. In addition, a secondary antibody only control was used to demonstrate minimal binding in the absence of a primary antibody. A positive antibody control against another known surface protein, EbpC, was also tested on the wild-type OG1RF and OG1RFΔ*salB* strains to show EbpC surface protein recognition by flow cytometry.

Since SalB shows limited surface association, I hypothesized that SalB is found in the supernatant. To resolve the localization of SalB, concentrated supernatants of wild-type OG1RF, OG1RFΔ*gelE,* OG1RFΔ*salB,* and OG1RFΔ*atlA* were evaluated for SalB expression using Western Blot analysis. Anti-SalB mAb 42 (10 μg/mL) was used as the primary antibody. The OG1RFΔ*salB* strain was utilized as a negative control to demonstrate that anti-SalB mAb 42 did not detect protein in the absence of SalB. SalB has been shown to be approximately 55

kDa in molecular weight (Muller *et al.*, 2006). In both the wild-type OG1RF and OG1RFΔ*atlA* (GelE+) strains, SalB appeared as fragments ranging in size from ~30 kDa to ~40 kDa. In the OG1RFΔ*gelE* strain, SalB detection showed a major band at ~55 kD and a minor band at ~45 kDa. Together, these results suggest that SalB is found in the supernatant and that the presence of GelE is important for the processing of SalB.

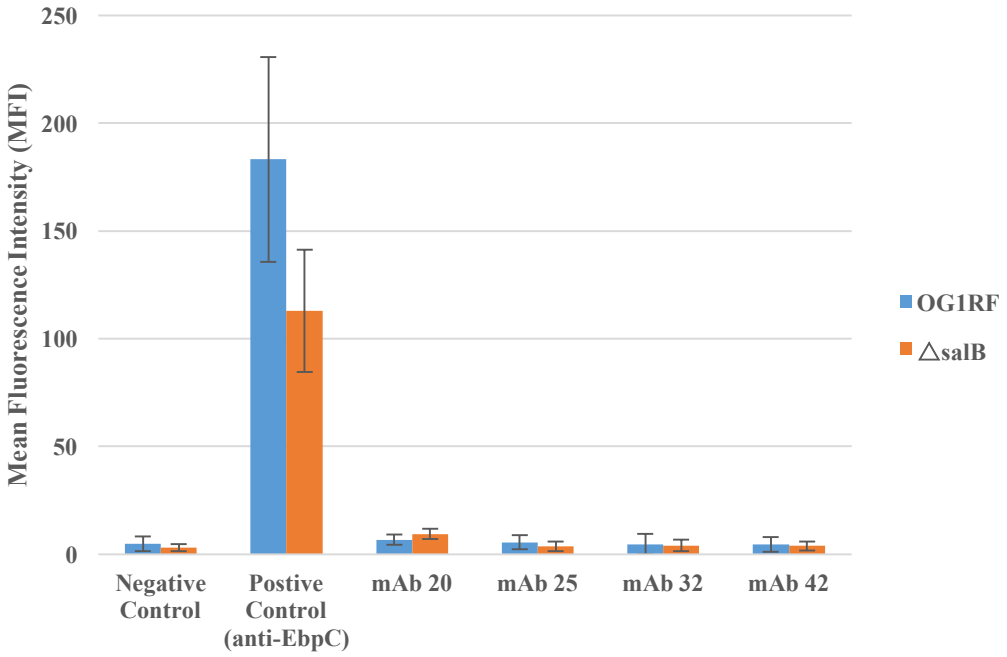

Figure 4.2: Detection of surface associated SalB using an anti-SalB antibody panel. Wild-type OG1RF, OG1RF△*gelE*, and OG1RF△*salB* strains were analyzed for SalB surface binding via flow cytometry with either anti-SalB mAb 20, mAb 25, mAb 32 or mAb 42. As a negative control, secondary antibody alone was used for each strain. As a positive control, anti-EbpC mAb 69 was analyzed for each strain. Results shown are an average of three independent experiments and error bars are one standard deviation from the mean.

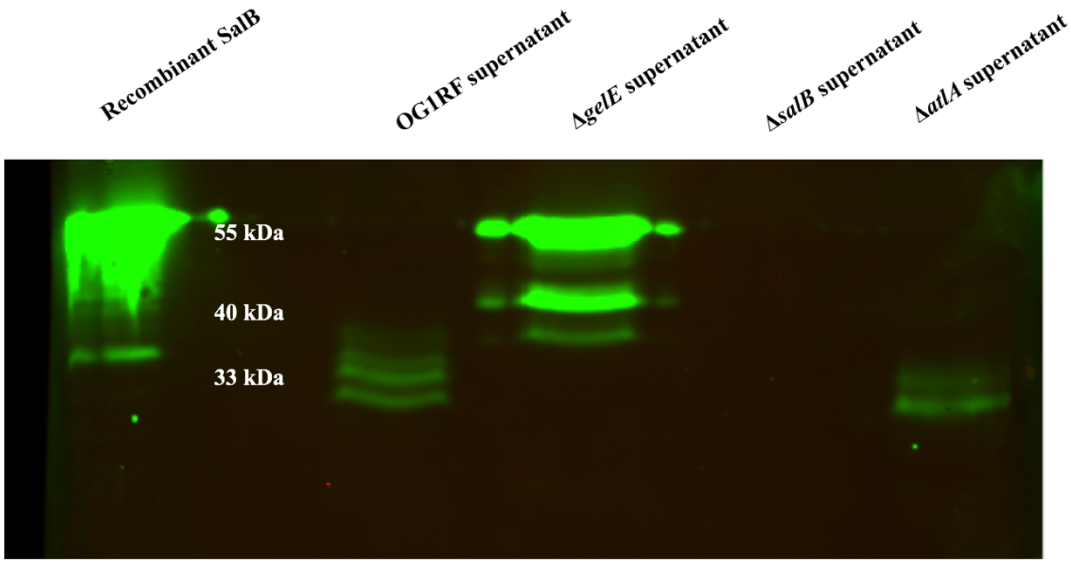

Figure 4.3: SalB is detected in media supernatants and processing of SalB as observed by differences in the banding pattern was impacted by the presence of GelE. The supernatants of exponentially grown OG1RF, OG1RF△*gelE*, OG1RF△*atlA*, and OG1RF△*salB* strains were collected and concentrated 20x. The concentrated supernatants were then boiled at 95ºC for 10 minutes in SDS-PAGE sample buffer and loaded onto a gel. Anti-SalB mAb 42 was used as the primary antibody. The OG1RF△*salB* strain was used as a negative control to show no binding in the absence of SalB. The left lane contains 10 µg/mL of recombinant SalB and was included as a positive control. Molecular mass standards are indicated in the second lane on the left in kilodaltons. This data represents three independent experiments.

Decreased levels of soluble SalB in the supernatant of the Δ*gelE* mutants compared to the wild-type supernatants was detected via ELISA analysis

Consistent with experiments by Shankar *et al.*, Western analysis demonstrated an increased intensity of SalB in the supernatant of OG1RFΔ*gelE* cells compared to the wild-type OG1RF cells (Figure 4.3) (Shankar *et al.*, 2012). In addition, the presence of GelE resulted in fragmented SalB (Figure 4.3). Together, these findings suggested that SalB is cleaved by GelE. In the supernatant of the OG1RFΔ*gelE* strain, SalB was detected as a major band at ~55 kD and a minor band at ~45 kDa, suggesting that another protease is responsible for the cleavage of SalB to the ~45 kDa fragment. To evaluate the impact of GelE and other proteases on SalB levels, an assay to detect SalB in the cell medium was developed. The schematic of the assay setup is shown in Figure 4.4A. To verify detection of SalB with this assay, media from wild-type OG1RF, OG1RFΔ*gelE,* OG1RFΔ*gelE*Δ*sprE*, and OG1RFΔ*fsrB* cells was evaluated for SalB levels. OG1RFΔ*salB* was used a negative control and data represents the net OD. In contrast with Western analysis, media from wild-type OG1RF cells showed the highest levels of SalB while media from strains that do not express GelE (OG1RFΔ*gelE* and OG1RFΔ*gelE*Δ*sprE*) displayed low levels of SalB. Media from OG1RFΔ*fsrB* cells displayed an intermediate level of SalB expression (Figure 4.4B). One possible explanation for the discrepancy between the Western and ELISA analysis could be the sample preparation. For the Western analysis, the supernatants were concentrated 20x while the media for ELISA analysis was not concentrated. To determine if this concentration impacted the results, TCA precipitation was used to evaluate SalB expression in the supernatants of wild-type OG1RF and OG1RFΔ*gelE* strains by Western analysis. Consistent with Western analysis that demonstrated increased levels of SalB in the Δ*gelE* strain (Figure 4.3), higher levels of full-length SalB was found in the OG1RFΔ*gelE* strain compared to the wild-type strain. Wild-type OG1RF displayed fragmented SalB that was at a lower intensity level compared to the OG1RFΔ*gelE* strain (Figure

4.5). This result is inconsistent with ELISA analysis that demonstrated higher levels of soluble SalB in the OG1RF strain compared to the Δ*gelE* strain (Figure 4.4).

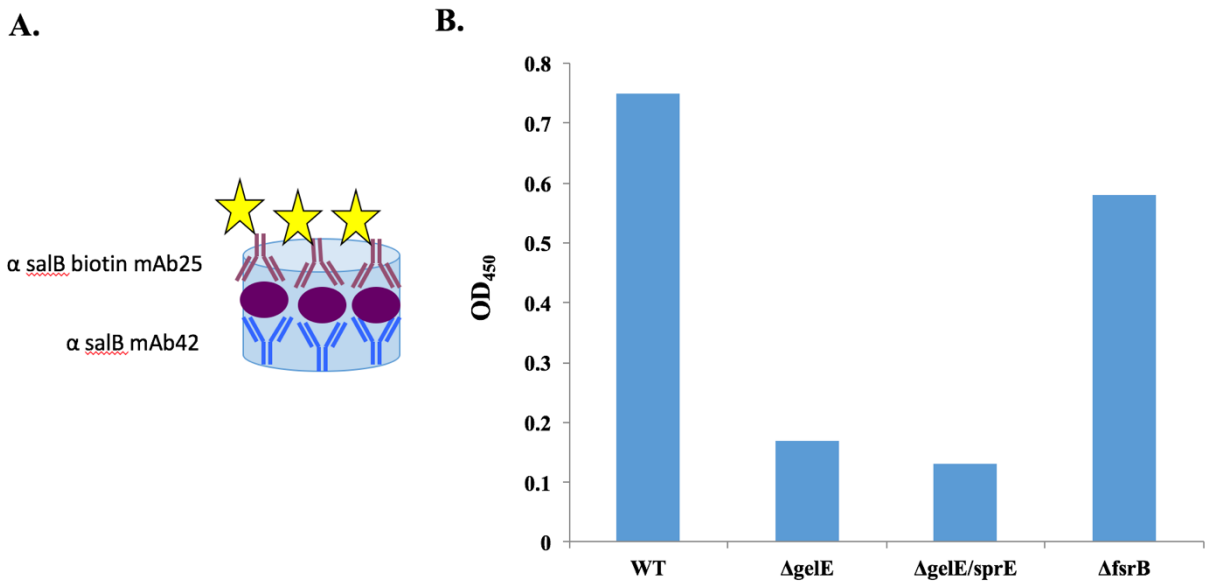

Figure 4.4: Sandwich ELISA detects increased levels of soluble SalB in wild-type supernatant from that of OG1RF△*gelE* mutants. (A) Schematic of the SalB detection assay setup. Anti-SalB mAb 42 was coated on a ELISA plate. The culture supernatant was incubated, allowing any SalB to bind to mAb 42. From there, biotin labeled anti-SalB mAb 25 was bound to any captured SalB. A streptavidin conjugated goat anti-mouse antibody was used to detect. (B.) Supernatants of exponentially grown wild-type OG1RF (WT), OG1RF△*gelE*, OG1RF△*gelE*△*sprE*, OG1RF△*fsr,* and OG1RF△*salB* strains were used to bind to mAb 42, in order to evaluate for the presence of SalB. Data represents the net OD, which was calculated by subtracting the absorbance readings of the OG1RF△*salB* strain. This result represents two independent experiments and was conducted by Ken Pinkston.

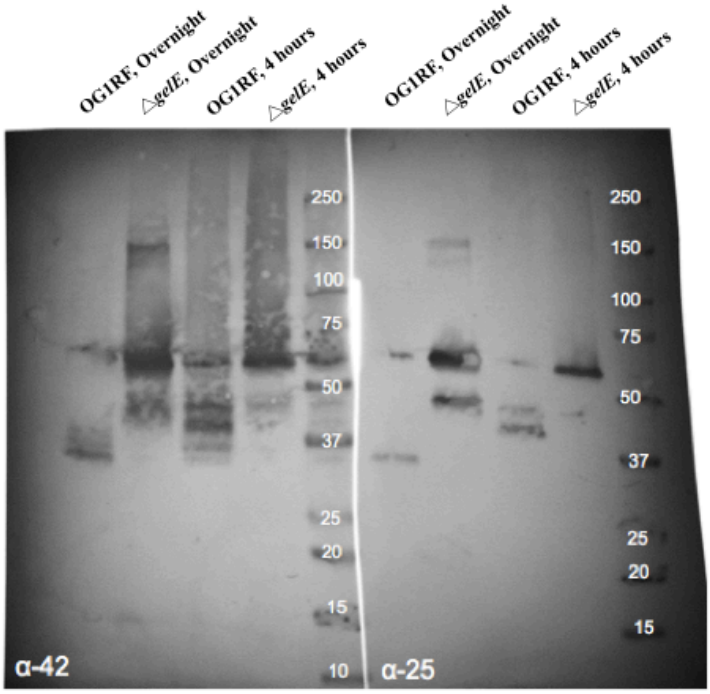

Figure 4.5: Western blot analysis of proteins that have been TCA precipitated from either OG1RF or OG1RF△*gelE* detects higher levels of SalB in OG1RF△*gelE* compared to OG1RF. The supernatants of either overnight or exponentially grown OG1RF or OG1RF△*gelE* were collected and subjected to TCA precipitation. The resulting pellets were incubated at 95ºC for 10 minutes in SDS-PAGE sample buffer and loaded onto a gel for electrophoresis. Anti-SalB mAb 42 (shown on the left) and mAb 25 (shown on the right) were used as the primary antibody. Molecular mass standards are indicated to the right in kilodaltons.

GelE cleaves recombinant SalB into multiple fragments

Detection of SalB in the supernatant of the wild-type OG1RF strain by Western blot detected fragmented pieces of SalB, while SalB detected in the supernatant of the OG1RFΔ*gelE* strain was detected at a molecular weight consistent with that of full-length SalB (Figure 4.3). This result strongly suggested that GelE is responsible for the processing of SalB. In an effort to determine at what concentration GelE cleavage of SalB occurs at, different concentrations of GelE (ranging from 0 to 15 μg/mL) were incubated with 30 μg/mL recombinant SalB at 37°C for 30 minutes and evaluated by SDS PAGE analysis (Figure 4.6). Upon the addition of purified GelE, recombinant SalB was cleaved into multiple small fragments. At lower GelE concentrations (0.015 to 0.45 μg/mL), recombinant SalB appeared as one major band at ~55 kDa, suggesting that GelE does not cleave SalB at low concentrations. However, at higher GelE concentrations (1.5 to 15 μg/mL), multiple SalB fragments are displayed ranging in size from ~37 kDa to ~50 kDa. In addition, the ~55 kDa full-length SalB band could not be detected at 15 μg/mL. This result suggested that SalB has multiple GelE cleavage sites.

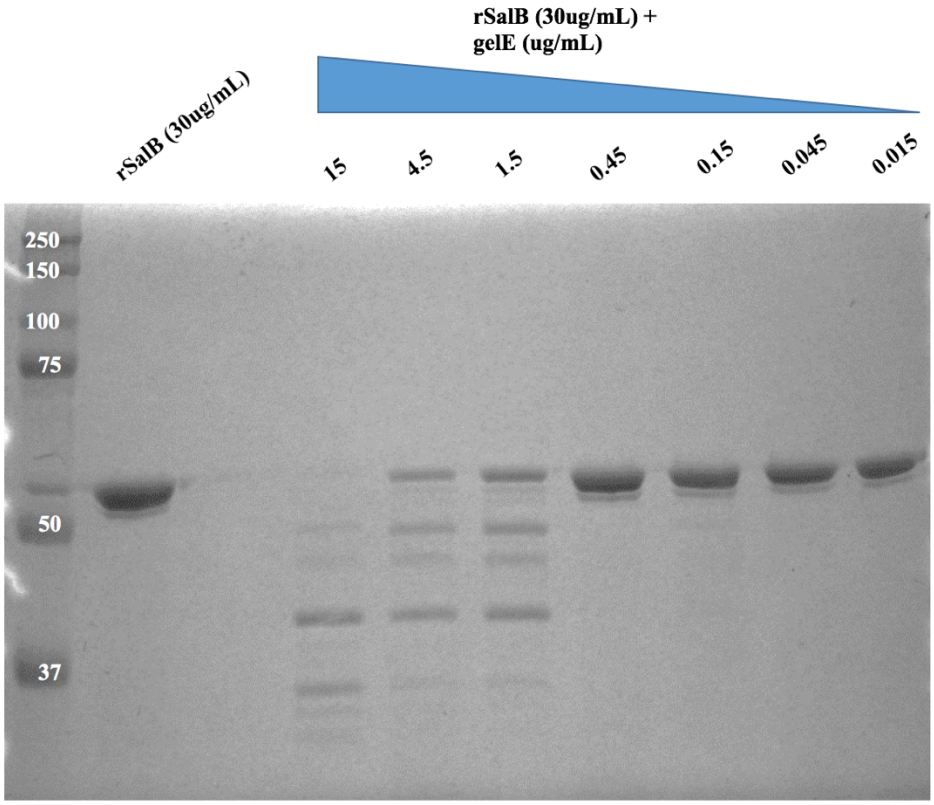

Figure 4.6: GelE cleavage of recombinant SalB. Sample lanes from right to left represent reaction mixtures containing different concentrations of purified GelE (0.015, 0.045, 0.15, 0.45. 1.5, 4.5, and 15 µg/ml) were incubated with 30 µg/mL of recombinant SalB for 30 minutes at 37ºC. Recombinant SalB (30 µg/ml) incubated alone is shown as a control. Molecular mass standards are indicated to the left in kilodaltons. This result represents three independent experiments.

Addition of recombinant SalB or native GelE-cleaved SalB to OG1RFΔ*salB* cells is unable to impact the cellular clumping phenotype

OG1RFΔ*salB* cells grow in clumps with misaligned cell septa. In addition, OG1RFΔ*gelE*Δ*salB* cells appeared with an increased cellular clumping phenotype and more frequently misaligned cell septa compared to the phenotypes seen in the OG1RFΔ*salB* cells (Shankar *et al.*, 2012). Our results have shown that GelE cleaves SalB into multiple fragments (Figures 4.3 and 4.6). These findings suggested that SalB plays a role in the cell division process, particularly in cell septum formation. Furthermore, the findings strongly suggested that the presence of GelE could impact the function of SalB in cell division. The impact of GelE-cleavage of SalB on cell division was examined under a light microscope by the addition of either native GelE-cleaved SalB or recombinant SalB to the OG1RFΔ*salB* strain. As controls, wild-type OG1RF and OG1RFΔ*salB* cells were first examined. Consistent with work done in Chapter 3, wild-type OG1RF cells appeared as either pairs or single cells (Figure 4.7A). Consistent with Shankar *et al.,* OG1RFΔ*salB* cells appeared in clumps (Figure 4.7B) (Shankar *et al.*, 2012). Upon the addition of recombinant SalB to the OG1RFΔ*salB* strain, the cells appeared as cell clumps, similar to that seen in OG1RFΔ*salB* cells alone (Figure 4.7C). Likewise, upon the addition of conditioned media from wild-type OG1RF strain (SalB+, GelE+) to the OG1RFΔ*salB* strain, cells also appeared as cell clumps (Figure 4.7D). These results suggested that neither the addition of recombinant SalB nor native GelE-cleaved SalB are able to impact the clumping phenotype. Taken together, these findings suggest that the clumping phenotype is irreversible.

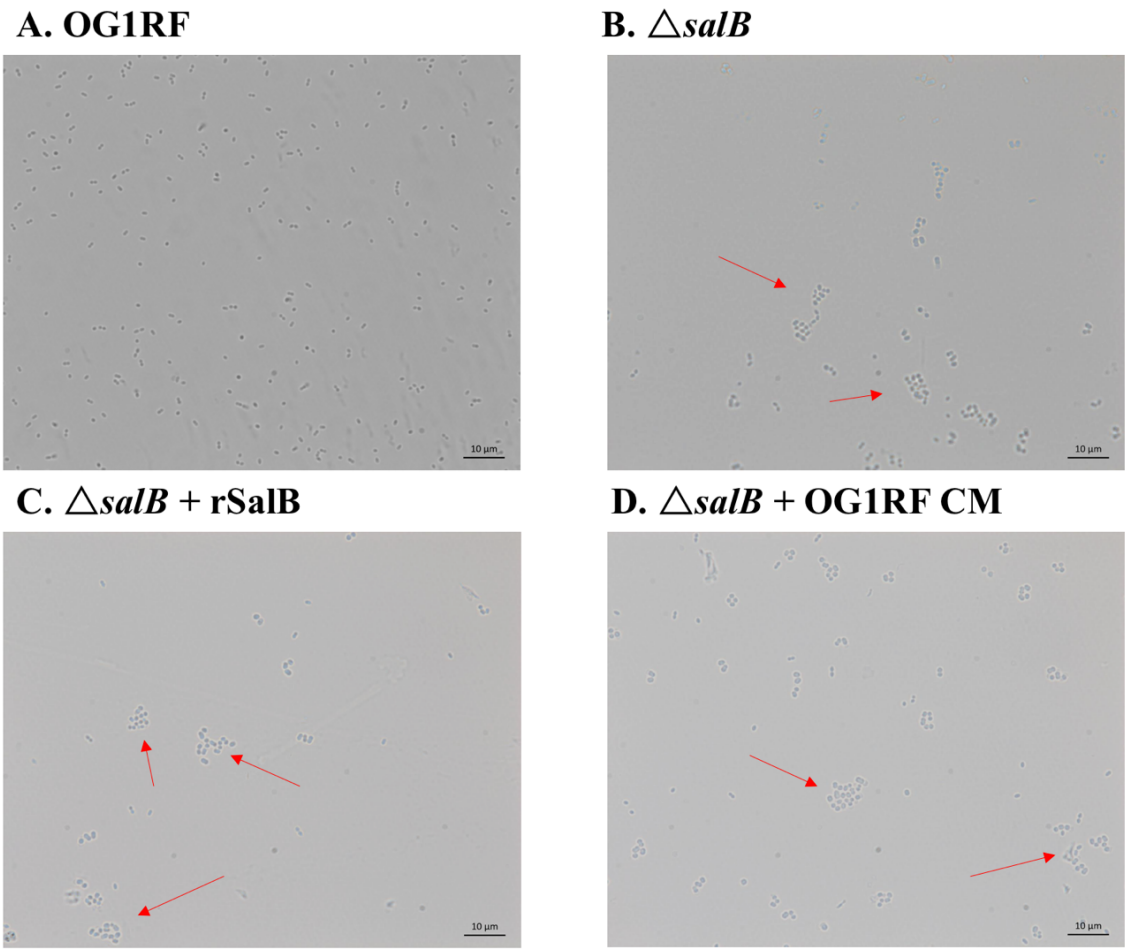

Figure 4.7: The addition of recombinant or native GelE-cleaved SalB to OG1RF△*salB* cells is unable to impact the clumping phenotype. OG1RF and OG1RF△*salB* cells were cultured overnight in BHI. In the morning, the overnight cultures were diluted to OD_{600} of 0.1 and incubated for an additional 3.5 hours. Where indicated, 5 µg/mL recombinant SalB or OG1RF conditioned media were incubated with the cultures for another 0.5 hours. Cells were harvested and resuspended in 0.9% saline and imaged on a light microscope at 1000X magnification. Red arrows point out cell clumps. (A) OG1RF (B) OG1RF△*salB* (C) OG1RF△*salB* + recombinant SalB (D) OG1RF△*salB* + OG1RF conditioned media (native SalB). Results represent three independent experiments. The scale bar in the lower right is 10 µm.

Inhibition of GelE activity by the addition of EDTA to OG1RFΔ*salB* cells does not increase the number of dead cells compared to the OG1RFΔ*salB* strain alone

Upon analysis by Shankar *et al.* with a Live/Dead viability assay, wild-type OG1RF showed 0.37% dead cells, OG1RFΔ*salB* showed 14.3% dead cells, and OG1RFΔ*gelE*Δ*salB* showed 53.9% dead cells, which suggested that there is a marked increase in the number of dead cells in the OG1RFΔ*gelE*Δ*salB* strain. Shankar *et al.* reasoned that as the clumping phenotype increased that the amount of oxygen, nutrients, and water would be less likely to reach the middle cells, resulting in cell death (Shankar *et al.*, 2012). To evaluate and verify the impact of GelE and SalB expression on viability, wild-type OG1RF and OG1RFΔ*salB* strains were evaluated by propidium iodine (PI), a DNA intercalating agent, staining via flow cytometry (Figure 4.8 and Table 4.1). EDTA, a chelating agent, was used to inhibit GelE activity. The strains were incubated with EDTA for 4 hours. Wild-type OG1RF and OG1RF + EDTA showed a small percentage of dead cells (0.32 and 0.5). OG1RFΔ*gelE* and OG1RFΔ*gelE* + EDTA also displayed low amounts of dead cells (0.34 and 0.32). The OG1RFΔ*salB* strain demonstrated an increase in percentage of dead cells, 29.2, compared to wild-type cells, 0.32. OG1RFΔ*salB* + EDTA showed an increase in percentage of dead cells, 15.2, compared to wild-type cells, 0.32. The percentage of dead cells seen in OG1RFΔ*salB* + EDTA was lower than that seen in OG1RFΔ*salB* alone (15.2 compared to 29.2). Although it was previously reported that OG1RFΔ*gelE*Δ*salB* cells displayed a marked increase in the number of dead cells compared to the OG1RFΔ*salB* cells (Shankar *et al.*, 2012), my PI staining analysis suggested that there was no increase in the amount of dead cells upon the inhibition of GelE in the OG1RFΔ*salB* strain.

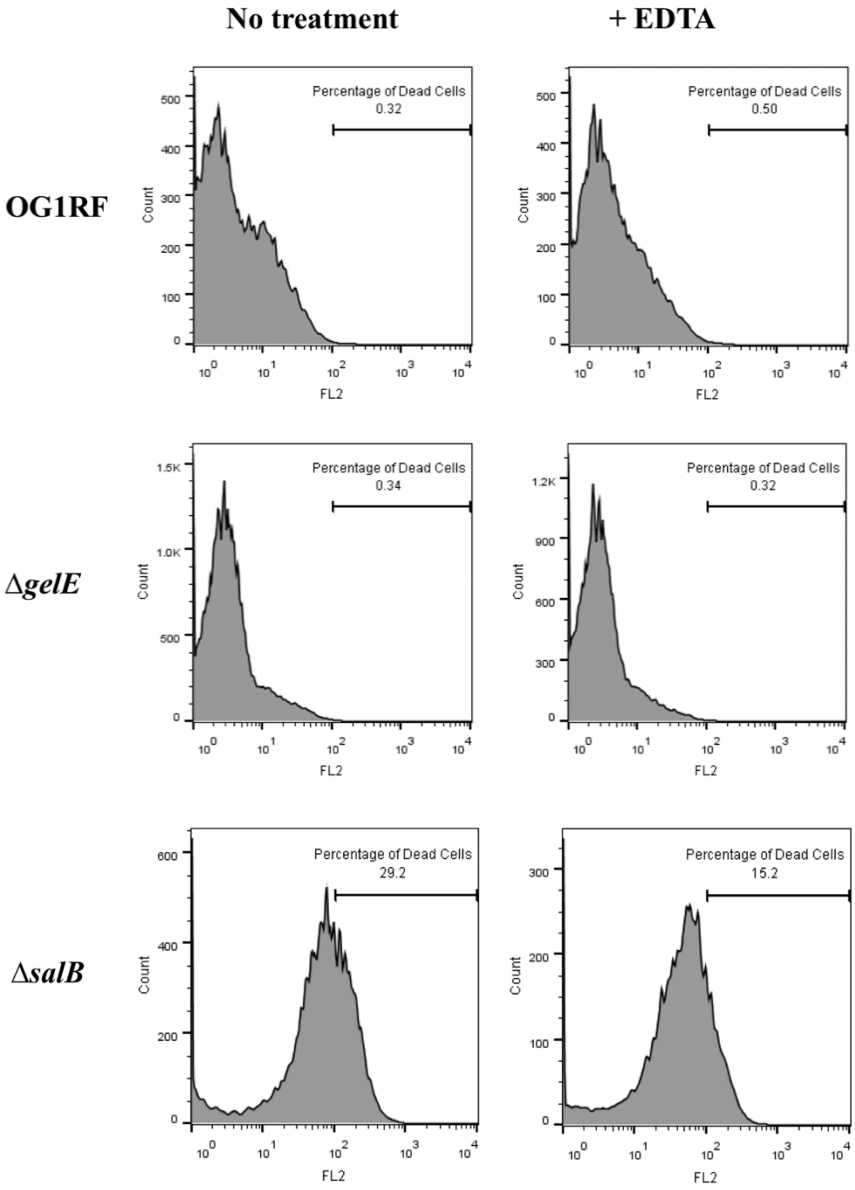

Figure 4.8: Inhibition of GelE activity in the OG1RF△*salB* strain did not impact the cell viability. Propidium iodide staining of the OG1RF, OG1RF△*gelE* or the OG1RF△*salB* strains with the addition of EDTA to block GelE activity. As controls, the addition of EDTA to OG1RF and OG1RF△*gelE* was also monitored. Horizontal lines represent percentage of dead cells. This result represents three independent experiments.

	% Dead cells/ fold difference relative to OG1RF	
	No treatment	**+ EDTA**
OG1RF	**0.32/1**	**0.50/1.56**
ΔgelE	**0.34/1.06**	**0.32/1**
ΔsalB	**29.2/91.25**	**15.2/47.5**

Table 4.1: Flow cytometry propidium iodine viability results.

Discussion

Upon environmental stress, bacterial cells are known to inhibit major cellular processes such as cell division. In *E. faecalis,* SalB is a protein secreted in response to certain environmental stressors (Muller *et al.*, 2006). Interestingly, a *salB* deletion strain appears as cellular clumps when viewed under the microscope (Le Breton *et al.*, 2003, Shankar *et al.*, 2012). These results suggested that SalB could play a role in cell division, possibly regulating division in response to stress. It is crucial to continue to seek a greater understanding of how SalB is regulated and the impact regulation has on cell division. The results presented in this chapter provide strong evidence that SalB is secreted into the supernatant and is cleaved in the presence of GelE.

The C-terminal region of SalB shows homology to proteins that contain a Lys7 domain, which allows for protein binding to cellular peptidoglycan (Muller *et al.*, 2006, Birkeland, 1994). Due to this homology, I hypothesized that SalB would be able to bind to the cell surface. Using an anti-SalB mAb panel to monitor whether SalB is surface associated on exponentially grown wild-type OG1RF and OG1RFΔ*gelE* strains, SalB does not appear to associate with the cell surface (Figure 4.3). Instead, upon examination of the supernatants of exponentially grown wild-type OG1RF and OG1RFΔ*gelE* strains, SalB was found to be in the supernatant (Figure 4.4). These findings demonstrate that SalB is a secreted protein that can be found in the supernatant during the exponential phase of growth.

While examining expression of SalB by Western blot analysis, a higher level of SalB in the supernatant of the OG1RFΔ*gelE* strain was detected, suggesting that the presence of GelE impacts the levels of SalB (Shankar *et al.*, 2012). The homologue of SalB in *E. faecium,* SagA, has been shown to be sensitive to proteinase K suggesting that SalB might also be sensitive to protease cleavage (Teng *et al.*, 2003). To determine whether GelE cleaves SalB and at what

GelE concentration cleavage occurs at, recombinant SalB was incubated with purified GelE. SalB was cleaved into fragments by purified GelE at high concentrations between 1.5 and 15 μg/mL, while no cleavage of SalB resulted from incubation of SalB with low concentrations of GelE (Figure 4.7). Since the physiological concentration of GelE in *E. faecalis* is ~ 30 nM (~1 μg/mL) (Hancock & Perego, 2004, Makinen *et al.*, 1989) and recombinant SalB is not cleaved by GelE at a concentration of 0.45 μg/mL and below (Figure 4.7), these findings suggested that SalB is cleaved by GelE. Using anti-SalB mAb 42 to detect SalB in the supernatant of wild-type OG1RF by Western blot analysis, fragments of SalB ranging in size from ~30 kDa to ~40 kDa were detected. Alternatively, detection of SalB in the supernatant of the OG1RFΔ*gelE* strain displayed a major band at ~55 kDa and a minor band at ~45 kDa (Figures 4.4 and 4.6). Consistent with Shankar *et al.*, a higher level of SalB was seen in the OG1RFΔ*gelE* strain (Figures 4.4 and 4.6)(Shankar *et al.*, 2012). Taken together, these findings suggested that in the presence of GelE SalB is cleaved.

OG1RFΔ*salB* cells grow in clumps and appear to have mislocalized division septa, suggesting that SalB impacts cell division, particularly during septum formation (Shankar *et al.*, 2012). In addition, it is known that OG1RFΔ*atlA* and OG1RFΔ*gelE* cells appear as cellular chains under light microscopy (Qin *et al.*, 1998, Waters & Bassler, 2005). Because deletions of *atlA, salB,* or *gelE* cause defects with regards to cell division, I hypothesized that SalB and AtlA could be directly interacting. To evaluate a potential interaction between SalB and AtlA or GelE-cleaved AtlA, SPR analysis was employed. No direct interaction between recombinant versions of SalB and full-length AtlA or cleaved AtlA was observed (Figure 4.1). Although SalB does not appear to impact septum formation through an AtlA interaction, it is still possible that SalB plays a pertinent role in cell septum formation and impacts AtlA localization.

In streptococcus, PcsB was found to bind to the essential cell division protein, FtsX (Sham *et al.*, 2011). In addition, PcsB was found to be a peptidoglycan hydrolase that becomes active only upon binding to FtsX (Sham *et al.*, 2011, Bajaj *et al.*, 2016). Similarly, SagA was also initially thought to have no peptidoglycan hydrolase activity, but later work showed that it did (Teng *et al.*, 2003, Rangan *et al.*, 2016). So far, SalB has shown no significant peptidoglycan hydrolase activity (Shankar *et al.*, 2012). Based on the homology of PcsB and SagA with SalB, I propose that SalB might also interact with one of the major cell division proteins causing SalB to exhibit peptidoglycan hydrolase activity. In fact, Shankar *et al.* proposed that the coiled-coil domain in the N-terminal region of SalB could interact with a cell division protein (Shankar *et al.*, 2012). In the future, I would like to determine whether or not SalB has any protein interaction partners such as one of the essential cell division proteins.

Purified GelE was shown to cleave recombinant SalB (Figure 4.7). The impact of GelE cleavage on the functions of SalB was evaluated. OG1RFΔ*salB* cells display a clumping phenotype, suggesting a role for SalB in cell division (Shankar *et al.*, 2012). To determine the impact of GelE-cleavage on the function of SalB in cell division, the addition of recombinant SalB or native GelE-cleaved SalB to OG1RFΔ*salB* cells was examined. Upon the addition of either recombinant SalB or native GelE-cleaved SalB, the cells grew in clumps. Since neither recombinant nor native GelE-cleaved SalB were able to restore the wild-type phenotype, this finding suggests that the clumping phenotype is irreversible (Figure 4.8). The absence of *gelE* and *salB* impacted the viability of cells (Shankar *et al.*, 2012). Contrary to Shankar *et al.*, the addition of EDTA to OG1RFΔ*salB* cells did not impact the viability of OG1RFΔ*salB* cells (Figure 4.9). Overall, these findings were unable to determine the impact of GelE cleavage on SalB function, particularly in regards to cell division and viability.

Based on the flow cytometry and Western analysis of wild-type OG1RF and OG1RFΔ*gelE* strains, SalB does not bind to the cell surface, but instead remains in the

supernatant (Figures 4.3 and 4.4). Although, SalB is found in the supernatant, due to the impact of SalB on cell division and septum formation, we propose that as SalB is secreted, a small amount of SalB remains bound to the cell at the cell septum (Figure 4.10), which would not be detected by flow cytometry. The discrepancy between our results and our model could also be due to SalB localization occurring at a different growth stage. Future work examining different time points and cell division interaction partners will determine if small amounts of SalB are found localized to the cell septum. The continued study of SalB and GelE will provide a deeper understanding of the impact that post-translational modification of SalB by GelE has on the functions of SalB, particularly in regards to cell division. Overall, this work deepens our understanding of the regulation of a stress induced protein by GelE post-translational modifications.

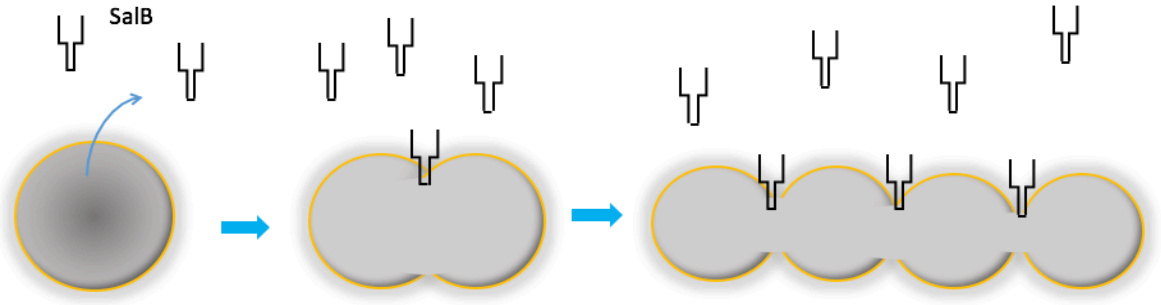

Figure 4.9: Model of SalB septum localization. SalB is expressed in response to cellular stress and secreted outside the cell. A large concentration of SalB is localized in the supernatant. I propose that a small concentration of SalB can bind to the cell septum via the Lys7 domain. The localization of SalB to the cell septum marks the cell septum placement for other proteins to bind.

Chapter 5: Conclusions and Final Remarks

Summary

Since the 1970s, the Gram-positive commensal bacterium, *Enterococcus faecalis,* has become a major healthcare associated pathogen (Murray, 1990, Sievert *et al.*, 2013). The emergence of *E. faecalis* as a major pathogen can be partially attributed to the bacterium's ability to form biofilms on both human tissues and on medical devices, making it difficult to eradicate (Donlan, 2002, Joyanes *et al.*, 1999, Arias & Murray, 2012). In addition, there has been a rise in antibiotic resistant enterococci isolates, making it difficult to treat enterococcal infections (Arias & Murray, 2012, Flemming & Wingender, 2010). Therefore, in order to develop better medical treatments, a greater understanding of the virulence-associated factors of *E. faecalis* is needed.

The major autolysin of *E. faecalis*, AtlA, is important in regulating cell separation during cell division (Tomasz, 1974, Shockman *et al.*, 1996). GelE, a secreted zinc metalloprotease, is a major virulence factor of *E. faecalis* (Engelbert *et al.*, 2004, Mohamed *et al.*, 2004). AtlA is affected by GelE. The impact of proteases on the autolytic profile was examined by Thomas *et al.* In cells that expressed GelE (OG1RF and OG1RF*ΔsprE*), a processed band at 62 kDa was displayed while cells that did not express GelE (OG1RF*ΔgelE,* OG1RF*ΔgelEΔsprE)* showed an AtlA band at 72 kDa (Thomas *et al.*, 2009). In this work, a monoclonal antibody panel whose members bind different domains of AtlA (Figure 3.2), was developed in order to study the impact of GelE-mediated AtlA cleavage. Western blot analysis and flow cytometry demonstrated that an N-terminally truncated form of AtlA existed only when GelE was present (Figure 3.1 and 3.3). Using N-terminal sequencing, the GelE cleavage site was determined to occur within Domain I, 7 amino acids from the start of Domain II, between Ala 173 and Leu 174 (Figure 3.5).

In this dissertation, the impact of GelE cleavage on the function of AtlA was evaluated. GelE-cleavage of AtlA did not significantly impact the ability of AtlA to cleave peptidoglycan compared to full-length AtlA (Figures 3.6 and 3.7). The interaction between AtlA and GelE was found to promote localization of AtlA to the cell septum. Specifically, in the wild-type OG1RF strain, AtlA was found to primarily localize to either the cell septum or to the cell poles while AtlA randomly localized across the cell surface in an OG1RFΔ*gelE* strain (Figure 3.9). Further analysis revealed that GelE-cleaved AtlA impacted the separation of dividing cells. The addition of recombinant GelE-cleaved AtlA (AtlA') to the OG1RFΔ*gelE*Δ*atlA* strain displayed single or paired cells while the addition of recombinant full-length AtlA to the OG1RFΔ*gelE*Δ*atlA* strain displayed long chains (Figure 3.8). Overall, these findings demonstrated that post-translational modification of AtlA by GelE regulates AtlA septum localization and impacts cell separation.

The second part of this dissertation focused on another *E. faecalis* secreted protein, SalB. SalB is a stress-induced protein with apparent importance in cell septum formation and in autolytic activity (Shankar *et al.*, 2012). In order to determine the localization and function of SalB, an anti-SalB monoclonal antibody panel was developed. Through sequence comparisons, it has been suggested that SalB is able to bind to peptidoglycan via the C-terminal Lys7 domain (Muller *et al.*, 2006). Using a combination of flow cytometry and Western blot analysis of different *E. faecalis* strains *in vitro*, SalB was detected in media supernatant but not associated with the cell surface (Figures 4.2 and 4.3). In addition, SalB was detected by Western blot analysis as cleaved fragments ranging in size from ~30 kDa to ~40 kDa in strains that express GelE (wild-type OG1RF and OG1RFΔ*atlA*) while strains that do not express GelE (OG1RFΔ*gelE*) displayed a major band at ~55 kDa and another at ~45 kDa (Figure 4.3). This finding suggested that the presence of GelE was responsible for the cleavage of SalB. In

addition, the presence of the minor ~45 kDa band in OG1RFΔ*gelE* suggested that another protease might impact the processing of SalB.

The impact of GelE cleavage on SalB function was evaluated. Since both AtlA and SalB influence cell division and autolysis, I studied the interaction between AtlA and SalB. Based on SPR analysis, AtlA and SalB showed minimal interaction (Figure 4.1). In addition, because deletion of *salB* demonstrated an effect on cell division (Shankar *et al.*, 2012), the addition of either recombinant SalB or native GelE-cleaved SalB to the OG1RFΔ*salB* strain was examined under a light microscope. Neither the addition of recombinant SalB nor native GelE-cleaved SalB impacted the cell clumping phenotype (Figure 4.7). This finding suggests that cellular clumping is irreversible. Since GelE-cleavage of SalB results in many SalB fragments, it is possible that post-translational modification does not impact the function of SalB, but rather degrades SalB from the environment when it is no longer needed. Further work will need to be conducted to completely understand the effect of GelE cleavage on SalB function.

The findings presented in this dissertation demonstrate GelE cleavage of two *E. faecalis* secreted proteins. In addition to the continued study of the impact of GelE cleavage on these two proteins, it will be important to determine if other *E. faecalis* secreted proteins are altered by the presence of GelE. For example, our laboratory has already shown that another surface protein, Ace, is cleaved by GelE (Pinkston *et al.*, 2011). Therefore, evaluation of other surface proteins post-translationally modified by GelE is necessary to fully comprehend how GelE can impact *E. faecalis* pathogenesis.

The work presented in this dissertation represents a significant contribution to understanding the impact of a major virulence factor, GelE, on two *E. faecalis* secreted proteins AtlA and SalB. These findings add to our knowledge of how the function of AtlA in cell division is regulated and suggests that the function of SalB is also regulated. The continuation of

this work will enhance our knowledge of cell division and the major regulators involved in the final stage of cell division, potentially providing mechanisms that antimicrobials can target to prevent the spread of *E. faecalis* infections.

Conclusions and Future Directions

Potential models of AtlA septal localization

Recall, that initial studies showed that AtlA has two forms: an active and a latent form. The active form was found to localize to the cell septum, while the latent form was found randomly across the cell surface (Shockman *et al.*, 1967a). In this work, GelE was discovered to cleave AtlA within Domain I, between Ala 173 and Leu 174 (Figure 3.5). GelE-mediated AtlA cleavage altered the localization of AtlA to the cell septum (Figure 3.9) and positively affected cell separation (Figure 3.8). In an OG1RF*ΔgelE* strain, full-length AtlA was found randomly across the cell surface, similarly to the latent form (Figure 3.9B). In the wild-type OG1RF strain, AtlA was primarily localized to the cell septum or the cell pole, similarly to the active form described by Shockman *et al.* (Figure 3.9A) (Shockman *et al.*, 1967a, Shockman & Martin, 1968, Higgins *et al.*, 1970). In this section, two models are proposed to understand how post-translational modification alters AtlA localization.

In *E. faecalis,* the secretory machinery has been found to be located near the site of cell division (Kline *et al.*, 2009). In the first model, I propose that as AtlA is secreted outside the cell, it attaches to the nearby peptidoglycan via Domain III, near the future cell septum region. As the cell continues to grow, new peptidoglycan is inserted at the septal region and the old peptidoglycan with attached AtlA migrates toward the cell pole (Raz, 2016, Wheeler *et al.*, 2011). At a later growth stage, GelE is secreted outside the cell and comes into contact with septum localized AtlA, cleaving it. In this model, I propose that post-translational modification by GelE would maintain AtlA at the cell septum instead of allowing truncated AtlA to migrate to other areas of the cell surface with the old peptidoglycan (Figure 5.1a). This putative model is consistent with my findings that truncated AtlA is found at the cell septum (Figure 3.9).

Alternatively, I propose that as AtlA is secreted outside the cell, it is released into the extracellular environment. Due to the presence of Domain III, AtlA will be able to bind anywhere along the cell surface (Figure 5.1B). As GelE is expressed and secreted, GelE cleaves the N-terminal domain from the surface associated AtlA, resulting in movement of AtlA towards the cell septum. At this point, it is unclear how cleaved AtlA is able to specifically localize to the cell septum. The next section discusses potential AtlA-cell septum interaction partners that could alter AtlA localization. This model is dependent upon the idea that the N-terminally truncated AtlA localizes to the cell septum.

In these studies, the localization of AtlA was difficult to determine because maximal GelE secretion occurs as the cells begin to enter stationary phase. Although, GelE expression and secretion is cell density dependent, there are always small amounts of GelE expressed during low cell densities (Nakayama *et al.*, 2001, Qin *et al.*, 2001, Pinkston *et al.*, 2011). Due to GelE expression, all imaging occurred at late exponential phase when AtlA would already be localized to the cell septum and theoretically cleaved by GelE.

In order to further evaluate AtlA septal localization, I propose the examination of AtlA localization pre-GelE cleavage at an early time point. This study would demonstrate whether AtlA is first localized to the cell septum, before GelE-cleavage, or if instead AtlA randomly binds across the cell surface. Although, as discussed above, small amounts of GelE will be present during an early time point and depending on the sensitivity of AtlA cleavage might cause AtlA to be cleaved. To examine localization pre-GelE cleavage, localization of AtlA in the OG1RFΔ*gelE* strain was examined and was found randomly dispersed across the cell septum, suggesting that AtlA does not first localize to the cell septum (Figure 3.9). Although, if GelE cleavage is important for the maintenance of AtlA at the cell septum, then AtlA would be randomly localized across the cell surface in a OG1RFΔ*gelE* strain.

Another potential way to evaluate AtlA localization would be to express fluorescently-tagged AtlA in the wild-type strain (Mellroth *et al.*, 2012). Using time lapse fluorescence microscopy, the AtlA localization could be monitored over a period of time, examining localization both pre- and post-GelE cleavage. To explore this idea further, I could also monitor fluorescently tagged GelE (with a different color than the tagged AtlA). This would allow us to determine if GelE localizes with AtlA only at the cell septum or localizes with AtlA across the cell surface. Taken together, in order to evaluate AtlA localization pre-GelE cleavage, I propose either monitoring AtlA by fluorescence microscopy or by examining localization at an early growth stage.

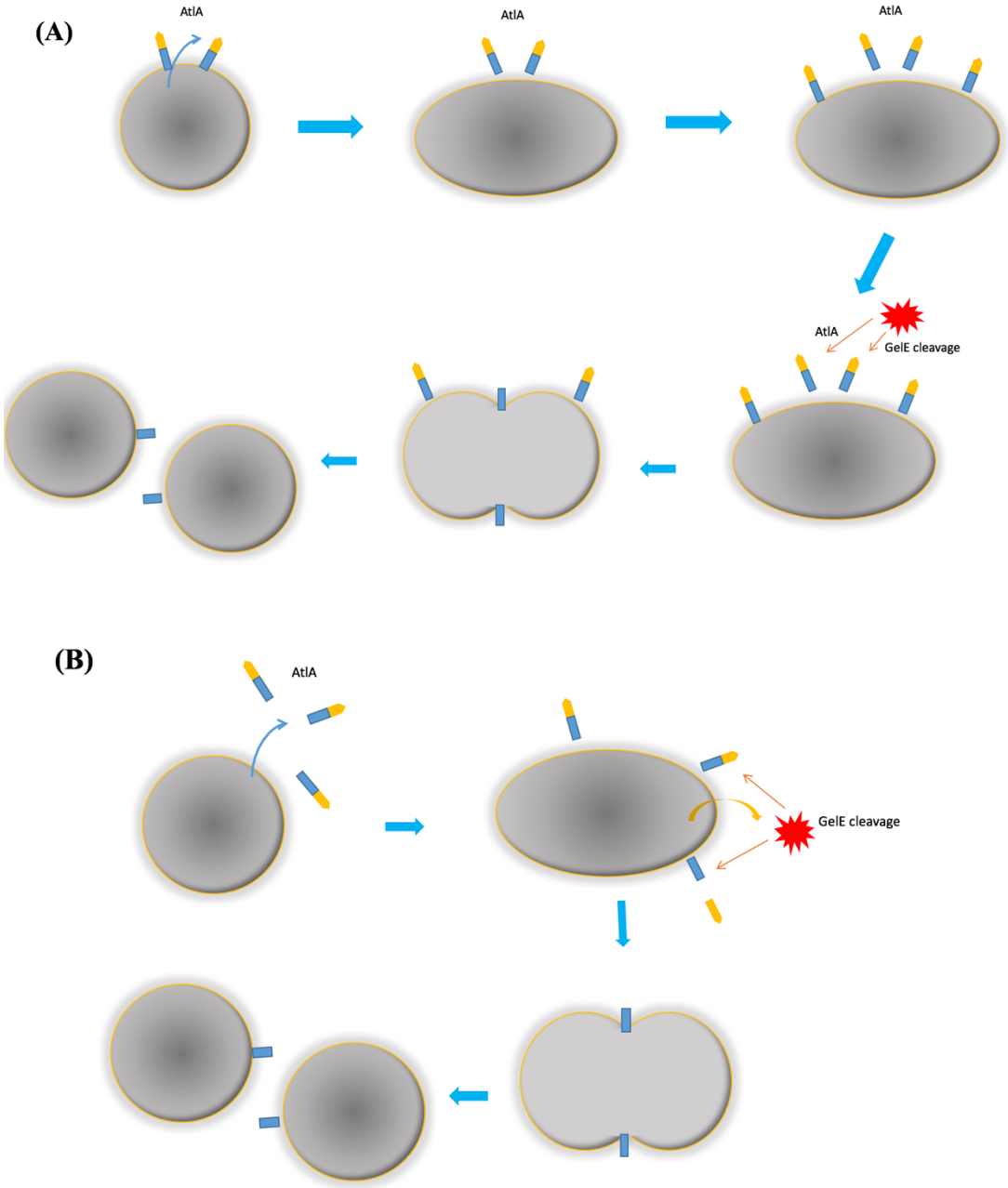
(A)
AtlA
AtlA
AtlA
AtlA
GelE cleavage
(B)
AtlA
GelE cleavage

Figure 5.1: Proposed models of AtlA cell surface localization. (A) AtlA is secreted outside the cell and binds to the surrounding peptidoglycan, near the future cell septum. As the new peptidoglycan is inserted, the old peptidoglycan with AtlA attached is pushed towards the cell pole. As GelE is secreted, it encounters the septum localized AtlA, cleaving AtlA. This cleavage allows the N-terminally truncated AtlA to remain at the cell septum and cleave the peptidoglycan, resulting in two separate cells. (B). AtlA is secreted outside the cell and binds anywhere along the cell surface. As GelE is secreted, it cleaves the N-terminal domain from surface bound AtlA. This cleavage localizes AtlA to the cell septum by some unknown mechanism. The N-terminally truncated AtlA localized to the cell septum cleaves the peptidoglycan, resulting in two separate cells.

Predicting potential partners that could interact with septum localized AtlA

As my results show, GelE-cleaved AtlA is localized to the cell septum (Figure 3.9). As described above, one of the proposed models implies that cleavage of AtlA by GelE is responsible for AtlA localization, yet it remains a mystery as to how only cleaved AtlA is septum localized. The first model proposes that GelE-cleavage retains AtlA at the cell septum, preventing it from migrating away from the septal region with the old peptidoglycan. Together, both of these models suggests that cleaved AtlA interacts with an unknown partner at the cell septum in order to either direct AtlA to the cell septum or to prevent cleaved AtlA from being pushed away from the septum region.

Recall that the major autolysin, Atl, in staphylococcus cells, is localized to the cell septum (Yamada *et al.*, 1996, Grilo *et al.*, 2014). Atl was found to bind to one of the cell wall components, lipoteichoic acid (LTA). Due to the increased amount of LTA at the cell septum, this interaction was proposed to allow Atl to become localized to the cell septum (Zoll *et al.*, 2012). In an effort to determine if AtlA binds to LTA in *E. faecalis*, an ELISA assay was used to evaluate binding interactions. Neither recombinant full-length AtlA nor D2D3 (representing truncated AtlA) demonstrated binding to LTA in the ELISA assay (Figure 3.14). Thus, although staphylococcus autolysin localization is determined by an interaction with LTA, it appears that *E. faecalis* AtlA does not localize based on an interaction with LTA. One caveat to this experiment was that the LTA used for analysis was from *Bacillus subtilis* instead of from *E. faecalis*. There are five different types of LTA, categorized by structure type (Fischer, 1994). Both *B. subtilis* and *E. faecalis* have Type 1 LTA (Schneewind & Missiakas, 2014). Hence, *B. subtilis* and *E. faecalis* have LTA with similar structures, suggesting that the LTA used in the evaluation would effectively mimic *E. faecalis* LTA. In order to further verify that LTA does not impact AtlA localization, an LTA deletion cell line (Kiriukhin *et al.*, 2001, Grundling & Schneewind, 2007, Lu *et al.*, 2009) could be examined for AtlA localization.

It was also proposed in staphylococcus cells that Atl is repelled from areas where the other major peptidoglycan polymer, wall teichoic acid (WTA), is found at high concentrations. The cell septum has been shown to have a lower abundance of WTA. Thus, due to the low concentration of WTA at the cell septum, Schlag *et al.* proposed that Atl would accumulate at the septum (Schlag *et al.*, 2010). Based on these findings, I hypothesize that *E. faecalis* AtlA could also be localized to the cell septum based on the decreased amount of WTA found there. Consistent with this hypothesis, Shockman *et al.* have examined the effect of the removal of WTA by TCA extraction from the cellular wall on the autolytic activity and found that activity decreased upon WTA removal, suggesting that the presence of WTA positively impacts the function of AtlA in autolysis (Shockman *et al.*, 1967b). One way to determine the impact of WTA on AtlA localization would be to generate a WTA deficient OG1RF cell line (Δ*tagB*) (Rigottier-Gois *et al.*, 2011, Schlag *et al.*, 2010, Biswas *et al.*, 2012) and monitor AtlA septal localization by transmission electron microscopy. Similarly, I could also monitor localization upon the removal of WTA by a TCA extraction. I anticipate that if WTA influences the localization of AtlA then a WTA deficient cell line would display random AtlA surface localization and a chaining phenotype.

Cell division proteins are known to mark the cell septum. Specifically, in *E. coli,* FtsZ produces the Z-ring that marks the cell septum (Bi & Lutkenhaus, 1991). Recall, that the ortholog of SalB, PcsB, is a peptidoglycan hydrolase and was found to interact with the essential cell division protein, FtsX (Sham *et al.*, 2011). Therefore, it is possible that AtlA, a peptidoglycan hydrolase, is interacting with an essential cell division protein in a similar manner as PcsB. To determine if AtlA interacts with a cell division protein, I propose conducting an AtlA co-immunoprecipitation assay. Another way to determine if AtlA interacts with any cell division proteins, would be to examine known cell division protein mutant cell lines and

examine AtlA localization. If one of these cell division proteins is important for AtlA localization, I would expect to see a random dispersal of AtlA across the cell surface in the absence of the cell division protein.

Another possibility is that AtlA could have a conformational change upon GelE-cleavage that exposes a new portion of one of the domains that was previously hidden or exposes an interaction site that allows only the cleaved AtlA to bind to the cell septum. In order to determine if there is a conformational change, a crystallography study examining full-length AtlA and GelE-cleaved AtlA could be conducted (Mesnage *et al.*, 2014). Thus, there are many different plausible mechanisms for how only cleaved AtlA is localized to the cell septum, while full-length AtlA randomly binds to the cell surface. Currently, because other autolysins have been shown to localize to the cell septum in this manner, I favor the model where the presence of WTA alters the localization of AtlA. Further studies will need to be conducted in order to determine the exact mechanism of GelE-cleaved AtlA septal localization.

Evaluating the major functions of SalB

The results presented in this dissertation demonstrated that SalB is detected *in vitro* in the media supernatant rather than associated with the cell surface during exponential phase (Figure 4.4). In addition, in the presence of GelE, SalB was detected as many fragments ranging in size from ~30 kDa to ~40 kDa (Figures 4.3 and 4.6). The impact of GelE cleavage on SalB function was evaluated, but not successfully determined. Further study of the interaction between GelE and SalB will need to be done to determine the impact of cleavage on SalB function.

Through sequence comparisons, it has been shown that SalB contains a C-terminal domain that is similar to the Lys7 domain, which is important in peptidoglycan binding (Muller *et al.*, 2006). Based on this region, I proposed that SalB would associate with the cell surface.

Upon flow cytometry analysis, however, surface associated SalB could not be readily detected (Figure 4.2). This lack of association was found to occur in exponentially grown cells. Since SalB has a peptidoglycan binding domain, SalB could be associated with the cell surface at an earlier or a later stage of growth. To evaluate this hypothesis, analysis of the association of SalB with the cell surface at different growth stages will need to be conducted.

OG1RFΔ*salB* cells grow in clumps and have misaligned septa (Shankar *et al.*, 2012), suggesting that one of the primary functions of SalB is in cell division, potentially in septum formation. To evaluate the impact of GelE cleavage on SalB function in cell division, either native GelE-cleaved SalB or recombinant SalB was incubated with OG1RFΔ*salB* cells and viewed under a light microscope. The cells maintained the clumping phenotype, suggesting that the formation of clumps is irreversible (Figure 4.7). The ortholog of SalB, PcsB, in streptococcus strains has been shown to interact with FtsX, a cell division protein (Sham *et al.*, 2011). Thus, I propose that SalB is interacting with a cell division protein, potentially at too low of an abundance to be visualized by flow cytometry. Shankar *et al.* proposed that SalB could interact with a cell division protein via the coiled-coil domain of SalB (Shankar *et al.*, 2012). To determine if SalB interacts with a cell division protein, I propose evaluation of potential SalB interaction partners via a co-immunoprecipitation assay.

Initially, it was proposed that SalB is a peptidoglycan hydrolase. PcsB was found to have peptidoglycan hydrolase activity, but only when bound to FtsX (Sham *et al.*, 2011). In work by Shankar *et al.,* SalB demonstrated no significant peptidoglycan hydrolase activity (Shankar *et al.*, 2012). It is possible that like PcsB, SalB might only have peptidoglycan hydrolase activity when bound to a specific partner. This cannot be determined until an interacting cell division protein is identified. Once potential SalB binding proteins are discovered by evaluating co-

immunoprecipitation assays, the peptidoglycan hydrolase activity of SalB with and without that cell division protein could be measured.

Proposed model of SalB, AtlA and GelE interaction

Recall that SalB is cleaved into many fragments in the presence of GelE (Figures 4.3 and 4.6). Although SalB was not associated with the cell surface (Figure 4.2), the presence of a peptidoglycan binding domain suggests a SalB-peptidoglycan interaction (Muller *et al.*, 2006). In my model, I propose that initially a small amount of SalB is bound to the cell septum. As GelE is expressed and secreted from cells when density of a cell population reaches a certain level, GelE processes SalB into small fragments, effectively removing SalB from the cell septum. Then, GelE-cleaved AtlA localizes to the cell septum, cleaving the peptidoglycan and completing cell division. Thus, in agreement with the results shown in this dissertation (Figure 4.1), SalB and AtlA do not interact, but instead compete for binding to the cell septum. Only after GelE processing of SalB can AtlA localize to the cell septum. Without SalB, AtlA randomly localizes across the cell surface instead of to the cell septum, potentially causing random cellular lysis as AtlA cleaves peptidoglycan across the surface. This proposal is consistent with work done that showed that OG1RFΔ*salB* had an increase in autolytic activity (Shankar *et al.*, 2012).

Although my results show that SalB does not bind to the cell surface, I propose that either SalB is only bound to the cell septum at an early stage of growth and thus not detected in my assay or only a small amount of SalB is bound to the cell septum at a given time, thus the bound SalB would be at too low of an abundance to be detected by flow cytometry. This model takes into consideration the idea that both AtlA and SalB seem to be important for both cell division and autolysis activity. At this time, this model has been untested, but it would be

interesting to determine the localization of AtlA in either a OG1RFΔ*salB* or OG1RFΔ*salB*Δ*gelE* deletion. If SalB impacts AtlA localization, then I would expect to see random AtlA surface localization or a lack of AtlA surface association in the OG1RFΔ*salB* or the OG1RFΔ*salB*Δ*gelE* strain. Unfortunately, this experiment could prove difficult because the cell septa are randomly located on the cell surface in a OG1RFΔ*salB* strain. It is likely that AtlA could localize to the various misplaced cell septa.

How is AtlA localization regulated when many clinical isolates lack GelE?

The *gelE* gene is found in about 60% of clinical isolates and only about 66.6% of those isolates were found to express GelE (Comerlato *et al.*, 2013). The results presented in this thesis suggested that the presence of GelE is necessary for AtlA septal localization and subsequent cell separation (Figures 3.8 and 3.9). Since GelE is required for proper cell division, this leads us to question how does the role of AtlA and possibly SalB in cell division get regulated when GelE is not present in all strains?

Originally, clinical isolates were found to contain *gelE* more frequently than non-clinical strains (Coque *et al.*, 1995). In contrast, a more recent study demonstrated that there was no significant difference in the presence of the *gelE* gene in *E. faecalis* from both normal flora and infective endocarditis (Johansson & Rasmussen, 2013). In addition, the presence of the *gelE* gene was examined in oral muscosal infections and deep infections and no significant difference in the presence of *gelE* was also observed between the two (Dahlen *et al.*, 2012). Thus, the site of infection does not appear to impact the presence of *gelE*.

Upon examining cellular chain lengths of different clinical isolates, it was found that the presence of GelE did not correlate with a short chain phenotype (Arias *et al.*, 2007). However, if AtlA is mutated and GelE impacts cell separation through AtlA as proposed in this thesis

(Figure 3.8), then regardless of the presence of GelE, a long chain phenotype would occur. Thus, disruption of AtlA could explain the lack of correlation seen between GelE expression and chaining. Another possibility could be that another protease acts on AtlA in the absence of GelE. For example, SprE has been shown to process AtlA differently than GelE (Figure 1.4) (Thomas *et al.*, 2009). It is also possible that in strains that do not express GelE that another autolysin, such as AtlB, could function similarly to GelE-cleaved AtlA. AtlB has been shown to be able to digest peptidoglycan and impact cell separation in an *atlA* deletion strain (Mesnage *et al.*, 2008, Emirian *et al.*, 2009). This suggests that AtlB has the ability to carry out similar functions to AtlA. Further work evaluating the presence of other proteases and other autolysins will need be done to determine the regulation of AtlA in the absence of GelE. The first step to evaluate these proposed mechanisms for cell division in the absence of GelE would be to analyze AtlA cleavage and the presence of AtlB in the clinical isolate strains.

Targeting of virulence factors as novel anti-microbial drugs

Over the last few decades, enterococci have dramatically risen to become one of the most frequent pathogens associated with hospital acquired infections (Arias & Murray, 2012, Olawale *et al.*, 2011). Antibiotic resistance, particularly vancomycin resistance, is rapidly spreading among the enterococcal species and has become a threat to current medical treatments (Hidron *et al.*, 2008). Thus, it is imperative to determine novel targets for anti-microbial drugs. One way to combat this problem is to develop drugs that target virulence associated surface proteins to inhibit functions such as biofilm formation, cell division, or host immune evasion. Particularly, in this dissertation, I examined two *E. faecalis* secreted proteins, AtlA and SalB, that appear to impact cell division, making these proteins or the pathways that they are involved in putative targets for anti-microbial drugs.

AtlA is necessary for completion of the last stage of cell division, cell separation, which makes AtlA an ideal target for antimicrobial drug design in order to prevent bacterial growth (Shockman *et al.*, 1967b, Kohanski *et al.*, 2010). Herein, AtlA was found to be cleaved by GelE (Figure 3.7), which was demonstrated to be necessary for the localization of AtlA to the cell septum and impacted the ability of dividing cells to separate (Figures 3.8 and 3.10). In *S. pneumoniae,* long chains have been associated with human epithelial cell adherence because there are more available adhesins per particle (Rodriguez *et al.*, 2012) while short chains have been shown to be important for evasion of host immune defenses, which promotes invasive disease (Weiser, 2010, Dalia & Weiser, 2011). Since results found in this thesis demonstrated that the post-translational modification of AtlA changed the cellular chain length, targeting of AtlA through an antimicrobial could impact chain length, altering how the bacterial cell interacts with the host cell. In addition, through its autolytic activity, AtlA is important in biofilm formation (Qin *et al.*, 1998). By targeting AtlA, drugs could inhibit biofilm formation, making it easier to eliminate bacterial infections with other antibiotics.

SalB is a stress-induced protein (Muller *et al.*, 2006) that impacts cell division (Le Breton *et al.*, 2003, Shankar *et al.*, 2012). Many studies have linked bacterial stress response with virulence (Brenot *et al.*, 2005, Cotter *et al.*, 1999, Teng *et al.*, 2002). For example, Gls24 is a stress protein that has been shown to impact the virulence in *E. faecalis* (Teng *et al.*, 2005, Giard *et al.*, 2000). In addition, anti-Gls24 antibodies were shown to protect mice against infection (Teng *et al.*, 2005). Although, the function of SalB in cell division is not known, I propose that SalB is secreted in response to stress in order to regulate the cell division pathway. Based on this hypothesis, the targeting of SalB will impact proper cell division, resulting in cellular clumps, which could impact the viability of cells. In agreement with this hypothesis, it has been shown that deletion of *salB* and *gelE* significantly impacts the viability of cells (Shankar *et al.*, 2012). Although the role of SalB in cell division is not yet defined, I believe that

the further study of SalB will provide an excellent target for drug design. As we increase our knowledge of both AtlA and SalB and their roles in cell division, we can determine more targets for rational drug design in the cell division pathway.

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