THE MOLECULAR CONTROL AND BIOLOGICAL IMPLICATIONS OF AUTOLYSIS IN ENTEROCOCCUS FAECALIS BIOFILM DEVELOPMENT

by

VINAI CHITTEZHAM THOMAS

B.Sc., University of Pune, India, 2000

M.Sc., University of Pune, India, 2002

M.Sc., University of Abertay Dundee, UK, 2004

AN ABSTRACT OF A DISSERTATION

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Abstract

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In the current study a mechanistic role for two Fsr quorum-regulated extracellular proteases- gelatinase (GelE) and its cotranscribed serine protease (SprE), were explored in biofilm development of *E. faecalis* V583. Confocal imaging of biofilms suggested that GelE$^-$ mutants were significantly reduced in biofilm biomass compared to V583, whereas the absence of SprE appeared to accelerate the progression of biofilm development. Culture supernatant and biofilm analysis confirmed that decreased biofilms observed in GelE$^-$ mutants resulted from their inability to undergo autolysis and release extracellular DNA (eDNA) in planktonic and biofilm cultures, whereas SprE$^-$ mutants produced significantly more eDNA as components of the biofilm matrix.

The governing principle behind GelE mediated autolysis and eDNA release in *E. faecalis* V583 was demonstrated to be fratricide. GFP reporter assays of V583 populations confirmed that GBAP (gelatinase biosynthesis-activating pheromone encoded by *fsrD*) quorum non-responders (GelE$^-$SprE$^-$) were a minority subpopulation of prey cells susceptible to the targeted fratricidal action of the quorum responsive predatorial majority (GelE$^+$SprE$^+$). The killing action is dependent on GelE, and the GelE producer population is protected from self-destruction by the co-production of SprE as an immunity protein. Targeted gene inactivation and protein interaction studies demonstrate that extracellular proteases execute their characteristic effects following downstream interactions with the primary autolysin, AtlA. Finally, comparison of virulence effects of isogenic extracellular protease mutants (Δ*gelE*, Δ*sprE* and Δ*gelEsprE*) relative to parental strain (V583) in a rabbit model of enterococcal endocarditis confirmed a critical role for GelE in the infection process.

In conclusion, the data presented in this thesis are consistent with significant roles for GelE and SprE in biofilm mediated pathogenesis of enterococcal infections.

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CHAPTER 1

Literature review: A case for death in enterococcal biofilm development

Introduction

Originally identified by Thiercelin (1899) as gram-positive intestinal diplococci capable of forming short chains (148, 149), enterococci were initially classified as members of the genus *Streptococcus*, and by 1984 they were formally recognized as a separate genus-*Enterococcus* (127). Interestingly, over the last two decades enterococci have emerged as both successful gut commensals and pathogens capable of infecting multiple sites within the human body (clinical incidence summarized in Table 1.1). The transition of enterococci from commensal to the pathogen state primarily requires an immunocompromised host. However extended treatments with broad-spectrum antibiotics (cephalosporins and aminoglycosides) that enterococci are inherently resistant to may disproportionately enhance its competitive advantage over other antibiotic susceptible gut colonizers, thus catalyzing its pathogenic potential (35, 36, 92, 139). Two of the more prominent clinical species from over twenty in this genus include *Enterococcus faecalis* and *Enterococcus faecium* (65). Although in recent times *E. faecium* is gaining much more notoriety due to acquisition of multiple antibiotic resistance traits including resistances to vancomycin and ampicillin, most infectious isolates are of the species, *E. faecalis* (>90%) (83, 123).

Table 1.1 Clinical incidence of enterococcal infections

Clinical infection	Disease frequency	References
Endocarditis	5-15%	(87)
Urinary tract infection	5-16 %	(17, 65)
Blood stream	10- 25%	(65)
Intra-abdominal infections	20%	(107)
Skin and soft tissue	5.1%	(72)
Wound or surgical site infections	12-19%	(17, 65, 95)
Pneumonia	2%	(65)

The increased frequency of infections resulting from pathogenic lineages of enterococci has often been associated with the acquisition of variable traits that confer enhanced virulence or antibiotic resistance over non-pathogenic isolates (48). Genome sequence analysis of the first reported vancomycin resistant isolate of *E. faecalis* from an intensive

care patient in the US (V583) confirms this argument as more than a quarter of its genome consists of mobile DNA elements of foreign origin that confer it unique infectious qualities. These foreign elements include a large pathogenicity island (~150-kb), three plasmids (pTEF-1, pTEF-2 and pTEF-3) that confer multiple antibiotic resistances, three integrated plasmid remnants, a transposon harboring vancomycin resistance, seven putative prophage regions and 38 insertion sequences (110). Besides accruing from variations in the content of mobile elements, phenotypic diversity and potentially infectious abilities of enterococcal isolates can also vary due to spontaneous modifications of chromosomal DNA. For instance, a 23.9-kb chromosomal deletion localized within the *fsr* region confers phenotypic differences in the expression patterns of extracellular proteases in different strains of *E. faecalis* (99).

Crucial to enterococcal colonization of the gut and pathogenesis is its ability to adhere to the gut epithelia and develop into infectious communities (30). Such communities also known as biofilms have been linked to the pathogenesis of multiple other bacterial pathogens (106). Biofilm bacteria are encased in a well hydrated matrix composed of secreted exopolymeric substances, proteins and nucleic acids from dead-lysed cells that affords protection against host immune clearance and antibiotic therapy (29). Besides tissue epithelia, bacterial biofilms can also develop on body implant devices (28). It is conceivable that the ever increasing use of antibiotics and implant devices in hospitals contribute to the enrichment of traits that promote biofilm development in clinical pathogens (28, 47, 60). Although a number of studies have dissected genetic determinants that influence *E. faecalis* virulence (summarized in Table 1.2), relatively little is known about its ability to form biofilms. Hence, the general aim of this introductory chapter is to review current evidence on genetic traits and mechanisms that are known to or may potentially aid the development of enterococci into biofilms during its infectious cycle. Further, as a direct precedence to the primary theme of this thesis, current evidence on the role of bacterial death and lysis (autolysis) in biofilm development and the factors that influence it will also be discussed.

Table 1.2 *Enterococcus faecalis* virulence factors

Virulence trait	Virulence effects	Animal model	References
Cytolysin	Lyses bacterial as well as eukaryotic cells	Rabbit endocaditis model Murine peritonitis model	(20, 25, 64, 66, 68)
Gelatinase	Secreted zinc metalloprotese; hydrolyses gelatin, collagen, casein, lactoglobulin; inactivates human endothelin, LL-37 and C3a; inhibits proteolytic activity inside the cells; modulates biofilm development	Rabbit endocarditis model rabbit endophthalmitis model Mouse and rat peritonitis model	(42, 115, 135)
Serine protease	Secreted serine protease; modulates biofilm development	Mouse peritoneal model	(42, 115, 135)
Hyaluronidase	Degrades hyaluronic acid	Not determined	(121)
Esp	Cell surface localized protein : Adhesin in colonization of the urinary tract, mediates biofilm formation	Mice transurethral infection model	(78, 131, 152)
Aggregation substance (Agg)	Cell wall anchored protein involved in conjugation and adhesion to eukaryotic cells	Rabbit endocarditis model	(20, 21, 59, 75, 128, 129)
Adhesin to collagen of *E. faecalis* (Ace)/ Adhesin to collagen of *E. faecium* (Acm)	Cell wall anchored protein collagen binding protein	Rat endocarditis model Murine macrophage model	(74, 79, 101, 122, 153)
Enterococcal polysaccharide antigen (Epa)	Cell surface polysaccharide; antiphagocytic	Mouse peritonitis model	(146, 172)
Cps	Capsular polysaccharide; antiphagocytic	Subcutaneous murine infection model	(53, 61)
Lipoteichoic acid (LTA)	Lipoteichoic acid; binding substance for conjugation; mediates biofilm development	Not determined	(129, 155)
Endocarditis and biofilm associated pili (Ebp)	Mediates attachment to eukaryotic cells and biofilm development	Rat endovascular infection model	(21, 136)

Stages of *Enterococcus faecalis* biofilm development

Multiple studies including ours (employing *E. faecalis*) suggest that bacterial biofilm development occurs in distinct stages (Fig 1.1) (43, 54, 62, 76, 151). The first stage involves attachment of planktonic bacteria to a substratum. Given that most abiotic/ biotic surfaces in nature, under physiological conditions display a net negative charge similar to the bacterial surface, primary attachment would be possible only if the bacteria can overcome electrostatic repulsion ensuing from the bacteria-substrate surface interface (159).

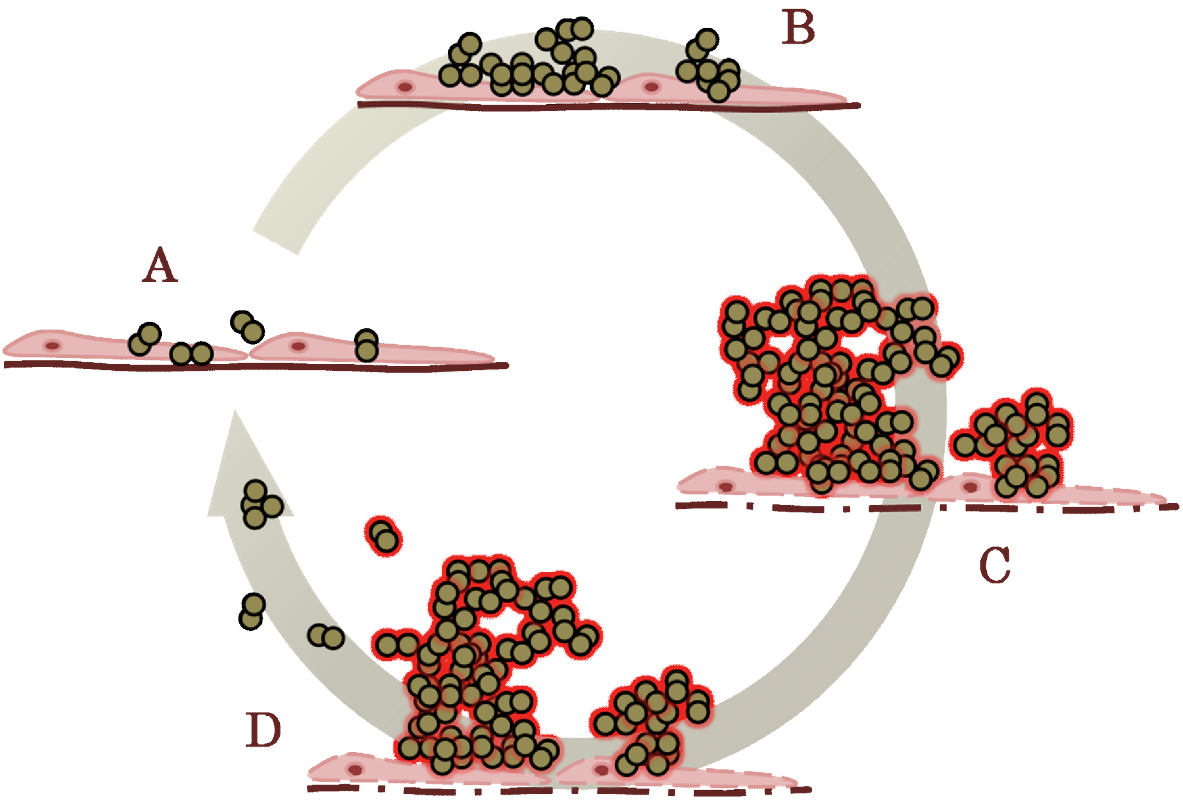

Figure 1.1 The four general stages of biofilm development.
A. Attachment phase: Bacteria overcome repulsive forces and attach to tissue surfaces (cells with intact borders and basal lamina). B. Microcolony phase: Bacteria interact with each other and the tissue surface to form layers leading to the development of microcolonies. C. Maturation phase: Microcolonies reach maximum thickness, and a broad shift in metabolic state is observed with excess production of exopolymeric substances (red glow). D. Dispersion phase: Bacteria within the mature biofilm are released and are free to infect fresh sites. The last two phases may be characterized by enhanced damage to tissue and basal lamina (broken borders).

Studies carried out using *E. faecalis* have identified at least two primary mechanisms by which this bacterium may overcome repulsive forces. Culture heterogeneity with respect to zeta potential (surface charge) influences surface adherence (157). Zeta potentials of most *E. faecalis* strains, display a bimodal distribution indicating subpopulations with differing cell surface charges within a biofilm population (159). Such populations are adept at forming prominent biofilms. In a recent study it was argued that culture heterogeneity played a role in the later stages of adhesion rather than the initial surface deposition which was characterized by attractive Lifshitz-Van der Waals forces (157). Consistent with this argument, homogenous *E. faecalis* strains with a unimodal zeta potential, although having similar deposition rates, formed relatively less dense biofilms compared to heterogeneous (bimodal zeta potential) biofilms. Complementing this observation, heterogeneous strains that were transiently enriched for a more homogenous subpopulation also lost their ability to form prominent biofilms (158). A second mechanism may relate to cell surface associated proteins that manipulate the overall hydrophobicity of the cell. *Enterococcus* surface proteins (eg. Esp, Agg) that increase overall cell surface hydrophobicity have been reported to play important roles in adherence to bile drain materials (163).

The second stage of biofilm development involves specific cell-cell interactions leading to the development of microcolonies (102). It may be noted that this stage follows the initial challenge of repulsive forces that bacteria overcome to attach to a surface and involves the development of incipient three dimensional communities. The primary mechanism involved at this stage is thought to be cell-cell adhesion and possibly other tactics that elevate bacteria from the surface. Ultra-structural studies of *E. faecalis* biofilms identified surface polymeric projections that interlink cells together, as well as cells with the substratum (43, 44). Such projections were specific to cells growing as biofilms and absent on planktonic cells (43). In *S. epidermidis* biofilms, a similar intercellular polymeric linkage was identified to be the polysaccharide intercellular adhesion (PIA) molecule (39). Consistent with this observation, multilayered biofilm microcolonies of *E. faecalis* intensely stained for neutral hexose sugars with periodic acid-Schiff (PAS) stain, while planktonic cells were only marginally detected with the same stain (43). Although a genetic locus responsible for the buildup of these polymeric projections hasn't been identified in *E. faecalis*, one possibility remains the *epa* (*Enterococus* polysaccharide antigen) locus (89, 171, 172). Antibodies against Epa have been reported in sera from several patients diagnosed with enterococcal endocarditis, yet interestingly these antigens have never been successfully localized to the

E. faecalis cell surface (146). One reason for this discrepancy may be that these polymers (as argued by Erlandsen et al.) are specific to the biofilm state and not to planktonic states usually tested in the laboratory (43). Consistent with this possibility, mutations in the *epa* loci rendered *E. faecalis* deficient in biofilm development (89). Erlandsen et al. also reported that these surface associated polymeric projections were able to elevate cells from the surface of the substratum (43). Although the available evidence suggests these surface projections to be carbohydrate in nature, a second possibility that these projections are at least partly composed of nucleic acids is also likely. Recently the biofilm matrices of *Psuedomonas aeruginosa* and a host of other organisms including *E. faecalis* have been demonstrated to contain copious amounts of nucleic acids (DNA), possibly accruing from dead lysed cells (3, 147, 151, 169). Such DNA termed extracellular DNA (eDNA) to distinguish it from genomic DNA, was shown to contribute to the structural stability of biofilms (3, 119, 151). Extracellular DNA was more predominant at the base of *P. aeruginosa* biofilms and may be crucial for elevating bacteria from surfaces (3). Consistent with a significance of autolysis and eDNA release in biofilm development, a role for the primary autolysin of *E. faecalis*, AtlA, has also been proposed (89).

More recently a locus in the core genome of *E. faecalis* encoding a heterotrimeric pilin named Ebp (endocarditis and biofilm associated pili) was shown to influence the initial attachment of cells to a surface (100). However the mechanism by which pilus promotes biofilm development remains unknown in the case of *E. faecalis* (24, 100). It is possible that the pilus may contribute to the ability of *E. faecalis* to interact with eDNA present within the matrix. Such an interaction has been established in the case of *P. aeruginosa* and the environmental isolate *Acidovorax temperans* (6, 56). At the second stage of biofilm development, following the initial establishment of a quorum of bacteria on a surface, quorum sensing mechanisms may be triggered to allow further biofilm development. Consistent with this argument, the Fsr quorum regulon including the extracellular proteases (gelatinase, GelE and serine protease, SprE) and a putative sugar binding transcriptional regulator called BopD (biofilms on plastic) has been attributed to play important roles in biofilm development of *E. faecalis* at this stage (12, 30, 54, 62, 90, 114).

Maturation of the microcolonies into full-fledged biofilm communities constitutes the third stage of development (102). This stage is characterized by an increased buildup of protective biofilm matrix layer and the multiplication of the bacteria within the biofilm (102). Genetic factors involved at this stage have rarely been determined in *E. faecalis*.

However it is highly likely that some of the factors discussed in the second stage of enterococcal biofilm development may functionally overlap into the third stage. Two recent studies have implied roles for additional genetic factors that may influence this stage (5, 77). Both studies together identified at least 68 genetic determinants located within the core genome of *E. faecalis* that possibly play a role in the maintenance of the mature biofilm (5, 77). However additional studies validating these reports are necessary to identify contributions of specific genes in the biofilm developmental process.

The final stage (4^{th}) of biofilm development involves the release of single or small clusters of cells from the established biofilm that may act as infectious niduses at alternate sites in the body (102). Autolysis of a subset of cells within the mature biofilm is thought to be necessary for dispersal. In the marine bacterium *Psuedoalteromonas tunicata*, an autolytic protein, AlpP, has been identified to aid dispersal (85, 86). Interestingly a filamentous phage, Pf1, is thought to influence autolysis and dispersal in biofilms of *Psuedomonas aeruginosa* (167). In *S. aureus* biofilms the *agr* quorum sensing system and two proteases, aureolysin and the V8 protease have been implicated to play a role in dispersal (11). Although *E. faecalis* secretes extracellular proteases- GelE and SprE, that are homologous to the staphylococcal proteases, any role they may have in biofilm dispersal is yet to be identified.

Factors that aid *E. faecalis* biofilm development

A number of environmental factors are known to influence bacterial biofilm development (88, 111). Such dynamism in the regulation of biofilm development is not astonishing considering that bacteria have the capability of rapidly sensing stimuli and adapting to a particular environmental niche. Fluxes in oxygen, pH, osmolarity, temperature, and nutrients can have varying effects on *E. faecalis* biofilm development (88). For example, *E. faecalis* reacts differentially to changing levels of glucose. At a concentration of 2g/ L (glucose), *E. faecalis* forms inferior biofilms compared to a control with no glucose, and better biofilms at concentrations between 5- 10g/ L (4, 76, 111). The genetic basis of this bimodality is unknown; however one study observes that since *fsr* or *gelE* mutants were also compromised in glucose-dependant biofilm development, a transcriptional regulator sensitive to glucose may mediate catabolite control over biofilm development via the *fsr* and downstream extracellular proteases (111). A destabilizing effect has also seen been reported

for *E. faecalis* biofilms developing at high salt concentrations of 20- 30g/ L and biofilms grown in complex media relative to more simple media (76). Interestingly serum at a concentration of 10% enhanced the adhesion of enterococci to glass and silicone surfaces (46). These observations reaffirm a concrete role for environmental stimuli in regulating biofilm development of *E. faecalis*.

A number of studies have attempted to dissect the genetic determinants of biofilm development in *E. faecalis*. However, most factors described till date relate to variable traits and very few studies have actually described core mechanisms (e.g. *ebp*) that contribute to biofilm development ubiquitously in all strains of *E. faecalis*. Taking into account the natural bias in research (most studies have focused on the first two stages of biofilm development involving adhesion and cell-cell contact); this suggests a greater role for variable traits in the initial stages of biofilm development. Such variability may result from differential abilities of *E. faecalis* strains to acquire foreign DNA with biofilm enhancing qualities or due to genetic instability leading to deletions of the core genome. More recently, Gary Dunny's group at the University of Minnesota has conducted a series of studies and identified 68 genes that are active specifically during biofilm development (5). Although a role for most proteins encoded by these genes awaits further validation in *E. faecalis*, we will attempt to speculate on their role along with other more established proteins that influence biofilm development.

Metabolic and stress related proteins in biofilm development

Active drivers of physiological heterogeneity promote a plethora of metabolically distinct states in biofilm bacteria, ranging from high metabolic activity to dormancy in cells (138). Accordingly biofilms display a metabolic proteome distinct from the mostly physiologically homogeneous, sessile states of bacterial existence (124, 126, 141). A number of studies have reported genes involved in nutrient stress, anaerobic development and pH homeostasis to be differentially regulated at different stages of biofilm development (138, 141). Studies on *E. faecalis* biofilms have also made similar observations (88).

Similar to *S. aureus*, biofilms formed by *E. faecalis* were observed to up-regulate the expression of specific glycolytic genes- alcohol dehydrogenase and triosephosphate isomerase (5, 8). It has been hypothesized that these enzymes may play a prominent role in deriving energy through fermentation processes carried out by bacteria in the depths of a biofilm (8). Alternately these proteins may have novel roles that may aid biofilm

development. In this regard, the localization of α-enolase (another glycolytic enzyme) on the surface of pathogenic streptococci and *Mycoplasma fermentans* is reported to allow strong binding to plasminogen (105, 175). In either case, fermentation by biofilm bacteria may result in acids that disturb the overall cellular pH homeostasis.

The maintenance of intra- and extra-cellular pH homeostasis is critical to optimal biofilm development and maturation. Biofilm bacteria, especially in the advanced stages of development respond to acidic environments, by the production of neutralizing compounds like ammonia (9). One important route for the production of ammonia is the arginine deiminase (ADI) pathway, wherein the amino acid L-arginine is converted to ammonia (31). Consistent with a role for ammonia in biofilm development, the gene encoding catabolic ornithine carbamoyltransferase (an enzyme in the ADI pathway) was activated in *E. faecalis* biofilms. A separate gene, *argR*, encoding an arginine repressor was observed to become active in biofilms as well (5). The activity of ArgR implies that the *E. faecalis* may use diamino acids like arginine as energy sources in the developing anaerobic environment of the biofilm. In the presence of arginine, ArgR is thought to repress anabolic ornithine carbamoyltransferase and induce the ADI pathway, thus satisfying ATP requirements of the growing biofilm in anaerobic environments (63).

Enterococci within biofilms up-regulate the expression of ABC transporters and other complex sugar uptake systems (PTS systems) (5). ABC transporters are known to be involved in the intracellular assimilation of a wide variety of substrates from their immediate environment that include amino acids, carbohydrates, oligopeptides, inorganic ions, bacteriocins and DNA (2, 32, 58). At least eight such ABC transporters are expressed on entry of *E. faecalis* into the biofilm state (5). However their specific roles in biofilm development still remain to be elucidated. An interesting finding reported by Ballering et al., suggested the involvement of specific PTS systems in *E. faecalis* biofilm development. How does uptake of complex sugars correlate with biofilm development? Although it is possible that bacteria within biofilms up-regulate the expression of PTS systems as a direct response to depletion of simple sugars (eg. glucose) over the course of growth, these very same systems (PTS) may also play an additional role in *E. faecalis* biofilm development. Evidence for this argument stems from our recent observation that an *rpoN* (the alternate sigma factor, σ^{54}) transposon mutant of *E. faecalis* forms significantly defective biofilms compared to its isogenic wildtype (Thomas and Hancock, unpublished observations). Interestingly besides a biofilm defect, this mutant also displayed pleiotropic phenotypes

that included increased hetero-resistance to cell-wall targeting antibiotics, resistance to autolysis and extracellular DNA release defects (Thomas and Hancock, unpublished observations). Consistent with a role for sugar availability, at least four PTS systems are linked to RpoN via specific activators (MptR, MphR, MpoR and LpoR) (55, 156). Given this linkage between RpoN and PTS pathways, it is conceivable that the up-regulation of PTS pathways and corresponding RpoN activators in response to depletion of simple energy sources may trigger RpoN mediated control of antibiotic hetero-resistance, autolysis and biofilm development (Fig 1.2). At least two observations are consistent with this hypothesis. Firstly, RpoN regulated PTS pathway components and activators are selectively expressed in *E. faecalis* biofilms (5). Secondly, autolysis a trait that RpoN controls is thought to significantly regulate biofilm development of *E. faecalis* and other organisms (Thomas and Hancock, unpublished observations).

A number of genes involved in general bacterial stress responses were also reported to be activated in *E. faecalis* biofilms. These include the chaperones- DnaK, DnaJ and their regulator HrcA, DNA repair enzymes and ClpC protease (5). Although all these proteins have been reported to be important for biofilm development in different organisms, their specific contributions remain vague (81). However in general these data are consistent with the observation that bacteria experience varying degrees of stress during biofilm development.

Cell surface associated and extracellular proteins in biofilm development

Similar to gram-negative organisms, a role for pili in biofilm development of gram-positive organisms including *E. faecalis* was recently demonstrated (100, 112, 130). At least two loci in *E. faecalis* are thought to encode for pili- *bee* (biofilm enhancer in enterococcus) and *ebp* (endocarditis and biofilm associated pili) (100, 130). Both these loci show similar genetic organizations and include the expression of five (three cell surface associated proteins and two sortases) and four proteins respectively (three cell surface associated proteins and one sortase). However unlike *ebp*, the *bee* locus is a variable trait that is encoded from and transmissible via a conjugative plasmid (145). The sortases form a class of membrane-bound transpeptidases that assemble pili and other cell wall proteins on the surface of the cell (103). Mutation of two genes predicted to encode sortases (*srtA* and *srtC*) in *E. faecalis* resulted in significantly lesser biofilm development (103). Biofilm defects were also mirrored in an *ebpR* mutant, a regulator of the *ebp* locus (13). Consistent with a role for the

pilin locus in biofilm development, a recent study showed that all genes from the *ebp* locus were expressed selectively in *E. faecalis* biofilms (5).

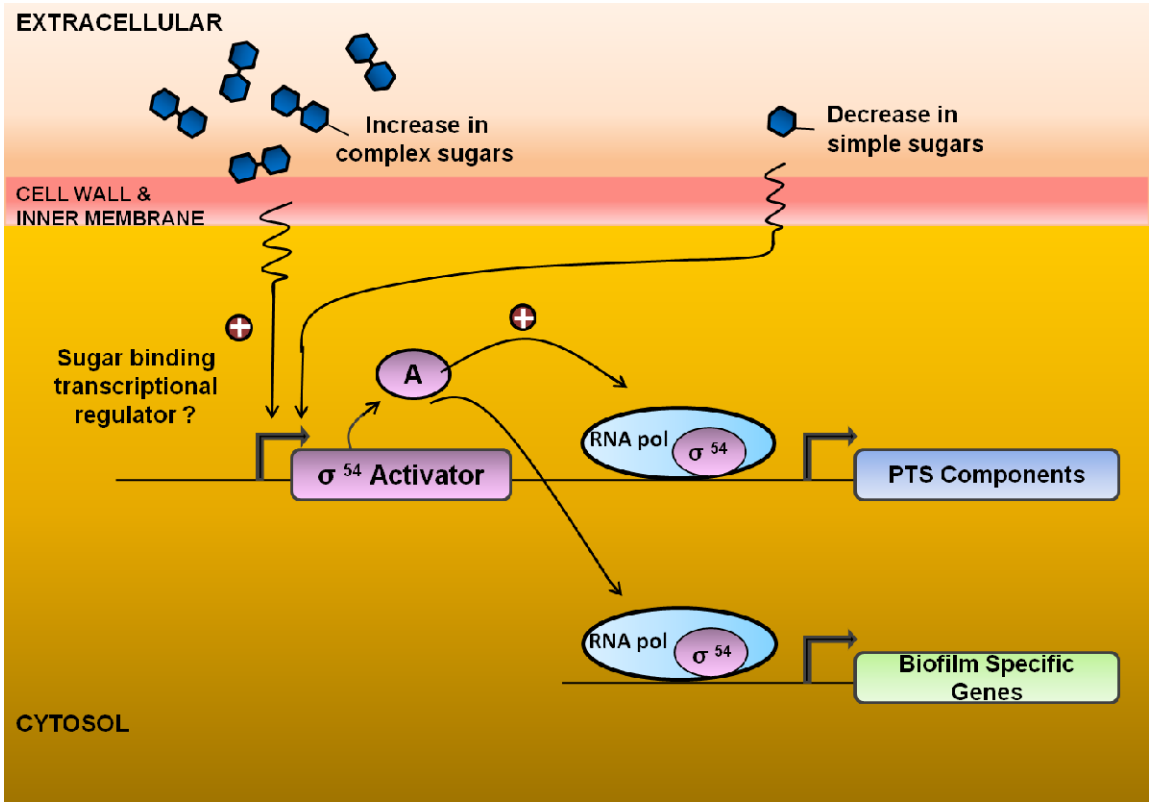

Figure 1.2 Link between PTS systems and biofilm development.
On depletion of simple sugars (eg. glucose), complex sugars may stimulate the expression of a σ^{54} activator that binds and activates RNA pol-RpoN complex to promote transcription of PTS components specific for the complex sugar. The triple component complex (RNA pol-RpoN-σ^{54} activator) may also cross-activate the expression of biofilm specific genes involved in the maturation and maintenance of biofilms during nutrient limiting conditions. Although the signaling cascade that activates expression of the σ^{54} activator is currently unknown, it may involve a sugar binding transcriptional regulator that binds the complex sugar, or alternately may be channeled through an unknown signaling cascade that senses the depletion of simple sugars in the environment.

The enterococcal surface protein (Esp) is a large surface protein of ~202 kDa that was initially observed to be enriched in clinical enterococcal isolates (132). Approximately 50% of this protein is made up of repeats whose function is currently unknown. However it has been predicted that the repeats may constitute a necessary feature to the structural stability of this protein or may serve to project the N-terminus of the protein away from the

surface, similar to a role predicted for the dipeptide repeat region of *S. aureus* clumping factor (132). Heterologous expression of Esp in two *esp* negative strains of *E. faecalis* (OG1RF and FA2-2) significantly enhanced the surface adherent capabilities of both strains suggesting a role in biofilm development (143). Consistent with this observation, mutation of *esp* in *E. faecalis* 11279 resulted in a significant decrease in biofilm development (152). Domain deletion mapping studies suggested that N-terminal region of Esp is sufficient for adherence and biofilm enhancement (144). At least three studies have attempted to address the mechanism by which Esp promotes biofilm development. Two independent studies have concluded that the expression of Esp on the cell surface enhances cell surface hydrophobicity an important factor that positively influences biofilm development (143, 152). A third study suggests that biofilm formation results from positive Esp mediated interactions with the surfaces like bile drain materials (163).

On entering the late log phase of growth, *E. faecalis* secretes two cotranscribed proteases- gelatinase (GelE) and serine protease (SprE) into their immediate external environment (12). GelE (30-kDa) belongs to the M4 family of zinc metalloproteases and is similar to thermolysin found in *Bacillus thermoproteolyticus*. SprE appears to be from the glutamyl endopeptidase I staphylococcal group of proteases and is similar to V8 protease from *S. aureus* (34, 69). A number of studies have observed a role for gelatinase in biofilm development (54, 89). Non-polar *E. faecalis* OG1RF mutants that lacked the ability to secrete GelE were deficient in biofilm development (89). Conditioned media derived from OG1RF and its isogenic SprE mutant, but not from an isogenic GelE mutant had biofilm enhancing capabilities (76). Interestingly contradicting these observations a subsequent etiological study comparing a large collection of clinical and fecal isolates failed to find a significant correlation between GelE expression and biofilm development (91). However trans-enzyme complementation studies carried out by Hancock et al. suggested that purified active GelE was crucial for biofilm development (54). Although the above observations suggest a limited role for SprE in biofilm development, a comprehensive mechanistic study on its role in biofilm formation remains to be addressed. It is not clear how GelE enhances biofilm development. One possibility is based on its cleavage specificity (Fig 1.3). As GelE cleaves its substrates primarily at hydrophobic residues, its activity on the cell surface may increase overall surface hydrophobicity and biofilm development (16). An alternate theory rests on its ability to activate a surface localized autolysin (Fig 1.3) (133, 165). Consistent with this theory a mutation of the primary autolysin (AtlA) of *E.*

faecalis resulted in a 30% reduction of biofilm development (89). Further supporting this theory, primary autolysins of a number of gram-positive organisms have been implicated in biofilm development (1, 10, 116). Besides their normal role in maintaining peptidoglycan turnover and cell division, autolysins are suspected to increase cell adherence to surfaces by some unknown mechanism.

Transcriptional regulators

Multiple novel transcriptional regulators have recently been demonstrated to be involved in *E. faecalis* biofilm development using a recombinase in vivo expression tag (RIVET) approach (5). One among these regulators, named EbrA (enterococcal biofilm regulator) displayed a 50-fold reduction in biofilm development relative to wildtype, when mutated. The genes regulated by EbrA are currently unknown (5).

The *fsr* locus has been described to be essential for optimal biofilm development of *E. faecalis* in a number of studies (54, 90). It comprises of four genes- *fsrA*, *fsrB*, *fsrD* and *fsrC* that are similar in organization and function to the *agr* quorum sensing system of *S. aureus* (114, 115). While FsrC is a membrane localized histidine kinase, FsrA constitutes a response regulator located in the cytoplasm (Fig 1.3). The C-terminus of FsrD comprises the quorum signaling molecule GBAP (gelatinase biosynthesis activating pheromone) that is an 11- residue peptide that is processed and secreted outside the cell with the aid of the membrane localized FsrB (97, 98). On reaching a threshold concentration, GBAP binds and activates FsrC which in turn activates the response regulator, FsrA (98). Activated FsrA differentially regulates the expression of over 300 genes involved in secondary regulatory cascades, virulence and metabolism (12). Mutations of the *fsrA-C* resulted in ~30% decrease in biofilm formation in an *E. faecalis* OG1RF background (89) and similar levels of biofilm defects were also observed in a V583 background following inactivation of the *fsr* genes (54).

How does Fsr regulate biofilm development? Transcriptional profiling analysis has shown that Fsr quorum sensing system regulates the expression of two secreted proteases (GelE and SprE) whose genes are located immediately downstream of the *fsr* locus (12). Hancock and Perego demonstrated that Fsr mediates its biofilm enhancing effects through the production of gelatinase, as a *gelE* mutation significantly abrogated biofilm development in strain V583 (54). Further this defect could be rescued back to wild type levels if *gelE* was expressed in trans (54). Consistent with this observation, the ability of an

fsr mutant to develop biofilms was restored to wildtype levels following its growth in media supplemented with physiological concentrations of purified GelE (54).

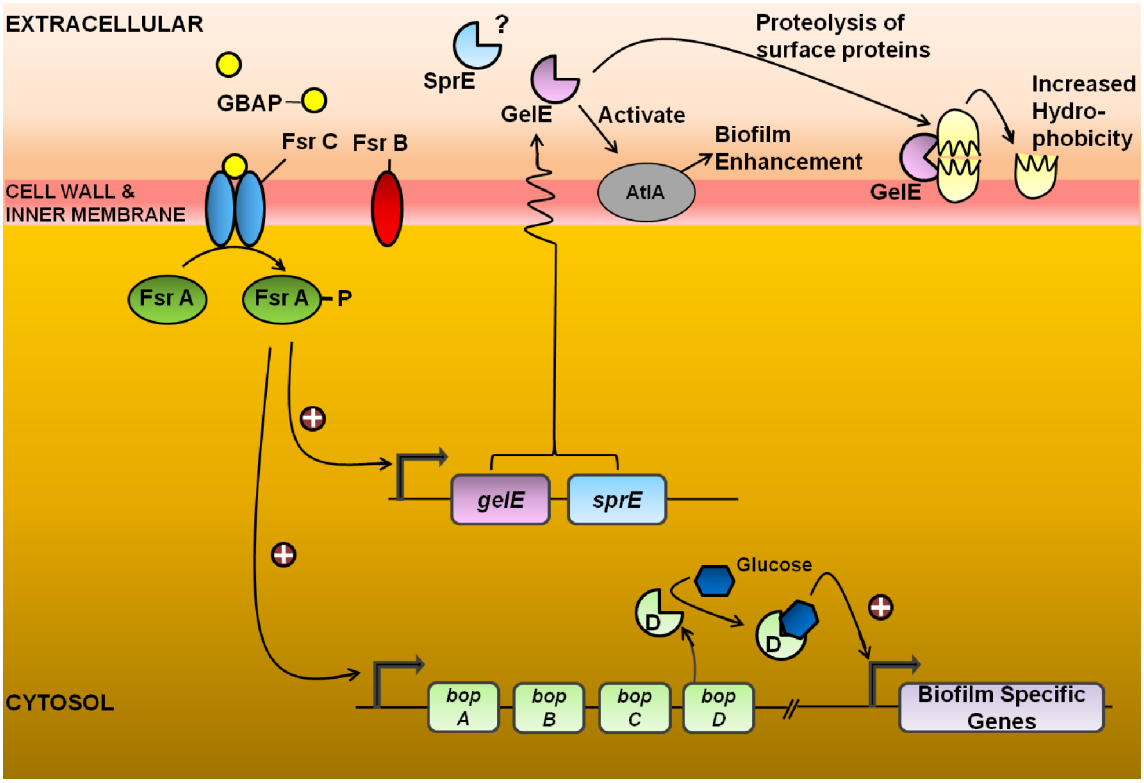

Figure 1.3 Fsr mediated control of biofilm development.
The *fsr* operon (*fsrABCD*) encodes a two component regulator with a membrane localized sensor histidine kinase (FsrC) and a cytosolic response regulator (FsrA). FsrB is involved in the cyclization and release of the quorum peptide GBAP into the immediate external environment of the cell. On quorum activation, activated FsrA~P, enhances biofilm development by at least two mechanisms- activating the expression of the extracellular protease- GelE and the sugar binding transcriptional regulator, BopD. GelE may enhance biofilm development by either increasing the overall hydrophobicity of the cells or by activating a surface localized autolysin, AtlA. A role for SprE in biofilm development has not been elucidated. BopD is thought to enhance biofilm in a glucose dependent manner.

Interestingly it has also been demonstrated that *fsr* regulates biofilm development in a gelatinase independent manner (90). This alternate route of biofilm regulation may be through activation of the four gene *bop* locus (*bopABCD*) which encodes components of a maltose specific PTS system (Fig 1.3) (12). Consistent with this argument, a transposon mediated mutation in *bopB* was demonstrated to reduce biofilm development due to polar

effects on *bopD* when grown in a 1% glucose supplemented medium (30, 62). BopD is a transcriptional regulator that is thought to act primarily as a repressor of *bopABC* and *malT*; both loci are thought to be involved in the cellular uptake of maltose. Interestingly, the same transposon mutant formed increased biofilms in the presence of 1% maltose (30). Based on these observations, it is evident that *bopD* is a sugar binding transcriptional regulator that mediates biofilm development following its interaction with glucose (Fig 1.3). However once glucose is exhausted, the consequent uptake and binding of maltose to *bopD* may inactivate its regulation of biofilm. In such conditions alternate pathways of biofilm development dependant on *bopABC* may be activated.

The biological role of death and lysis in biofilm development

In recent times, cell death and lysis has been ascribed to play important biological roles in bacterial developmental processes including biofilm development (7, 23, 117). However, such lytic events are usually limited to subpopulations within a larger bacterial community. It is our contention that the driving forces of population heterogeneity within biofilms may contribute to limit bacterial autolysis to specific subpopulations.

The rise of variant subpopulations within a community of clonal bacteria resembles differentiation and division of labor in multicellular eukaryotic organisms (96, 166). One of the primary hallmarks of multicellular developmental strategies is cell death (73). In eukaryotes cell death plays a crucial role in sculpting developmental processes and in the elimination of altered cells (damaged, pathogen infected or rogue cells with altered cellular characteristics) from the population (73). Stolp and Starr (1965) defined bacterial autolysis as lytic events that develop due to the cell's own intracellular autolytic enzymes and observed that such lysis could be mediated by endogenous and exogenous factors. While uncontrolled activity of cell-surface localized autolytic enzymes could lead to cellular suicide (endogenous), it was also likely that released autolytic enzymes mediated lysis of neighboring cells (exogenous) (140). More recently, these same autolytic mechanisms have been observed among bacterial populations and have been implicated to be crucial for their developmental programs (23).

What benefits might autolysis bring about to bacterial biofilm development? As in eukaryotic development, wherein cell death is crucial for the survival of the organism as a whole, in bacteria, autolysis may play an analogous role in maintenance of communities

and social behavior. One benefit of autolysis in bacterial communities is the release of genomic DNA into the immediate external environment of cells. Such DNA also called extracellular DNA (eDNA) contributes to antibiotic resistance and biofilm development of a variety of gram-negative and gram-positive bacteria, including *P. aeruginosa, S. aureus*, *B. subtilis* and *E. faecalis* (147, 151, 162, 169). While eDNA in the biofilm matrix layers is generally thought to act as a cellular scaffold contributing to structural stability of the biofilm (3), the negative charge of the phosphate backbone also promotes cation chelation in the immediate vicinity of cells resulting in the induction of PhoPQ and PmrAB regulated cationic antimicrobial peptide resistance operons in *Pseudomonas aeruginosa* (94). Bacterial autolysis has also been implicated to play important roles in biofilm dispersal, colonization of new surfaces and also contribute towards nutrients (85, 86, 167). The marine bacterium *Psuedoalteromonas tunicata* produces an autolytic protein, AlpP that promotes cell death and seeding dispersal following biofilm development (86). After 8 days of biofilm development, wild-type *P. tunicata* exhibited a dramatic increase in dispersal of viable cells (86). The characteristic dispersal was seen from the internal portions of the biofilm microcolonies of wildtype, but not Δ*alpP* mutants. It has been proposed that AlpP mediated autolysis may destabilize the biofilm microcolony structure leading to dispersal of internally trapped cells. The Δ*alpP* mutants relative to wildtype *P. tunicata* were also compromised in their phenotypic diversity critical for successful colonization of new surfaces (86). Interestingly survivors within biofilm lysis zones seemed to display increased metabolic activity suggesting an additional nutritional benefit for autolysis (86).

The driving forces of population heterogeneity influence autolysis

Although biofilm bacteria are clearly physiologically different from their clonal planktonic counterparts, it should be noted that a plethora of phenotypically distinct states exist within a mature biofilm, thus contributing to overall heterogeneity (138). This spatio-temporal diversity (heterogeneity) in bacterial phenotypes among clonal cells of the same community results from unique adaptations of bacterial subpopulations to specific challenges within the local biofilm microenvironment or may also occur due to noise in gene expression (41, 138, 142). Existence of diverse phenotypic subpopulations within biofilms is thought to primarily enhance chances of survival at the community level during stressful times (45).

What drives the genesis of subpopulations within biofilms? Bacterial differentiation into physiologically distinct subpopulations within biofilms primarily arises due to the response they elicit toward chemical concentration gradients within the biofilm (138). These responses may be classified as being of an epigenetic (transient) or genetic (long-lasting) nature (33, 160, 161). Epigenetic responses that result in differentiation of subpopulations can arise due to environmental influences where in bacteria physiologically adapt to specific challenges in their immediate environment (138), cell density fluctuations that trigger bacterial quorum sensing mechanisms (71) or noise in gene expression leading to bistability (38, 160).

Within a biofilm environment, the dynamics of gaseous diffusion, nutrients and other byproducts of bacterial metabolism are constantly changing resulting in complex concentration gradients (138). As most bacteria efficiently sense external stimuli and are able to elicit an adaptive physiological response that involves differential gene regulation and expression, it remains to reason that bacteria may transiently differentiate into several physiological states within the biofilm depending on their immediate microenvironment. Consistent with this argument, in *Pseudomonas*, genomic and proteomic analysis has identified a number of proteins involved in general biosynthesis and metabolism whose expression is altered following their transition into the biofilm state (125, 170).

A variety of secreted signaling molecules can also aid bacteria in their physiological adaptation to specific biofilm microenvironments. For instance indole a secreted stationary phase signal was recently shown to regulate biofilm development by inducing acid resistance and motility in *E. coli* (80). Cell density dependent signaling (quorum sensing) leads to the activation of social behaviors in subpopulations that are beneficial to the community as a whole (67). Such signals can often have diverse physiological effects in different bacteria. For example the LuxS enzyme (S-ribosylhomocysteinase) responsible for the production of the quorum signal, AI-2, is required for biofilm development of *Salmonella typhimurium* (113), but plays a negative regulatory role in the attachment of *Helicobacter pylori* to a substratum (26). Bistability represents stochastic fluctuations in gene expression at the cellular level often resulting in the bimodal distribution of bacterial populations and constitutes yet another major driving force for the segregation of subpopulations in biofilms (38). The rise of persisters in *Staphylococcus aureus* and *Escherichia coli* populations that are resistant to antibiotic mediated killing and the development of competence in *Bacillus subtilis* populations have been attributed to bistability (38, 93). While noise in gene

expression may lead to bistability it may be argued that quorum sensing may have evolved to prevent certain developmental processes from being under such a passive control.

Genetic mutations and phase variations can also contribute to the rise of biofilm sub-populations (138). Hypermutable clinical strains (mutators) of *P. aeruginosa* have previously been reported to emerge within biofilm environments (37). Mutators are generally thought to arise due to spontaneous mutations that occur in DNA repair genes (eg. *mutS and mutL)* (104). The resulting high frequency of mutations may select for variants that are optimized for specific environments (19). Phase variations involve reversible genetic rearrangements within a subset of cells in the population (18). Such rearrangements are often stochastic but stable through generations as the genetic material is conserved and leads to ON/ OFF switching of gene expression. Phase variation has been attributed to regulate a number of genes involved in biofilm development of *S. aureus* and *S. epidermidis* (27, 70, 154). Reversible inactivation of biofilm associated genes (*ica*, *sarA*, *bap*) in these organisms by the insertion sequence IS256 results in the generation of biofilm negative variants (27, 70).

An increasing body of evidence suggests that the very driving forces of population heterogeneity may also contribute to the genesis of bacterial subpopulations that are differentially susceptible to autolysis in multiple developmental programs including biofilm development. In *Pseudomonas aeruginosa*, quorum sensing via the quinolone signaling (PQS) and lactone signaling (LasI and RhlI) pathways regulate autolysis and release of extracellular DNA (eDNA), a critical component of the biofilm matrix (3, 84). Accordingly *lasIrhlI* double quorum sensing mutants had significantly less eDNA localized to the stalks of the *Psuedomonas* biofilms relative to wildtype. Similarly, a *pqsA* mutant deficient in PQS signaling showed a decrease in eDNA release, and a *pqsL* mutant that overproduced PQS displayed a corresponding increase in eDNA release. PQS signals are thought to regulate autolysis and eDNA release within biofilms by the induction of a prophage (3). Bistability leading to the activation of Spo0A (Spo0A-ON), the master regulator that controls entry of cells into the sporulation state has been shown to regulate cannibalistic behavior (a form of autolysis) in *B. subtilis* (15, 22, 40, 50). Consequently the Spo0A-ON bacteria release toxins that are instrumental in lysing their siblings (Spo0A –OFF) (50). Finally phase variation in gene expression has also been reported to influence autolysis. The *lytA* gene of *S. pneumoniae* is considered to be phase variable, leading to spontaneous

reversible variations in its surface expression within a population. Increased expression of LytA in colonies was correlated to a corresponding increase in autolysis rates (168).

Biofilm death mode: altruistic suicide vs. fratricide

The nature of bacterial lysis in a number of developmental processes, including biofilm development exhibits broad commonalities. Following initial adherence, bacterial biofilm communities differentiate into relatively lysis-susceptible and lysis-resistant subpopulations (150). This differentiation as argued earlier may be dependent on any number of mechanisms that promote heterogeneity. Following differentiation, the lysis susceptible population undergoes autolysis through two major mechanisms- altruistic suicide (7, 117) or fratricide (49, 150) (also refer Fig 5.1).

Altruistic suicide involves the ability of an individual bacterial cell to terminate itself for the common good of the larger community (7, 117). Self-destruction of the individual cell in bacterial communities has often been compared to apoptosis or programmed cell death (PCD) in higher eukaryotes (82). Analogous to apoptosis, altruistic suicide in bacteria is thought to result in the elimination of cells (damaged or phage infected) that might potentially be detrimental to the population as a whole. Such elimination may reduce competition for nutrient resources within a population and also allow nutrient recycling from dead lysed cells (82, 117). More recently altruistic suicide was argued to be responsible for the lysis of a bacterial subfraction and cause the release of extracellular DNA in *Staphylococcus aureus* biofilms (119). Contrary to the self- initiated destruction of an individual cell (apoptosis), fratricide involves the death of a cell due to corruptive mechanisms initiated by a genetically identical sibling (23, 150). In this regard, fratricide may appear similar to necrosis in higher eukaryotes, where in cells die not primarily due to their suicidal decisions but rather due to external cues (e.g., toxins, chemical injury, etc.) that coerce or force them to die (57). Accordingly, in bacterial developmental processes (biofilm development, sporulation and competence) that utilize fratricide, mechanisms of death involve toxic compounds or extracellular killing factors secreted by individual cells (attackers) against their siblings (targets). Interestingly, in this chemical warfare attackers are shielded from harmful effects of their secreted killing factors due to specific immunity proteins they alone produce (23, 150).

Molecular regulation of autolysis during biofilm development

The effectors of bacterial autolysis are mostly murein hydrolases- enzymes that hydrolyze cell wall peptidoglycan (134). Although these proteins are normally involved in peptidoglycan turnover and cell separation, subpopulations of bacteria succumb to murein hydrolase mediated autolysis within biofilms and spill cell constituents into their immediate environment (119, 151). Contrary to what has been assumed previously, recent evidence suggests that autolysis (altruistic suicide and fratricide) is a highly regulated phenomenon in biofilms (7). In this regard much insight has been gained from studies involving altruistic suicide in *S. aureus* biofilms.

The molecular control of altruistic suicide in *S. aureus* is directly dependent on two operons, *cid* and *lrg* that encode proteins structurally similar to the bacteriophage holins and antiholins (14, 164). The holins constitute a class of proteins that are predicted to oligomerize on the membrane and form protein channels allowing endolysins that accumulate in the cytoplasm to translocate across the membrane to the peptidoglycan layer, their primary site of activity (52, 164). The *cid* operon consists of three genes, *cidA-C* of which at least *cidA* is confirmed to directly aid autolysis (118). CidC was recently demonstrated to function as a pyruvate oxidase that promoted cell death only in the presence of excess glucose (108). Consistent with its predicted role, a mutation in the putative *S. aureus* holin, *cidA* resulted in decreased murein hydrolase activity and increased tolerance to rifampin, penicillin and vancomycin relative to wild type (118, 120). The role of CidB is currently unknown, but hypothesized to aid CidA in its holin function (7, 118). Curiously, the primary murine hydrolases (encoded by *atl* and *lytM*) are thought to be secreted independent of the holin pathway and hence *cidAB* may have additional ways by which it regulates murein hydrolase activity (118). For example, the activity of CidA may result in the dissipation of membrane potential and trigger lysis (52, 109). Inhibitory to holin function and corresponding holin-dependent murein hydrolase activities are the antiholins (164). The deletion of the *lrgAB* operon led to an increased murein hydrolase activity and increased tolerance to penicillin in cells entering stationary phase (51). Hence *lrgAB* operon was predicted to encode proteins that function as antiholins and inhibit CidAB dependent murein hydrolase activity (117).

From a molecular context, at least two separate pathways that regulate *cid-lrg* dependent autolysis have been described in *S. aureus* (109). It may reasonably be assumed

that both these pathways may influence the release of eDNA crucial for the maintenance of *S. aureus* biofilm architecture. The first depends on the metabolism of carbohydrates wherein cells that utilize excess glucose may accumulate acetic acid in the culture supernatant (120). The efficiency of this process is dependent on functional CidC (pyruvate oxidase) that promotes the conversion of pyruvate resulting from glucose metabolism to acetic acid (108). Based on the differential diffusion kinetics in biofilms (137), acetic acid may enter cells and trigger the activation of CidR, a putative LysR type transcriptional regulator that induces the expression of *cidABC* and *lrgAB* operons, catalyzing the decision of the cell to commit suicide (174). Consistent with this argument, mutation of *cidR* was reported to not only abolish expression of both the *cid* and *lrg* operons but also result in the reduction of murein hydrolase activity and survival in the stationary phase (174). Interestingly, microarray analyses identified the expression of *alsSD* to be regulated by CidR in addition to *cid* and *lrg* operons (173). The proteins encoded by the alsSD operon are predicted to be important in the conversion of pyruvate to acetoin (173). Curiously disruption of *alsSD* resulted in increased cell death with minimal autolysis (173). The phenotype may be explained based on the observation that an *alsSD* mutant was abrogated in CidR mediated- expression of *cidABC* operon, even in the presence of excess glucose. These observations also argue for certain metabolites of acetic acid catabolism being necessary for CidR-mediated induction of the *cidABC* operon (173). The second pathway that could affect altruistic suicide is independent of CidR or the *cidABC* operon, but involves the control of *lrgAB* operon by the LytSR two component system (109). LytS is predicted to be a hydrophobic sensor kinase localized on the membrane and LytR is thought to be its cognate response regulator (14). Agents that dissipated membrane potential were shown to induce the expression of *lrgAB* in a LytS dependent manner (109). This suggests that the LytSR two component system may sense fluctuations in membrane potential ($\Delta\Psi$) of damaged or injured cells within a population and influence cell death and autolysis (117).

Scope of the thesis

In a study that involved 163 *Enterococcus faecalis* isolates (including 51 isolates from other countries) of clinical and faecal origin, it was observed that 93% were proficient in biofilm development (89). The same study also concluded that strains responsible for infective endocarditis produced significantly more biofilms than non-endocarditis isolates (89). This

ability of enterococci to develop into biofilms is of great concern to public health not only from an infectious disease standpoint, but also from an antibiotic resistance perspective. This is so as biofilms not only dramatically increase resistance up to 1000- fold, but are also considered to be hot-spots for inter- and intra- species dissemination of antibiotic resistance traits (16).

With the broad goal of defining basic mechanisms that aid enterococcal pathogenesis, especially during infective endocarditis, the specific contributions of two co-transcribed extracellular proteases- GelE and SprE in biofilm development of *E. faecalis* V583 was examined. As presented in the literature review (**Chapter 1**), there is ample evidence to suggest a significant role for GelE in biofilm development. However a role for the co-expressed SprE was only speculated. Accordingly **Chapter 2** gives insight on how both extracellular proteases of *E. faecalis* V583 regulate autolysis and extracellular DNA release in opposing ways and contribute to biofilm development. In **Chapter 3**, the fundamental principle of cell death and lysis in *E. faecalis* biofilm development is demonstrated as fratricide. Further, the relationship between extracellular proteases and the primary autolysin of *E. faecalis* is also explored. In **Chapter 4** we test whether the general contributions of GelE and SprE to in vitro biofilm development also hold true in a rabbit model of enterococcal endocarditis (in vivo). Finally, in **Chapter 5** we summarize the major findings of this thesis and discuss it in the broader light of bacterial developmental processes.

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CHAPTER 2

Regulation of autolysis-dependant eDNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development

Abstract

Enterococci are major contributors of hospital-acquired infections and have emerged as important reservoirs for the dissemination of antibiotic resistance traits. The ability to form biofilms on medical devices is an important aspect of pathogenesis in the hospital environment. The *E. faecalis* Fsr quorum system has been shown to regulate biofilm formation through the production of gelatinase, but the mechanism has been hitherto unknown. Here we show that both gelatinase (GelE) and serine protease (SprE) contribute to biofilm formation by *E. faecalis*, and provide clues to how the activity of these proteases governs this developmental process. Confocal imaging of biofilms suggested that GelE$^-$ mutants were significantly reduced in biofilm biomass compared to the parental strain, whereas the absence of SprE appeared to accelerate the progression of biofilm development. The phenotype observed in a SprE$^-$ mutant was linked to an observed increase in autolytic rate compared to the parental strain. Culture supernatant analysis and confocal microscopy confirmed the inability of mutants deficient in GelE to release extracellular DNA (eDNA) in planktonic and biofilm cultures, whereas cells deficient in SprE produced significantly more eDNA as a component of the biofilm matrix. DNaseI treatment of *E. faecalis* biofilms reduced the accumulation of biofilm implying a critical role for eDNA in biofilm development. In conclusion, our data suggests that the interplay of two secreted and co-regulated proteases- GelE and SprE, is responsible for regulating autolysis and release of high molecular weight eDNA, a critical component for the development of *E. faecalis* biofilms.

Introduction

Bacteria are often found in nature as communities of sessile surface-adherent populations covered in a slimy matrix composed of exopolysaccharides, protein and DNA (9, 18, 22). Bacteria present within these communities (also referred to as biofilms) exhibit social behavior analogous to that found in higher organisms, in that they can communicate and rapidly adapt to changing growth environments (5, 22).

The gram-positive opportunistic pathogen, *Enterococcus faecalis* develops persistent biofilm like vegetations on implant devices including othropaedic implants, urethral stents, catheters and heart valves making it a leading cause of nosocomial infection (28). Enterococci are becoming increasingly resistant to many conventional antibiotics (21). Compounding the drug-resistance phenotypes displayed by clinical isolates is the observation that enterococci growing as biofilms are more resistant to vancomycin, ampicillin, and linezolid compared to their planktonic counterparts (44). Epidemiological data also suggest enterococci to be important reservoirs for the transmission of antibiotic resistance genes among different species of bacteria (7, 57).

Of the factors reported to be important for *E. faecalis* biofilm formation (28), the enterococcal surface protein (Esp), and the secreted metalloprotease, gelatinase (GelE) are known to be expressed as variable traits (32, 47). More recently, Tendolkar et al. (52) identified a locus from a clinical *E. faecalis* urinary tract isolate that they termed biofilm enhancer in *Enterococcus* (*bee* locus). The genes from this locus resemble the pilin biosynthetic genes identified by Nallapareddy et al. (33) and have been shown to contribute to biofilm formation, but were found to be present in less than 5% of clinical isolates. It is noteworthy that Arciola et al. (3) recently correlated the presence of the *esp* gene and high phenotypic expression of gelatinase with the ability of *E. faecalis* epidemic clones from orthopaedic implant infections to form biofilms. The *esp* gene that encodes the surface associated Esp is located on a 153-kb pathogenicity island and its expression significantly increases the bacterial cell surface hydrophobicity and attachment on a substratum (51, 53). The expression of GelE is dependent on the *fsr* regulatory system (38, 39) and is known to vary among strains of *E. faecalis* due to a defined 23.9-kb deletion in the genome that encompasses the *fsr* genes. (32). The *fsr* locus consists of four genes, designated *fsrA*, *fsrB, fsrC* and *fsrD* (31). The *fsrC* and *fsrA* genes encode a two component sensor kinase-

response regulator pair (39). The *fsrD* codes for a peptide lactone that functions in a cell-density dependent manner (30). FsrB is thought to be responsible for proteolytic cleavage and cyclization of FsrD (31). It is likely that FsrC sensor histidine kinase senses the accumulation of the FsrD peptide in the extracellular space, leading to activation of the response regulator FsrA. The gene encoding GelE is located immediately adjacent to the 3' end of *fsrC* and is co-transcribed with *spr*E, which encodes a secreted serine protease (38, 39). Mutations in the *fsr* locus and its downstream target *gelE* resulted in poor biofilm forming capabilities, indicating that biofilm formation in *Enterococcus* is dependent on quorum sensing (19, 29, 36). Mutants defective in *fsr* quorum signaling were restored to wild-type biofilm levels by the addition of purified GelE, indicating that GelE alone is a major contributor to biofilm development (19).

The mechanism by which GelE positively regulates biofilm formation has hitherto been unknown. It was hypothesized that GelE, like Esp may be able to modify the bacterial cell surface hydrophobicity by virtue of its ability to cleave substrates at hydrophobic residues (6, 26, 27). An alternate hypothesis involves the ability of GelE to activate cell wall autolysins (48, 55). SprE has also been shown to be an important virulence factor as an *sprE* gene disruption resulted in decreased virulence in a mouse peritonitis model (39, 50), *Caenorhabditis elegans* model (15, 49) and a rabbit endopthalmitis model (14)

In this study, we investigated the role of both extracellular secreted proteases in biofilm formation by comparing isogenic single Δ*gelE* and Δ*sprE* and double protease Δ*gelE-sprE* deletion mutants of *Enterococcus faecalis* V583. Further, the ability to regulate autolysis with the concomitant release of extracellular DNA was shown to be a key contributor to the overall development of *E. faecalis* biofilms.

Results

Construction and complementation of isogenic protease mutants

Kristich et al. recently developed a *pheS* counterselectable vector system, pCJK47 to generate markerless inframe isogenic deletion mutations in *E. faecalis* OG1RF (24). However, this vector system was unsuitable for studies with strain V583 due to the unavailability of selectable resistance markers, as well as difficulty associated with conjugal mating of strains possessing multiple plasmids. In the present study, we

employed the plasmid pLT06 (a derivative of pCJK47), which encodes resistance to chloramphenicol and contains a temperature sensitive replication origin from pWV01 (25).

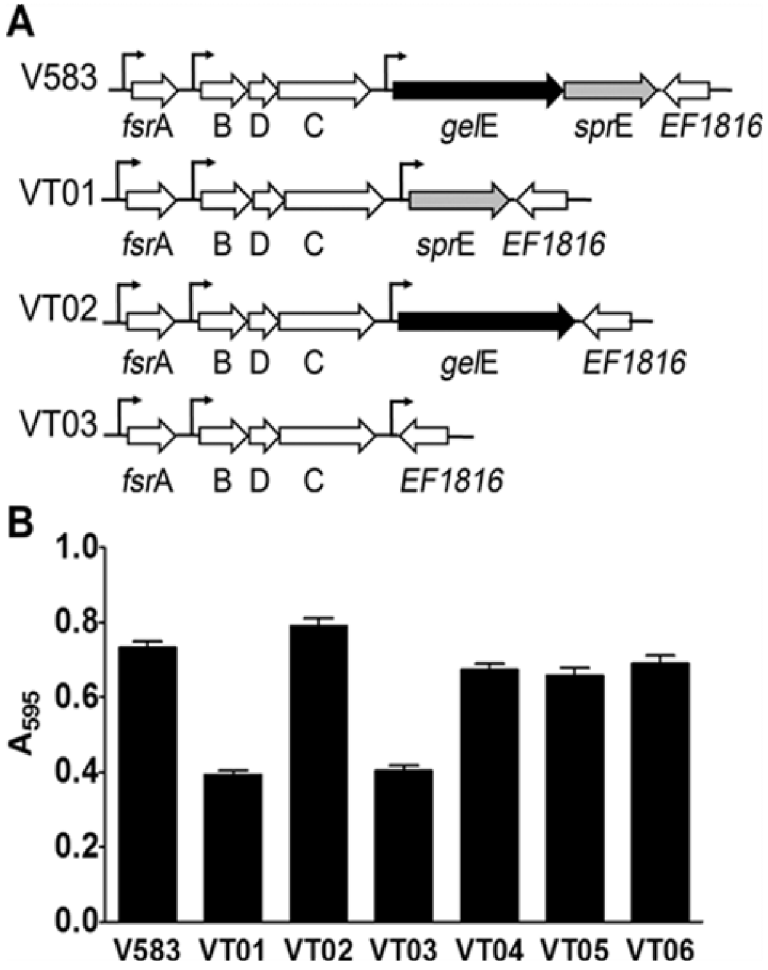

Figure 2.1 Extracellular protease deletion mutations affect *E. faecalis* V583 biofilm development.

A) Diagrammatic depiction of extracellular protease deletions. VT01, VT02 and VT03 correspond to *E. faecalis* V583 strains harboring Δ*gel*E, Δ*spr*E and Δ*gel*E*spr*E protease deletions, respectively. Solid lines indicate chromosome; boxed arrows indicate genes and curved arrows indicate promoter regions. Schematic is not drawn to scale. B) Biofilm formation of extracellular protease mutants on polystyrene microtiter plates. The biofilm density within microtiter plate wells were assayed as a function of crystal violet stain retained by the biofilm biomass. Mutant strains complemented with *gelE, sprE,* and *gelE-sprE* are designated as VT04, VT05 and VT06, respectively. Assays were performed in triplicate and error bars indicate standard error of the mean.

Extracellular protease deletion mutants- VT01 (Δ*gelE*), VT02 (Δ*sprE*) and VT03 (Δ*gelE-sprE*) (Fig. 2.1A) were constructed using the markerless exchange vectors pVT01, pVT02 and pVT03, respectively. The respective plasmids were integrated into the V583 genome by homologous recombination. Subsequent plasmid excision was counterselected by plating on media containing DL-p-chlorophenylalanine as described (24). Roughly 50% of the isolates growing in the presence of DL-p-chlorophenylalanine yielded the expected gene deletion for each of the plasmid constructs. The proteolytic phenotypes of the mutants were compared with V583, and were consistent with previous reports (23). Strains VT01 and VT03 lacked a zone of proteolysis on 1.5% skim milk and 3% gelatin agar, whereas strain VT02 showed a smaller zone compared to V583 (data not shown).

E. faecalis V583 isogenic protease mutants (VT01, VT02 and VT03) were complemented with full-length genes of *gelE*, *sprE* and *gelE-sprE in trans* under the control of the native *gelE* promoter. Complementation confirmed that the protease negative phenotypes were a result of targeted protease deletions and not due to polar effects of gene mutations elsewhere on the chromosome (data not shown).

Biofilm formation of *E. faecalis* V583 isogenic protease mutants

Quantitative analysis of biofilms formed by the protease deletion mutants on polystyrene confirmed previous findings (19). VT01 (Δ*gelE*) and the double protease deletion strain VT03 (Δ*gelE-sprE*) were significantly reduced in biofilm biomass compared to strain V583 (Dunnetts test, $P < 0.05$) (Fig. 2.1B). Interestingly, deletion of *sprE* (VT02) marginally increased the biofilm biomass, although this did not appear to be statistically significant (Dunnetts test, $p=0.30$). Complementation of the protease negative strains restored biofilm formation to near wild-type levels suggesting no polar effects for the deletion mutations (Fig. 2.1B).

Given the differences in biofilm biomass on polystyrene, we asked whether mutant cells exhibited any differences in primary biofilm matt formation on a glass substrate. Confocal laser scanning microscopic analysis of the structural and spatial organization of 24–hour old biofilms (Fig. 2.2) showed a dense and compact parental V583 biofilm (VT09). Consistent with our earlier observations, VT10 (Δ*gelE*) and VT12 (Δ*gelE-sprE*) displayed poor biofilms (decreased by ~60% and 50% respectively, when compared to VT09; Table 2.1) and was composed mainly of isolated and sparse distributions of cells on the glass surface.

In contrast, biofilms of VT11 (Δ*sprE*) were more dense when compared to those formed by the parental strain (increased by ~55%; see Table 2.1) and appeared to have a rugged, mountainous surface terrain consistent with an early initiation of microcolony development.

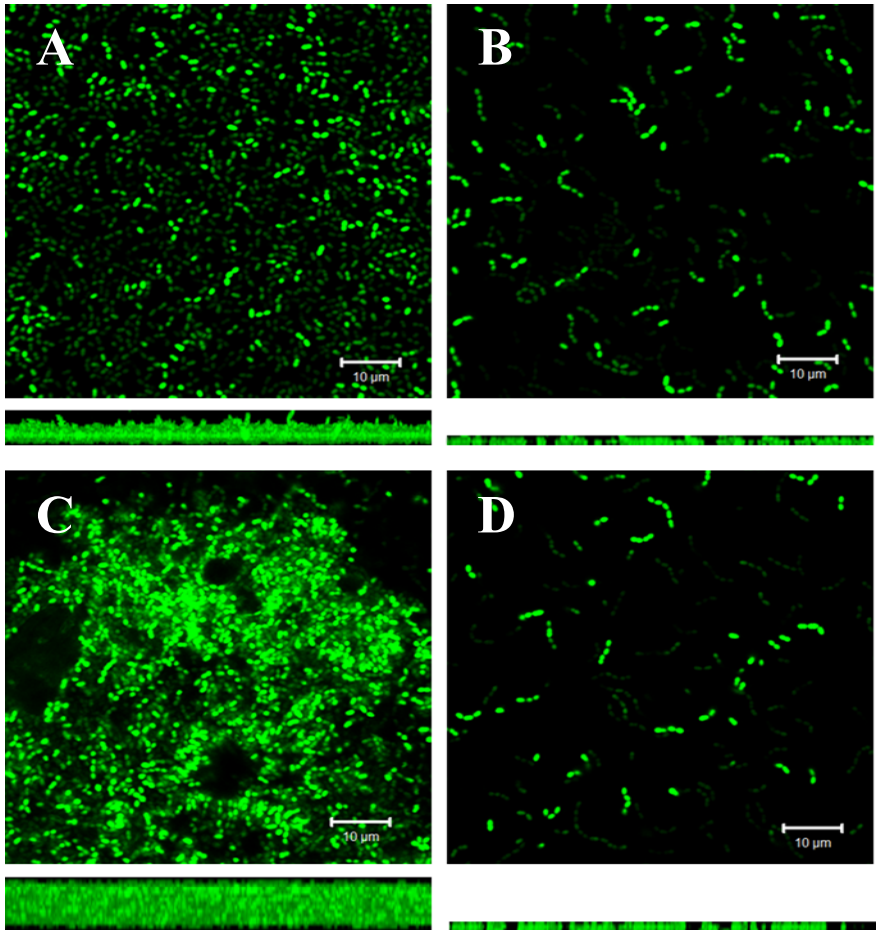

Figure 2.2 Confocal analysis of one day old biofilms of *E. faecalis* wild type and isogenic protease deletion mutants.
All strains constitutively expressed Gfp from pMV158GFP (see materials and methods) and were grown on glass coverslips in M17 media. Panel A, B, C and D are representative biofilm projections of VT09, VT10, VT11 and VT12 respectively. Below each panel is the z-projection for the corresponding image and the depth of the biofilm is indicated by the height of the z-stack (see Table 2.1). Inset scale bar represents 10µm.

Table 2.1 COMSTAT analysis of wild-type and isogenic protease mutant biofilm images

Day	Biofilm	Biomass ($\mu m^3/\mu m^2$) ± SD	Mean thickness µm ± SD	Maximum thickness µm ± SD
1	VT09	6.7 ± 0.93	6.3 ± 0.98	6.3 ± 0.98
	VT10	2.6 ± 0.37	2.4 ± 0.41	2.45 ± 0.49
	VT11	10.5 ± 0.33	10.1 ±0.48	10.15 ± 0.49
	VT12	3.0 ± 0.48	2.4 ± 0.49	2.45 ± 0.5
4	VT09	5.6 ± 0.38	7.9 ± 1.04	11.2 ± 0.0
	VT10	0.035 ± 0.04	0.022 ± 0.027	8 ± 2.26
	VT11	11.61 ± 0.46	19.9 ± 0.01	20.8 ± 0.0
	VT12	0.194 ± 0.04	0.28 ± 0.03	7.2 ± 2.26

Extracellular proteases do not affect cell surface hydrophobicity

To determine if the ability of GelE to enhance biofilm formation resulted from an increase in overall cell surface hydrophobicity, we tested whether a Δ*gelE* mutation would decrease the overall hydrophobicity of cells. The assay was carried out by quantifying the population of bacteria that were able to separate into an organic phase (n-hexadecane) depending on the degree of cell-surface hydrophobicity displayed. The presence or absence of either protease in both single and double deletion protease mutants did not result in significant differences in partitioning into the n-hexadecane phase relative to the wildtype V583 strain (Fig. 2.3, Dunnetts test, $P < 0.05$).

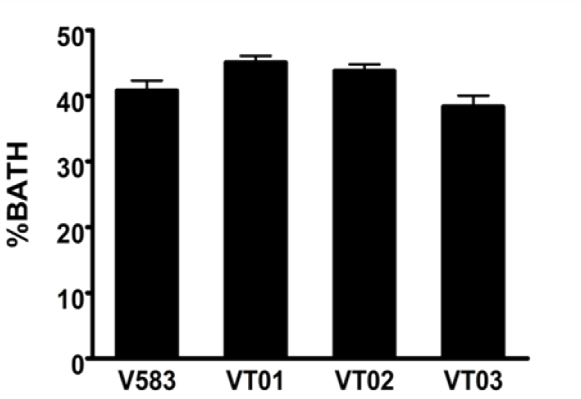

Figure 2.3 Cell surface hydrophobicity of *E. faecalis* V583 and protease mutants.
The overall measure of hydrophobicity of wildtype and mutant populations were calculated as the percent bacteria that adhered to hydrocarbon (BATH). Assays were performed in triplicate and error bars represent the standard error of the mean.

Extracellular proteases modify the rate of *E. faecalis* V583 autolysis

Given the ability of enterococcal proteases to modify autolysins (48), and based on the observations seen using confocal imaging of biofilms, we hypothesized that GelE and SprE may differentially regulate autolysis rates of *E. faecalis*. We observed that VT01 (Δ*gelE*) and VT03 (Δ*gelE-sprE*) exhibited a decrease in the rate of autolysis compared to V583 (Fig. 2.4A), consistent with observations reported by Waters et al. (55). In contrast, VT02 (Δ*sprE*) displayed a significant increase in the rate of autolysis compared to V583 (Fig. 2.4A, Student's t-test, $P < 0.05$).

Extracellular DNA in *E. faecalis* V583 culture supernatants

Based on the altered rates of autolysis, we hypothesized that extracellular DNA (eDNA) resulting from cell lysis would be more abundant in culture supernatants of *E. faecalis* V583 compared to mutants deficient in GelE production. Concentrated (20-fold) supernatant fractions were assessed for the presence of eDNA by agarose gel electrophoresis. High molecular weight DNA was detected in V583 and VT02 (Δ*sprE*) fractions (Lanes 1 and 3, Fig. 2.4B), but not in mutants VT01 and VT03 (Lanes 2 and 4, Fig. 2.4B), consistent with a decreased rate of autolysis in strains lacking GelE.

Densitometric determination of band intensity between DNA present in V583 and VT02 culture supernatants indicated a ~2-fold increase in the amount of eDNA from an SprE$^-$ mutant, consistent with a role for SprE as a negative regulator of autolysis.

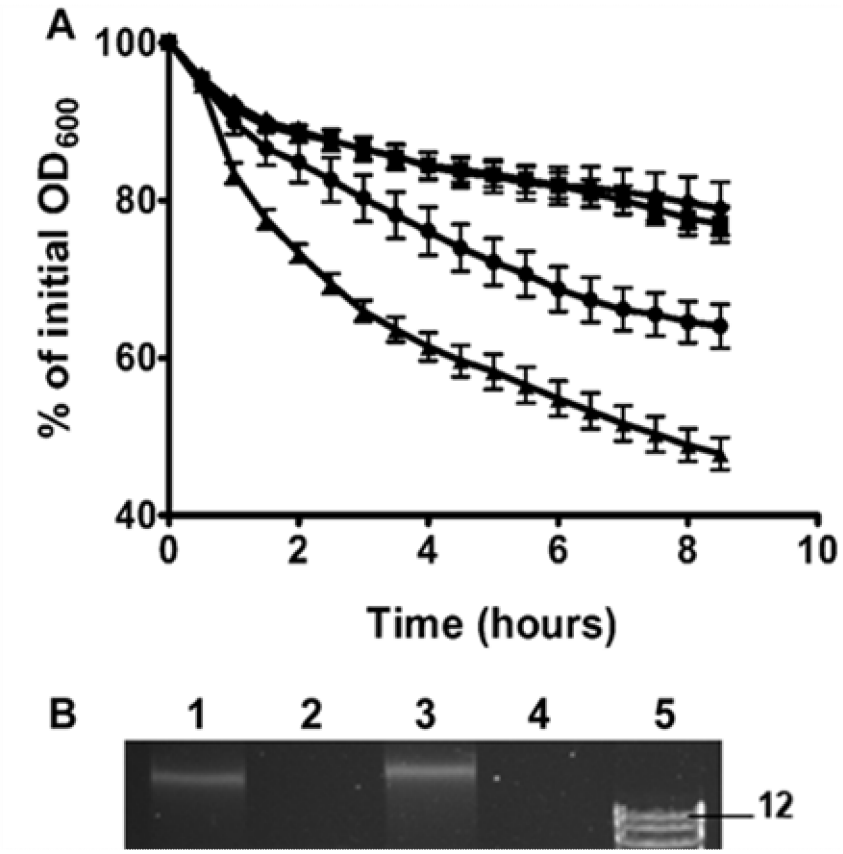

Figure 2.4 Extracellular proteases influence autolysis rates and extracellular DNA release. **A)** Differences in autolysis rates of V583 (circles) and extracellular protease mutants- VT01 (squares), VT02 (triangles) and VT03 (diamonds) are exhibited as percent values of initial OD_{600}. Assays were performed in quadruplicate and error bars denote the standard error of the mean calculated from three independent assays. **B)** High molecular weight bacterial chromosomal DNA was detected by ethidium bromide staining, after 20-fold concentration of 24-hour old culture supernatants.V583 (Lane1); VT01 (Lane 2); VT02 (Lane 3); VT03 (Lane 4) and 1 Kb DNA ladder showing the 12, 10, 8-kb bands, the 12 kb band is labeled (Lane 5).

Initiation of DNA release in V583 culture supernatants followed expression of GelE in the transition to stationary phase (data not shown) consistent with the earlier observation that GelE initiates autolysis. Finally, comparative PCR using eDNA and chromosomal DNA as templates confirmed that eDNA was indeed chromosomal in nature as amplification with primer pairs targeted to randomly distributed regions of the V583 genome could be amplified from both templates (Fig. 2.5).

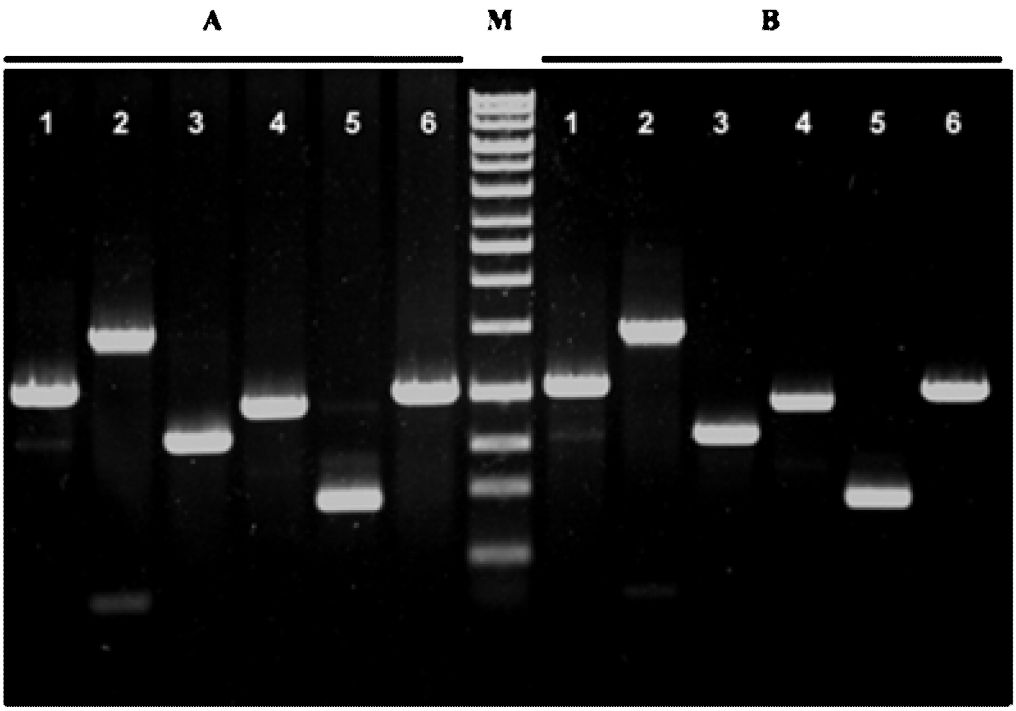

Figure 2.5 Comparative PCR analysis of chromosomal DNA and extracellular DNA of *E. faecalis*.
PCR of purified *E. faecalis* choromosomal DNA (panel A) or extracellular DNA (panel B) with primers targeting regions within the genes, *gelE* (lane 1), EF1091 (lane 2), EF0887 (lane 3), EF2490 (lane 4), EF2488 (lane 5) and EF2194 (lane 6). M, 1kb ladder

Tracking cell death in enterococcal biofilms

Because autolysis and biofilm formation of *E. faecalis* was directly dependent on the presence of gelatinase, we questioned if biofilms formed by the parental strain would contain foci of lysed cells compared to VT01 (Δ*gelE*). To test this, biofilms of V583 and VT01 expressing Gfp (VT09 and VT10, respectively) were grown over a period of three days and were stained for the presence of DNA and dead cells using propidium iodide (PI). Regions within the biofilm of VT09 contained concentrated foci of DNA (as detected by PI staining) in contrast to the few random dead cells in VT10 biofilms (Fig. 2.6, compare panels A and B). Image J analysis software was used to quantify the amount of PI-stained volumes within the biofilm as a measure of eDNA present in the biofilm. From this analysis, it is apparent that a common feature shared by both GelE$^-$ mutant and wild-type

cell populations is the presence of damaged cells capable of taking up propidium iodide, and this cell population is accounted for in our analysis.

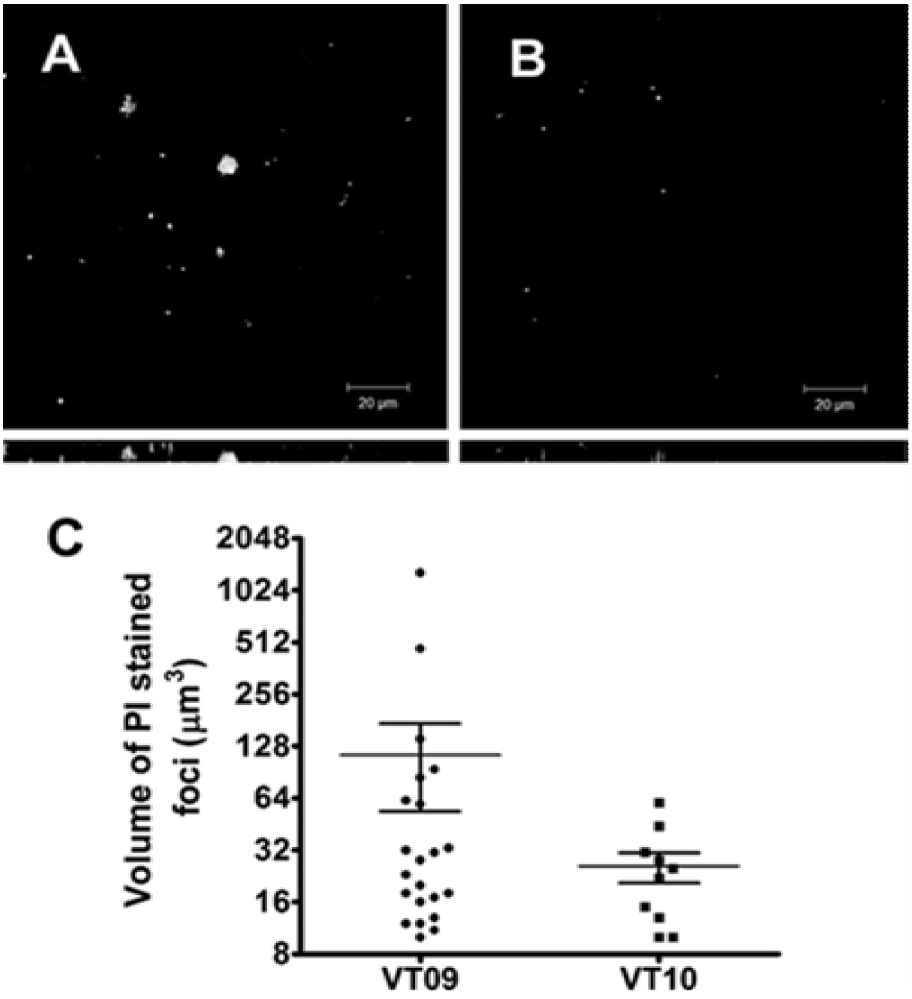

Figure 2.6 Bacterial cell death and eDNA release in three day old biofilms.
E. faecalis biofilms grown in M17 media over a period of three days were stained with PI (1µM) before being visualized by CLSM. Panel A represents a top-down view of VT09 biofilm displaying discrete foci of lysed bacteria while panel B shows isolated dead bacterial cells within the VT10 biofilm. Below each panel is the z-projection for the corresponding image. Inset scale bar represents 20µm. Panel C depicts the volumetric analysis of propidium iodide stained foci for VT09 and VT10 biofilms. Vertical scatter plots with each of the values of stained foci (cubic microns) are shown along with the mean and standard error of the mean.

A property unique to the wild-type cells compared to the GelE⁻ mutant is the presence of larger volumes of PI-staining associated with lysed cells. The mean values for PI-stained volumes is ~4.4-fold higher in the wild-type strain (113.5 ± 59.88) compared to the GelE⁻ mutant (25.80 ± 5.09), and this was shown to be statistically significant (p=0.0004) using an unpaired t-test, after data transformation, to account for the fact that stained volumes present in the V583 biofilms were not normally distributed compared to VT01 biofilms. A graph of this analysis is shown in figure 2.6C, and the Z-stack image comparing V583 and VT01 biofilms stained with PI is also shown in figure 2.6 (A and B). Collectively, these results suggest that GelE enhances biofilm formation by inducing lysis in discrete pockets of cells that appear to initiate biofilm development.

As the expression of GelE and SprE may only be optimally activated following the establishment of a quorum of bacteria on a surface, we hypothesized that we would see more prominent defects in the differentiation of the biofilm at later stages of development rather than the initial stages of attachment and proliferation. Consistent with this hypothesis, we observed that VT10 (Δ*gelE*) and VT12 (Δ*gelE-sprE*) were able to form a primary biofilm matt on a glass surface within 48- to 72-hours of growth (data not shown). However unlike the parental VT09 or VT11 (Δ*sprE*) strains, even after 96 hours of growth they were not able to differentiate into microcolonies (Fig. 2.7). Propidium iodide staining of the dead bacteria and eDNA in 4-day old biofilms revealed clusters of dead bacteria around the base and stalk of a microcolony whereas live bacteria interspersed with DNA frequently occupied the top of microcolonies within biofilms (Fig. 2.7). This suggested that pockets or clusters of dead cells which are dependent on the expression of GelE visualized at an earlier phase of biofilm development (Fig. 2.6A) may actually be sites of initial microcolony development. Consistent with a role for SprE in negatively regulating GelE activity, we observed significantly more biofilm biomass (107% increase compared to wild type) in an SprE⁻ mutant after 96 hours of growth compared to the parental strain (Fig. 2.7, Table 2.1).

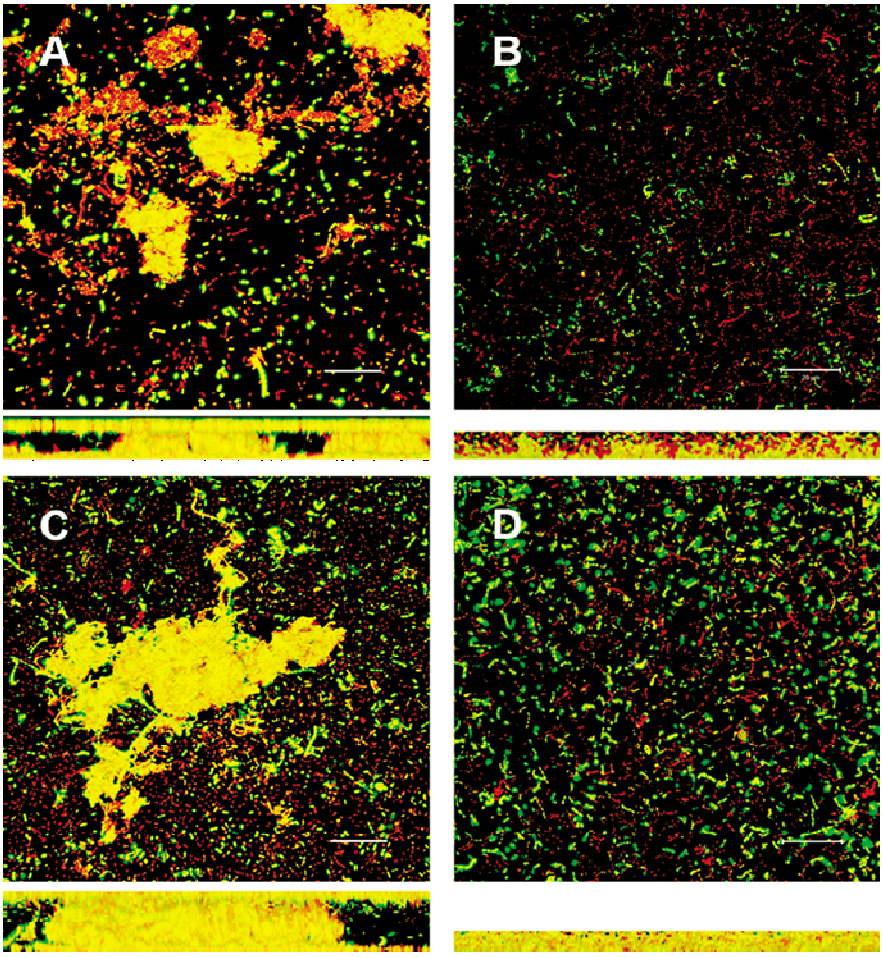

Figure 2.7 Comparison of biofilm architectures and relative eDNA localization.
Four day old Gfp expressing strains of *E. faecalis* V583 and isogenic protease mutants were grown in M17 and stained for the presence of eDNA with propidium iodide (1μM) as indicated in materials and methods. Live bacteria are green and eDNA and dead cells are visualized in red. High concentrations of eDNA laced among live bacteria present on each raised microcolony and surroundings appear in shades of yellow. Panel A, B, C and D are representative biofilm projections of VT09, VT10, VT11 and VT12 respectively. Below each panel is the z-projection for the corresponding image and the depth of the biofilm is indicated by the height of the z-stack (see Table 2.1). Inset scale bar represents 20μm.

Functional role of eDNA in enterococcal biofilms

To determine whether eDNA of *E. faecalis* played a structural role in biofilm development, we analyzed the affect of DNaseI on biofilm formation. Static biofilms grown on glass substrates were treated with DNaseI after 6, 12 and 24 hours of growth. Biofilm defects were most pronounced after early treatments of DNaseI at 6 and 12 hours of biofilm growth

(Fig. 2.8). The affect of DNase I treatment at later stages of development was less significant as exhibited by the 24 hour treatment.

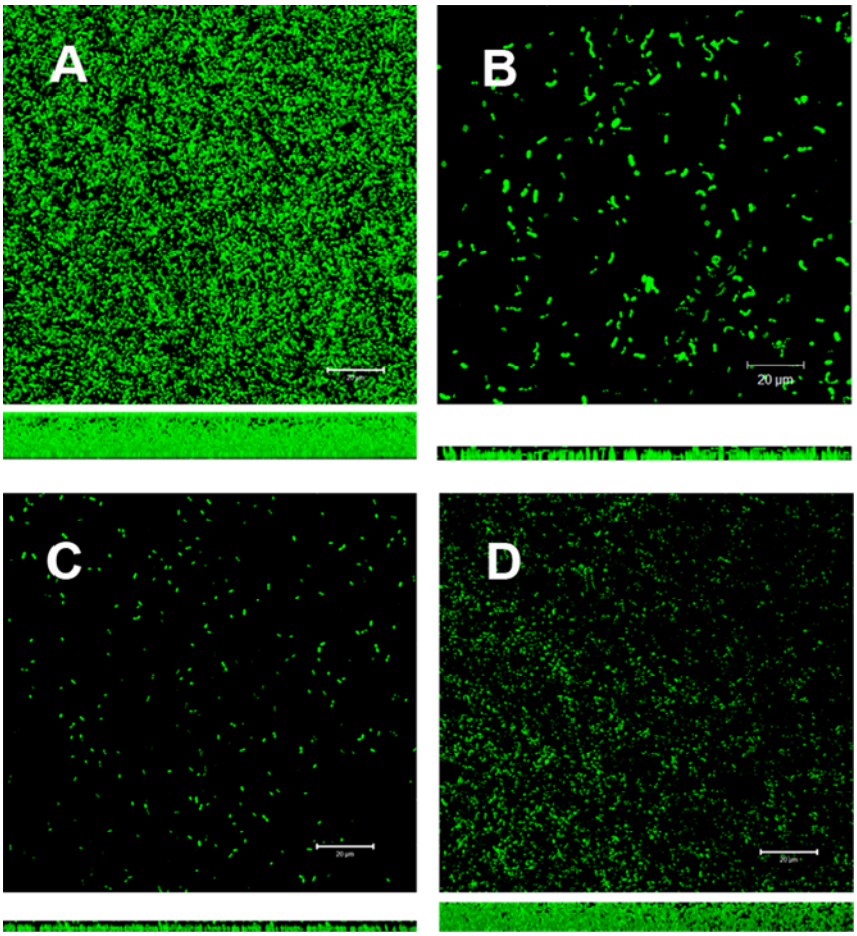

Figure 2.8 DNase I inhibits biofilm formation at early stages of development.
V583 biofilms grown on glass coverslips were treated with DNase I after 6, 12 and 24 hours of growth (represented in panels B, C and D, respectively) and analyzed after 26 hours by CLSM. The biofilm micrograph on the far left (Panel A) shows a control experiment with heat inactivated DNaseI introduced after 6 hours of biofilm development. Below each panel is the z-projection for the corresponding image, indicating the depth of the biofilm. Inset scale bar represents 20µm.

Discussion

The importance of extracellular proteases of *E. faecalis* in pathogenesis has been well demonstrated in a number of biological models (14, 15, 49, 50). Components of the host innate immune response are known to be cleaved by the proteolytic activity of gelatinase

and include LL37 (45), α-defensin (46), and complement components C3a and C3b (35) providing a mechanism for host immune evasion. GelE has also been shown to cleave fibrin possibly enhancing efficient dissemination of the organism in vivo (55). Aside from its proteolytic affects on host factors, gelatinase has also been shown to have a positive role in *E. faecalis* biofilm development (19, 24). Hence our present focus was to elucidate the mechanism behind GelE dependent biofilm development and to further examine the role of SprE in that process.

A speculative role for GelE in biofilm development included its potential ability to increase cell surface hydrophobicity by cleaving surface polypeptides at hydrophobic residues (6, 26). Although cell surface hydrophobicity has previously been proposed to be a key factor in the initial attachment of bacteria to a substratum (12), our analysis of the different protease mutants does not support a role for GelE or SprE in altering cell surface hydrophobicity as the deletion of either protease singly or in tandem resulted in minimal changes. A second hypothesis centered on the ability of GelE to alter rates of autolysis, based on observations by Shockman et al. (48) and Waters et al. (56). Our data appear to confirm the importance of autolysis in driving the development of *E. faecalis* biofilms, as we observed altered rates of autolysis, changes in eDNA release and differences in biofilm development in mutants defective in extracellular protease production. The contributions of both proteases to the process of biofilm development was readily observed only after confocal analysis. We did not initially observe a contribution for SprE in the microtiter plate biofilm assay. The apparent discrepancy between the two assays is consistent with observations reported by Tendolkar et al. (51) in which the plate assay significantly underestimated biofilm biomass compared to confocal imaging and COMSTAT analysis.

The major autolysin, AtlE of *Staphylococcus epidermidis* was recently shown to contribute to biofilm development through the generation of eDNA upon autolytic activation (40). A role for muramidase 2, a major autolysin of *E. faecalis*, in biofilm formation was reported by Mohamed et al. (29), and these authors concluded that it played a major role in the initial adherence phase of biofilm development. The findings reported by Qin et al. (40) that eDNA is an integral component of the biofilm matrix in *S. epidermidis* biofilms may warrant a re-evaluation of the role of autolytic processes in biofilm development in *E. faecalis*. The observed alterations in eDNA release that are dependent on protease activity and appear to mediate the ability of *E. faecalis* to develop microcolonies within biofilms suggest that autolytic processes may govern not only initial

attachment but also the subsequent development of the biofilm. Our findings have not only confirmed the role for GelE in activating autolysis as its deletion resulted in autolysis and biofilm defects, but also provide direct evidence that SprE is involved in negatively regulating autolysis, eDNA release, and biofilm maturation.

Previous reports have identified and characterized SprE as a virulence factor whose activity is altered in the presence of GelE (23). This activity is similar in nature to that reported for the corresponding homologous extracellular proteases of *S. aureus* where the metalloprotease aureolysin processes the cotranscribed SspA (V8 protease) (41). In *S. aureus*, SspA is known to alter the autolytic profile (41), which is consistent with our observations for the role of GelE and SprE in regulating autolysis. Because our data suggest that SprE prevents early maturation of biofilms by negatively regulating GelE activity, we postulated if there would be a fitness cost associated with the bacterial cell in the absence of SprE. Our observations suggest that the quick biofilm maturation phenotype of VT02 is associated with cell surface perturbations that may be disadvantageous at a planktonic level of existence. For instance, the SprE$^-$ mutant is at least 4-fold more sensitive to vancomycin as compared to wildtype V583 (data not shown). Hence it would seem that the trade-off for rapid biofilm development is costly and in an evolutionary sense, unstable.

It has been observed in several model systems that extracellular DNA serves as an important matrix component of microbial biofilms (2, 40, 42, 58). Consistent with a role for eDNA as a matrix component, we observed that treating a developing biofilm with DNase I at 6 and 12 hours post-inoculation resulted in diminished biofilm accumulation compared to a heat-inactivated DNase I control. In contrast, addition of DNase I at 24 hours showed only a marginal reduction in biofilm accumulation, suggesting that changes in the matrix composition may take place at later stages of development. Consistent with our findings, the observation that disrupting biofilms with DNase I-treatment works better at earlier stages of development has been reported for *Pseudomonas aureginosa* (58) and *S. aureus* (42) biofilms.

Although the factors regulating the spatial death of a subpopulation of bacterial cells in a biofilm are not clear, the extracellular nature of the proteases and their opposing phenotypes may play a role in this process. Our current model (Fig. 2.9) proposes two possible means by which these proteases may exert their regulatory affects on biofilm development. The first mechanism involves an autolytic pathway, wherein GelE localizes

to the cell wall of the producing cell to activate autolysis. If insufficient levels of SprE are present to control the autolytic activation induced by GelE, then that cell will likely undergo autolysis. The second mechanism would involve an allolytic or

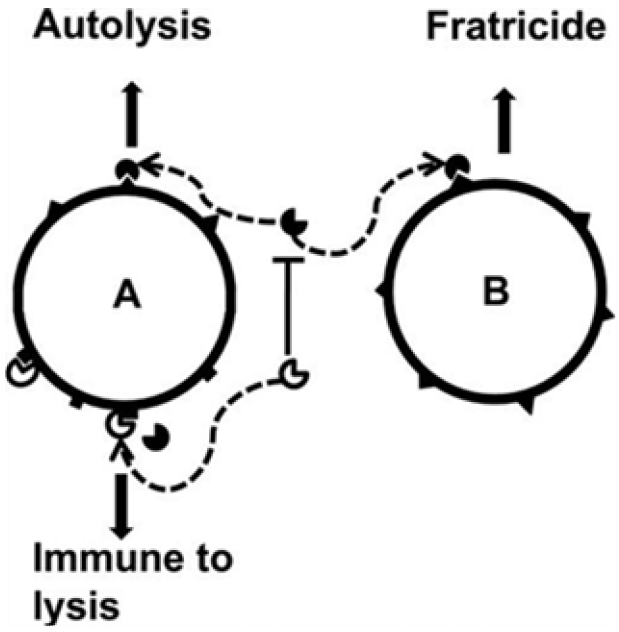

Figure 2.9 Model of GelE mediated lysis in *E. faecalis* biofilm development.
The model presents two mechanisms by which GelE could mediate lytic activity. The first mechanism is referred to as autolysis, and gelatinase (◔) from the producer cell (A) could activate a putative autolysin (▲) on the cell surface resulting in autolysis. The presence of SprE (◷) is predicted to regulate the GelE –mediated autolysin activation. The second mechanism referred to as fratricide (allolysis) allows for the diffusion of GelE (◔) from the producer cell (A) to a susceptible sibling (B), wherein the sibling cell undergoes lysis following autolysin (▲) activation by GelE. The extent of bystander or sibling lysis would potentially be regulated by the presence of SprE (◷) in the environment. The mechanism of SprE-mediated regulation is unknown, but may involve alteration of the putative autolysin, rendering it to an inactive form (■).

fratricidal event, wherein GelE freely diffuses from the producer cell to a target sibling cell to activate autolysins present on the sibling cell wall. A delay in responding to the quorum signal by siblings would render them susceptible to the action of autolysins activated by GelE secreted from another cell. SprE would also likely be present in this extracellular environment, but differences in diffusion and affinity for the cell wall may likely give rise to regions in the biofilm where GelE could act independent of SprE activity. In those rare

instances in which GelE would function independent of SprE, a sibling cell would lyse providing the necessary eDNA scaffold on which a developing biofilm could form. Consistent with the above model is the fact that only a few pockets within the observed biofilms give rise to cell lysis, indicative of the fact that the process is highly regulated.

In recent times, bacterial death in biofilms has been compared to programmed cell death in eukaryotes (4). Often such comparisons propagate the idea that defective cells within a biofilm population are eliminated in response to environmental challenges due to their altruistic suicidal acts (4). Our model adds to this complexity by proposing that GelE-mediated lysis appears to be an important aspect of biofilm development by *E. faecalis*. The cytotoxic activity of GelE toward the producer cell (autolysis) or sibling cells (allolysis) by the activation of autolysins may result in the release of extracellular DNA crucial for the early development of biofilms. Although in this case, a sub-population of cells may not be defective 'per se', their inability to produce the immunity factor (SprE) would result in their death. Allolysis has also recently been referred to as microbial fratricide ("sibling killing sibling") and this term has been applied to the competence developmental program in *Streptococcus pneumoniae* (18), and a model was proposed on how this process might contribute to the development of biofilms (16). Consistent with other fratricidal systems; allolysis (17) and cannibalism (16) that regulate differentiation of competent cells in *Streptococcus pneumoniae* and sporulation in *Bacillus subtilis* respectively, a model for how fratricide in *E. faecalis* mediated through the concerted action of the two proteases, GelE and SprE, to regulate differentiation of biofilms is also proposed (Fig. 2.9) . Interestingly, all three processes of differentiation may be considered as attributes of multicellularity resulting from cell-cell communication and involve quorum sensing, killing factors and immunity proteins (8, 13). In *E. faecalis* biofilm development, quorum sensing is mediated through a peptide lactone (FsrD) originally characterized as the gelatinase biosynthesis-activating pheromone (GBAP) (30). The extracellular accumulation of the peptide triggers expression of both GelE (the effector) and SprE (the regulator). Both proteases are co-transcribed suggesting an equal number of both molecules in the extracellular milieu. For this reason, we would anticipate that most cells would be protected from autolysis or allolysis. However, within discrete foci, the balance of these two proteases may not be the same giving rise to effector mediated processes in the absence of regulatory control. Ongoing studies will better clarify which of the two mechanisms (autolysis vs. fratricide) plays the dominant role in *E. faecalis* biofilm development.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The different strains and plasmids used in this study are listed in Tables 2.2 and 2.3, respectively. Strains were cultured in Todd-Hewitt broth (THB) or M17 medium (Difco Laboratories) and grown at 37°C unless otherwise indicated. *Escherichia coli* EC1000 was used for plasmid constructions. Antibiotics used for selection in *E. coli* were chloramphenicol, kanamycin and spectinomycin at concentrations of 10 µg/ ml, 50 µg/ ml and 150 µg/ml, respectively and for *E. faecalis* included chloramphenicol, tetracycline and spectinomycin at concentrations of 15 µg/ ml, 15 µg/ ml and 750 µg/ ml, respectively.

Construction of *E. faecalis* V583 in-frame protease deletion mutants

In-frame deletions of *gelE*, *sprE* and *gelE-sprE* were constructed using pLT06, an *E. coli*-enterococcal temperature sensitive cloning vector that had selectable and counter selectable markers that aided in selection of mutants containing the targeted deletions (Thurlow, L. and Hancock, L. E., unpublished results). The vector pLT06 is a derivative of pCJK47 (25), retaining the counter-selection properties on DL-p-chlorophenylalanine containing agar due to presence of the *pheS* dominant negative allele (25). In addition, pLT06 contains a chloramphenicol resistance marker and origin of replication from pWV01 (26).

Flanking regions (~1kb) from both the 5' and 3' end of the targeted proteases were PCR amplified with primers listed in Table 2.4. For construction of pVT01 (*gelE* deletion), primers GelEP1 and GelEP2 were used to amplify the region 5' to *gelE* on the V583 genome. Primers GelEP3 and GelEP4 were used to amplify the region 3' to *gelE*. GelEP1 and GelEP2 contained EcoRI and XhoI sites, respectively and GelEP3 and GelEP4 contained SalI and BamHI restriction sites to facilitate cloning. Each PCR product was digested with the corresponding restriction enzymes and both products ligated into pLT06 cut with EcoRI and BamHI, prior to electroporation into *E. coli* EC1000. The correct constructs were identified by selection on LB agar plates containing chloramphenicol at 10 µg/ ml and screened by restriction digest analysis and further sequenced for verification. A similar approach was employed in the construction of pVT02 (*sprE* deletion) using primer pairs SprEP1 and SprEP2 as well as SprEP3 and SprEP4; and for pVT03 (*gelE-sprE* deletion) primer pairs GelEP1 and GelEP2 were used along with SprEP3 and SprEP4. The

isolated plasmids were electroporated into electrocompetent *Enterococcus faecalis* V583 (10). *Enterococcus faecalis* V583 transformants were selected by growth at 28°C on THB agar containing chloramphenicol at 15 µg/ ml and X-gal at 120 µg/ ml. Blue colonies were inoculated into fresh THB containing chloramphenicol at 15 µg/ ml. Cultures were grown overnight at 28°C, diluted 1:100 into fresh media and grown for an additional 2 ½ hours at 28°C and then shifted to 42°C for an additional 2 ½ hours to favor single site integration of plasmids into the *E. faecalis* V583 genome. Serial dilutions of the integrants were plated onto THB agar plates supplemented with 15 µg/ ml chloramphenicol. Colony PCR was used to confirm single site integration for each construct using vector specific primers OriF or SeqR along with primers targeted to regions 5' or 3' to the site of insertion (GelEUp, GelEDown, SprEUp or SprEDown). A positive colony was then cultured in the absence of selection until the culture reached stationary phase (~2 x 10^9 CFU/ ml). Serial dilutions were prepared and fresh media (THB) was inoculated such that it contained 100 CFU/ ml. Serial dilutions (1:500 and 1:1000) were plated on MM9YEG agar supplemented with 10mM DL-p-Cl- Phe and X-Gal at 120µg/ ml. Counter selection using DL-p-Cl- Phe has been shown to favor the selection of colonies that have lost the plasmid (24). Colony PCR using primers GelEUp and GelEDown for VT01, SprEUp and SprEDown for VT02, and GelEUp and SprEDown for VT03 were used to confirm the gene deletion in the genome. Phenotypic confirmation of the protease deletions were also visualized on THB agar containing 1.5% skim milk and 3% gelatin.

Complementation of *E. faecalis* V583 in-frame protease deletion mutants

In-frame protease deletions of *E. faecalis* V583 were complemented with full-length *gelE*, *sprE* and *gelE-sprE,* each with the native *gelE* promoter region in a pAT28 vector (54) and were denoted pVT05, pVT08 and pVT07 respectively. The *gelE* complement insert was cloned by PCR amplification of V583 genome with primers GelEprom and SprEP2 and subsequently inserted as an EcoRI/ XhoI fragment into pAT28 cut with EcoRI/ SalI. The *gelE-sprE* double protease complement construct was cloned by PCR amplification of V583 genome using primers GelEprom and GelEP4 and inserted as an EcoRI/ BamHI fragment into EcoRI/ BamHI cut pAT28 vector. Plasmid pVT08 was constructed by digesting pVT07 with AflII and NcoI, followed by Klenow treatment to make it blunt-ended and then the molecule was circularized by self-ligation to obtain an in-frame deletion of *gelE*. These

constructs were transformed into the corresponding protease deletion mutants and phenotypic complementation was confirmed by zymography using skim-milk at a final concentration of 0.02% as substrate (23).

Table 2.2 *E. faecalis* strains used in this study

Strain	Relevant genotype	Complementation in pAT28	Relevant phenotype	Origin
V583	Parental		GelE$^+$ SprE$^+$	Clinical isolate
VT01	Δ*gelE*		GelE$^-$ SprE$^+$	V583
VT02	Δ*sprE*		GelE$^+$ SprE$^-$	V583
VT03	Δ*gelE-sprE*		GelE$^-$ SprE$^-$	V583
VT05	*gelE*	*gelE* promoter *gelE*	GelE$^+$ SprE$^+$ Spec^R	pVT05→ VT01
VT07	*gelE sprE*	*gelE* promoter *gelE sprE*	GelE$^+$ SprE$^+$ Spec^R	pVT07→ VT03
VT08	*sprE*	*gelE* promoter *sprE*	GelE$^+$ SprE$^+$ Spec^R	pVT08→ VT02
VT09	Parental		GelE$^+$ SprE$^+$ Gfp Tet^R	pMV158gfp→ V583
VT10	Δ*gelE*		GelE$^-$ SprE$^+$ Gfp Tet^R	pMV158gfp→ VT01
VT11	Δ*sprE*		GelE$^+$ SprE$^-$ Gfp Tet^R	pMV158gfp→ VT02
VT12	*ΔgelE-sprE*		GelE$^-$ SprE$^-$ Gfp Tet^R	pMV158gfp→ VT03

Biofilm assay on polystyrene microtiter plates

Biofilm formation on polystyrene was quantified with crystal violet staining method as previously described (19). Each assay was performed in octuplicate and repeated 5 times. Statistical significance was calculated using Dunnets test (GraphPad Software, San Diego, CA).

Table 2.3 Plasmids used in this study

Plasmid	Description	Source/ Reference
pLT06	Integration vector, Cm^R derivative of pCJK47	L. Thurlow, unpublished
pVT01	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *gelE* deletion	This study
pVT02	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *sprE* deletion	This study
pVT03	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *gelE sprE* deletion	This study
pAT28	Broad-host range shuttle vector, spectinomycin resistance	(54)
pVT05	pAT28 containing 1748-bp EcoRI/XhoI fragment containing the native *gelE* promoter along with full-length *gelE*	This study
pVT07	pAT28 containing 2687-bp EcoRI/BamHI fragment containing the native *gelE* promoter and full-length *gelE sprE*	This study
pVT08	pAT28 containing 2123-bp EcoRI/BamHI fragment containing the native *gelE* promoter, a truncated *gelE* and full-length *sprE*	This study
pMV158gfp	Gram-positive replicative vector expressing a Gfp reporter	(1)

Table 2.4 Oligonucleotides used in this study

Primer	Sequence
GelEP1	5' GAGAGAATTCGATTGGCTTAGTCATTGAAGC T
GelEP2	5' CTCTCTCGAGAAAGATGCCTGTACCTAAAATG
GelEP3	5' GAGAGTCGACCAGGTAAACCAACCAAGTGAAT
GelEP4	5' CTCTGGATCCCCGTGATTCTGGAAATTCCGAG
SprEP1	5' GAGAGAATTCGTGAACGCTACAGATGGAACAA
SprEP2	5' CTCTCTCGAGTTCATTCATTGACCAGAACAGA
SprEP3	5' GAGAGTCGACCTCGGAATTTCCAGAATCACGG
SprEP4	5' CTCTGGATCCAGGTTCAGCGTTATCTACTAAG
GelEUp	5' CGCCAGAGATTTCACCTGACT
GelEDown	5' GGTACTTTTATCGTAACTTACAC
SprEUp	5' CAATCGGTTGATGCAATCGGTG
SprE Down	5' GTACGAGCATTCGCAGTAAATTC
GelEprom	5' GAGAGAATTCGCTATGGTATTGAGTTATGAGG
Ef1091-5'	5' GCTATGTTGGCTACTCAAGTG
Ef1091-3'	5' TGTCCTGCAGGTGCTTTTAC
Ef0887-5'	5' GAGAGAATTCGAAGGAATTGTCATTGTGCG
Ef0887-3'	5' CAAAACGGATCCGACTCGC
Ef2490-5'	5' GAGAGTCGACAGCAAAAGGGTTGTTGAAATA
Ef2490-3'	5' CTCTGCATGCCCTACTTTCTCTGTTACTTAAT
Ef2488-5'	5' GAGAGGATCCTAAACAGGGGAGTGTGTGACATG
Ef2488-3'	5' GAGAGCATGCCAAACACCAAATGCATTATTTA
Ef2194-5'	5' GGAACAACGCTAAACTTTTAC
Ef2194-3'	5' CGCTCCTATTCTGCTGCTAA
OriF	5' CAATAATCGCATCCGATTGCA
SeqR	5' CCTATTATACCATATTTTGGAC

Cell surface Hydrophobicity assay

The cell surface hydrophobicities of *E. facalis* V583 and isogenic protease mutant strains were carried out as previously described (43). The percentage of bacterial adhesion to hydrocarbon was calculated as follows: [1- (OD_F/ OD_I)] ×100, where OD_I and OD_F are the ODs of cells resuspended in PUM buffer determined at the beginning and the end of the experiment, respectively. Statistical signifance was computed using the Dunnett's test (GraphPad Software, San Diego, CA).

Autolysis assay

Autolysis assay was carried out as previously reported (11).

Isolation of extracellular DNA from *E. faecalis* planktonic culture supernatants

Supernatants from 24 hour old grown cultures were passed through a sterile 0.2 μm syringe filter (Nalgene) and concentrated approximately 20-fold using a 10 kDa cut-off membrane (YM-10 Centricon centrifugal filter devices, Millipore) according to the manufacturer's instructions. The concentrated samples were loaded on a 1% agarose gel and stained with ethidium bromide to visualize high molecular weight DNA. Densitometric spot comparisons were performed using Alphaimager software (Alpha-Innotec, San Leandro, CA)

Extracellular DNA from culture supernatants was isolated using the Wizard genomic DNA purification kit, according to the manufacturer's instructions and chromosomal DNA was isolated as previously described (37). For comparative PCR, primers listed in Table 2.4 were designed to amplify genes from regions of the *E. faecalis* V583 genome, including Ef0887, Ef1091, Ef2194, Ef2488, Ef2490, and Ef1818 (*gelE*).

Laser scanning confocal microscopy

E. faecalis strains V583, VT01, VT02 and VT03 were transformed with pMV158GFP (34) to constitutively express Gfp for confocal imaging. The resulting strains were designated VT09, VT10, VT11, and VT12, respectively. Confocal microscopy was performed on *E. faecalis* biofilms grown on glass coverslips. Sterile glass coverslips were placed on the bottom of 6-well tissue culture plates and submerged with 5 ml of M17 broth, seeded with a

1: 100 dilution from an overnight culture (approximately 5-10 x 10^6 CFU), and grown for 24 hours at 37°C. For 2, 3 and 4 day old biofilms, the culture supernatants were replaced with fresh media daily. Just prior to imaging, biofilms were gently rinsed three times with sterile PBS, followed by 10 minute staining with 5 mL propidium iodide (1 μM). The coverslips were mounted on a microscope slide and sealed with clear nail polish to prevent dehydration. Slides were visualized by using a Zeiss LSM 5 PASCAL (Laser Scanning Confocal Microscope). The LSM 5 system was equipped with a Zeiss Axioplan 2 MOT research microscope, a fully motorized stage, a Plan Apohromat objective (63x/ 1.4 oil) and differential contrast interference (DIC). Dual fluorescence emission imaging of GFP and propidium iodide (PI) was accomplished using 488nm line of 458/ 488/ 514 Argon gas ion laser to excite GFP and 543nm line of HeNe laser to excite PI. A secondary HFT 545 dichroic was used to split the emission signals into two signals, the shorter wavelengths passed through a band pass 505- 530nm filter to image GFP fluorescence and the longer wavelength passed through a long pass 560nm filter to image PI fluorescence. For z- series, the Airy units of the longer and shorter wavelengths were adjusted to give an optical slice thickness of 0.7μm and this thickness was used as slice interval. Biofilm quantification was carried out using the COMSTAT analysis package (20). Volumetric analysis (μm^3) of representative confocal images portraying regions within the biofilm stained by propidium iodide were carried out using the 3D Object counter plug-in, for the NIH Image J software. For determination of statistical significance, the data was natural log transformed and unpaired t-test was performed using GraphPad (GraphPad software, San Diego, CA)

DNaseI treatment of biofilms

To assess the significance of extracellular DNA for *E. faecalis* biofilms, 6 hour, 12 hour and 24 hour old biofilms were treated with 100 Kunitz units per mL DNaseI. The control contained denatured DNaseI that was heated at 100 °C for 15 minutes. The biofilms were imaged using confocal laser scanning microscopy.

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CHAPTER 3

A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*

Abstract

Extracellular DNA (eDNA), a by-product of cell lysis was recently established as a critical structural component of the *Enterococcus faecalis* biofilm matrix. Here, we describe fratricide as the governing principle behind gelatinase (GelE) mediated cell death and eDNA release. GFP reporter assays confirmed that GBAP quorum non-responders ($GelE^{-}SprE^{-}$) were a minority subpopulation of prey cells susceptible to the targeted fratricidal action of the quorum responsive predatorial majority ($GelE^{+}SprE^{+}$). The killing action is dependent on GelE, and the GelE producer population is protected from self-destruction by the co-production of SprE as an immunity protein. Targeted gene inactivation and protein interaction studies demonstrate that extracellular proteases execute their characteristic effects following downstream interactions with the primary autolysin, AtlA. Finally, we address a mechanism by which GelE and SprE may modify the cell wall affinity of proteolytically processed AtlA resulting in either a pro- or anti-lytic outcome.

Introduction

Opportunistic enterococcal infections have traditionally been perceived as serious clinical threats due to rising incidence of antibiotic resistance and lateral transfer of resistance traits (7, 33). More recently, these same threats have been associated with the existence of bacteria as surface adherent communities called biofilms (14). The close residential proximity of organisms within biofilms and altered genetic responses primarily driven by density dependent mechanisms may aid the observed heightened tolerance to antibiotics and dissemination of resistance traits (22). Observations of clinical enterococcal biofilms on endodontic surfaces, bilary stents, urinary catheters, heart valves and tissue surfaces suggest a correlation of this pathogen's community lifestyle and virulence (6).

Analogous to developmental processes (biofilm development, competence regulation and sporulation) displayed by some bacteria, biofilm formation by enterococci involves quorum signaling (18, 27). The quorum signal is derived from the *fsr* gene cluster of *E. faecalis* (*fsrABDC*) and results from the processing and secretion of FsrD by FsrB to yield an 11-amino acid cyclized peptide lactone termed gelatinase biosynthesis-activating pheromone (GBAP) capable of density dependent signal transduction via the FsrAC two-component histidine kinase-response regulator pathway (30). Unlike the peptide pheromones involved in conjugal mating and plasmid transfer in *Enterococcus*, the formation of a cyclized lactone is thought to protect GBAP from proteolytic turnover by enterococcal proteases (30). Transcriptional profiling has indicated strong expression of two co-transcribed secreted extracellular proteases, gelatinase (GelE) and serine protease (SprE) following Fsr signal activation (5, 35). A number of independent studies have observed a critical role for gelatinase in enterococcal biofilm development (18, 23, 27). Gelatinase is proposed to activate lysis of a sub-population of bacteria and thereby catalyze the release of genomic DNA (eDNA), a critical component of the biofilm matrix (45). Intriguingly the phenotypic effects of SprE opposed those of GelE with higher rates of lysis, eDNA release and increased biofilm development observed upon its inactivation (45). Earlier observations by Shockman and others suggested the cellular target of GelE to be an autolysin (42, 47). Although a direct interaction of SprE with a cell surface autolysin is yet to be established, evidence suggests it can modify rates of autolysis (45). At least three autolysins (AtlA, AtlB and AtlC) have been identified to be secreted by *E. faecalis* of which

AtlA is thought to be crucial for biofilm development (24, 26). Based on sequence similarity, AtlA was proposed to be made up of three domains. The central catalytic domain is responsible for the glucosaminidase activity (12). The C-terminal domain is composed of six LysM modules that afford peptidoglycan affinity and possibly target autolysins to the division septum and poles (44). No known function yet exists for the T/ E rich N-terminal domain (12).

The existence of a bimodal bacterial population was hypothesized to explain the nature of enterococcal cell death leading to biofilm development (45). The first sub-population would be a predatory population that produces GelE as an effector of cell death and is immune to self-inflicted GelE toxicity by virtue of the co-transcribed SprE protease while the second, a lysis susceptible prey population that does not produce either extracellular protease. Such bimodality in an otherwise isogenic bacterial population during growth is plausible given that cell density dictates those cells that respond to the GBAP quorum peptide and activate protease expression. Bacteria that do not respond to quorum signaling would then be susceptible to GelE mediated cell death due to their inability to produce the immunity factor, SprE. Although the above hypothesis is consistent with bacterial fratricide (8), wherein a specific cell is responsible for predation of its clonal neighbor, an alternate hypothesis of altruistic suicide or programmed cell death of the quorum responder remains a possibility (2, 39).

In the current study, we show that extracellular proteases regulate enterococcal fratricide leading to eDNA release and biofilm development. Further AtlA is demonstrated to be the target of both GelE and SprE and this interaction is critical to the regulation of enterococcal fratricide and biofilm development.

Results

SprE prevents altruistic suicide in enterococcal populations

Programmed cell death was recently hypothesized to be critical for *S. aureus* biofilm development (2, 39). To investigate whether extracellular proteases (GelE and SprE) controlled an altruistic nature of cell death and lysis in *E. faecalis* populations, a GBAP responsive GFP-reporter construct (pVT31) was introduced into V583, VT01(Δ*gelE*), VT02 (Δ*sprE*) and VT03 (Δ*gelE sprE*) isogenic protease deletion mutant backgrounds resulting in

strains VT15, VT16 (Δ*gelE*), VT17 (Δ*sprE*) and VT18 (Δ*gelEsprE*) respectively. The plasmid pVT31 contains a 1055-bp fragment composed of a *gelE* promoter-*gfp* gene fusion, in a high copy vector (pAT28) background. As the expression of GelE in native circumstances is dependent on GBAP levels, the coupling of the *gelE* promoter to the *gfp* gene in pVT31 would enable the expression of GFP to be dependent on the extracellular concentration of GBAP. Hence enterococci (bearing plasmid pVT31) that respond to the quorum peptide (GBAP) would switch on the expression of GFP. However cells that do not respond to GBAP would retain a GFP^- phenotype. This phenotypic segregation in GFP expression effectively facilitated tracking of cells that responded to the quorum peptide within the wild type and each of the mutant populations by flow cytometry. The stringency of pVT31 as a reporter construct was determined using *E. faecalis* VT14, a derivative of V583 harboring pVT30 (reporter construct lacking functional promoter fusion) and VT13, an *fsrA* insertion mutant that contains pVT31. The absence of a functional FsrA would prevent VT13 from responding to the accumulation of quorum peptide (GBAP). Accordingly figure 3.1A depicts a clear shift in fluorescence intensities of VT15, VT16, VT17 and VT18 relative to VT14. Both the above controls (VT13 and VT14) exhibited a signal noise less than ~6% relative to VT15 (V583 pVT31) (Fig. 3.1B). Fluorescent signal generated by VT13 likely arises from a basal level of expression from the *gelE* promoter, which is known to occur independent of FsrA (43). The relatively weak signal observed in strain VT14 is likely attributed to use of a weak promoter in the vector sequence, as plasmid pVT30 lacks the sequence for the *gelE* promoter.

Quantitative estimates of GFP^+ cells (GBAP responders) in wild type and extracellular protease mutant backgrounds were achieved by flow cytometry. The settings were adjusted such that GFP^- cells were within the first log decade while cells with higher intensities were considered GFP^+ (Fig. 3.1A). Estimation of GFP^+ cells (GBAP responders) by flow cytometry indicate (Fig. 3.1B) that although the majority of the wild-type (VT15) *E. faecalis* population under the present assay conditions responded to quorum signaling at the stationary phase of growth, approximately 15% correspond to GBAP non-responders. The independent effects of GelE and SprE on GBAP responders were characterized with respect to relative numbers of GFP^+ responders within isogenic extracellular protease mutant populations and their geometric mean fluorescence intensities (GMFI). Relative numbers of GFP^+ cells (Fig. 3.1B) in VT16 (85.68 ± 7.02%) and VT18 (87.09 ± 6.5%) were similar to those found in *E. faecalis* VT15 (87.26 ± 4.37%).

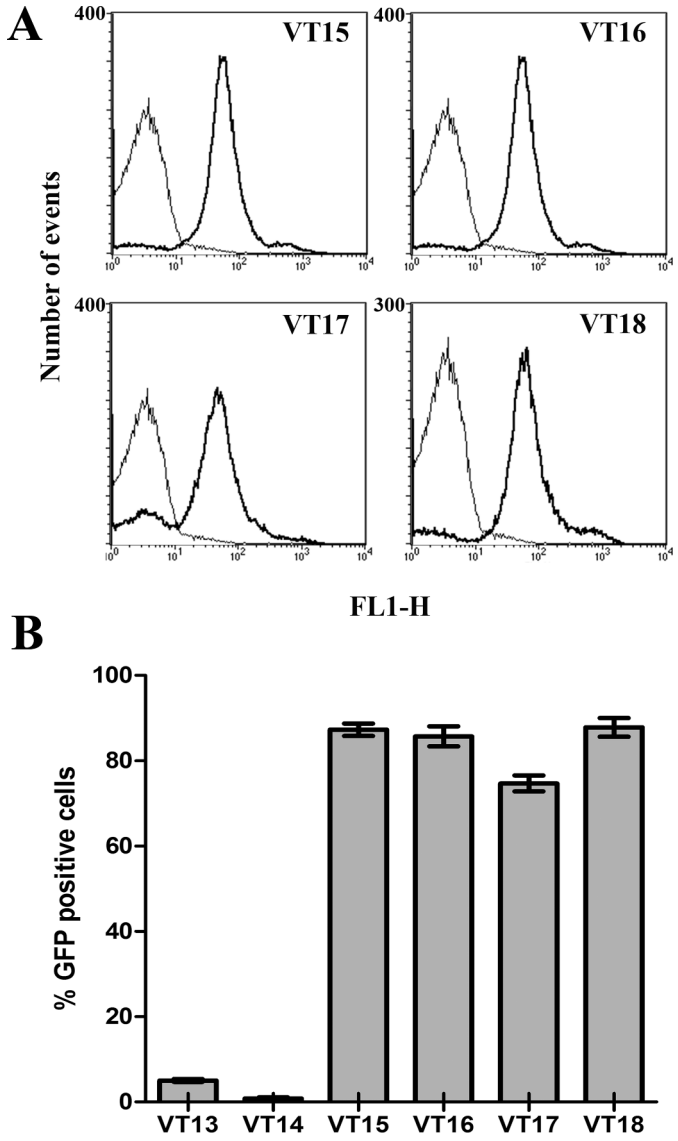

Figure 3.1 Estimation of altruistic suicide in enterococcal populations.
A. Representative flow histograms of GBAP responders from wild type VT15, and isogenic protease mutants- VT16 (Δ*gelE*), VT17 (Δ*sprE*) and VT18 (Δ*gelEsprE*) cultured to stationary phase. The pVT31 reporter plasmid in these isogenic strains activates GFP expression in response to GBAP quorum signaling. GFP fluorescence (FL-1H) corresponding to VT15, VT16, VT17 and VT18 are plotted (thick solid lines) and are contrasted in each instance with auto-fluorescence (thin solid lines) from VT14 (V583 pVT30, reporter construct lacking functional *gelE* promoter fusion). B. Percent GFP^+ cells (GBAP responders) following overnight growth of VT13 (V583::*fsrA*), VT14, VT15, VT16, VT17 and VT18. The percent estimates were derived from histograms of seven independent trials ± SE using the CELLQuest program (version 3.1, Beckton and Dickinson).

Interestingly, the absence of SprE expression in VT17 resulted in a significant decrease in GFP$^+$ responder cells to 74.66 ± 5.63% as compared to parental strain ($P< 0.05$, Bonferronis test). This suggested that GBAP responders within a population underwent significant lysis only in the absence of SprE. Consistent with this observation the geometric mean fluorescence intensity (GMFI) of GFP$^+$ responder cells of the VT17 (Δ*sprE*) population was significantly lower (59.23 ± 2.64) compared to that of V583 and mutants deficient in GelE expression (V583, 74.64 ± 13.11, VT16, 81.61 ± 6.97 and VT18, 83.22 ± 3.55). These observations are also consistent with earlier reports suggesting a pro-lytic activity for GelE and an anti-lytic activity for SprE (45).

Interplay of GelE and SprE in enterococcal fratricide

In order to assess the role of extracellular proteases of *Enterococcus faecalis* in fratricide, predator-prey co-culture lysis assays were performed. Parental and isogenic protease deletion mutants (predators) - V583, VT01 (Δ*gelE*), VT02 (Δspr*E*) and VT03 (Δ*gelEsprE*) were co-cultivated in the presence of VT12, a double protease mutant strain that constitutively expressed GFP from plasmid pMV158GFP (32). This previously characterized prey strain is deficient in lysis due to its inability to produce GelE (45). However as the downstream cellular target of GelE that affects autolysis is still intact within these cells; their lysis may be activated when they are exposed to diffusible GelE. Consequently we reasoned that fratricidal control of the GFP$^+$ prey population by diffusible extracellular proteases produced by the co-cultivated predator strains may be assayed by tracking the levels of GFP released into the culture supernatant. Neither of the purified extracellular proteases (GelE or SprE) exhibited an effect on GFP turnover at the concentrations assayed (data not shown) and hence any signal detected in culture supernatants should be a true reflection of bacterial lysis. Western blot analysis of culture supernatants, using anti-GFP antibody clearly showed that the lysis of the prey (VT12) population is dependent on the diffusible gelatinase (GelE) produced by the predator (Fig. 3.2A; compare lanes 1 and 3 to lanes 2 and 4). Whereas co-cultivation of the parental V583 (GelE$^+$SprE$^+$) predators resulted in wild type levels of target lysis, GelE mutant predators (VT01 and VT03) demonstrated no detectable lysis of VT12 (prey). Interestingly, the use of VT02 (SprE$^-$) as the predator resulted in an increased lysis of prey (Fig. 3.2A, compare lanes 1 and 3), suggesting that diffusible SprE was able to modulate GelE dependent fratricide.

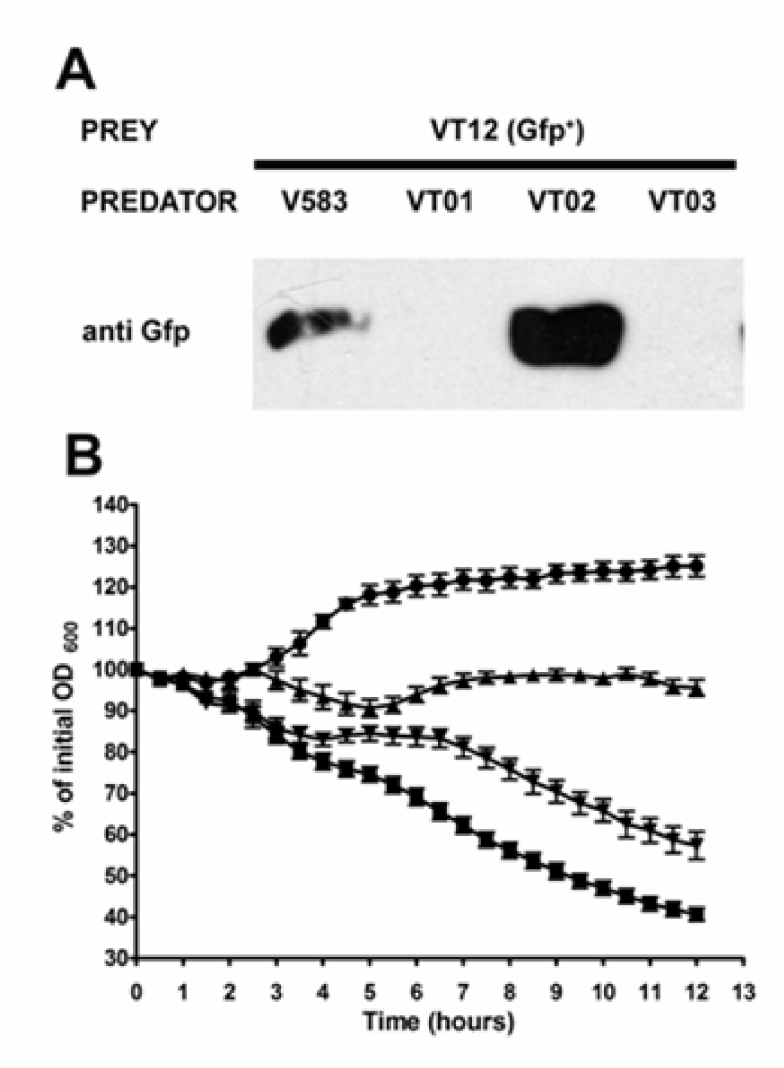

Figure 3.2 Extracellular proteases regulate enterococcal fratricide.
A. Detection of GFP immunoreactivity in stationary phase predator-prey co-culture supernatants. Predator (V583, GelE$^+$SprE$^+$; VT01, GelE$^-$SprE$^+$; VT02, GelE$^+$SprE$^-$; and VT03, GelE$^-$SprE$^-$) and prey (VT12, GelE$^-$SprE$^-$ GFP$^+$) were co-inoculated at a 20:1 ratio of prey: predator and grown overnight at 37°C prior to immunodetection of GFP. B. Effect of extracellular proteases on lysis of VT03 (GelE$^-$SprE$^-$). Differences in lysis rates of VT03 in the absence or presence of extracellular proteases (GelE and SprE) are exhibited as percent values of initial optical density at 600 nm. VT03 was incubated alone (●) or in the presence of 100 ng of GelE (■), SprE (▲) or GelE and SprE (▼) together over a period of 12 hours at 37°C. Data are mean ± SE of three independent trials with each performed in triplicate.

As expected, spectrofluorometric measures of GFP fluorescence emanating from the prey population that survived the fratricidal action mediated by the predators showed a

significant reduction in cell fluorescence when co-cultivated with VT02 (Δ*sprE*) compared to V583, VT01 and VT03 (data not shown).

Consistent with these results, we also observed that the addition of purified extracellular proteases (GelE and SprE) in physiological concentrations (~30nM) (18, 25) resulted in the differential lysis of VT03 (Δ*gelEsprE*) (Fig. 3.2B). Incubation with purified GelE alone caused a rapid increase in the lysis-rate of VT03 (Δ*gelEsprE*). While SprE by itself did not affect VT03 (Δ*gelEsprE*) rates of lysis, it prevented the initial increase in optical density observed in the VT03 strain without exogenous proteases. Furthermore, co-incubation of SprE with an equal concentration of purified GelE reduced the rate of GelE mediated lysis (Fig. 3.2B). These findings not only point to the ability of GelE to initiate fratricide but also of SprE to modulate GelE activity.

Comparison of autolysin profiles among enterococcal strains

Based on previously reported autolytic rates of protease mutants (45) and on observations by Shockman reflecting the role of GelE in activating an autolysin (42), we hypothesized enterococcal cell death to be a direct consequence of extracellular proteases manipulating cell surface associated autolysin(s). To determine potential autolysins regulated by extracellular proteases, we subjected cell wall protein extracts of GelE mutants from *E. faecalis* V583 and OG1RF strain backgrounds to zymography (Fig. 3.3).

Surface autolysin profiles of V583 and OG1RF exhibited significant differences. While a minor high molecular weight autolysin (~66kDa) was apparent in protein extracts derived from OG1RF, this activity was not visualized in VT20 (OG1RFΔ*gelE*) or V583 cell wall extracts (Fig. 3.3; compare lane 1 to lanes 2, 3 and 4). However, a novel 38kDa autolytic band that seemed to be immune to the proteolytic activity of GelE was uniquely found in the V583 strain background and not in OG1RF (Fig. 3.3; compare lane 1 and 2 to lanes 3 and 4). Similarly, a 50kDa autolytic band could only be detected in VT01 cell wall extracts, suggesting sensitivity toward GelE activity (Fig. 3.3; compare lanes 3 and 4). In light of a recent study by Bourgogne et al. (4) the absence of the 38kDa and 50kDa autolytic bands from OG1RF is not surprising as this strain lacks the prophage-associated endolysins present in V583 (34).

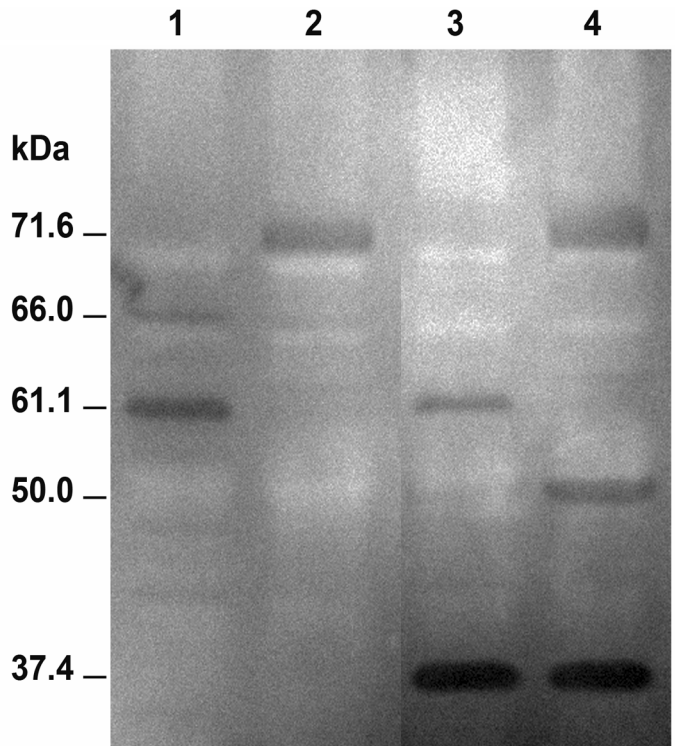

Figure 3.3 *Enterococcus faecalis* cell surface autolysin profiles.
Surface proteins were extracted from overnight grown cultures by boiling bacterial pellets in SDS-PAGE sample loading buffer. Samples were resolved on an 8% SDS polyacrylamide gel containing 0.1% purified OG1RF cell wall as substrate. Lanes 1 through 4 represents cell wall protein extracts from OG1RF ($GelE^+SprE^+$), VT20 (OG1RFΔ*gelE*; $GelE^-SprE^+$), V583 ($GelE^+SprE^+$) and VT01 (V583Δ*gelE*; $GelE^-SprE^+$), respectively. Calculated molecular weights are indicated on the left.

Interestingly, only two autolytic bands (71.6 kDa, found in VT01 (V583Δ*gelE*) and VT20 (OG1RFΔ*gelE*); 61.1 kDa in V583 and OG1RF) remained common among the two strains. Consistent with previous reports (12, 26), we identified and mapped the 71.6 kDa activity to AtlA by MALDI-TOF mass spectrometry. Targeted mutagenesis of *atlA* by insertion inactivation (see Materials and Methods) in V583 and OG1RF strain backgrounds (VT19 and VT21, respectively) demonstrated the 61.1 kDa activity to correspond to the proteolytically processed form of AtlA (Fig. 3.4). Interestingly, autolysin profiles of VT02 (Δ*sprE*) when compared with V583 appeared to display a considerable increase in the intensity of the 61.1 kDa band (Fig. 3.5). These results collectively suggest that in parental strains, GelE and SprE are capable of differentially regulating the turnover of AtlA.

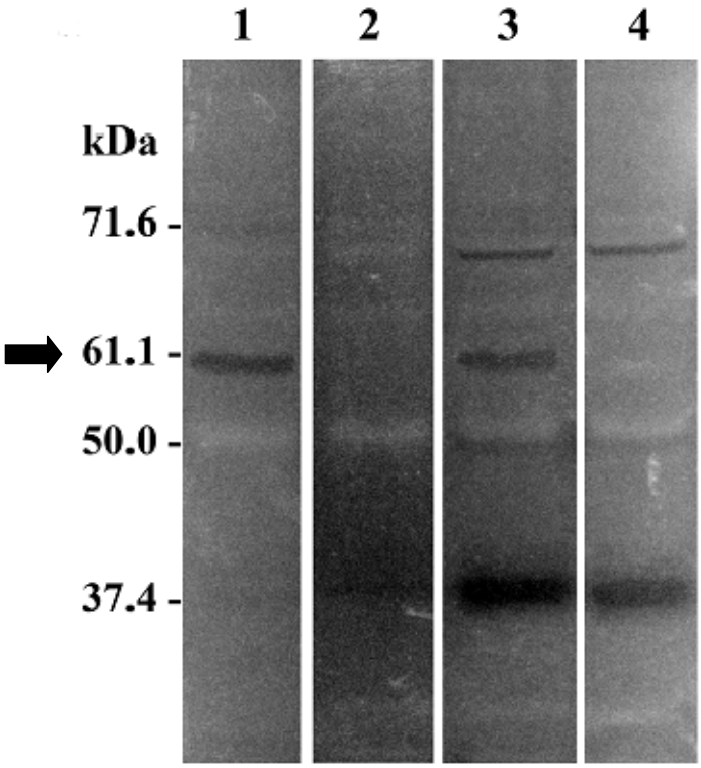

Figure 3.4 Targeted mutagenesis of *atlA* in *E. faecalis* V583 and OG1RF.
Targeted mutation of AtlA by insertion inactivation was carried out using the suicide vector system pVT10. Surface-associated proteins were extracted from overnight grown cultures by boiling cell wall pellets in SDS-PAGE sample loading buffer. Samples were resolved on an 8% SDS polyacrylamide gel containing 0.1% purified V583 cell wall as substrate. Lane 1, OG1RF; lane 2, VT21 (OGIRF::*atlA*); lane 3, V583; lane 4, VT19 (V583::*atlA*). Block arrow points to the 61.1 kDa form of AtlA present on *E. faecalis* cell surface. Calculated molecular weights are indicated on the left.

AtlA is critical for eDNA release and biofilm development

Recent studies have observed that inactivation of AtlA was detrimental to biofilm development of *E. faecalis* OG1RF (24). Given that, AtlA was the sole autolysin whose activity was demonstrated to be regulated by both proteases, we tested for differences in eDNA release and biofilm forming abilities of VT19 (V583::*atlA*) and VT21 (OG1RF::*atlA*) against parental strains. Culture supernatants of overnight grown *E. faecalis* V583 and OG1RF parental strains and their corresponding AtlA mutants were assayed for eDNA using a DNA specific dye, SYTOX green (Molecular Probes). As expected and confirming

our previous observations (45) deletion of *gelE* in V583 (VT01) and OG1RF (VT20) backgrounds resulted in a significant 4- and 12-fold decrease in eDNA release as compared

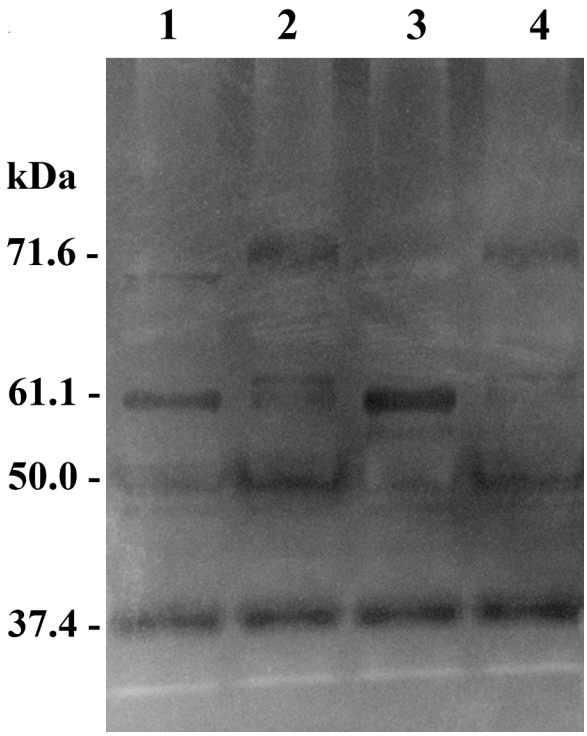

Figure 3.5 Cell surface autolysin profiles of extracellular protease mutants.
Surface-associated proteins were extracted from overnight grown cultures by boiling cell wall pellets in SDS-PAGE sample loading buffer. Samples were resolved on an 8% SDS polyacrylamide gel containing 0.1% purified V583 cell wall as substrate. Lanes 1 through 4 represents cell wall protein extracts from V583 ($GelE^{+}SprE^{+}$), VT01 (V583Δ*gelE*; $GelE^{-}SprE^{+}$), VT02 (V583Δ*sprE*; $GelE^{+}SprE^{-}$) and VT03 (V583Δ*gelEsprE*; $GelE^{-}SprE^{-}$), respectively. Calculated molecular weights are indicated on the left.

to their respective parental strains (Fig. 3.6A, $P< 0.0001$, t-test). More importantly, the decrease in eDNA release due to the inactivation of *atlA* paralleled GelE mutants from both strain backgrounds. This strongly suggested that AtlA may be the major target of extracellular proteases on the cell surface, that mediates fratricide and eDNA release.

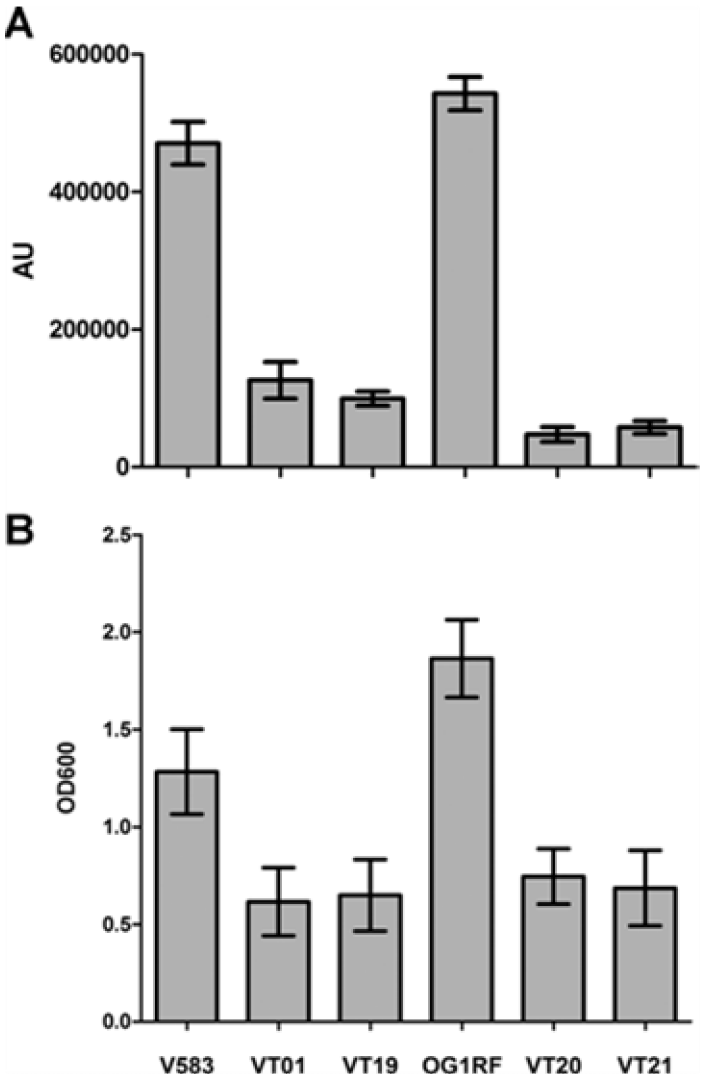

Figure 3.6 AtlA is critical for eDNA release and biofilm formation.
A. Detection of extracellular DNA in culture supernatants. Cell free culture supernatants of V583 (GelE$^+$), VT01 (V583Δ*gelE*), VT19 (V583::*atlA*), OG1RF (GelE$^+$), VT20 (OG1RF Δ*gelE*) and VT03 (OG1RF::*atlA*) were supplemented with the DNA specific dye, SYTOX green to a final concentration of 1 μM before being assayed spectrofluorometrically as described. Fluorescence intensity data are represented as arbitrary units (AU). B. Biofilm formation of *E. faecalis* strains on polystyrene. Biofilm formation was assayed as a function of crystal violet stain (measured at 550 nm) retained by the biofilm biomass grown for 24 hours. Data are mean ± SE of three independent trials. The strain designations shown in panel B correlate with panel A.

Because AtlA mutants are deficient in eDNA release, we further hypothesized these strains to be defective in biofilm formation. Quantitative analysis of biofilms grown on polystyrene surfaces revealed that AtlA mutants, similar to GelE mutants formed

significantly less biofilm biomass as compared to parental strains (Fig. 3.6B, P< 0.0001, t-test). The observed deficiencies in biofilm formation were not a result of growth defects as all mutants displayed similar growth kinetics compared to the parental strains (data not shown). These observations are consistent with a critical role for AtlA in eDNA release and biofilm formation.

Role of AtlA in fratricide

Given that extracellular proteases and AtlA contribute to similar biological pathways in *E. faecalis*, we hypothesized that fratricidal effector- modulator functions of GelE and SprE, are channeled through their downstream interactions with AtlA. However, prey-predator co-culture lysis assays using VT23 (an *atlA* mutant derivative of VT12 (Δ*gelE-sprE,* GFP$^+$) as prey showed no abrogation of V583 and VT02 (Δ*sprE*) predator-mediated fratricide (Fig. 3.7A; lanes 1 and 3), although maintaining GelE dependency (Fig. 3.7A; lanes 2 and 4).

In an attempt to address these seemingly confounding observations, we tested the autolytic rates of the *atlA* mutant derivative of VT03, designated VT22, in the presence of purified enterococcal extracellular proteases. Addition of physiological concentrations of GelE or SprE (~30 nM) individually or together did not lead to any significant differences in lysis of VT22 as compared to protease negative controls (Fig. 3.7B). This suggested that GelE dependent fratricide of VT23 observed earlier was possibly due to predator specific factors (in addition to GelE) that interacted with the prey population. As AtlA has previously been shown to be a diffusible enzyme capable of hydrolyzing the cell walls of *Micrococcus lysodeikticus* (36) and *E. faecalis* (26), we hypothesized that AtlA may fulfill the role of a predator factor responsible for the lysis of prey cells in the population. Such diffusible AtlA may possibly be released from a subpopulation of predator cells (~15%, refer Fig. 3.1B) that have not responded to the quorum signal and hence are susceptible to the effector functions of GelE. To test this hypothesis we co-cultured VT19 (V583::*atlA*) as a predator population against VT23 (Δ*gelEsprE*,::*atlA*, GFP$^+$) prey population. Fig. 3.7A (lane 5) clearly indicates the absence of any detectable fratricide in the prey population upon inactivation of the predator AtlA, suggesting a crucial role for soluble (diffusible) forms of AtlA in fratricide.

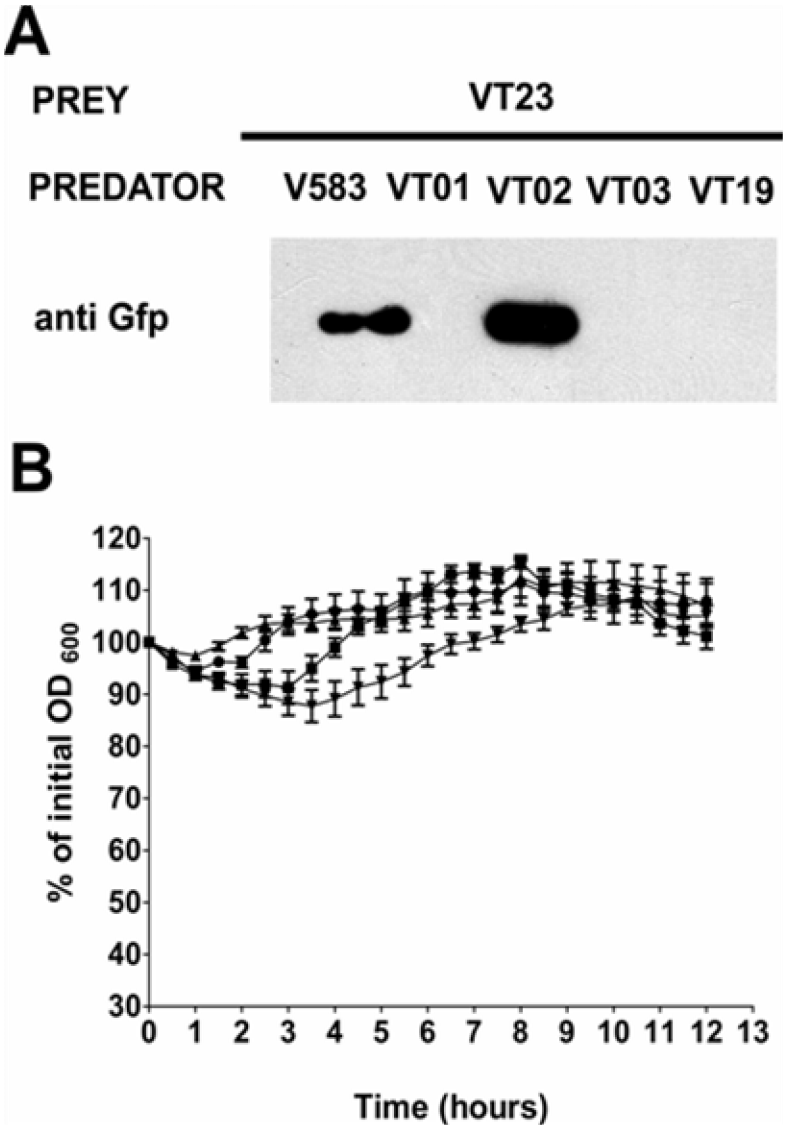

Figure 3.7 *Enterococcus faecalis* AtlA is a critical determinant of protease mediated fratricide.

A. GFP immunoreactivity from predator-prey co-culture supernatants. Predators represented by V583 (GelE$^+$SprE$^+$), VT01 (GelE$^-$SprE$^+$), VT02 (GelE$^+$SprE$^-$), VT03 (GelE$^-$SprE$^-$) and VT19 (GelE$^+$SprE$^+$AtlA$^-$) were each individually co-cultured with prey (VT23, GelE$^+$SprE$^+$AtlA$^-$GFP$^+$). B. Effect of extracellular proteases on lysis of VT22 (VT03::*atlA*, GelE$^-$SprE$^-$AtlA$^-$). Lysis rates are exhibited as percent values of initial optical density at 600 nm. VT22 was incubated alone (●) or in the presence of 100 ng of GelE (■), SprE (▲) or GelE and SprE together (▼) over a period of 9 hours at 37°C. Data are mean ± SE of three independent trials with each performed in triplicate.

Characterizing the effects of GelE and SprE on recombinant AtlA

To further investigate the interaction between extracellular proteases and AtlA, we subjected recombinant histidine tagged AtlA (rAtlA, Fig. 3.8A) expressed and purified from *E. coli* cultures to GelE and SprE treatment. GelE treated rAtlA was multiply processed into minor forms within 30 minutes (Fig. 3.8B; lane 2) and was completely turned over by 5 hours (data not shown). Zymogram analysis showed that most processed forms of rAtlA were active on V583 cell wall (Fig. 3.8C; lane 2) suggesting that GelE preferentially cleaves initially at non-catalytic sites within AtlA. The full-length 72.1 kDa autolytic band seemed to show activity only after extended periods of incubation (data not shown), suggesting an enhancement in activity upon proteolytic cleavage. Interestingly SprE action on rAtlA resulted in a single major 62kDa form and a minor higher molecular weight form (Fig. 3.8B and C, lane 3). From the current experimental conditions, although it would seem that rAtlA has a faint auto-catalytic activity and processes itself into the 62kDa form, the action of SprE clearly augments the rate of this processing while that of gelatinase seemed to affect the turnover of rAtlA.

Comparison of the molecular weights of GelE and SprE processed recombinant AtlA fragments (Fig. 3.8B) to their corresponding spectrograms (generated from MALDI TOF MS/MS peptide mass mapping) empirically identified regions within rAtlA that were susceptible to protease attack. As compared to the full length recombinant AtlA (72.3 kDa, includes 6XHis tag, but devoid of signal peptide), the 62kDa (Fig. 3.8B, lanes 2 and 3) form maintained an intact C-terminal domain inclusive of the histidine tag, suggesting that both GelE and SprE specifically cleaved within the N-terminal T/ E rich domain of rAtlA. Additionally, GelE also displayed cleavage specificities within the C- terminal domain. This was evident as the smaller rAtlA forms (55.3, 49.2, and 43.7kDa; Fig. 3.8B, lane 2) shared a common N-terminus but exhibited clear differences in their C- terminal peptide fingerprints (see appendix A).

Given that the C-terminal domain of AtlA is composed of multiple units of LysM modules (Fig. 3.8A) it seemed plausible that the action of GelE on the C-terminal domain of AtlA may catalyze its release from the cell surface. To test this hypothesis and to investigate any effect SprE may have toward rAtlA's affinity to the cell surface, we assayed the cell wall affinities of full-length and proteolytically processed forms of rAtlA.

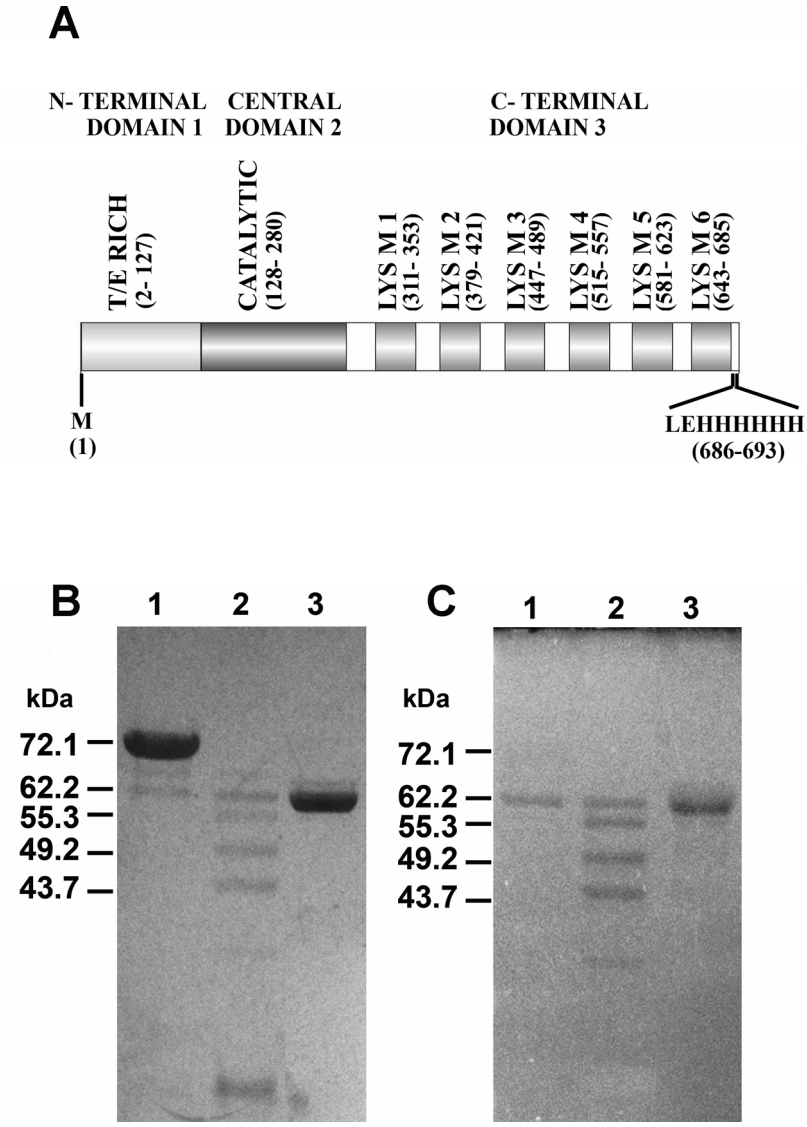

Figure 3.8 GelE and SprE differentially process AtlA.

A. Schematic representation of domains present within recombinant AtlA. Domain 1, Threonine and glutamic acid rich N-terminal domain; domain 2, N-acetyl glucosaminidase central catalytic domain; domain 3, C-terminal domain containing six LysM modules predicted to be crucial for peptidoglycan adherence. The recombinant protein was engineered to start with the residue, methionine and end with a 6 x histidine tag. Schematic was graphed using protein domain illustrator program (38), DOG 1.0.3. B. SDS PAGE analysis of proteolytically processed forms of AtlA. Purified AtlA (10 μg) was incubated alone (lane 1) or in the presence of 30 nM GelE (lane 2) or SprE (lane 3) for 30 minutes at 37°C. C. Zymogram analysis of proteolytically processed AtlA. Lanes represent AtlA alone (lane 1), AtlA incubated with GelE (lane 2) or SprE (lane 3). Calculated molecular weights are indicated on the left.

Recombinant AtlA was initially allowed to bind *E. faecalis* V583 cell walls, followed by individual treatments with GelE and SprE proteases. Relative affinities of rAtlA forms processed by GelE and SprE to the cell wall was determined as a function of their ability to remain in the supernatant fraction on centrifugation as opposed to the pelleted cell wall bound fraction. Although it was clearly evident from the analysis that cell wall bound rAtlA displayed altered processing by GelE compared to the unbound form (compare proteolytic pattern from Fig. 3.9, lane 5 and Fig. 3.8B, lane 2), the supernatant fractions revealed that GelE treatment resulted in processed soluble forms of rAtlA (Fig. 3.9, lane 2).

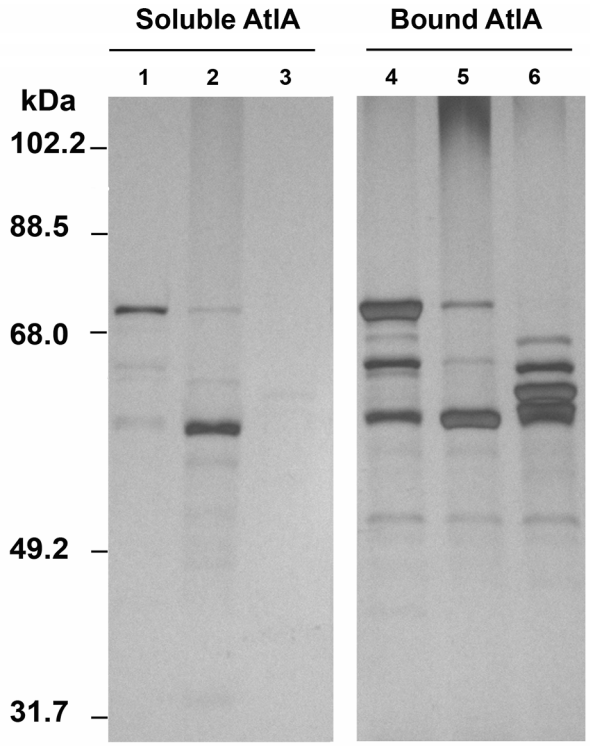

Figure 3.9 Differential affinities of GelE and SprE processed recombinant AtlA forms to cell wall peptidoglycan.
Following incubation with purified cell wall peptidoglycan, rAtlA was treated with GelE (lanes 2 and 5) and SprE (lanes 3 and 6). Lanes 1 and 4 contained rAtlA that was not treated with either GelE or SprE. Lanes 1-3 represent the soluble fraction of rAtlA after cell wall recovery, and lanes 4-6 represents the cell wall bound forms of rAtlA.

Notably, SprE treatment resulted in the absence of any detectable soluble forms of AtlA in the supernatant fraction. Complementing these observations, comparison of the GelE and

SprE processed cell wall bound forms of rAtlA displayed significant differences (Fig. 3.9; compare lanes 5 and 6). Cleavage of rAtlA by SprE resulted in processed forms with molecular masses approximately equal to and greater than 62kDa (Fig. 3.9 lane 6) that showed significantly higher affinity to peptidoglycan compared to the ~62kDa band resulting from GelE treatment (Fig. 3.9, lane 5). Given that cleavage of rAtlA by SprE did not result in the loss of LysM modules (a known peptidoglycan binding and localization domain) it stands to reason that AtlA may be shuttled to the septum optimally after SprE processing.

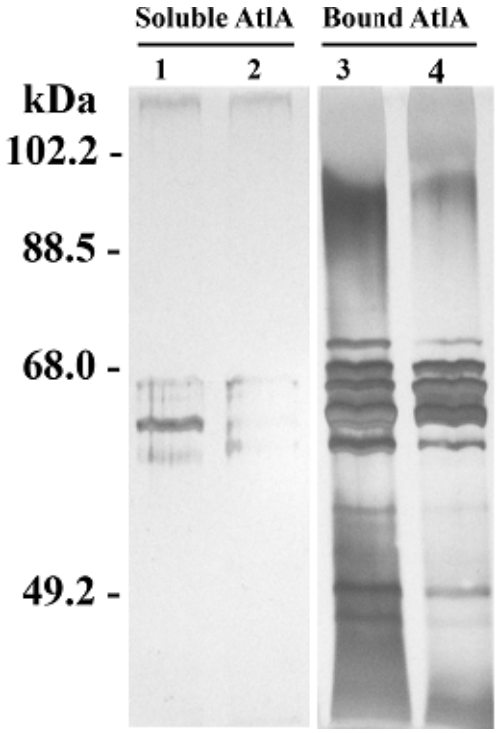

Figure 3.10: Effect of GelE and SprE co-treatments on the cell wall affinity of recombinant AtlA.
Following incubation (20 minutes, 37°C) with purified cell wall peptidoglycan (15 mg wet weight), AtlA (10 µg) was treated with either 30 nM GelE (lane 1 and 3) or 30 nM SprE (lanes 2 and 4) for 10 minutes. This was followed by an equi-molar addition of the complementary protease for 30 minutes. The mixture was centrifuged (13,000 x g; 20 minutes) and cell wall bound AtlA (pellet fraction) was extracted from peptidoglycan while the supernatant fraction containing unbound AtlA (soluble forms) was directly loaded on 10% acrylamide gel.

We reasoned that optimal proteolytic activity of SprE toward rAtlA may prevent GelE from subsequently mediating its (rAtlA's) release from the cell surface, thus arguing for the observed resistance of attacker subpopulations ($SprE^+$) to GelE mediated lysis. To test this hypothesis, we pretreated rAtlA bound to peptidoglycan with physiological concentrations of SprE and then subjected it to the proteolytic action of an equimolar concentration of GelE. Figure 3.10 (pg. 97) clearly demonstrates the inability of GelE to release SprE processed rAtlA from the peptidoglycan fraction into the supernatant.

Further, SprE was unable to prevent the release of peptidoglycan bound rAtlA into the supernatant if GelE acted on the autolysin first. This suggests that SprE prevents lysis of attacker populations by efficiently competing with GelE for the primary autolysin, AtlA. However, the exact mechanism of competition by the extracellular proteases toward AtlA remains to be elucidated. Collectively these results suggest that in wild-type populations the role of SprE may be to prevent altruistic suicide of quorum responders by limiting the release of AtlA from the cell surface.

Discussion

The results presented in this study demonstrate extracellular protease mediated fratricide to be responsible for governing eDNA release and biofilm development of *E. faecalis*. Death of sibling cells (fratricide) mediated by isogenic cells within the same population has previously been implicated in developmental processes (competence and sporulation) of *Streptococcus pnuemoniae* and *Bacillus subtilis* (13, 15-17, 20). Cell-cell signaling during these developmental processes results in the coordinated production of specific killing factors and immunity proteins within a subpopulation of cells (predators). Killing factors which include bacteriocins and murein hydrolases target the death of a small susceptible isogenic population (prey) (8). Susceptibility of prey to the killing factors is largely due to the absence of immunity proteins in this population, a cost these cells possibly pay for not participating in the signaling process. Death of the prey ultimately benefits the surviving population either in the form of nutrients in nutritionally stressed *B. subtilis* or as released genomic DNA for naturally competent *S. pneumonia* (8). In analogy with these model systems, we have identified two prominent driving forces of cell death (killing factors) responsible for biofilm development in enterococci; secreted gelatinase (GelE) and the soluble autolysin, AtlA (Fig. 3.11). Although cell wall peptidoglycan is refractory toward

GelE's ability to affect turnover of cell surface localized AtlA as compared to a relatively quick turnover in solution, it nevertheless allows limited cleavage potentially resulting in its release from the cell surface. We propose that released AtlA may result in bystander cell death especially in high cell density biofilms, although arguably the extent of such bystander effects will be limited by GelE to small sub-populations within the biofilm. Consistent with this hypothesis, we earlier reported the development of pockets filled with dead/ lysed cells within the primary biofilm matt (45).

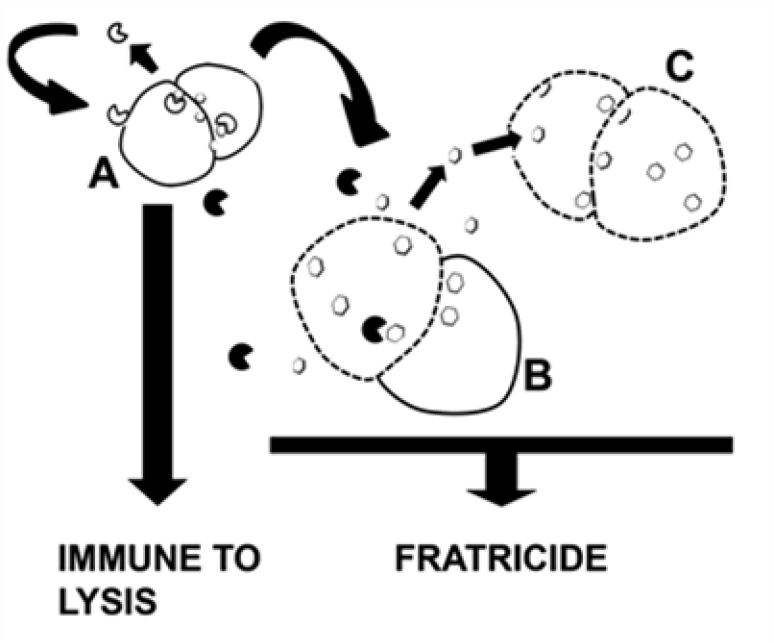

Figure 3.11 Model of GelE mediated fratricide in *Enterococcus faecalis*.
In response to GBAP quorum sensing, predators (A) co-secrete GelE (◔) and SprE (◔) extracellular proteases into the immediate environment. GelE and SprE diffuse from the producer cell and if GelE hits the target prior to SprE this event may result in lysis of sibling cells that have not responded to the quorum peptide, prey (B) resulting in the release of AtlA (○) from their surface. Alternatively, unbound, but enzymatically active AtlA could bind and lyse neighboring cells (C). The extent of such bystander cell lysis may be directly dependent on the rate of AtlA turnover by GelE. SprE primarily acts as an immunity proteins in predator (protected cells depicted as solid lines) cells by increasing affinity of AtlA to localized subcellular locations on the cell surface (septum) to prevent GelE from activating autolysis. Cells that are damaged by AtlA are depicted with discontinuous lines. Cells that are protected against the lytic action of AtlA are depicted with solid lines.

Biofilm development in *Staphylococcus aureus* was recently demonstrated to be dependent on cell death and eDNA release (39). However, the nature of cell death observed in this case was proposed to be due to altruistic suicide or programmed cell death (PCD) (2). Altruistic suicide of GelE$^+$ enterococci would be conceptually improbable as over 85% of the population in stationary phase show evidence of their participation in Fsr signaling (Fig. 3.1B) and the Fsr quorum response is known to differentially regulate over 300 genes involved in secondary regulatory cascades, virulence and metabolism (5).

How do cells producing GelE ensure their own safety against self-inflicted lysis? Several lines of evidence suggest the co-transcribed serine protease, SprE, to be an immunity protein. First, GFP reporter assays confirm a significant increase in death of predator populations in an isogenic SprE mutated strain compared to the parental strain. Consistent with this observation, co-culture lysis assays which detect solubilized GFP from lysed cells also indicate that secreted SprE has significant trans-protective activities toward prey cells. Second, purified SprE was able to significantly reduce the rate of GelE induced cell lysis. Finally, pretreatment of peptidoglycan bound AtlA with purified SprE significantly reduced its GelE mediated release (Fig. 3.10), a necessity for cell death. Consequently, it may be reasoned that SprE protects predator cells from lysis by modifying surface localized AtlA against further proteolysis by GelE.

Autolysins have consistently been described as regulators of cell death and biofilm development in different gram-positive species, although mechanistic details at a molecular level remain vague. Inactivation of the primary autolysins of *Streptococcus mutans*, *Staphylococcus aureus* and *Staphylococcus epidermidis* have been shown to decrease their abilities to form biofilms, presumably due to a defect in eDNA release (1, 3, 21, 37, 41). GelE has previously been implicated in the proteolytic processing of a latent high molecular weight *E. hirae* muramidase 1 (137 kDa) to the active 87 kDa form (42). Although *E. faecalis* muramidase activities have been characterized for AtlB and AtlC (26), their prophage origins and existence in the active state as low molecular weight lysins with predicted molecular masses (50 kDa and 47 kDa respectively) suggest that GelE is not required for their activation from latency. On the contrary, zymogram analysis suggests that GelE targets a ~50 kDa autolysin of V583 (Fig. 3.3, compare lanes 3 and 4). Whether AtlB identified by Mesnage et al. (26) corresponds to the ~50 kDa band that is only present in the absence of GelE in strain V583 awaits further characterization. However it is tempting to speculate based on protein size, as well as enzymatic activity that GelE may be

turning over AtlB. Furthermore, the affect of protease processing on AtlB and AtlC by GelE or SprE would not have been observed in the study by Mesnage et al. (26) as this study used strain JH2-2, known to be deficient in protease production because of the absence of the *fsr* locus (49). In spite of a recent study that suggested AtlA, the primary N-actyl glucosaminidase of *E. faecalis* as not being prone to proteolytic activation (12), we clearly demonstrate that purified GelE activates cellular autolysis in an AtlA dependent manner (compare Fig. 3.2B and Fig. 3.7B). We propose that the observed activation of cellular autolysis may possibly be due to the altered affinity of AtlA to the cell wall of *E. faecalis* in the presence of GelE and SprE, rather than the activation of latent AtlA. In agreement with this hypothesis, our observations suggest that soluble forms of AtlA are critical to the process of enterococcal fratricide. Of the three domains within AtlA, the C-terminal LysM domain is critical for cell wall localization. Consistent with this observation, we noted that all C-terminal truncated forms of AtlA (that lost one or more LysM modules) resulting from GelE proteolysis also displayed reduced affinity to cell wall peptidoglycan (data not shown). It is noteworthy that the C-terminal truncated forms of AtlA still appear to retain at least four functional LysM modules, based on detectable tryptic fragments from MS analysis. Intriguingly, we also observed a 62 kDa N-terminal form of AtlA with an intact C-terminus that resulted from GelE proteolysis, but displayed significantly decreased peptidoglycan affinity. Although at physiological concentrations, SprE was capable of generating the 62kDa form of AtlA, the presence of cell wall seemed to significantly alter this processing into higher molecular weight truncations (> 62kDa). This is consistent with the predicted cleavage specificity of SprE, given the high glutamic acid content of the N-terminal TE-rich domain.

In light of the present findings, it is likely that in *E. faecalis* the N-terminal domain of AtlA is also required for efficient adherence to peptidoglycan and that GelE mediates the release of active AtlA from prey cell surfaces by proteolytically processing the N- and C-terminus of AtlA. Furthermore, processing of AtlA by GelE to generate soluble forms of the autolysin may allow localization to cell regions other than the septum leading to the lysis of prey cells. The ability of SprE to process AtlA on predator cell walls may alter its structural conformation potentially targeting AtlA to the septum where its activity would govern cell division rather than lysis. The affinity of SprE-processed AtlA for the cell wall would also prevent GelE from further processing AtlA. (Fig. 3.11). Future studies will address whether proteolytic processing affects AtlA localization on the cell wall and this

may provide additional clues as to the role of SprE and GelE in triggering a pro or anti-lytic response. It may also be noted that the relative concentration of GelE in a biofilm would be highest in the vicinity of the predators, and solubilized forms of AtlA have been shown to be much more susceptible to GelE turnover, which may provide an additional control point to ensure that the GelE producer population is not killed by soluble forms of AtlA.

The *fsr*-dependent quorum sensing may be considered a coordinate cooperative behavior that enterococci employ to produce costly public goods (e.g. proteases) that benefit the whole population. However as with any population that employs cooperative strategies, a threat in the emergence of cheaters that does not contribute to the cost of public goods, but benefit from them is very high (10, 48). The occurrences of quorum sensing cheaters have previously been reported among different bacterial communities (10, 40). Within the limits of our experimental conditions (see Materials and Methods) we have estimated approximately 15% of the population in the stationary phase of growth to be comprised of cheaters (Fig. 3.1B) that do not respond to GBAP (as these quorum non-responders potentially benefit from nutrients generated from proteolytic activity of the remaining majority). This relatively high percentage of GBAP non responders is surprising considering the fact that by the time enterococci enter into the log phase, they have already secreted nano-molar concentrations of GBAP enough to activate *fsr* signaling within every cell of the population (29). Although, previous studies have implied the absence of a 23.9 kB region within the *fsr* locus (11, 31) as a possible contributor to the rise of cheaters, this is unlikely to be the case in our experimental set up and hence more investigations are necessary to further characterize the mechanisms of cheater development among enterococcal populations. Although arguably cheaters may enjoy a fitness advantage over their cooperative siblings (as they do not share the same metabolic burden), our observations suggest the contrary wherein co-culturing the wild-type V583 with its isogenic FsrA quorum sensing mutant (cheaters) or a double protease mutant , led to ~12% decrease in the cheater population after overnight growth (Table 3.1). Based on these observations, it is tempting to speculate that fratricide may have evolved among cooperative enterococci as a way to police cheaters and prevent them from taking over the entire population.

Table 3.1: Percentage bacteria remaining after overnight co-culture of predator and prey initially mixed in a 1:1 cell ratio (value ± S.D)

A	V583 (Predator)	62.47 ± 1.636	**B**	V583 (Predator)	61.98 ± 1.295
	KS10(Δ*fsrA*) (prey)	37.53 ± 1.636		VT03(Δ*gelEsprE*) (prey)	38.02 ± 1.295

Method/ Results of Co-culture experiments:

V583 and KS10 (V583Δ*fsrA*) were cultured in THB medium overnight at 37°C without shaking in separate culture vessels. Cells were enumerated by plate counting to determine the number of viable organisms, and equal cell numbers from both strains were co-inoculated into a single vessel at a 1:100 dilution. This co-culture was incubated overnight at 37°C and cells were serially diluted and plated onto THB-Skim Milk agar to differentiate proteolytic from non-proteolytic colonies. The experiment was performed in triplicate with two experimental replicates. Similarly, V583 was also co-cultured with VT03 (V583Δ*gelEsprE*). In both instances, V583 was significantly enriched in the co-culture assay compared with the non-proteolytic strains KS10 or VT03 following 24 hour growth ($P < 0.05$, Student t-test)

Materials and Methods

Bacterial strains, plasmids and growth conditions

Relevant bacterial strains and plasmids used in the present study are listed in Table 3.2. Strains were cultured in Todd-Hewitt Broth (THB) or M17 media and grown as standing cultures at 37°C unless otherwise indicated. *Escherichia coli* Electro-Ten Blue was used for maintenance and propagation of plasmid constructions. Clones were cultured aerobically in Luria-Bertani broth at 37°C. The antibiotics used for selection included chloramphenicol at

Table 3.2 Bacterial strains and plasmids

Strains	Genotype/ relevant phenotype	Origin
E. faecalis		
OG1RF	Clinical isolate	(28)
V583	Clinical isolate, TIGR sequence strain; Vn^R	(45)
VT01	V583Δ*gelE*; $SprE^+$	(45)
VT02	V583Δ*sprE*; $GelE^+$	(45)
VT03	V583Δ*gelE-sprE*	(45)
VT12	VT03 (pMV158GFP); GFP^+ Tet^R	(45)
VT13	V583::*fsrA* (pVT31); Cm^R, Spc^R	This study
VT14	V583 (pVT30); Spc^R	This study
VT15	V583 (pVT31); GFP^+ Spc^R	This study
VT16	VT01 (pVT31); GFP^+ Spc^R	This study
VT17	VT02 (pVT31); GFP^+ Spc^R	This study
VT18	VT03 (pVT31); GFP^+ Spc^R	This study
VT19	V583:: *atlA*; Cm^R	This study
VT20	OG1RFΔ*gelE*; $SprE^+$	This study
VT21	OG1RF:: *atlA*; Cm^R	This study
VT22	VT03::*atlA*; Cm^R	This study
VT23	VT12::*atlA* (pMV158GFP); GFP^+, Cm^R, Tet^R	This study
VT24	VT03 (pVT08); $SprE^+$	This study
KS10	V583Δ*fsrA*	Gorman, M. and Hancock, L.E.,(unpublished)
E. coli		
Electro Ten Blue	General plasmid maintenance strain	Stratagene
BL21 (DE3)	Protein expression strain	Stratagene
VT25	BL21(DE3) (pVT21; $AtlA^+$ * Amp^R	This study
Plasmids		
pAT28	Broad host range shuttle vector; Spc^R	(46)
p3CAT	p3TET derivative (19), suicide vector; Cm^R, used for targeted gene inactivation	Hancock, LE (unpublished)
pMV158GFP	Gram-positive replicative vector expressing a GFP reporter	(32)
pML115	p3CAT derivative carrying an internal fragment of *fsrA*; generates *fsrA*::*cm* mutation in *E. faecalis*	Hancock, L.E (unpublished)
pVI01	Low copy *gelE promoter- gfp* fusion reporter construct	Iyer, V and Hancock, L.E (unpublished)
pVT08	pAT28 derivative carrying a 2123-bp EcoRI/BamHI fragment (native *gelE* promoter and full-length *sprE*); leads to the over expression of SprE	(45)
pVT10	p3CAT derivative carrying a 1006-bp EcoR1/BamH1 internal fragment of *atlA*; generates *atlA*::*cm* mutation in *E. faecalis*	This study
pVT21	pET21b derivative carrying a 2056-bp Nde1/Xho1 *atlA* fragment devoid of signal peptide; used for the expression of AtlA in *E. coli*	This study
pVT30	pAT28 derivative carrying a 759-bp SalI/SalI *gfp* fragment; promoterless *gfp*	This study
pVT31	pAT28 derivative carrying a *gelE promoter-gfp* fusion; reporter construct that allows discrimination of predators within *E. faecalis* population	This study

^{R} Resistant

Vn, vancomycin; Cm, chloramphenicol; Spc, spectinomycin; Tet, tetracycline; Amp, ampicllin

* AtlA devoid of signal peptide (53 amino acid residues from the N-terminus)

10 μg/ ml and spectinomycin at 750 μg/ ml. Electrotransformations of *E. faecalis* were performed as previously described (9).

Targeted gene mutagenesis

Construction of VT01, VT02 and VT03 are as described previously (45). AtlA mutants were constructed by targeted insertional mutagenesis. An internal fragment of *atlA* was PCR amplified using primers Aut2f and Aut2r (Table 3.3) and cloned EcoRI/ BamHI into the suicide vector (p3CAT). The resulting construct was verified by restriction analysis and was electroporated into *E. faecalis* V583 and OG1RF. Growth of transformants on antibiotic selective media (chloramphenicol, 10 μg/ ml) followed by colony PCR with primers Auto2Up and M13R, confirmed the targeted mutation.

Table 3.3 Primers used in this study

Primer name	**Sequence (5' →3')**
M13R	CAGCTATGACCATGATTACG
Aut2-f	GAGAGAATTCTGGGGAGCAAGTACGCTATC
Aut2-r	CTCTGGATCCCCACCGGTGTTACCTGAAGT
Auto2Up	GAGACACCAACAACAGAA
Ef0799-SP-NdeI	GAGACATATGACAGAAGAGCAGCCAACAAATGC
Ef0799XhoI	GAGACTCGAGACCAACTTTTAAAGTTTGAC
VLAC1	GTTGAATAACACTTATTCCTATC
Gfp3'SalI	CTCTGTCGACTACGAATGCTATTTGTATAG

Flow Cytometry analysis

A 1119-bp fragment composed of a *gelE* promoter *gfp* fusion, was PCR amplified from pVI01 (Table 3.2) using primers VLAC1 and Gfp3'SalI (Table 3.3). The fragment was either restricted with EcoRI resulting in a 1055-bp fragment or with SalI giving rise to a 759-bp fragment. The 759-bp fragment containing the *gfp* was cloned into SalI restricted pAT28 resulting in pVT30, and the 1055-bp fragment was cloned into EcoR1/ SmaI restricted pAT28 giving rise to pVT31. *E. faecalis* V583, VT01, VT02 and VT03 were transformed with pVT31 to track cells within the population that responded to GBAP and expressed GFP.

The resulting strains were designated VT15, VT16, VT17 and VT18 respectively. pVT30 which contains promoterless *gfp* was electroporated into V583 as a negative control. Flow cytometric analysis was performed using one-day old stationary phase cultures of *E. faecalis* on a FACSCalibur flow cytometer (Beckton and Dickinson, San Jose, California). Cell samples were washed twice and diluted to a final concentration of 10^6 cells per ml in PBS. Cells were stained for 10 minutes with propidium iodide (2 μM) and analyses was carried out at a flow rate of ~2000 cells per s. In total 50,000 events were collected for each sample and FSC, SSC, FL-1 and FL-2 signals were measured using logarithmic amplifications. Bacteria were discriminated from background using a combination of FSC and SSC. Data were analyzed with the CELLQuest program (version 3.1, Beckton and Dickinson).

Zymography

An 8% SDS PAGE gel containing 0.1% (w/ v) of prepared *E. faecalis* V583 cell wall as substrate was used to analyze autolysin activity. Gels were run under constant voltage (200V). Following electrophoresis, gels were washed extensively with deionized water to remove SDS and then in renaturation buffer (25 mM sodium phosphate pH 7.0, 1 mM $MgCl_2$ and 1% TritonX-100) four times at intervals of one hour each and then incubated in fresh buffer overnight at 37°C. Activity was detected as clear bands after counter-staining the gel with 0.1% methylene blue in 0.01% KOH solution followed by destaining in deionized water.

Protein expression and purification

Gelatinase (GelE) was purified as previously described (18). For purification of serine protease (SprE), two liters of THB was inoculated with 20 ml of an overnight culture of *E. faecalis* strain VT24 (ΔgelEsprE, pVT08, $SprE^+$) and allowed to grow as a standing culture at 37°C for 24 h. Following separation of bacteria by centrifugation (30 min at 27,500 x g at 4°C), SprE was precipitated from the cell free culture supernatant by gradual addition of ammonium sulfate to a final saturation of 70% and incubated overnight at 4°C with constant agitation. The precipitants were removed by centrifugation (30 min at 27,500 x g at 4°C) and resuspended in 200 ml of 200 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.6. The 200 ml sample was filter-sterilized (0.2μm) and extensively dialyzed (2 weeks) against dialysis

buffer (50 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.6). The sample was concentrated (Pierce ICON™ concentrator) down to 2 ml and was resolved at room temperature on a TSK G3000SW gel filtration column (7.8 mm x 300 mm; Tosoh Bioscience, Montgomeryville, PA) at a flow rate of 1 ml/ min, using TSK buffer (0.06 M sodium phosphate, 0.1 M sodium sulfate, pH 7.0) and stored at 4°C.

Histidine tagged AtlA (devoid of signal sequence) was purified from *Escherichia coli* BL21 (DE3) harboring the expression construct pVT21 and grown in 1 L LB medium containing ampicillin (100µg/ ml). Expression of recombinant protein was induced by the addition of 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) to the *E. coli* cultures at an optical density (at 600 nm) of 0.6. Following IPTG treatment cultures were incubated for an additional 3 hours at 37°C after which bacterial cells were harvested by centrifugation and resuspended in 40 ml lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 20 mM -mercaptoethanol (pH 7.9). Cells were disrupted by ultra sonicaton (5 cycles of intermittent 20 sec pulse and 20 sec cooling) and the supernatant retrieved after ultracentrifugation at 45,000 rpm for 1 h at 4°C to remove cell debris. The supernatant was passed through a 0.2µm filter prior to being loaded onto a nickel nitrilotriacetic acid-agarose column (Qiagen). The His-tagged AtlA was eluted with a step-wise gradient of imidazole (20, 40, 100, and 250 mM) in lysis buffer and optimal purity obtained at 100 mM concentration of imidazole in lysis buffer. Purified AtlA was concentrated and buffer exchange (25 mM sodium phosphate buffer, pH 7) carried out using an ICON™ concentrator (Pierce). Purified protein was stored at 4°C for short term or -80°C for long term storage.

Protein purity was analyzed by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis (>95% for all purified proteins) and tryptic digests of the proteins followed by MALDI-TOF mass spectrometry confirmed their identities.

MS and MS/ MS analysis

After staining gels with Coomassie R-250, selected gel bands were excised manually as 1-2 mm slices and transferred to 1.5 ml Eppendorf tubes. An equivalent slice from the protein-free region of the gel was excised as a background control. The control and test gel sections were then destained using three 30 min washes of 60 µl 1:1 acetonitrile: water at 30°C. Gel pieces were then dried for 10 min under vacuum. The gels were subjected to reduction and alkylation using 50 mM Tris (2-carboxyethyl) phosphine (TCEP) at 55°C for 10 min

followed by 100 mM iodoacetamide in the dark at 30°C for 60 min. The carboxymethylated samples were thoroughly washed and redried in vacuo, then incubated with sequencing grade trypsin (Trypsin Gold, Promega, Madison,WI), 20 ng/ µl in 40 mM ammonium bicarbonate, in 20 µl. Upon rehydration of the gels, an additional 15 µl of 40 mM ammonium bicarbonate and 10% acetonitrile was added, and gel sections incubated at 30°C for 17 h in sealed Eppendorf tubes. The aqueous digestion solutions were transferred to 1.5 ml clean Eppendorf tubes, and those tryptic fragments remaining within the gel sections were recovered by a single extraction with 50 µl of 50% acetonitrile in water containing 2% trifluoracetic acid (TFA) at 30°C for 1 h. The acetonitrile-water fractions were combined with the previous aqueous fractions and the liquid removed by speed vacuum concentration. The dried samples were resuspended in 10 µl of 30 mg/ ml 2,5-dihydroxylbenzonic acid (DHB) (Sigma, St. Louis, MO) dissolved in 33% acetonitrile/ 0.1% TFA and 2 µl of peptide/ matrix solution was applied to a Bruker aluminum target plate for MALDI-TOF and TOF/ TOF analysis.

Mass spectra and tandem mass spectra were obtained on a Bluker Ultraflex II TOF/ TOF mass spectrometer. Positively charged ions were analyzed in the reflector mode. MS and MS/ MS spectra were analyzed with Flex analysis 3.0 and Bio Tools 3.0 software (Bruker Daltonics). Measurements were externally calibrated with 8 different peptides ranging from 757.39 to 3147.47 (Peptide Calibration Standard I, Bruker Daltonics) and internally re-calibrated with peptides from the autoproteolysis of trypsin. Peptide ion search was performed using MASCOT software (Matrix Science) against the expected recombinant AtlA protein sequence. The following parameters were used for the database search: MS and MS/ MS accuracies were set to < 0.6 Da. Trypsin as an enzyme, missed cleavages 1, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as a variable modification.

GelE and SprE mediated proteolysis of AtlA

GelE or SprE (30nM) were incubated with 10µg of AtlA separately in 100 µl of reaction buffer (50 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.6). After 10, 30, 60 and 300 mins of incubation at 37°C, aliquots (25µl) were withdrawn and the reaction was stopped by the addition of SDS-PAGE sample loading buffer and boiling for 10 minutes. Samples were analyzed by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis and silver

staining. MALDI-TOF MS and MS/ MS analysis was carried out as previously described, to empirically map regions of AtlA that were susceptible to proteolytic attack.

Biofilm assay

Biofilm assays were carried out as described previously (45).

eDNA release assay

Measurements of eDNA release were carried out on stationary phase cultures using the nucleic acid stain SYTOX green (Invitrogen). Overnight grown cultures of *E. faecalis* (37°C) were centrifuged (13,000 x g; 3 minutes) to pellet cells and 200 µl of the culture supernatants were transferred to microtiter plate wells in triplicate. SYTOX green was supplemented to these wells to a final concentration of 1 µM and incubated for 10 minutes before being spectrofluorometrically measured with excitation at 485 nm and emission at 535 nm on a Perkin Elmer Victor 3 fluorescent plate reader.

Fratricide assay

Co-culture lysis assays were carried out using V583, VT01 (Δ*gelE*), VT02 (Δ*sprE*), VT03 (Δ*gelEsprE*) and VT19 (V583::*atlA*) as attacker strains and either VT12 (Δ*gelEsprE*, GFP^+) or VT23 (Δ*gelEsprE*, ::*atlA*,GFP^+) as targets. Attacker strains grown overnight were diluted 1:1000 in fresh Todd Hewitt broth (THB) media and were co-inoculated with target strains diluted 1:20 from an overnight culture grown for an equivalent period at 37°C in THBG (THB + 2% glycine). Following 24 hours of co-culture at 37°C, GFP (released as a result of lysis of target) from 250 µL of supernatant was precipitated using 4 volumes of cold acetone (-20°C) and resolved by SDS-PAGE. Detection of GFP in the supernatant was carried out by western blot analysis using anti-GFP antibodies.

Lysis assay

Lysis assays were carried out as described previously with the following modifications (45). Briefly, pre-cultures of VT03 (Δ*gelEsprE*)or VT22 (Δ*gelEsprE*, ::*atlA*) were grown overnight at 37°C in 2.5 ml THB and diluted 100-fold in SM17 media supplemented with 3% glycine. Following overnight growth at 37°C, 1.5 mL cultures were centrifuged at 13,000 rpm for 3 minutes and washed thoroughly, thrice in ice-cold sterile distilled water. After the third

wash, the cells were resuspended in 10 mM sodium phosphate buffer (pH 6.8) and supplemented with 1 mM $CaCl_2$. Two hundred microliters of the suspended cells were dispensed into a 96 well plate and the optical density at 600 nm was monitored for 9 h at 30-min intervals either in the presence or absence of protease treatments (30 nM of either GelE or SprE or both).

Peptidoglycan affinity assay

Purified recombinant AtlA (10 μg) was allowed to bind with 20 mg wet-weight of isolated *E. faecalis* V583 cell wall for 15 minutes at 37°C in 100 μL of buffer (10 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.6). Approximately, 30 nM of either GelE or SprE was supplemented separately into each sample mix. For co-protease treatments recombinant AtlA bound to V583 peptidoglycan (20mg wet-weight) were either pretreated with 30 nM GelE or SprE for 10 minutes. This was followed by the immediate addition of the second complementary protease (30 nM; SprE or GelE) for 30 minutes. Samples (25 μL) were withdrawn at intervals of 10, 30, 60 and 300 minutes. Supernatants containing unbound proteolytically processed AtlA derivatives were retrieved after centrifugation (13,000 x g; 10 minutes). The pellet was washed twice in fresh buffer to remove loosely adherent AtlA from pellet and the cell wall bound fraction eluted in SDS PAGE sample loading buffer. Unbound and bound AtlA derivatives were resolved by SDS PAGE and visualized by silver staining.

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CHAPTER 4

Characterization of the virulence effects of *Enterococcus faecalis* extracellular proteases in a rabbit model of endocarditis

Abstract

Infective endocarditis is a disease characterized by the formation of bacterial biofilm-like vegetations on the aortic valve and adjacent areas within the heart. Based on previous reports that implicated *E. faecalis* extracellular proteases in biofilm development, we in the current study, compared the pathogenic potential of the vancomycin resistant *E. faecalis* V583 and three isogenic protease mutants (Δ*gelE*, Δ*sprE* and Δ*gelEsprE*) in a rabbit model of enterococcal endocarditis. The bacterial burdens displayed by *gelE* mutants (Δ*gelE*, Δ*gelEsprE*) in the rabbit heart were significant lower than V583. Valve areas infected with *gelE* mutants also showed significantly increased deposition of fibrinous matrix layer and increased chemotaxis of inflammatory cells. Unexpectedly the *sprE* mutant (Δ*sprE*) showed lower bacterial burdens than V583. These data suggest a critical role for GelE, but less so for SprE in the pathogenesis of infective endocarditis.

Introduction

The wide-spread ever increasing use of antibiotics has resulted in the frequent emergence of antibiotic resistant clinical pathogens (2). The enterococci constitute one such class of pathogens due to their inherent and acquired resistances to commonly used antibiotics and their ability to persist in the clinical environment (20). Among the different species of the genus *Enterococcus*, *E. faecalis* is reported to cause up to 90% of infections (18). Although a normal commensal of the human gastrointestinal tract, experiments in animal models have indicated that at elevated ratios relative to other gut commensals *E. faecalis* possess the ability to translocate across the gut epithelium and cause infections (39, 40). Accordingly, broad spectrum antibiotic therapies that enterococci are inherently resistant to have been considered as significant risk factors of *E. faecalis* infections. Enterococci are currently considered to be among the three most common nosocomial pathogens and are a frequent cause of bacteremia, surgical site-infections, urinary tract infections and endocarditis (13).

Infective endocarditis is a disease that results from bacterial infection of damaged endothelial lining of the heart and commonly occurs on native heart valves. However, in rare circumstances prosthetic heart valves may also attract bacterial pathogens (29). Enterococci account for up to 20% of native valve and 7% of prosthetic valve endocarditis cases in the US (17). The infection begins as circulating enterococci that evade host immune defenses adhere to and encase themselves within the damaged heart endothelium coated with fibrin and platelets, resulting in characteristic lesions called vegetations (8). Sections of vegetations are prone to embolization. Emboli resulting from left sided endocarditis (vegetations on the aortic valve or mitral valve) often lead to pathological complications and secondary infections within the kidney, spleen, brain and extremities (8). Till date very few virulence factors (mostly related to the organism's ability to adhere to the endothelium) have been studied in relation to enterococcal endocarditis and little mechanistic information exists on how these factors may aid the development of endocarditis (16). However as *E. faecalis* is less adherent than most other endocarditis associated pathogens (e.g. *Staphylococcus aureus*), adherence factors may not play as crucial a role in enterococcal pathogenesis (5). As the characteristic architecture of enterococci within endocardial vegetations resembles biofilm microcolonies encased in platelets and fibrin, the mechanisms that aid enterococcal biofilm development may be of critical importance in

endocarditis. Of particular interest in the endothelial infection process is the role of two co-transcribed enterococcal secreted proteases, gelatinase (GelE) and serine protease (SprE) (16). Both proteases have previously been implicated in biofilm development of *E. faecalis*. GelE, a zinc metalloprotease secreted by enterococci in response to GBAP mediated quorum sensing, enhances biofilm development by promoting fratricide (lysis of siblings) of a small subset of quorum non-responders (34, 35). Such cell lysis results in the release of extracellular DNA (eDNA) a key structural component of the biofilm matrix. The co-expressed serine protease (SprE) protects quorum responders ($GelE^+$) from GelE mediated self-destruction, thus regulating cell lysis and biofilm development. This is possible as both secreted proteases differentially regulate proteolysis and possibly cell surface localization of the primary autolysin, AtlA (34).

Apart from biofilm development, GelE also exhibits broad substrate specificities towards proteinaceous host tissue structural components and immunity factors. GelE has been shown to hydrolyze collagen, fibrin, endothelin-1, bradykinin, LL-37 and complement cascade components in vitro, all of which could potentially reflect on the complex pathology of enterococcal endocarditis (14, 15, 22, 23, 38). Although the specific role of SprE on host immune defenses still remains to be elucidated, it is evident from a number of studies employing animal models that SprE may have a crucial role in virulence of enterococci (6, 27, 31, 33). In the current study we investigated the pathogenic potential of secreted entercococcal proteases (GelE and SprE) and their overall contribution to *E. faecalis* virulence in a rabbit model of enterococcal endocarditis.

Results

Histopathological examination of infected tissues

Vegetations (sterile or infected) were observed on the aortic valve, aorta and at times extended into the myocardium. Grossly, the lesions appeared irregular, friable, exhibited variable sizes ranging from 1- 4 mm in length and displayed a light pink tan (Fig. 4.1).

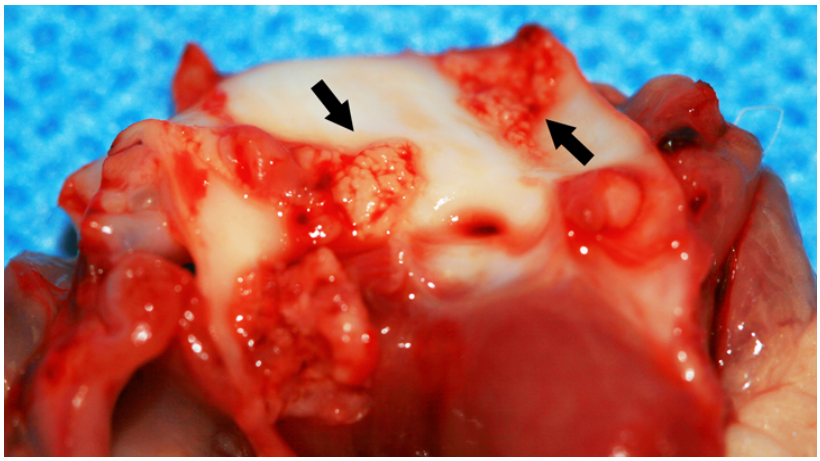

Figure 4.1 Gross morphology of aortic vegetations colonized by *E. faecalis* V583.
The left side of the heart was surgically dissected lengthwise from the proximal aorta to the base of the left ventricle. Black arrows point to small, irregular friable vegetations on the ascending aorta.

Histological examination of infected lesions showed bacterial colonization on the endothelial lining of the ascending aorta, that mostly appeared as a smooth layer (approx. 25µm- 50µm in thickness) closely interspersed with tower-like projections that arose up to ~150µm (Fig. 4.2). Additionally, aortic vegetations also showed a relatively uniform deposition of a matrix layer (ML, generally thought to be composed of host fibrin, fibronectin, plasma proteins and platelets) across the bacterial biomass (19). The overall biofilm architecture of *E. faecalis* V583 (devoid of the ML) is consistent with that reported earlier (35), although aorta associated biofilms exhibited ~10-fold increased thickness when compared to a 4-day old biofilm grown on a glass substratum (data not shown). The relative thickness of the ML was variable among different extracellular protease mutants (Fig 4.3). VT01 ($GelE^{-}SprE^{+}$) and VT03 ($GelE^{-}SprE^{-}$) displayed a ML ~11-fold thicker than V583 ($P< 0.05$) suggesting a critical role for GelE in regulating ML turnover. This observation is consistent with earlier reports of GelE's ability to hydrolyze fibrin, a major constituent of the ML (38). Interestingly, when compared to the parental strain (V583), VT02 ($GelE^{+}SprE^{-}$) exhibited a ~3.4- fold increase in the thickness of the ML ($P< 0.05$), suggesting a role for SprE in degradation of the ML layer. However as the relative thickness of the ML layers of VT01 ($GelE^{-}$ $SprE^{+}$) and VT03 ($GelE^{-}SprE^{-}$) were not significantly different, the observed role of SprE in ML turnover may arguably be minor.

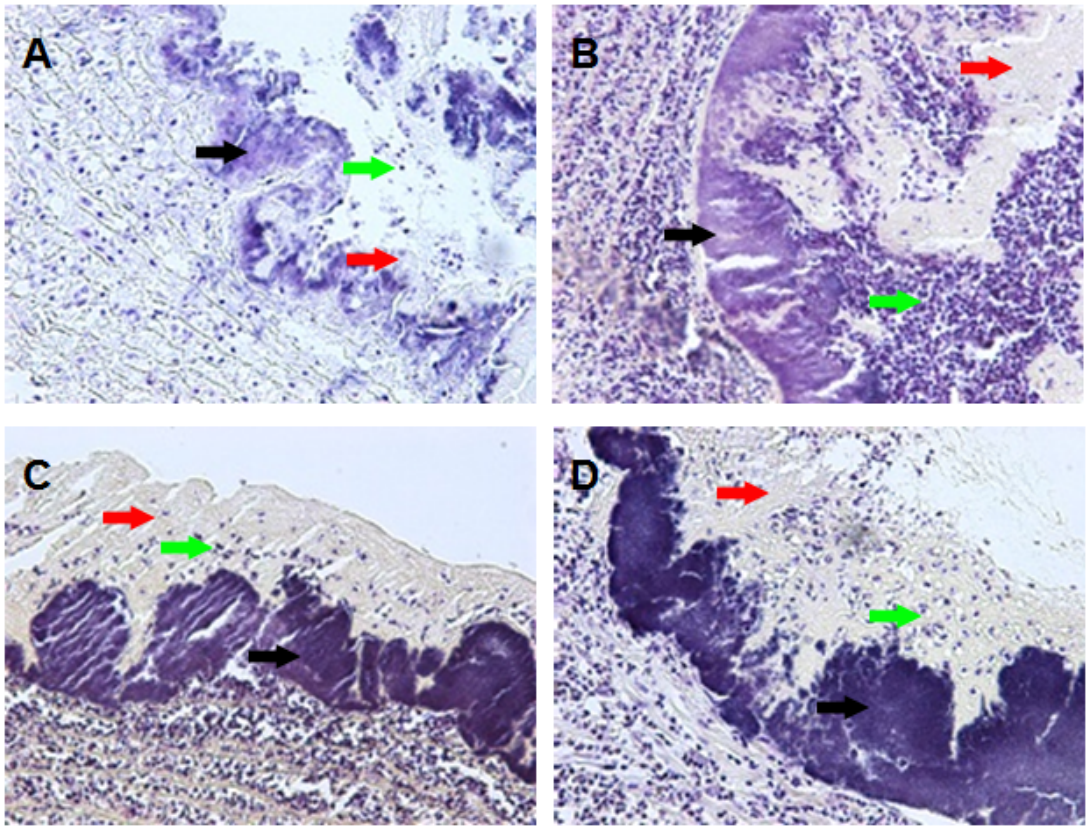

Figure 4.2 Histology of aortic vegetations.
Panels A, B, C and D are representative images of gram-stained cross-sections (5µm) of vegetations formed on the ascending aorta of rabbits infected with V583, VT01, VT02 and VT03 respectively (magnification, x 200). Black arrows point to *E. faecalis* biomass on the surface of the endothelium. Red arrows point to deposited matrix layer (ML) composed mostly of platelets and fibrin. Green arrows point to influx of heterophils and other immune cell infiltrates.

As bacterial proteases are known to regulate localized tissue inflammatory responses, the rate of leukocyte chemotaxis to the vegetation was measured as a function of circulatory heterophils trapped within an area of 10000 μm^2 of ML layer for each of the extracellular protease mutants (Fig. 4.4). Heterophil migration across the ML toward the areas of biofilm development displayed clear dependence on protease expression. V583 and VT02 ($GelE^+SprE^-$) showed similar levels of heterophils in the ML where as VT01 ($GelE^-SprE^+$) and VT03 ($GelE^-SprE^-$) displayed a ~4- fold increase in the total number of heterophils

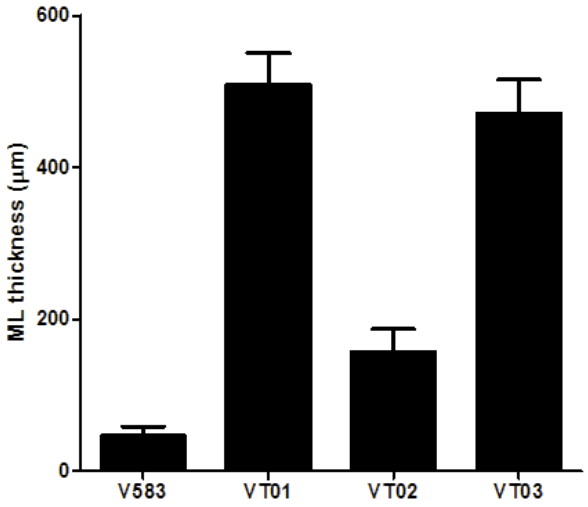

Figure 4.3 Matrix layer (ML) of extracellular protease mutants.
Differences in the ML thickness were determined from histological images of vegetations (magnification, x 400). The lengths between the *E. faecalis* biomass layer and the upper edges of ML from eight random regions of vegetations from each strain were measured and reported as mean thickness (µm, Mean ± SEM). VT01, *ΔgelE*; VT02, *ΔsprE*; VT03, *ΔgelEsprE*.

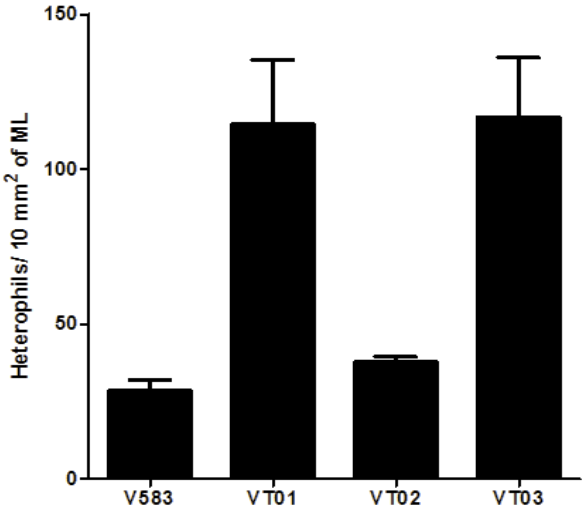

Figure 4.4 Heterophil chemotaxis at the sites of *E. faecalis* infection (vegetation).
Differences in the number of heterophils that have migrated to the bacterial vegetations were determined from histological images (magnification, x 400) and were normalized to the area of ML surrounding them. Heterophils were counted using Image J software from 4 random images of vegetations from each strain and reported as the total number of heterophils trapped per 10 mm^2 of ML (Mean ± SEM). VT01, *ΔgelE*; VT02, *ΔsprE*; VT03, *ΔgelEsprE*.

when compared to GelE$^+$ enterococci (P< 0.05). These observations suggest that GelE may play an important role in preventing heterophil chemotaxis to areas of enterococcal biofilm infections. Curiously despite the increased heterophil influx toward GelE$^-$ mutants (VT01 and VT03), their relative biofilm thickness (devoid of the ML) was similar to GelE$^+$ cells (Fig. 4.2) suggesting a resistance of biofilm enterococci to clearance by heterophils.

Tissue bacterial burden

To quantitatively assess viable bacteria within endothelial vegetations, bacterial burdens were determined from the aorta and heart tissues (Fig. 4.5). Additionally, a general estimate of embolization from the primary vegetations was determined by quantifying the bacterial numbers from the kidneys, liver, spleen and blood following necropsy. Bacterial burdens were reported as $\log_{10}$ CFU per gram of tissue or $\log_{10}$ CFU per ml of blood. In general, the mean relative bacterial counts of wildtype (V583) and extracellular protease mutants (VT01, VT02 and VT03) present in each tissue followed a specific pattern with V583 and VT02 being more abundant than either VT01 or VT03, although these trends did not always reflect statistical significance (Fig. 4.5). Analysis of V583 abundance in tissues suggested the aorta to be significantly more infected (9.4± 0.2) than the heart (7.3± 0.3, P< 0.05) followed by the spleen (4.8± 0.7), liver (4.5± 0.5), right kidney (4.5± 0.6), left kidney (4.4± 0.4) and blood (2.3± 0.7). *E. faecalis* V583 burdens were not significantly different between the spleen, liver and kidneys (Fig. 4.5). These observations are consistent with the primary lesions being in the aorta and the heart whereas the spleen liver and kidneys being secondary sites of infection due to embolization from the former sites.

Based on the biofilm regulatory and immunomodulatory effects of extracellular proteases in vitro, we hypothesized VT01, VT02 and VT03 infections of the heart and aorta to display notable differences in overall bacterial loads. Heart tissue homogenates of rabbits displayed maximum differences in total bacterial burden based on the extracellular protease profile of colonizing enterococci. Both GelE mutants, VT01 (GelE$^-$SprE$^+$) and VT03 (GelE$^-$SprE$^-$) displayed a significant 1.7 log and 1.4 log reduction in CFU/ gm of heart tissue when compared to V583, respectively (Fig. 4.5). Interestingly, VT02 mutants displayed a 0.6 log reduction in CFU/ gm relative to the wildtype *E. faecalis* (V583) in the heart (Fig. 4.5), indicative of a reduced fitness for these mutants in vivo. As bacterial loads of VT03 parallel VT01, it may be argued that the observed decrease in bacterial burden of

VT02 is possibly due to auto-toxic effects of GelE in this mutant rather than a specific role for SprE in pathogenesis. Consistent with this argument we have earlier shown that GelE is able to cause autolysis in an AtlA dependent manner (34). Curiously, but in agreement with histological observations of similarly sized biofilms (Fig. 4.2), no significant differences in the relative $\log_{10}$ bacterial burdens of V583 (9.4± 0.2), VT01 (9.0± 0.4), VT02 (9.2± 0.5) and VT03 (8.7± 0.8) were observed in the aorta (Fig. 4.5).

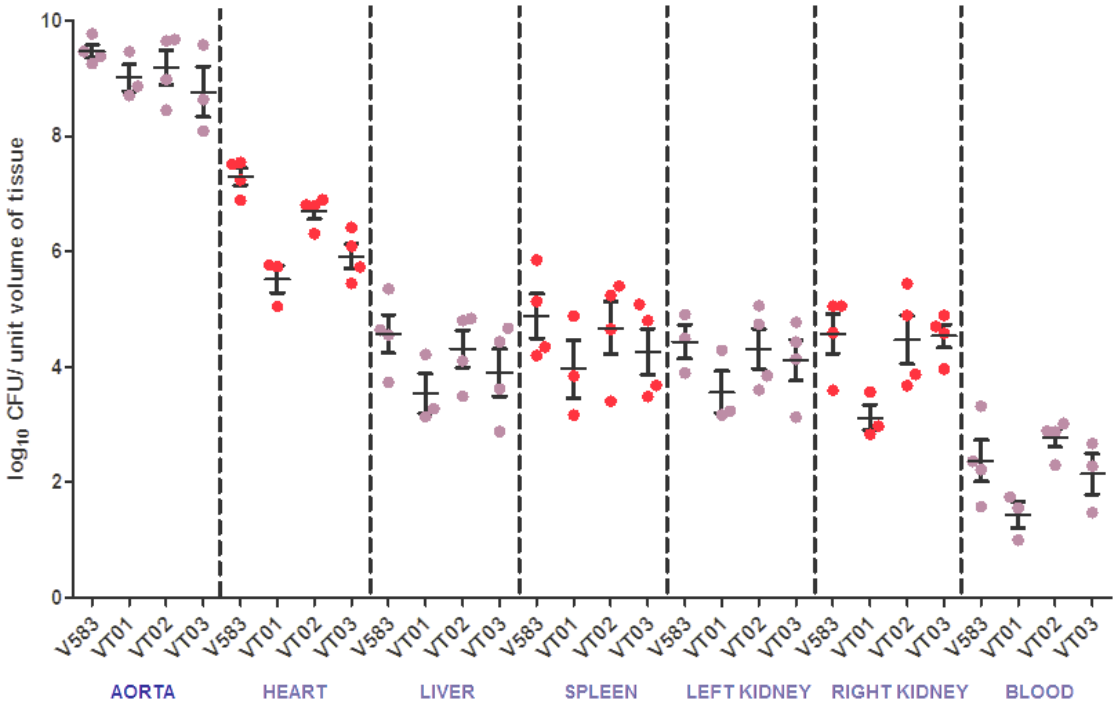

Figure 4.5 Scatter dot plots of tissue bacterial burdens.
Whole organs or tissues (aorta, heart, liver, spleen, kidneys and blood) from rabbits that developed enterococcal endocarditis were homogenized and serially track diluted on THB agar. Following overnight incubation of these plates at 37^0C, *E. faecalis* were counted and expressed as $\log_{10}$ CFU/ gm for all tissues and $1\log_{10}$ CFU/ ml in blood. Every dot represents a rabbit and lines among any single column of dots represent the Mean± SEM. VT01, *ΔgelE*; VT02, *ΔsprE*; VT03, *ΔgelEsprE*.

Given that intravenously injected bacteria are rapidly cleared from blood circulation by the reticuloendothelial system of the liver and spleen (1, 9, 10), we reasoned that viable bacterial burdens in these tissues after 48 hours may reflect the degree of embolization from cardiac vegetations. Consistent with this argument, emboli were detected following

histological examination of kidney sections from rabbits infected with V583, VT01, VT02, and VT03 (data not shown). However surprisingly $\log_{10}$ bacterial burdens from the spleen, liver and kidneys were relatively higher (ranged between 3.1± 0.3 to 4.8± 0.7) compared to that observed in the blood circulation (ranged between 1.4± 0.3 to 2.7± 0.3) suggesting a relative resistance of emboli to clearance in these organ tissues (Fig. 4.5). The relative mean bacterial burdens between tissues (liver, spleen, kidney and blood) infected with V583, VT01, VT02 and VT03 did not exhibit any significant differences.

Discussion

A recent study described a vancomycin resistant *E. faecalis* infective endocarditis isolate that failed to respond to linezolid treatment (36). The strain was shown to have *gelE* among other virulence traits (36). Mechanisms of virulence related to protease activity of vancomycin resistant enterococci in infective endocarditis are poorly understood. Etiological evidence on the importance of extracellular proteases in enterococcal endocarditis remains contradictory. While Pillai and colleagues initially noted a significant enrichment of the *fsr* locus (that regulates protease expression) in *E. faecalis* endocarditis isolates relative to fecal isolates (25), subsequent studies employing larger libraries of isolates have disputed this observation (11, 30). Although the latter two studies reach a consensus on the lack of selective preference for the *fsr* locus or extracellular proteases in disease incidence, they however fail to address the relative differences in severity of the disease caused by strains variable for this trait. In this regard, Gutschik et al. demonstrated that non-isogenic proteolytic strains of enterococci affect more severe outcomes of endocarditis with increased frequency of embolization and significant reductions in animal life span (7). Consistent with this observation, Singh et al. concluded that an *E. faecalis* double extracellular protease mutant ($GelE^{-}SprE^{-}$) was significantly more attenuated in its ability to induce endocarditis than its isogenic wild type ($GelE^{+}SprE^{+}$)(32). The results presented in the current study demonstrate that both secreted proteases, gelatinase (GelE) and serine protease (SprE) of the vancomycin resistant type strain, *E. faecalis* V583 contribute to the complex pathology of endocarditis in a rabbit model.

V583 lacking *gelE* (VT01) was shown to be attenuated in all tissues, except aorta by at least ~1.0 log compared to wildtype, suggesting a crucial role for GelE in virulence. However the observed phenotypic effects of a GelE mutation in this strain (VT01) may be

overrated as the double protease mutant (VT03) mostly phenocopied VT02 (*ΔsprE*) at the secondary sites of infection (kidneys, liver and spleen) in vivo, suggesting that SprE by itself (in the absence of GelE) may have a detrimental role to the fitness of *E. faecalis*. Consistent with this argument, the SprE homologue of *S. aureus*, SspA (V8 protease) was shown to inactivate α1-proteinase inhibitor, the major inhibitor of neutrophil elastase (26). A potential indirect activation of neutrophil elastase by SprE in the absence of GelE could augment the release of kininogen-derived anti-enterococcal peptides (21), resulting in the increased detrimental effect observed in VT01 relative to VT03.

In vitro biofilm studies demonstrated that VT02 (Δ*sprE*) forms a better biofilm with a higher biomass than V583 (35). Interestingly this was not the case in vivo as VT02 biofilms formed on the aorta were similar in thickness to those formed by V583 (Fig. 4.2). Viable counts of bacteria in the aorta and heart suggested that VT02 was 0.3 and 0.6 log lesser than wildtype (V583) respectively. This suggests that while increased death of a subpopulation of bacteria mediated by GelE may be beneficial for the whole community in vitro (in terms of eDNA and biofilm development), the opposite may be true in vivo as the additional stress afforded by the immune system may have an additive effect on bacterial death or viability in addition to GelE mediated autotoxicity. Interestingly, the quantity of immune infiltrates (primarily heterophils) seemed to increase ~4-fold around GelE mutants. This observation is in agreement with recent reports on GelEs ability to turn over C3a (22, 23) and C5a anaphylatoxins (Thurlow, L.T., unpublished results) and modulate heterophil chemotaxis.

Contradicting previous in vitro biofilm observations histopathological analysis suggested that biofilm thickness of *gelE* mutants (VT01 and VT03) on the aorta were comparable to V583. One possible explanation for this observation is that V583 exhibits a more metastatic phenotype by virtue of GelE's ability to cleave proteins (e.g. fibrin) in the ML (7), hence resulting in a smaller and more dynamic biofilm biomass than originally expected. The other possibility is based on more recent observations that neutrophils and heterophils upon activation are capable of releasing extracellular chromosomal traps (NETs or HETs) intended to clear bacterial infection (3, 4, 37). Although NETs are fairly effective against planktonic bacteria, their role in biofilm clearance has been debated. (12, 24, 28). Given that enterococci in the biofilm state are potentially resistant to heterophil mediated clearance, the released HETs may substitute enterococcal eDNA and aid VT01 and VT03 develop into better biofilms.

In conclusion, we report that both extracellular proteases of *E. faecalis* (GelE and SprE) contribute to the increased fitness of V583 in endocarditis. Lower bacterial burdens in the heart for VT01 were consistent with a crucial role for GelE in enterococcal pathogenesis. The presence of GelE also retards chemotaxis of neutrophils to the site of infection consistent with its ability to modulate and evade host immune defenses (22, 23). Although the role of SprE remains unclear, the current evidence suggests that it may be beneficial to *E. faecalis* only when coexpressed with GelE. Additional information is required to understand the specific role of SprE in pathogenesis. Given the decreased fitness of VT01 in vivo, GelE may be considered an alternative therapeutic target for the treatment of enterococcal endocarditis.

Materials and Methods

Bacterial strains and growth conditions

E. faecalis strains used in this study were previously characterized (35) and their relevant characteristics are summarized in Table 1. Growth of *E. faecalis* was routinely carried out in Todd-Hewitt broth (THB) at 37°C and after overnight growth attained a cell density of ~2 x 10^9 cfu/ ml of culture.

Experimental endocarditis

Pathogen-free female New Zealand White rabbits (2- 2.5 kg) were anesthetized (35 mg/ kg ketamine and 1.5 mg/ kg xylazine) and their left carotid arteries in the neck regions were exposed for catheterization. To induce valve damage, catheters with an internal diameter of 0.86 mm (Becton Dickinson, MD) were introduced through a small incision on the left carotid artery and advanced until it traversed the aortic valve of the heart. Catheters were left in place and the rabbits were monitored for the entire period of the experiment (3 days). Following catheterization, the surgical sites were sutured and the animals allowed a day of recuperation. Enterococci (V583, VT01, VT02 and VT03) were grown up to stationary phase, washed twice and diluted to a final cell density of 10^7 cfu/ ml in sterile saline. Diluted cultures (1ml) were injected into the left marginal ear veins (2^{nd} day) and the rabbits were sacrificed by lethal injection of sodium pentobarbital (100 mg/ kg), 48 hours after bacterial challenge.

Post-necropsy, the aortic valves and adjacent regions in the heart were examined for vegetations. Parts of the left kidney and walls of the aorta and valves exhibiting vegetations from representative rabbits infected with V583, VT01, VT02 and VT03 were fixed in 10% buffered formalin for histopathology. For general morphology, tissues were embedded in paraffin and serial sections (5μm thick) were stained with either with HE (hematoxylin and eosin) or gram stain. Bacterial burdens were determined by plate count from whole kidneys, spleen, liver and heart of each rabbit.

Determination of bacterial burden

Harvested organs were introduced into defined volumes of sterile saline and thoroughly homogenized. Tissue homogenates were track diluted on THB agar and colonies counted after overnight growth at 37°C. For determining the bacterial load in circulation, blood samples were drawn by cardiac puncture post euthanization, serially diluted and plated similar to other tissues. Bacterial loads were expressed as $\log_{10}$ CFU/ gm of infected tissue or $\log_{10}$ CFU/ ml of infected tissue.

Image analysis and statistical analysis

Images were obtained at a final magnification of 400X and analyzed using imageJ software. For quantitative analysis of heterophils in the matrix layer (ML), images were initially converted to 8-bit and a threshold was applied to contrast heterophils from the background. Heterophils were counted from thresholded images using dimensions obtained from a training dataset. In cases where heterophils overlapped, the watershed algorithm was applied to delineate heterophil boundaries before counting particles. The total number of heterophils from each bacterial treatment was normalized to the area of the ML layer and reported as the number of heterophils per 10 mm^2 (10000 μm^2) area of ML. The average thickness of the ML layer and bacterial biofilm biomass was determined from regions of the vegetation that were adherent to the underlying endothelial layer. Measures of four randomly picked regions from the base of the endothelial layer to the tip of the bacterial biomass and from the tip of the biofilm biomass to the edge of the ML layer were considered as respective thicknesses of the biofilm bacterial biomass and ML layer. Thicknesses were averaged and expressed as mean± SD.

Statistical analysis of heterophil counts, ML thickness and bacterial tissue burdens was carried out with GraphPad software. One way analysis of variance followed by Neuman-Keuls post test was carried out to determine statistical significance. A $P < 0.05$ was considered to be statistically significant.

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CHAPTER 5

Summary & Discussion

General conclusions

Enterococci are currently considered to be the second leading cause of hospital acquired infections in the US (35). Multiple factors are thought to contribute to its emergence as a pathogen. Firstly, its ability to inherently resist bactericidal action of commonly used antibiotics due to a wide and diverse repertoire of resistance traits it possesses offers it competitive advantage over other nosocomial pathogens (23). Secondly, enterococci are very effective in their ability to acquire and disseminate infectious traits necessary for colonization of atypical host environments (25). Finally, its ability to effectively adhere and colonize artificial implant devices, which are increasingly being used in the hospital environment may contribute to its recent rise as a pathogen (1). All the above factors are in many ways related to its core ability to develop into biofilms. Once in the biofilm mode, enterococci can effectively adhere to surfaces through its selective expression of adherence factors, display increased tolerance and resistance to antibiotics, engage in the promiscuous exchange of infectious traits and display numerical advantage over commensal colonizers (4).

In the current study we attempted to dissect the role of two extracellular proteases of enterococci- gelatinase (GelE) and serine protease (SprE), in biofilm development and pathogenesis. Consistent with a crucial role in pathogenesis, multiple studies have reported extracellular protease mutants to display attenuated virulence in animal and plant infection models (11, 14, 19, 29, 33, 34). However a role in biofilm development was inferred only for GelE, from in vitro studies examining the ability of *gelE* mutants to adhere to plastic surfaces relative to wild-type (17, 21, 22). Given that SprE is co-transcribed along with GelE, we reasoned a potential role for this protease in biofilm development as well. Mechanistic evidence on how extracellular proteases regulated biofilm development were hypothesized to either relate to an increase in overall cell hydrophobicity or involve specific interactions with a cell surface autolysin (alluded to in **Chapter 1, Chapter 2** and depicted in **Fig 1.3**). The results presented in **Chapter 2** suggest that GelE was capable of increasing rates of autolysis and extracellular DNA (eDNA) release in enterococcal populations. However, interestingly GelE-dependent autolysis was limited to a small sub-fraction of cells in the developing biofilm. Given that a nonpolar isogenic deletion mutation of *sprE* resulted in an increased rate of autolysis compared to parental strain, we reasoned that SprE was

possibly responsible for limiting GelE mediated lysis in enterococcal biofilms. Further we also demonstrated that eDNA released as a byproduct of autolysis was a critical matrix component of the developing biofilm. The crucial role of autolysis and eDNA release in *E. faecalis* biofilms is globally similar to that described in a number of other gram-positive and gram-negative bacteria.

In **Chapter 3** we further explored the role of both extracellular proteases in affecting autolysis. Based largely on studies carried out in *Staphylococcus aureus*, bacterial autolysis in biofilms has been assumed to be a form of altruistic suicide and has been compared to eukaryotic apoptosis (2, 30, 31). However an additional form of autolysis, termed fratricide has been implicated in other developmental processes like sporulation and competence (5). While altruistic suicide involves the suicidal death of a cell for the benefit of its siblings, fratricide implies a process by which a sub-fraction of cells are killed due to harmful effects orchestrated by genetically identical siblings in the population. In this dissertation we demonstrate for the first time, that fratricide is the predominant form of GelE-mediated autolysis in *E. faecalis* biofilm development. Employing in vitro approaches, GelE was shown to be capable of proteolytically processing the peptidoglycan localization domain (LysM) of the primary autolysin and, thus releasing it into the supernatant. As the released autolysin is still active, it potentially causes unregulated bystander cell death and eDNA release. Supporting these observations, we also demonstrated that an *atlA* mutation abrogated eDNA release, biofilm development and GelE mediated fratricide. Further, SprE was demonstrated to significantly protect $GelE^+$ cells (~85%) within the population from self destruction, possibly through its ability to modify GelE's target- AtlA.

Given the mechanistic role of GelE and SprE in regulating fratricide and biofilm development, we speculated that such a role may also be applicable in vivo. Accordingly we employed a rabbit endocarditis model (**Chapter 5**) for studying enterococcal biofilm development and related pathogenesis due to extracellular proteases. However our attempt to dissect a biofilm-specific role for either protease in vivo has been riddled with complications arising from their ability to differentially regulate host immunity in addition to biofilm development. The $GelE^-$ mutants were reduced in cell density by a factor of ~1.55-log-fold compared to wild type strain in the rabbit heart. Interestingly and contradictory to our previous observations (Fig. 2.2 and Table 2.1) the $SprE^-$ mutants were also partially compromised (0.6-log reduction) compared to wild type. The effects of these proteases were not additive as reported earlier in a rabbit endopthamitis model, as the

double protease mutant (GelE⁻SprE⁻) displayed a slightly higher cell density than the GelE⁻ mutant (1.4-log vs. 1.7-log). These observations are consistent with an in vivo infectious fitness advantage for *E. faecalis*, only in the presence of both proteases.

Global similarities of autolytic processes in bacterial developmental programs

Bacterial cell death and lytic pathways are commonly activated during social behavior- be it sporulation or cannibalistic behavior in *B. subtilis*, competence development of *S. pneumoniae* or biofilm development of *E. faecalis* (5, 36, 37) (Fig 5.1). Interestingly all three pathways can be initiated by stressful environments (nutrient deprivation and other environmental stimuli including changes in pH and antibiotics), suggesting a common evolutionary root for these developmental programs (5).

The initial stages in the induction of all three developmental processes involve cell-cell communication followed by the differential activation of a master regulator among members of its community resulting in a bimodal distribution, with some in the active state (master regulator-ON) and others in the inactive state (master regulator-OFF)(5, 36, 37). In *B. subtilis*, the master regulator Spo0A regulates sporulation following its increased expression and phosphorylation (Spo0A~P) (12). The phosphorylation of Spo0A is dependent on the import of the quorum dependent PhrA, PhrC, PhrE and PhrH pentapeptides into the cell. These pentapeptides block activity of the Rap family of phosphatases (specifically, RapA, RapB, RapE and RapH) which ultimately regulate phosphorylation states of key components of the KinABCDE-Spo0FBA phosophorelay pathway (3). In *S. pneumoniae*, competence is dependent on a two-component regulatory system encoded by the *comABCDE* operon (6, 7). CSP (competence stimulating peptide) encoded by *comC*, is secreted out of the cell with the help of ComA and ComB (secretory apparatus). Once a critical concentration of CSP has reached outside the cell, it binds to the membrane bound histidine kinase, ComA which in turn gets autophophorylated and subsequently trans-phosphorylates ComE to the active state (ComE~P) (5). Similar to this process and as described in **Chapter 1**, the *fsrABDC* operon of *E. faecalis* encodes a quorum sensing system that activates the response regulator FsrA by phosphorylation (FsrA~P) (24, 29).

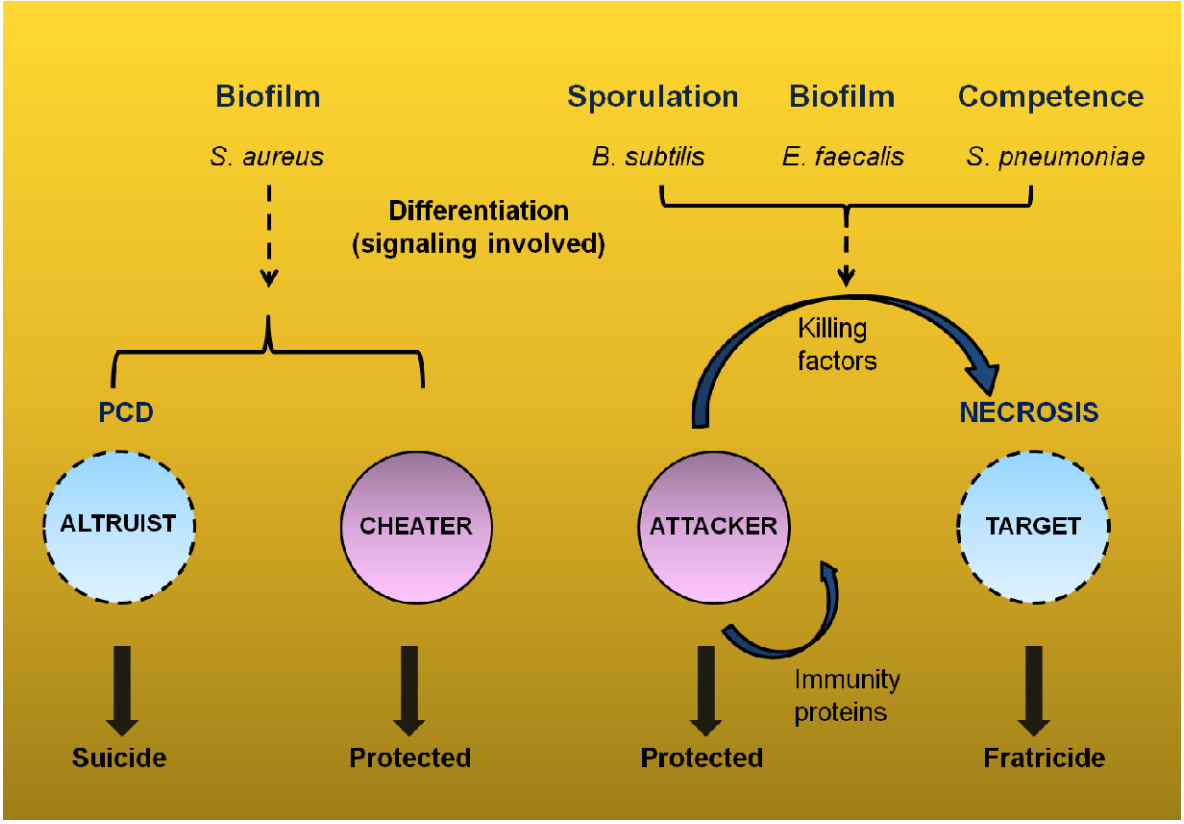

Figure 5.1 Characteristic features of altruistic suicide and fratricide in bacterial development.
Common to sporulation, competence and biofilm development autolysis is initiated in differentiating cultures and involves signaling pathways (cell-cell signaling, signaling mediated by environmental stimuli, etc.). In case of altruistic suicide (*S. aureus*), cells differentiate into altruists and cheaters. Altruists commit suicide by programmed cell death (PCD) and benefit the cheaters. In the case of fratricide (*E. faecalis*, *B. subtilis* and *S. pneumoniae*), cells differentiate into attackers and targets. Attackers release killing factors that kill targets. Attackers themselves are protected from self-destruction due to specific immunity proteins they express.

Once activated, the master regulators control the expression of developmental genes including those involved in cell death and lytic pathways. As discussed earlier (**Chapter 1**), such activation of autolytic pathways has distinct advantages in promoting developmental programs. Mechanistically, all three developmental pathways exhibit broad similarities in their autolytic pathways and involve killing factors and immunity proteins (5, 36). Autolytic effectors in these developmental processes constitute specific bacteriocins and/ or

autolysins. While autolysins are primarily proteins that have murein hydrolase activities, bacteriocins comprise a class of narrow spectrum bactericidal factors that range from small peptides that primarily dissipate membrane potential of target cells to large proteins with peptidoglycan hydrolyzing activity (8, 39). Activated Spo0A (Spo0A-ON) governs the expression of two operons- *skf* (sporulating killing factor) and *sdp* (sporulating delay protein) in *B. subtilis* (15). Both operons encode bacteriocins (SkfA, SdpC) in attacker cells of *B. subtilis* populations and are exported outside the cell to kill sibling cells (targets; Spo0A-OFF) (6, 15). The genes that encode effectors that promote cell death in *S. pneumoniae* include *cbpD* (autolysin), *lytA* (primary autolysin), *lytC* (autolysin) and *cibAB* (two peptide bacteriocin)(13, 16, 18). Effectors are only produced by competent cells (ComE~P) in the population and are instrumental in the lysis of non-competent cells (5). Analogous to these systems, biofilm development of *E. faecalis* involves the lysis of a sub-fraction of cells that have not participated in Fsr signaling (36). Attackers (FsrA~P) activate the expression of *gelE* whose product is secreted outside the cell and in turn causes lysis of sibling cells in an AtlA (primary autolysin) dependent manner (36).

Strikingly in all three developmental programs, attackers that secrete toxic effectors (autolysins and bacteriocins) are also protected from self-inflicted damage or suicide by immunity proteins that are selectively expressed only in attackers, but not target populations (5). Accordingly the co-expression of SprE (immunity protein) in *E. faecalis* along with GelE possibly prevents GelE from modifying surface localized AtlA in attacker populations. In *B. subtilis* immunity against SkfA and SdpC is offered by other members of these operons. SkfE and SkfF resemble ABC transporters that may act as a SkfA-specific efflux pump (15). Further, SkfD (a protein with broad homology to type II CAAX prenyl endopeptidases) may proteolytically inactivate bacteriocins (5, 26). Immunity to SdpC is afforded by the membrane localized SdpI and is reportedly a tightly regulated process (10). In *S. pneumoniae*, two membrane localized proteins are thought to mediate resistance to autolysin and bacteriocin mediated killing. CibC confers immunity against its cognate two-peptide bacteriocin (CibAB) and ComM is thought to mediate immunity to all three effector autolysins (CbpD, LytA and LytC) (16, 18).

The autolytic process that we have described in biofilm development of *E. faecalis* is fundamentally different from that reported for *S. aureus* (**Chapter 1**, Fig. 5.1) in that, a subfraction of cells commit suicide for the benefit of the entire population in case of the latter organism compared to fratricidal killing in the former (2, 36). Interestingly, despite

this difference, both processes-altruistic suicide and fratricide are mediated by murein hydrolases and result in the accumulation of eDNA in the biofilm matrix. From an evolutionary viewpoint it may be possible to reason how individual bacterial cells would benefit by committing suicide only when one considers the whole bacterial population as a 'multicellular organism'. Yet even evolutionary biologists struggle to explain mechanisms that may limit the rise of cheaters that benefit from the sacrifice of altruists without paying the price of cooperation, in such populations (9, 27, 32). Fratricide on the other hand, not only endorses multicellularity, but may also play a major role in policing cheaters (that do not participate in quorum sensing but derive the benefit there of) within a population (36, 38).

Future directions

The current study has generated a plausible hypothesis on how GelE and SprE may regulate fratricide during biofilm development. According to this hypothesis, GelE secreted by attackers in the population, is thought to release/ mislocalize AtlA from the surface of target cells resulting in by-stander cell death. SprE on the other hand is hypothesized to promote proper localization of AtlA to the septum and prevent GelE mediated release of AtlA from the surface of attacker populations. Ongoing studies will test this hypothesis using antibodies specific to the primary autolysin, AtlA. Given our ability to sort attackers from targets, one would expect to find a quantitative increase in the amount of AtlA on the surface of attackers compared to targets. Further using fluorescence microscopy it would also be possible to confirm localization/ mislocalization of AtlA to the septum in attacker and target populations.

One further avenue of interest is with respect to the timing of GelE (effector of death) and SprE (immunity protein) production. As with other fratricidal systems, one would expect the immunity protein to be active before the lytic effector. However this simplistic interpretation does not seem to be the case for *E. faecalis* as both proteases are co-transcribed and secreted presumably at the same time (28). Interestingly evidence to the contrary exists, where in GelE is required for the proteolytic activation of SprE (20) and yet we have observed that SprE is able to significantly deter GelE mediated fratricide (36). One possibility remains the involvement of additional immunity proteins that may act in concert

with SprE to protect cells from autolysis. However, given these paradoxical observations, it becomes necessary to further test these hypotheses.

Finally, evidence on the role of other regulators in *E. faecalis* that affect autolysis and biofilm development is at best fragmentary. A number of regulators including one that was identified by us (the alternate sigma factor, RpoN) in a transposon screen have been demonstrated to affect biofilm development (Thomas and Hancock, unpublished results). Given that autolysis plays a major role in biofilm development, it becomes necessary to determine whether any of the previously identified regulators of biofilm development display significant cross-talk with death/ lytic pathways. One important development in this regard is the finding that RpoN can not only regulate biofilm development but also autolysis and eDNA release. Interestingly RpoN mediated regulation was independent of protease activity. Future studies will dissect out the roles of these additional pathways in regulating cell death and lysis in *E. faecalis*.

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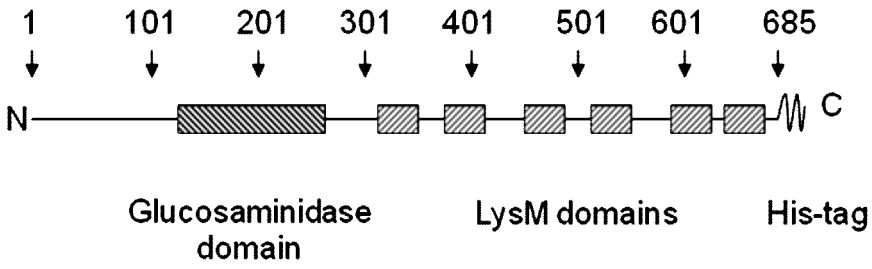
Appendix A- MALDI-TOF peptide mass mapping



Band name	Matched pep. number	Peptide region
Lane 1.		
72.1 kDa :	27	113-693C
62.2 kDa :	21	171-693C
Lane 2.		
62.0 kDa :	28	171-693C
55.3 kDa :	21	171-623
49.2 kDa :	23	171-604
43.7 kDa :	20	171-623
Lane 3.		
62.2 kDa :	31	113-693C

Comments: The molecular weights of AtlA cleavage products described in Lanes 1, 2 and 3 correspond to those found in Figure 3.8B. 'C' represents detection of an intact C terminus (His-tag) for the specific AtlA cleavage product by MALDI-TOF MS analysis.

Appendix B- Permission to release copyrighted material

In

CHAPTER 2

Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development

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