Role of Enterococcal Membrane Proteins in Cell-Cell Communication and Stress Adaptation

By

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Submitted to the graduate degree program in the Department of Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date approved: 12/15/2014

ABSTRACT

Enterococcus faecalis has recently emerged as one of the leading causes of nosocomial infections. The ability to tolerate a variety of antibiotics and environmental stresses (biotic and abiotic) combined with a plethora of host adaptation strategies allows this bacterium to transition from its sessile state as gut commensal into an opportunistic pathogen that can colonize multiple niches in a host to cause persistent infections. Pheromone-mediated conjugation facilitates *E. faecalis* in acquiring a variety of antibiotic resistance genes and virulence factors which are often encoded by the pheromone-responsive plasmid that undergoes cell to cell transfer during conjugation. Although a lot is known about the mechanisms by which pheromones regulate gene expression in these systems, the transport of these pheromones (export and import) across the cell membrane is poorly understood. In the current study we have identified the ABC transporter (PptAB) that is required for the secretion of these peptide sex pheromones. Using 3 distinct pheromone-responsive plasmids systems we demonstrated that the deletion of this transporter (*pptAB*) resulted in a severe conjugation defect. We also identified a second oligopeptide permease (Opp) that is involved in the import of these secreted peptide sex pheromones into the cell. Deletion of the genes encoding these peptide transporters (*pptAB, opp1* and *opp2*) resulted in a severe biofilm defect suggestive of a role played by these peptide pheromones in the development of these surface adherent structured communities.

We also identified the additional roles played by the membrane protein Eep in the stress adaptation pathways of *E. faecalis*. Eep was originally described as a protein that was involved in the processing of lipoprotein derived peptide sex pheromones. We showed that Eep is the site-2 protease that is involved in the regulated intramembrane proteolysis of an anti-sigma factor called RsiV which in turn results in the activation of an extracytoplasmic function sigma factor called SigV that confers high level resistance to lysozyme. We also showed that the deletion of eep increases the susceptibility of *E. faecalis* towards high temperature, low pH and ethanol stresses indicative of a role played by Eep in multiple stress adaptation pathways.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Lynn Hancock for being an exceptional mentor. He has set an example of excellence as a researcher, mentor, instructor, and role model. He sets high standards for his students and works tirelessly with them to meet those standards. The enthusiasm he has for his research was contagious and inspirational for me, even during the tough times in the Ph.D pursuit. I'd like to next thank Nathan Harms who made imperative contributions to the initial stages of the PptAB project. I would also like to thank our collaborators Dr. Michael Gilmore (Harvard Medical School) and Dr. John Tomich (Kansas State University) who made significant contributions to the PptAB and Opp project. I'd like to personally thank Dr. Manisha Diaz who have made my time in the lab such an intellectually stimulating and enjoyable experience.

None of this would be possible without the support of some special people over the past few years. A special thank you to Aanya Jagdish, Manoaj Vijayarengan and Ian Huck for their amazing friendships, love, and support which made getting through graduate school possible. I take this opportunity to thank my loving uncle and aunt, Mr. & Mrs. N.S. Ragunathan for their continued love and support. I would like to thank my mother Vaidehi Varahan who has instilled the value of ambition, dedication, hard work, and integrity in me and for teaching me that education and knowledge are the two most precious treasures of all. I'd also like to thank my grandparents N.S.Sundararajan and Saroja Sundararajan, for instilling confidence and values that have made me into the person I am today.

Last but not the least my brothers Veeraraghavan Varahan, Naga Sundar, Keshav Krishna and Premanand Satgunam and my sisters Dr. Premnandhini Satgunam and Dr. Shruthakirthe Ragunathan whose love and kindness makes everything possible.

DEDICATION

This work is dedicated to my grandparents, N.S.Sundararajan and Saroja Sundararajan and my mother Vaidehi Varahan.

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CHAPTER 1:

INTRODUCTION

Enterococcus faecalis:

Thiercelin (1899) identified enterococci as a gram-positive intestinal commensal capable of forming chains. Enterococci were originally classified under the genus *Streptococcus*, however in 1984 it was recognized as a separate genus due to its distinct differences from the streptococci (1). Over the last two decades, enterococci have emerged among the vanguard of antibiotic resistant strains due to extensive acquisition of mobile genetic elements and this coupled with the extended use of broad-spectrum antibiotics in clinical settings has allowed them to transition from gut commensals to leading nosocomial pathogens (2). Enterococci, currently are the second leading cause of nosocomial infections after *Staphylococcus aureus*. Based on a recent study on reported hospital acquired/associated infections, enterococci account for 11.6% of surgical site infection, 18.1% of central line associated blood stream infection and 15.1% of urinary tract infections (3). The two prominent and clinically relevant species of this genus are *Enterococcus faecalis* and *Enterococcus faecium* (4). Although *E. faecium* is gaining a lot more notoriety in recent times due to its high level resistance to vancomycin, ampicillin and other last line antibiotics, most enterococcal infections are still caused by *E. faecalis* (5) (6).

The acquisition of mobile genetic elements (~25% of the genome) has endowed the pathogenic lineages of enterococci with virulence traits and antibiotic resistance cassettes providing an advantage for these strains over the non-pathogenic isolates (7). A recent study showed that the presence of these mobile genetic elements correlates with the absence of clustered, regularly interspaced short palindromic repeats (CRISPR) system in multidrug resistance enterococci (8). CRISPR system provide bacteria and archaea with sequence-specific, acquired defense against plasmids and phages and their absence provides a rational explanation for the presence of a

plethora of mobile genetic elements is these pathogenic enterococci. This argument is further validated as the first sequenced vancomycin resistant *E. faecalis* strain V583, which lacks a functional CRISPR system (8) contains mobile genetic elements of foreign origin that endow it with potent infectious qualities. These foreign mobile elements include two pheromone-responsive plasmids pTEF1 and pTEF2 which encode for antibiotic resistance genes and virulence factors, a third plasmid pTEF3, a large pathogenicity island (~150 kb) (~15% of the isolates), a transposon harboring vancomycin resistance, three integrated plasmid remenants, seven putative prophage regions and 38 insertion elements (9).

The success of enterococcal colonization depends on its ability to compete with host commensal flora for nutrients and survive the different stresses (host factors) experienced during host colonization (10). Hospital lineages often have to confront and survive a multitude of antibiotics stresses to establish persistent infections (10). An arsenal of strategies are employed by enterococci to adapt to these host niches and establish successful infections amongst which biofilms have been shown to be an important lifestyle of enterococci in a host (10). Biofilms are complex three dimensional multicellular units wherein the bacteria are encapsulated in a well hydrated exo-polymer matrix composed of polysaccharides, proteins and nucleic acids from dead cells and provide protection against host immune components and antibiotic therapy (11). Enterococci are also innately resistant to a growing number of antibiotics and exhibit high level resistance to host derived factors like lysozyme, bile and other antimicrobial proteins (2). The pheromone-responsive plasmids encode for antibiotic resistance and virulence factors such as aggregation substance and cytolysin which have been shown to enhance the pathogenicity of these bacteria and their ability to survive antibiotic treatments (12). As a direct precedence to the primary theme of this

dissertation, proteins involved pheromone-mediated cell signaling and gene regulation events in *E. faecalis* and other closely related gram-positive bacteria will be discussed along with how *E. faecalis* responds to lysozyme and other relevant stresses.

CELL-CELL COMMUNICATION IN *Enterococcus faecalis*:

The ability of bacteria to communicate to each other via secreted signaling molecules is an important aspect of microbiology. Several multicellular events like biofilm formation, light production, competence and other development processes are coordinated by these cell-cell communication events (13-15). Quorum sensing is a type of cell signaling wherein the bacterial population is sensed with the help of signaling molecules. In gram-negatives these are usually homoserine lactones and in gram-positives these are peptides. Enterococci communicate to each other via two distinct cell signaling mechanisms:

i) Gelatinase Biosynthesis Activating Pheromone (GBAP) Signaling

ii) Lipoprotein Derived Peptide Pheromone Signaling

GBAP Signaling:

A number of studies have described the critical role played by the *fsr* operon in regulating biofilm formation in *Enterococcus faecalis* (16). The *fsr* locus is analogous to the *agr* quorum sensing system in *Staphylococcus aureus* with respect to organization and function (17, 18). It comprises four genes: *fsrA, fsrB, fsrD* and *fsrC*. FsrC and FsrA are part of a bacterial two component system where FsrC is a membrane localized histidine kinase and FsrA is the cognate cytoplasmic response regulator. The C-terminus of FsrD encodes for the 11-residue quorum signaling peptide,

GBAP, that is processed and secreted outside the cell with the help of the membrane protein, FsrB (19, 20). Once a concentration threshold of GBAP is reached outside the cell, it then binds to the FsrC histidine kinase which in turn activates the response regulator, FsrA (19). Once activated, FsrA differentially regulates the expression of ~300 genes involved in metabolism, virulence and other regulatory cascades (21, 22). With respect to biofilm formation, Fsr system regulates the expression of two secreted proteases (GelE and SprE). GelE has been shown to be an important player in enterococcal biofilm development and has also been shown to be important for *E. faecalis* pathogenesis in multiple animal studies (23, 24). Although SprE has been shown to be an important player in the fratricide model of biofilm development (25, 26) not much is known about the potential roles it could be playing in enterococcal pathogenesis and deducing these is one of the current focuses of ongoing studies in the laboratory.

Lipoprotein Derived Peptide Pheromone Signaling:

Originally described by Dunny *et al.* in 1978, peptide pheromone signaling is a well characterized cell signaling phenomenon that occurs in *E. faecalis* (27). These heptapeptide or octapeptide signaling molecules are usually derived from processing of lipoproteins (28) and serve as signaling molecules to mediate transfer of unique plasmids called pheromone-responsive plasmids (29). Lipoprotein precursors of peptide pheromones are usually encoded in the chromosome and the function of most of these lipoproteins are hitherto unknown (28). A simple diagram to illustrate the pheromone-mediated conjugation is shown in Figure 1-1.

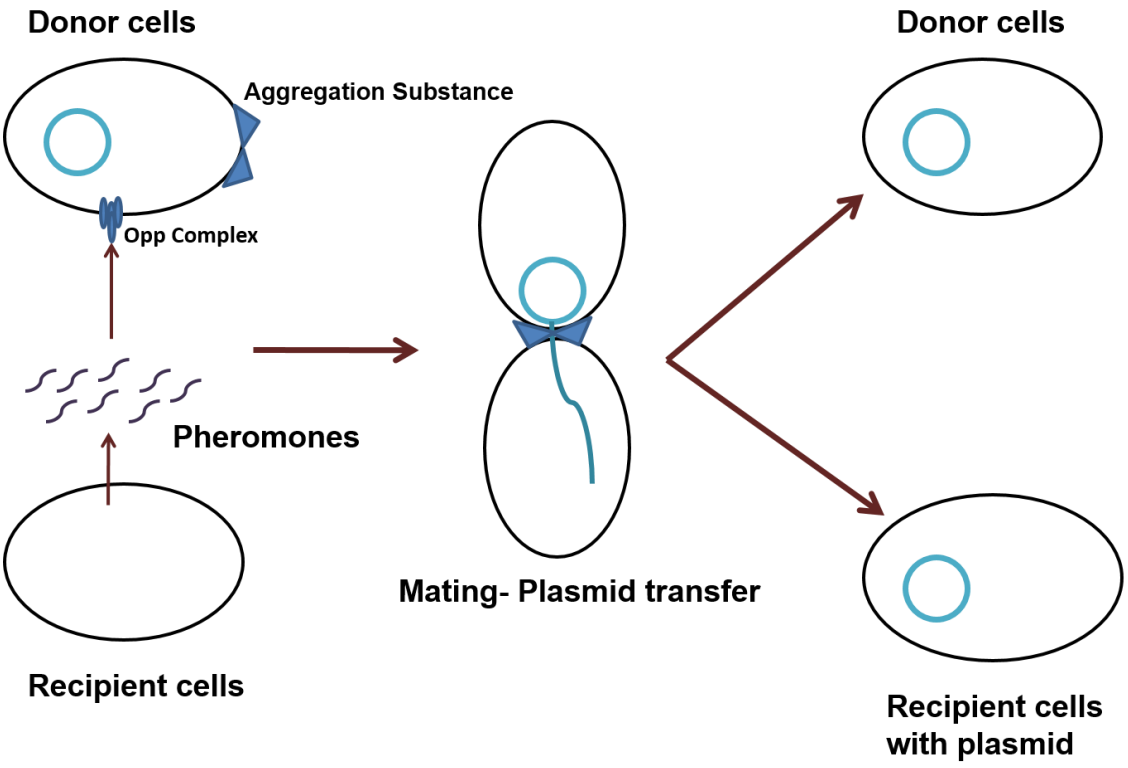

Figure 1-1: Pheromone-mediated conjugation.

Recipient cells that lack pheromone-responsive plasmids secrete these peptide sex pheromones which are usually hydrophobic in nature. Donor cells that harbor these pheromone-responsive plasmids import these peptides via a dedicated oligopeptide permease complex. This peptide elicits a response in the donor that results in the expression of the aggregation substance, a large membrane anchored protein that results in the aggregation of donor and recipient cells. Other pheromone-responsive plasmid encoded genes aid in the replication and transfer of a copy of this plasmid to the recipient (12). Pheromone-mediated conjugation is a high frequency DNA transfer event, meaning that in a span of 3-4 hrs most recipients that lack plasmids acquire these plasmids. These plasmids usually encode for antibiotic resistance genes and endow the cells with a plethora of virulence factors like the aggregation substance, cytolysin toxin to name a few (12, 29). Several

regulatory cascades control the production of pheromones in *E. faecalis* and these pheromones in turn regulate the gene expression of the donor cells to enable the transfer of a copy of this pheromone-responsive plasmid to the recipient. In other gram-positives, peptide pheromones regulate the expression of genes involved biofilm formation, competence, sporulation and virulence (30). Peptide pheromone-mediated regulation of these aforementioned processes will be discussed in detail in the subsequent sections of this introduction.

PEPTIDE PHEROMONE SIGNALING:

Recent years have seen a dramatic increase in the number of studies that describe peptide pheromone-mediated signaling and gene regulation in gram-positives. Although all of the cell signaling peptides in gram-positives are ribosomally synthesized, the mechanism by which they regulate gene expression, their processing and secretion vary widely. Figure 1-2 depicts the conventional peptide signaling systems in gram-positives. Gram-positive pheromones are often produced from precursors which are processed by multiple proteins and ultimately get secreted out of the producer cells (30). Once in the external environment, these pheromones exert their effects on neighboring cells either by:

i) Directly getting imported into the cell and binding to a transcriptional regulator to regulate gene expression.

ii) Interacting with a surface exposed sensor histidine kinase which in turn activates a cognate response regulator that alters gene expression.

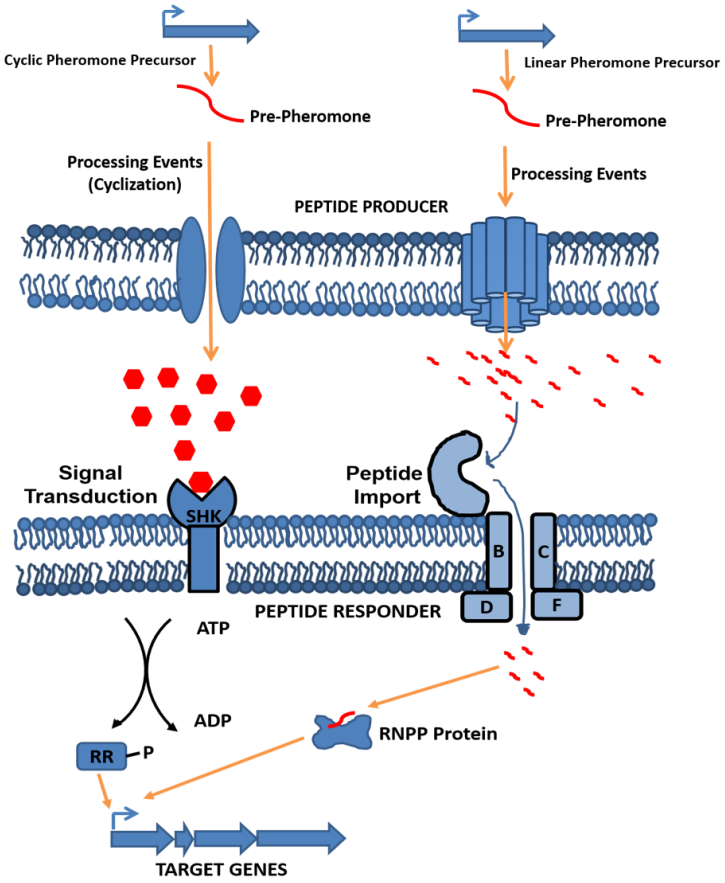

Figure 1-2: Pheromone signaling in gram-positives.

Several well characterized proteins and their cognate peptide pheromones regulated a variety of important cell functions in gram-positives including biofilm formation, competence and virulence gene expression. The Agr system of *Staphylococcus aureus*, The Fsr system of *Enterococcus faecalis* and the Com system of *Streptococcus pneumoniae* possess two component system that sense the presence of the cognate cyclic peptide pheromone via a sensor histidine kinase and regulates gene expression via a response regulator (RR) (30). The RNPP protein family (Rap, Npr, PlcR and PrgX) alter gene expression after binding to their cognate linear peptide pheromone that get processed and re imported into the cells via a dedicated oligopeptide permease complex. RNPP proteins contains motifs related to the tetratricopeptide repeats (TPRs) which are known to be

involved in protein-protein interactions (31, 32). These proteins and their regulatory functions along with the peptides they interact with are listed in Table 1-1. For the purposes of my dissertation, the subsequent sections of this introduction will focus on peptides pheromones in gram-positives that interact with RNPP family proteins to exert their gene regulatory effects in these bacteria.

Protein	Cognate peptides	Function	Reference
Two component system signaling			
AgrC	Auto Inducing Peptide (**AIP**)	Regulation of exotoxin production, biofilm formation and virulence.	(33-36)
ComD	Competence Stimulating Peptide (**CSP**)	Competence, bacteriocin production, biofilm formation.	(37-39)
FsrC	Gelatinase Biosynthesis Activating Peptide (**GBAP**)	Biofilm formation, exoprotease production and virulence.	(16, 18-20)
RNPP family protein signaling			
Rap	**Phr**	Regulation of sporulation in *B. subtilis*.	(40)
NprR	**NprX**	Regulation of exoprotease production.	(41, 42)
PlcR	**PapR**	Regulation of phospholipaseC, proteases and hemolysin. production	(43, 44)
PrgX	**cCF10**	Pheromone-mediated conjugation in *E. faecalis*.	(45-47)
Rgg	Short Hydrophobic Peptide (**SHP**)	Biofilm formation, extracellular DNAase release and virulence.	(48-51)

Table 1-1: Pheromone-mediated gene regulation in gram-positives.

PhrA peptide signaling in *Bacillus subtilis*:

Bacillus subtilis is a sporulating gram-positive rod shaped bacteria and is considered as the model Gram positive bacteria for the study of developmental gene regulation. Sporulation in *B. subtilis* is controlled by the master regulator Spo0A. Phosphorylation of Spo0A is preceded by the phosphorylation of Spo0F which in turn forms a part of complex phosphorelay system that governs sporulation. Rap phosphatases are a family of protein aspartate phosphatases that dephosphorylate the phosphorelay intermediates in the sporulation cascade (52). Rap proteins have been exhaustively studied in *Bacillus subtilis*, which expresses 11 chromosomal- and five plasmid-encoded members (53). Several Rap proteins block the signaling mediated by the two-component system by interacting with RRs. However, two completely different ways of accomplishing this function have been reported for members of this family. One subset of Rap proteins, including RapA, RapB, RapE, RapH, and RapJ, displays phosphatase activity to their target RR, Spo0F or Com (54-56). The second subgroup, comprising RapC, RapF, RapG, RapH, and RapK, blocks the action of the target RR (Spo0F or ComA) by a direct interaction with their DNA binding domain, and works as an anti-activator (56-59). Interestingly, RapH possesses both activities (59). For Rap proteins, regulatory peptides are called Phr and their mature active form is a penta- or hexa-peptide generated from a ~40 amino-acid precursor by means of a post-transcriptional export-import process (60, 61). Phr peptides are commonly linked to their target Rap proteins in such way that *phr* genes are situated immediately downstream of the genes encoding the Rap proteins to form Rap-Phr signaling cassettes, which are concurrently transcribed. Eight (RapA, RapC, RapE, RapF, RapG, RapH, RapI, and RapK) of the 11 genome-encoded Raps in *B. subtilis* form rap-phr signaling cassettes. RapB is regulated by the RapC (PhrC) peptide, while RapD and RapJ remain as Phr orphan Raps (62).

The first described Rap-Phr system was the RapA-PhrA system in *B. subtilis*. Perego and Hoch in 1996 showed that RapA phosphatase activity was controlled by a small 48 amino acid peptide called PhrA which was encoded on the same transcript as RapA phosphatase. The authors showed that PhrA gets secreted outside the cells and undergoes proteolytic processing and the C-terminal portion of PhrA which comprises of 19-amino acids then re-enters the cell via the oligopeptide permease complex. Upon re-entry this peptide negatively regulates the expression of *rapA* which in turn allows the phosphorelay system to phosphorylate Spo0A ultimately leading to cell sporulation (40). The authors used *phrA* deletion mutants and showed that this mutant had an uncontrolled *rapA* activity and was sporulation defecient and this defect could be complemented by the exogenous addition of purified PhrA peptide. The involvement of the oligopeptide permease complex for the transport of the PhrA peptide into the cell was confirmed by the observation that a *opp* deletion mutant was sporulation defecient and this defect could not be complemented by adding purified pheromone but only by mutationally inactivating *rapA*. Using a series of truncated peptides, the authors also showed that last six amino acids (NH_2-Ala-Ala-Arg-Asn-Gln-Thr-OH) retains the same activity as that of the proteolytically processed 19-amino acid C-terminal portion native PhrA peptide.

Stephenson *et al.* in 2003 showed that a single proteolytic processing event is responsible for the production of the active pentapeptide form of PhrA in *Bacillus subtilis*. They also showed that this processing event ocurred between residues 38-40 of the of the native PhrA peptide (63). However, the protease that is responsible for this cleavage event still remains to be identified since none of the previosly characterized signal peptidase-I deletion mutants affected PhrA processing.

Although the strucutre of RapA remains to be elucidated, two recent studies determined the three-dimensional structures of RapF and RapH in complex with their cognate binding partners (RRs: ComA and Spo0F) (55, 56). Del sol *et al.* in 2013 solved the structure of RapF bound to its cognate PhrF peptide. The authors in this study showed that 6 TPR domain helices of RapF interacts with the PhrF peptide resulting in a confirmation change which inactivates RapF (64). Although a lot is known about the gene regulatory effects exerted by Phr peptides in *B. subtilis* the mechanism by which these peptides exit the cell before reimportation by the Opp complex is still unknown. Chapter 2 of this dissertation will provide a possible mechanism by which this cell signaling peptide is exported from the cell.

NprX peptide signaling in *Bacillus cereus*:

Bacillus cereus is an oppurtunistic pathogen that causes a variety of human ailments including food-borne gastroenteritis and more severe infections like pneumonia and endophthalmitis (65-67). Several virulence factors contribute to the pathogenesis of *B. cereus* including hemolysins and phospholipase C. The metalloprotease NprA of *B. cereus* group was recently described as a potential virulence factor due to its host tissue degradative properties (68). Perchat *et al.* in 2011 showed that in *B. anthracis*, *B. thuringienesis* and *B. cereus*, NprA maps close to NprR, a predicted transcriptional regulator belonging to the RNPP protein family (42). The authors show that the levels of NprA in the supernatant are minimal when these bacteria are grown in enriched medium and these levels significantly go up when these bacteria are grown in sporulating media confirming the role played by NprA during sporulation (69). A closer look at the genetic locus of *nprA* revealed the presence of a short peptide encoding gene, NprX. The authors showed that the interaction

between NprX and NprR is critical for NprR to activate the gene expression of *nprA*. The native NprX peptide contains 43 amino acids and based on a series of peptide truncation expreiments the authors showed that the active peptide has 7 amino acids (NH_2-Ser-Lys-Pro-Asp-Ile-Val-Gly-OH) and retains the same biological acivity of the native NprX peptide. The authors also show that this heptapeptide binds to NprR with high affinity with a Kd of around 50 nM and this complex binds tightly to the -35 box of the *nprA* promoter.

A recent study solved the crystal structure of NprRΔHTH (lacking the helix-turn-helix motif) bound to the active NprX heptapeptide (70). NprR in its inactive form exists as a dimer. The binding of the NprX heptatpeptide results in the a confirmation and the dimers dimerze to form a tetramer which is thought to then bind to the *nprA* promoter region. The authors also show that the NprX peptide binds in a deep cleft in the middle of the TPR domain of the protein. It is speculated that the tetrameric active form of NprR bound to NprX binds to DNA at two distinct locations and this would result in DNA binding similar to the DNA binding that occurs when PrgX tetramers in *E. faecalis* bind to the DNA (71). A great degree of NprR-NprX polymorphism exists among the different members of the *B. cereus* group. As a result NprR-NprX proteins in *B. cereus* are classified into seven distinct groups and the NprR proteins in each group vary significntly from each other with respect to their amino acid sequence. The corresponding NprX peptide of each group also have distinct amino acids with the exception of Aspartic acid which is the central amino acid of all the NprX heptapeptides. Although a lot is known about the strucutre of NprR and its interaction dynamics with its cognate NprX peptide, not much is known about the genes that this RNPP family member regulates.

PapR peptide signaling in *Bacillus cereus*:

Transcription of a plethora of virulence factors including the transcription of the phosopholipase C encoding gene *plcA*, in *B. cereus* is controlled by the master transcriptional regulator, PlcR (43). PlcR is a prototypic member of the RNPP family of transcriptional regulators and positively regulates its own expression (43). Slamti *et al.* in 2002 showed that the activation mechanism of PlcR is under the control of a small 48 amino acid peptide called PapR (44). The *papR* gene is part of the PlcR regulon and is located 70 bp downstream from *plcR*. The authors showed that the deletion of *papR* completely abolished the expression of *plcR* and this in turn resulted in severly reduced hemolysis and virulence.

Although *papR* encodes for a 48-amino acid peptide, it has been shown the biological activity of PapR can still be retained with just the last five amino acids containing an initiator methionine sequence (NH_2-Met-Leu-Pro-Phe-Glu-Phe-OH). Subsequent studies have confirmed this and have shown that PapR gets processed to its pentapeptide form which in turn binds to the PlcR transcriptional regulator. Pomerantsev *et al.* in 2009 showed that the neutral protease NprB in *B. cereus* extracellularly processes the native PapR into 5, 7, 8 and 27 amino acids residues all of which retain biological activity (72). Once the PapR is extracellularly processed, it gets re imported into the cell via the oligopeptide permease complex. Binding of PapR to the PlcR protein induces a confirmation change that in turn is essential for PlcR to bind to DNA and initiate transcription of its regulon (44). The crystal structure of the biologically active form of PlcR bound to the pentapeptide PapR was solved in 2007. The 34 KDa PlcR protein has an N-terminal Helix-Turn-Helix DNA binding domain and a C-terminal regulatory domain composed of 11 helices which in turn form the 5 tetraticopeptide repeat (TPR) domain. Two PlcR molecules associate with each

other through their TPR domains to form a fuctional DNA binding complex. The PapR peptide binds to the concave side of the TPR domains (5^{th} and 7^{th} helices) to functionally activate the PlcR dimers which inturn binds to the *plcR* binding box ($TATGNAN_4TNCATA$) (73, 74).

Although a several studies have investigated the structure of PlcR and the genes that are controlled by this transcriptional regulator, the mechanism by which the native PapR is exported still remains to be elucidated. Chapter 2 of this dissertation attempts to explain a potential mechanism by which the PapR peptide might be transported outside the cell.

SHP peptide signaling in *Streptococcus pyogenes*:

Streptococcus pyogenes which is often referred to as Group A streptococci (GAS) is a bacterial pathogen known to cause a wide variety of human ailments ranging from the pharyngitis to the very severe necrotizing fasciitis. Several virulence factors aid in the pathogenesis of *S. pyogenes* in a host and the cysteine proteinase, SpeB is one such important factor. Several studies have shown that SpeB is important for host colonization and virulence and about a decade ago, it was shown that *speB* gene expression is regulated by an Rgg family transcription regulator (75). Originally described in *Streptococcus gordonii* as a regulator gene of glucosyltransferases (76), Rgg family proteins are well conserved among the members of the genus *Streptococcus* and control a variety of cellular functions including lantibiotic bacteriocin production in *Streptococcus mutans* (77) and virulence gene expression in *Streptococcus agalactiae* (78). Only in recent years were Rgg family proteins shown to interact with short hydrophobic peptides (SHP) resulting in their inclusion into the broader RNPP family proteins.

In a bioinformatics study that analyzed the intergenic regions of a few streptococcal genomes it was found that *rgg*-like genes were frequently found adjacent to small genes which were predicted to encode for SHPs (79). The evidence that these SHPs interact with the Rgg family proteins in *S. pyogenes* was demonstrated in a study in 2011 where the authors showed that two Rgg/SHP systems, Rgg2/SHP2 and Rgg3/SHP3 act as competing regulators with Rgg3 as a repressor and Rgg2 as an activator, both working on the promoters for the two adjacent *shp* genes (50). The interaction with SHP was critical for the functioning of both these regulators. The authors also showed that these peptides (SHP2 and SHP3) are processed by Eep before they exit out of the cell and are re-imported back into the cell via the oligopeptide permease (Opp) complex. In the absence of SHP3, Rgg3 acts as a repressor of gene expression and blocks the transcription of target promoters. The binding of SHP3 is thought induce a conformational change which displaces Rgg3 from the operator site alleviating repression. Interestingly, this de-repression event alone is not enough to drive the expression of the Rgg regulon in *S. pyogenes*. Rgg2 interaction with its cognate SHP-2 peptide is critical for the activation of various target genes (50). Although the Rgg2/SHP2 and Rgg3/SHP3 systems contributes to in-vitro biofilm formation and virulence of *S. pyogenes,* not much is known about the genes that are regulated by these proteins (50, 80).

Amongst all the Rgg proteins of *S. pyogenes*, Rgg1 better known as the regulator of proteinase B (RopB) is the most well characterized protein. Although a lot is known about the genes that RopB regulates and its role in virulence (75, 81), the cognate peptide that binds to this transcriptional regulator is not known. RopB also does not encode any divergently transcribed short peptide encoding gene around its genetic locus and this has made it harder to identify the peptide that it interacts with. However, the fact that deletion of the *opp* complex in *S. pyogenes* significantly

reduces the levels of SpeB combined with the fact that RopB shares significant structural homology with PrgX and PlcR proteins would suggest that RopB is activity is dependent on the interaction with its cognate peptide. A recent study showed that RopB-dependent activation of SpeB in late exponential growth is negatively affected by a peptide originating from a protein, Vfr. It is possible that this peptide might indeed be required for the functioning of RopB (82).

cCF10 signaling in *Enterococcus faecalis*:

Enterococcus faecalis is an opportunistic pathogen found as a commensal microorganism in the gastro-intestinal tract of most mammals including human beings. One of the hallmarks of enterococcal biology is their ability to transfer genes within and between species via conjugative/pheromone-responsive plasmids (27, 29). Conjugation in *E. faecalis* is controlled by two counteracting pheromones where one pheromone acts as the activator of genes essential for conjugation while the other pheromone acts as an inhibitor that prevents self-mating and unnecessary mating between cells that already harbor these pheromone-responsive plasmids (12). The activator pheromones are usually derived from lipoprotein precursors that are chromosomally encoded while the inhibitor peptides are plasmid encoded. A series of proteolytic events are essential for the processing of the lipoprotein precursor to yield the active form of the activator pheromone and this cascade is depicted in Figure 1-3. **1)** The lipoprotein precursors are thought to be secreted in Gram-positive bacteria through a Sec-dependent pathway (83). **2)** It is also known that the prolipoprotein diacylglyceryl-transferase (Lgt) is important in lipoprotein membrane anchorage in *E. faecalis* as it adds two fatty acid tails to the conserved cysteine of the protein (84).

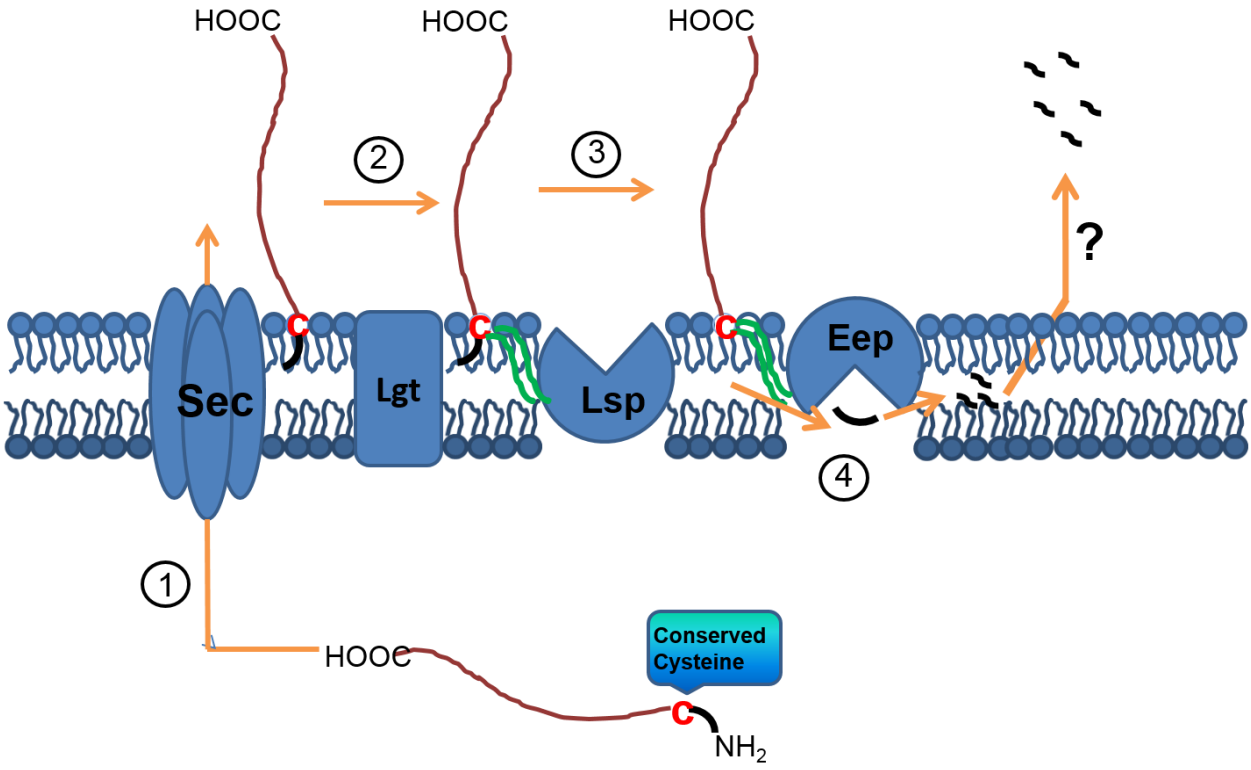

Figure 1-3: Pheromone production in *E. faecalis*.

3) Lipoprotein signal peptidase (LspA), which is a type II signal peptidase is also important for N-terminal processing of lipoproteins and acts after Lgt and cleaves the hydrophobic N-terminal tether of the lipoprotein (85). **4)** An *et al.* showed this N-terminal tether that is released is further processed by the membrane embedded zinc metalloprotease, Eep which results in the production of active pheromone (86). Several well characterized peptide pheromones in *E. faecalis* are generated via this sequential proteolytic cleavage events and are highly hydrophobic in nature with a length of 7-8 amino acids. These pheromones share common aspects of signaling and examples include the well-studied pAD1 system and pCF10 systems (87). The pAM373 system is an exception to this since cAM373 synthesis does not require the proteolytic processing of Eep (86).

For the purposes of my dissertation, the processing events that lead to the production of active cCF10 and its interaction with the RNPP family member PrgX will be discussed.

The activator pheromone of the pheromone-responsive plasmid pCF10 is derived from the constitutively expressed lipoprotein CcfA whose function is still unknown (88). A schematic diagram of the proteolytic events that result in the production of active cCF10 is shown in Figure 1-4.

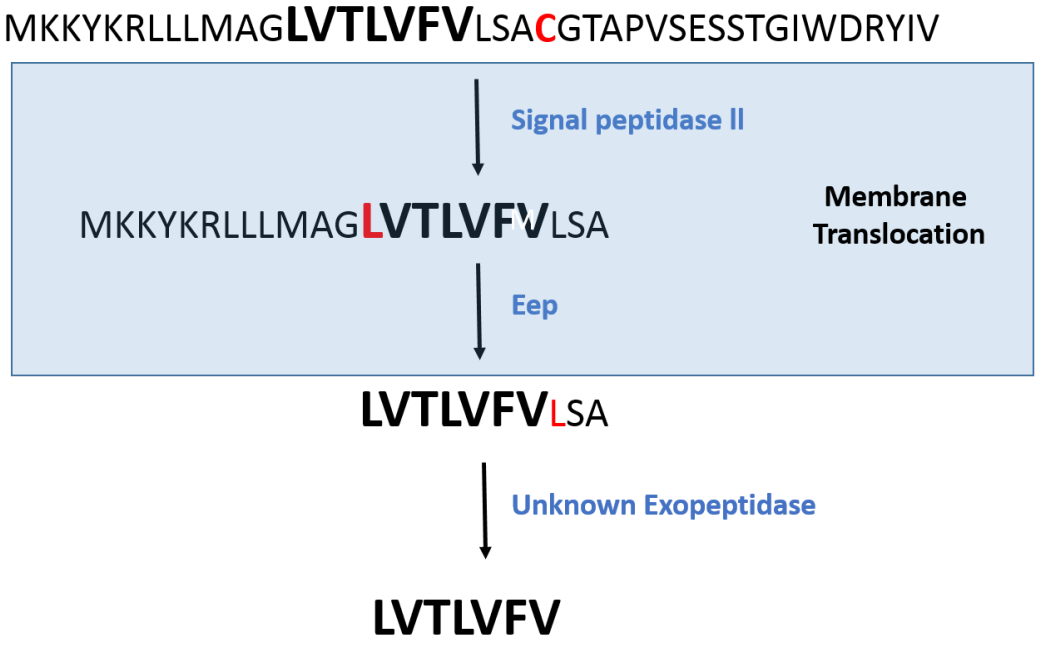

Figure 1-4: Proteolytic cleavage of CcfA to yield active cCF10 (Adapted from (88)).

Once produced in the cytosol, CcfA is exported outside the cell via the secretory apparatus and anchored to the membrane via the action of Lgt. The signal peptides-II, LspA cleaves this lipoprotein at the conserved cysteine resulting in the release of the hydrophobic N-terminal tether. This N-terminal tether contains 22 amino acids and is further processed by Eep and the resulting

10 amino acid peptide is released from the cell. This is then thought to be further processed by an unknown exo peptidase to yeild the active cCF10 pheromone (12). Once cCF10 is secreted by cells, it is percieved by cells that harbor the cognate pheromone-responsive plasmid pCF10. The plasmid pCF10 encodes for a protein called PrgZ with homology to OppA, the substrate-binding subunit of the oligopeptide permease (Opp) system (89). PrgZ is thought to hijack the chromosomally encoded permease subunits (OppBCDF) forming a transport complex (89). Although it is known that the presence of PrgZ increases the donor cell response to the cCF10 pheromone, it is not esssential for pheromone import, presumably because the chromosomally encoded OppA can fulfill this role (89). However OppA displays much lower affinity to cCF10 compared to PrgZ and this is true with respect to all other pheromone-responsive plasmids systems wherein the plasmid encoded OppA homolog binds to the cognate pheromone with much higher affinity compared to the chromosomally encoded OppA. Following import, mature cCF10 binds to the master regulator of conjugation, PrgX, a member of the RNPP family of regulators (90).

In the absence of the activating pheromone cCF10, PrgX binds to two target sites (Qa and *prgX* mRNA) in pCF10 that is located upstream of the Pq promoter (91). The protein structure of PrgX has been solved and this study has shown that the tetrameric form of PrgX is essential for its DNA binding and subsequent repression of genes encoding essential conjugation factors like the aggregation substance (AS) (71). The stabilization of this PrgX tetramer occurs via its interaction with iCF10, antagonist of cCF10 pheromone. The inhibitor pheromone, iCF10, is encoded by the plasmid encoded gene *prgQ* and PrgQ, similar to CcfA is processed by Eep and re-imported back into the cell as actives iCF10 via the Opp system in conjuction with PrgZ (92). The cCF10 activating pheromone and the iCF10 inhibitor pheromone are thought to compete with each other

for PrgX binding and it is speculated that binding of cCF10 to PrgX breaks up the tetramer by destabilizing the PrgX complex and the dimeric interface and this results in the alleviation of PrgX repression (71).

PHEROMONE-RESPONSIVE PLASMIDS IN *Enterococcus faecalis*:

Pheromone-responsive plasmids are a group of very large plasmids (~50 Kbp) and are characterized by the ability of the plasmid harboring cells (Donor cells) to form aggregates when mixed with plasmid free cells (Recipient cells) (27). Donor cells undergo self clumping or self aggregation when exogenous cognate pheromone is added to the donor cell culture or when they are exposed to culture supernatants containing the cognate pheromone. Once the recipient cells acquire the pheromone-responsive plasmids via the process of conjugation, there is a shut down of the cognate pheromone production; however, unrelated pheromones that are not associated with the pheromone resposive plasmid are still produced (29). Two distinct mechanisms are employed by plasmid bearing cells to prevent self aggregation i) Competitve binding by plasmid encoded inhibitor pheromone ii) Pheromone sequestration by TraB/PrgY (pAD1/pCF10 plasmids) proteins (29).

The inhibitor pheromones which are also derived from protein precursors via Eep processing competitevely bind to TraA/PrgX (pAD1/pCF10 plasmids) proteins or their equivalents in other pheromone-responsive plasmids and this results in the repression of pheromone-responsive genes enabling the desensitization of cells to the cognate pheromones (93). It has been shown that iCF10, which is the inhibior pheromone encoded by pCF10 stabilizes the tetrameric form of PrgX which allows this RNPP family protein to repress the expression of genes essential for pheromone-

mediated conjugation (71). It is known that the amount of inhibitor pheromone produced is much higher than the amount of activating pheromone (80:1) and hence these plasmid bearing cells remain desenstized until an external source of these activating pheromones are encountered by the plasmid bearing cells. The second distinct mechanism preventing conjugation is mediated by the plasmid encoded protein TraB/PrgY (92, 94). Although the exact mechanism by which TraB/PrgY dampenes the pheromone response is not known, it is known that this process is independent of Eep and involves the direct interaction between the activating pheromone cAD1/cCF10 with TraB/PrgY. Chandler *et al.* in 2008 showed that PrgY recognizes specific residues in cCF10 heptapeptide and specific residues in PrgY are important for this recognition (92). It is hypothesized that TraB/PrgY sequesters and possibly degrades the activating pheromone with high affinity and this in-turn prevents the re-entry of these pheromones into the plasmid bearing cells. Interestingly, a recent study by Bazan *et al.* showed that the metazoan Wnt protease and signaling inhibitor TIKI shares significant sequence homology with bacterial TraB/PrgY proteins It is coherent to speculate that the inhibitor pheromones and TraB/PrgY family of proteins act in tandem to prevent self aggregation of plasmid bearing cells (29). A partial list of the well characterized pheromone-responsive plasmids, the cognate activating pheromones and inhibitor pheromones associated with them and their cellular functions are listed in Table 1-2. Some of the well characterized pheromones amongst these include pAD1, pCF10 and pAM373 (12). Although each of these pheromone-responsive plasmids are distinctly different from each other with respect to the pheromones they respond to, the induction of a protein termed as “aggregation substance” (AS) in response to these cognate pheromones occurs in all these pheromone-responsive plasmid systems.

Plasmid (Size)	Activating/Inhibitor pheromones	Function	Reference
pAD1 (60 kb)	cAD1/iAD1	Encodes for cytolysin and UV resistance, Virulence enhancement.	(95, 96)
pCF10 (65 kb)	cCF10/iCF10	Tetracycline resistance, Virulence enhancement.	(47, 97)
pPD1 (56 kb)	cPD1/iPD1	Bacteriocin21 production.	(98-100)
pAM373 (36 kb)	cAM373/iAM373	Interspecies cell signaling.	(101-105)
pOB1 (71 kb)	cOB1/iOB1	Encodes for Hemolysin/Bacteriocin.	(106, 107)
pAMγ1 (60 kb)	cAD1/iAD1	Encodes for cytolysin and UV resistance.	(108)
pAMγ2 (60 kb)	cAM γ2/iAM γ2	Bacteriocin production.	(108)
pAMγ3 (60 kb)	cAM γ3/iAM γ3	Unknown function.	(108)
pAM323 (66 kb)	cAM323/iAM323	Erythromycin resistance.	(109)
pYI17 (58 kb)	cYI17/iCY17	Bacteriocin31 production.	(110)

Table 1-2: Pheromone-responsive plasmids in *Enterococcus faecalis*.

It has been shown that aggregation substance encoded by pAD1 and pCF10 is a 1296 amino acid protein is anchored to the cell wall of the donor cells with the help of the sortase system and interacts with the lipoteichoic acid (LTA) expressed on the surface of the recipients (111, 112). It is interesting to note that the aggregation substance encoded by pAM373 termed as Asa373 is

significantly different from the AS encoded by pAD1 or pCF10. Asa373 is much smaller in size compared to the pAD1/pCF10 encoded AS and does not interact with the LTA of recipient cells (113). The interaction partner of Asa373 still remains to be elucidated. Many of the genes encoded by these conjugative plasmids serve as virulence factors of *E. faecalis* and significantly enhance their diesease pathogenesis in a host (29).

Ike *et al*. in 1984 was the first to show that the cytolysin encoded by pAD1 significantly enhanced peritonitis in a mouse model (114). Following that report, several clinical isolates of *E. faecalis* from humans were found to harbor pAD1 or pAD1-like pheromone-responsive plasmids that encoded for cytolysin (115). Several follow up studies have shown that the pAD1 encoded cytolysin increases *E. faecalis* virulence in experimental models of endocarditis (116) and endophthalmitis (117). Another plasmid encoded gene product that has been shown to enhance enterococcal virulence is the aggregation substance. Presence of pheromone-responsive plasmids that express this aggregation substance has been shown to enhance the internalization of *E. faecalis* cells by an human epithilial cell line (118) and increse the adherence to an kidney epithileal cell line (119). Aggregation substance expressed by pCF10 and pAD1 have also been shown to improve the pathogenicity of *E. faecalis* in a rabbit model of experimental endocarditis (116, 120). It is important to note that when *E. faecalis* cells harboring pCF10 encounter host environmental factors like serum, there is in an increase in the expression of pheromone-responsive genes including aggregation substance. A study by Chandler *et al.* in 2005 showed that the inhibitor pheromone iCF10 gets sequestered by the host serum and this results in the resensitization of the pCF10 bearing donor cells to its own activating pheromone (cCF10), resulting in the expression of the aggregation substance and other pheromone resposive genes (121). This study provides a

rational explanation of how plasmid bearing cells express pheromones responsive genes that enhance virulence in a host even in the absence of an exogenous source of activating pheromone.

In addition to the significant contributions to virulence, pheromone-responsive plasmids also play an important role in the emergence and dissemination of antibiotic-resistant nosocomial pathogens (4). pCF10 was originally identified in a clinical isolate of *E. faecalis* that was resistant to both tetracycline and erythromycin (Strain SF7) from a patient in New York in 1980 (97). Interestingly when vancomycin resistant *E. faecium* emerged a decade later, a percentage of them harbored a conjugative vancomycin resistance plasmid called pHKK702. Interestingly some isolates of *E. faecium* harbored another distinct plasmid called pKHH703 which contained a gene that shared >90 % sequence similarity to PrgX of pCF10 and *E. faecium* strains that harbored this plasmid elicited an aggregation response to cCF10 (122, 123). Other sequence analysis studies have shown that pCF10 and pHKK703 are closely related. It is possible that pCF10 is the genetic predecessor of pHKK703 and that exchange of genetic information pCF10 and pHKK703 via transposition could have lead to the existence of this new plasmid. Another noteworthy observation is the ability of enterococci to exchange genetic information with *S. gordonii* and *S. aureus* by responding to the peptide pheromones that these species produce. Both *S. gordonii* and *S. aureus* produce cAM373 like pheromones that slightly differ from the enterococcal cAM373 pheromone (*S. gordonii* by three amino acids and *S. aureus* by one amino acid). Weigel *et al.* in 2003 showed that vancomycin resistant enterococci (VRE) can transfer this resistance (encoded by pheromone-responsive plasmids harboring Tn1547) to *S. aureus* strains that produce the cAM373 like pheromone via the process of conjugation resulting in VRSA strains (101). Vickerman *et al.* in 2010 showed that interspecies gene transfer events can occur between enterococci and *S. gordonii*

that produce cAM373 like pheromone (102). Few cases of VRSA have been reported in the United States (124) and the first reported case in Europe was only identified in 2013 (125). However, it is of paramount importance to prevent the dissemination of vancomycin resistance to these virulent pathogens since vancomycin is used as a drug of last resort to treat several gram-positive bacterial infections. The spread of antibiotic resistance is a dangerous problem and dissecting the mechanisms of transfer in *E. faecalis* will be essential in preventing further dissemination of resistance determinants to other bacterial species that are already multi-drug resistant.

STRESS ADAPTATION:

As a bacterium that can colonize the intenstine of a wide variety of fauna including humans, *E. faecalis* experiences a number of stresses in a given host environment and has evolved several mechanisms to survive and cause persistent infections. As a gut commensal, *E. faecalis* experiences a wide variety of stresses including oxidative stress, bile salt stress, osmotic stress and acid stress (low pH) to name a few (126, 127). Studies have shown that enterococci can quickly adapt to the aformentioned stresses when they are pre-exposed to sublethal doses of the agents that cause these stresses . One can speculate on the presence of transcriptional factors that efficiently control gene expression to ensure that the response will be rapidly induced after exposure to these stresses. Although *E. faecalis* lacks a master stress regulator like the *Escherichia coli* RpoS, studies have shown that several two-component systems in *E. faecalis* and other orphan regulators are involved in the general stress response (128). A common stress experienced by many bacteria that colonize the intestines in mammals is the bile salt stress. *Listeria monocytogenes* and *E. coli* have specific efflux pumps that are activated in the presence of bile and offer protection against this stress (129, 130). Several studies have shown that *E. faecalis* alters its gene expression and protein

synthesis when grown in the presence of bile and pre-exposure to bile increases the tolerance of *E. faecalis* to other stresses including increased tolerance to daptomycin (131). In 2011, Michaux *et al.* showed that the SlyA transcriptional regulator was involved in the *E. faecalis* bile salt stress response by regulating the expression of a bile salt hydrolase (EF_3005) (132). Interestingly the pathogenicity island present in certain clinical isolates of *E. faecalis* also encodes for a second bile salt hydrolase (EF_0521) that is though to contribute to the resistance towards bile salts (9). A recent study showed that the incorporation of exogenous fatty acids contributes towards the resistance of *E. faecalis* to bile salts adding another layer of complexity to the stress response paradigm (131).

E. faecalis can cause a variety of infections including blood stream infections and surgical site infections wherein it encouters a variety of stresses including oxidative stress and this occurs due to the production of hydrogen peroxide, hydroxyl radicals and superoxide by phagocytes present in blood and other neighbouring tissues (133). Several antioxidative enzymes in *E. faecalis* enable this bacterium to persist in these harsh conditions. The highly conserved mangenese containing superoxide dismutase (MnSOD) is encoded by the *sodA* gene in *E. faecalis* and this enzyme is critical for *E. faecalis* survival in host environments (macrophages) that generate free radicals. *sodA* deletion mutants have been shown to be attenuated in *in-vivo* animal models implicating its importance in *E. faecalis* pathogenesis (134). *E. faecalis* also encodes for catalase that degrades hydrogen peroxide; however, the production of active catalase protein is heme–dependent (135). A recent study identified several uncharacterized genes essential for the functioning of catalse in *E. faecalis* and showed that biogenesis of catalase was independent of the cytochromeABCD complex (136). The exact mechanism by which catalase is activated in *E. faecalis* still remains to

be elucidated since this bacterium does not synthesize its own heme. *E. faecalis* strains that harbor the pathogenecity island encode for a regulator known as PerR. Although the exact mechanism by which PerR contributes towards oxidative stress resistance is unknown, it has been demonstrated that PerR is important for survival inside macrophages and other phagocytes (137).

Another important host immune component that *E. faecalis* frequently encouters during infection is lysozyme. This enzyme is found in a wide variety of body fluids, such as tears, breast milk, respiratory and saliva secretions, as well as in cells of the innate immune system, including neutrophils, monocytes, macrophages, and epithelial cells (138, 139). Lysozyme usually causes bacterial cell lysis by hydrolyzing the glycosidic linkage between *N*-acetylmuramic acid (MurNAC) and *N*-acetylglucosamine (GlucNAC) present in the cell wall peptidoglycan. Other studies have shown that lysozyme can also function as a cationic anti-microbial peptide (CAMP) and this leads to bacterial death likely through the destabilization of the cytoplasmic membrane (140, 141). Two main mechanisms are employed by bacteria to survive and persist in the presence of lysozyme. Both of them counteract antibacterial activity of lysozyme: i) the modification of different sites of the PG structure by two kinds of enzymes such as the *N*-acetylglucosamine deacetylase (PgdA) of *Streptococcus pneumoniae* (142), or the peptidoglycan-specific *O*-acetyltransferase (OatA) of *S. aureus* (143) prevents the binding of lysozyme to its substrate and contributes to the muramidase resistance; ii) the modification of the net negative charge of the bacterial cell surface by adding positively charged residues (D-alanine esterification through *dlt* genes) to teichoic and lipoteichoic acids helps bacteria to avoid being killed by antimicrobial peptides or CAMP activity of lysozyme (144, 145). Although *E. faecalis* were known to be extremely resistant to lysozyme, the exact mechanism that confers this high level resistance was

not known. Le Jeune *et al.* in 2010 showed that the extracytoplasmic function (ECF) sigma factor SigV, was a cruicial player in the lysozyme resistance pathway . The *sigV* deletion mutant displayed an attenuated phenotype in a murine urinary tract infection (UTI) model indicative of the importance of lysozyme resistance in establishing a persistent infection (146).

ECF sigma factors are proteins that are activated in bacteria in response to an external (extracytoplasmic) signal. Some bacteria posses multiple ECF sigma factors that are activated in response to different stresses. The activation of ECF sigma factors is a complex process and is described in Figure 1-5. Under normal conditions, ECF sigma factors are sequestered to the membrane by a membrane spanning protein called the anti-sigma factor. The presence of the activating stress leads to a confirmation change in this anti-sigma factor making it susceptible to the action of membrane proteases (147). Two proteases are usually involved in the degradtion of the ant-sigma factor. The Site-1 protease cleaves first and this cleavage usually happens outside the intra-membrane space and a large portion of the C-terminus of the anti-sigma factor is cleaved. This exposes this protein to the cleavage of the Site-2 protease (148). Site-2 proteases are membrane embeded metallo-proteases and usually cleave the anti-sigma factor in the intramembrane space (149). This second cleavage event releases the ECF sigma factor into the cytoplasm where it is further processed by the cytoplasmic proteases (Clp proteases) and this active form of the ECF sigma factor regulates gene expression nesecarry for the bacterial cell to develop resistance towards the activating stress (148). ECF sigma factor SigV is also conserved in *B. subtilis* and *Clostridium difficile* and gets activated in the presence of lysoyme (150, 151). Since *C. difficile* and *E. faecalis* frequently colonize the intestine of the host, it is possible that SigV provides a survival advantage for these pathogens in these lysozyme rich environment.

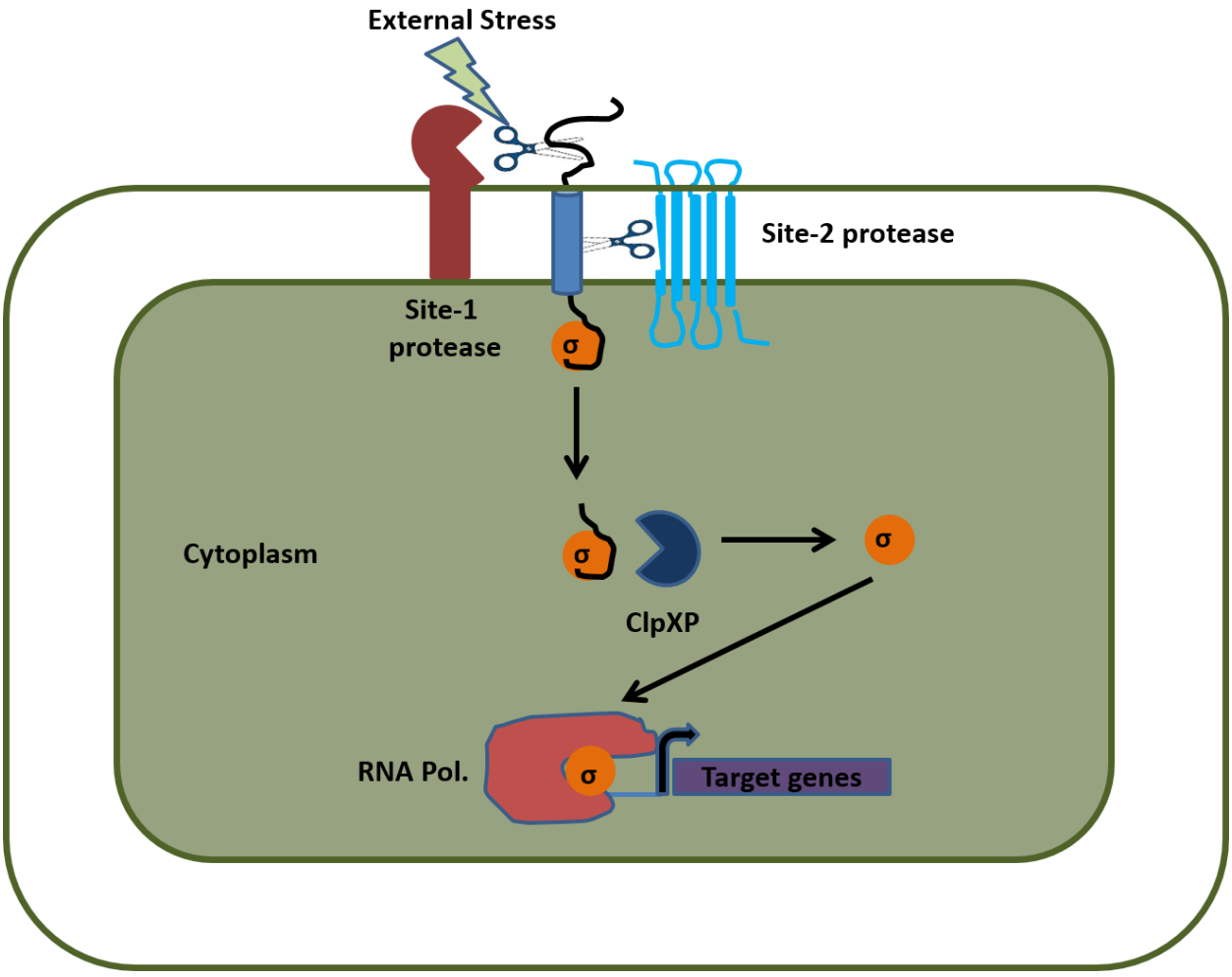

Figure 1-5: ECF sigma factor activation in bacteria.

Although not much is known about the SigV regulon (genes regulated by SigV) or the proteases that activate SigV, it is interesting to note that in *E. faecalis*, *pgdA* (gene encoding for a peptidoglycan deacytelase) expression is under the direct control of SigV (146). However, the expression of other cell wall modifying enzymes like *oatA* and *dlt* in *E. faecalis* (common cell wall modifying enzymes that confer lysozyme resistance to other Gram positives like *S. aureus* and *B. subtilis* (143, 152)) is not regulated by SigV. Interestingly, Le Jeune *et al.* showed that when *pgdA* alone is deleted in *E. faecalis*, this does not affect the lysozyme resistance of the mutant (146).

Taking all this account, it is possible that a novel mechanism exists and confers lysozyme resistance to *E. faecalis*. Whether this is completely under the regulation of *sigV* still remains to be elucidated.

SCOPE OF THE DISSERTATION:

In recent years, *E. faecalis* has emerged as one of the leading causes of nosocomial infections due to its ability to tolerate a variety of stresses including antibiotic stress and other host derived stresses. Several virulence factors have also been shown to contribute to the pathogenesis of *E. faecalis* (10). Pheromone-responsive plasmids in particular have been shown to encode for both antibiotic resistance genes and virulence factors and strains that harbor these plasmids display significantly enhanced virulence in a host (29). Although *E. faecalis* peptide sex pheromones and the mechanisms involved in the transfer of these pheromone-responsive plasmids is well characterized (12), very little is known about the proteins that are involved in the transport of these highly hydrophobic signaling molecules (89). In the second chapter of my dissertation, I have identified an ABC transporter that is essential for the secretion of peptide sex pheromones in *E. faecalis*. This transporter is highly conserved in other Gram positives and likely serves the same function in these *firmicutes*. In the third chapter of my disseratation, I have characterized a second oligopeptide permease (Opp) complex in *E. faecalis* and demonstrated that in the absence of the first complex, the second complex can serve as a backup transporter and import peptide sex pheromones secreted by pheromone producing cells. I have also demonstrated that the deletion of these transporters in tandem affects biofilm formation in *E. faecalis* suggestive of a possible role played by pheromone-mediated cell signaling in biofilm formation. In the fourth chapter of my dissertation, I have identified an additional role for the membrane-embeded zinc metalloprotease,

Eep, in the biology of *E. faecalis*. Eep was orginally described as a protein that processes the N-terminal tether of cell wall anchored lipoproteins and generates active peptide sex pheromones (86). Here, we show that Eep is the Site-2 protease that is essential for the activation of the ECF sigma factor, SigV in *E. faecalis*. SigV confers high levels of lysozyme resistance to *E. faecalis* and plays a role in *E. faecalis* pathogenesis in a murine model of urinary tract infection. Overall, my dissertation has identified and characterized membrane peoteins that contribute to cell signaling and stress adaptation in *E. faecalis*. In the dawn of post antibiotic era, a better understanding of these aformentioned membrane proteins may aid in the development of new therapeutics that could potentially curb antibiotic resistance resistance dissemination, stress resistance and virulence of this hardy bacterium.

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CHAPTER 2

AN ABC TRANSPORTER IS REQUIRED FOR SECRETION OF PEPTIDE SEX PHEROMONES IN *Enterococcus faecalis*

ABSTRACT:

Enterococci are leading causes of hospital-acquired infection in the United States, and continue to develop resistances to new antibiotics. Many *Enterococcus faecalis* isolates harbor pheromone responsive plasmids that mediate horizontal transfer of even large blocks of chromosomal genes, resulting in hospital adapted strains with over a quarter of their genome consisting of mobile elements. Pheromones to which the donor cells respond, derive from lipoprotein signal peptides. Using a novel bacterial killing assay dependent on the presence of sex pheromones, we screened transposon mutant library for functions that relate to the production and/or activity of the effector pheromone. Here we describe a previously uncharacterized, but well conserved ABC transporter that contributes to pheromone production. Using three distinct pheromone-dependent mating systems, we show that mutants defective in expressing this transporter display a 5-6 order of magnitude reduction in conjugation efficiency. In addition, we demonstrate that the ABC transporter mutant displays an altered biofilm architecture, with a significant reduction in biofilm biomass compared to its isogenic parent, suggesting that pheromone activity also influences biofilm development. The conservation of this peptide transporter across the *Firmicutes* suggests that it may also play an important role in cell-cell communication in other species within this important phylum.

INTRODUCTION:

Enterococcus faecalis is an opportunistic pathogen that has emerged as a leading cause of hospital-acquired infection in the United States (1). Enterococci exhibit resistance to many antibiotics, and heavily colonize patients after antibiotic therapy (2). *E. faecalis* is particularly facile at exchanging mobile elements, having evolved a pheromone mediated conjugation system (3). Recipient cells release an array of pheromones into the environment and a potential donor specifically responds to a unique pheromone by inducing a conjugative mating response that includes the production of a plasmid-encoded adhesive protein called aggregation substance that promotes effective mating pair formation in liquid cultures, leading to efficient plasmid transfer from donor to recipient (4-8). Distinct pheromone responsive plasmid systems have been identified in *E. faecalis* (9) and are known to harbor traits that contribute to the severity of infection including cytolysin, aggregation substance and a wide variety of antibiotic resistance genes (10-12). Peptide pheromones derive from lipoprotein signal peptides and are generally hepta or octamer peptides comprised of predominantly hydrophobic amino acids (13). Eep, a zinc-dependent membrane metalloprotease, has been shown to be involved in processing the lipoprotein precursor, leading to production of the active pheromone in most but not all pheromone systems (14, 15). A recent study by Hancock *et.al* (submitted PNAS) showed that production of a specific pheromone, cOB1, by commensal *E. faecalis* in the gastrointestinal tract consortium, inhibits the vancomycin resistant hospital-adapted *E. faecalis* strain V583. A transposon screen to identify additional functions involved in this peptide-mediated antagonism led to the identification of mutants that were defective in inhibiting V583. A series of independent transposon mutants whose insertion localized to well-conserved genes encoding a predicted ABC transporter that belongs to the EcsAB family (16) were identified.

It was therefore of interest to further characterize this mutant to determine the extent to which the well-conserved transporter contributes to generalized pheromone production in *E. faecalis*. We have designated this ABC transporter as the Peptide Pheromone Transporter (PptAB) to reflect its global role in peptide secretion.

MATERIALS AND METHODS:

Bacterial Strains, Plasmids & Growth Conditions:

Bacterial strains and plasmids used in the current study are listed in Table 2-1 and Table 2-2.

Relevant Strain	Genotype, Resistance	Strain Origin/Plasmid
V583	*pptAB*$^+$*eep*$^+$	(17)
FA2-2	*pptAB*$^+$*eep*$^+$*fsr*$^-$, Rif^r	(18)
OG1RF	*pptAB*$^+$*eep*$^+$*fsr*$^+$, Rif^r	(19)
SV02	V583Δ*pptAB*	This study
SV18	V583Δ*pptAB*::*pptAB*	This study, pSV06→SV02
SV04	FA2-2Δ*pptAB*, Rif^r	This study
SV05	FA2-2Δ*eep*, Rif^r	(20)
SV06	FA2-2Δ*pptAB*::*pptAB*, Rif^r	This study, pSV06→SV04
VT09	V583 (pMV158GFP), GFP$^+$ Tet^r	(21)
SV20	SV02 (pMV158GFP), GFP$^+$ Tet^r	This study
SV23	SV18 (pMV158GFP), GFP$^+$ Tet^r	This study
OG1SSp (pCF10)	Spec^r Strep^r Tet^r	pCF10§, (22)
OG1SSp (pAM714)	Spec^r Strep^r Erm^r	pAM714§, (23)
OG1SSp (pAM378)	Spec^r Strep^r Tet^r	pAM378§, (24)

[a] Rif^r , rifampicin resistance ; Spec^r , spectinomycin resistance ; Strep^r , streptomycin resistance ; Tet^r , tetracycline resistance ; Erm^r , erythromycin resistance, § - Pheromone responsive plasmids

Table 2-1: Bacterial strains.

Strains were cultured in Todd-Hewitt broth (THB) and grown at 37˚C unless otherwise indicated. *E. coli* Electro-Ten Blue (Stratagene, La Jolla, CA) was used for maintenance and propagation of plasmid constructs.

Plasmid	Description
pLT06	**Integration Vector, Derivative of pCJK47, Chloramphenicol resistance (25)**
pSV02	**pLT06 containing a ~2Kb EcoRI/SphI fragment comprising engineered *pptAB* deletion**
pSV03	**pLT06 containing a ~2Kb BamHI/SphI fragment comprising engineered *eep* deletion**
pSV06	**pLT06 containing a ~4Kb BamHI/SphI fragment encompassing *pptAB* the ~1Kb regions flanking the 5' and 3' ends of *pptAB***
pMV158GFP	***gfp*-containing plasmid, Tet^r (26)**
pCF10§	**Pheromone responsive plasmid harboring tetracycline resistance (22)**
pAM714§	**Pheromone responsive plasmid harboring erythromycin resistance, Derivative of pAD1§ (23)**
pAM378§	**Pheromone responsive plasmid harboring Tetracycline resistance, Derivative of pAM373§ (24)**
pCAM45	**Plasmid used for the transposon mutagenesis. Contains Himar transposon (27)**

§ - Pheromone responsive plasmids

Table 2-2: Plasmids.

Antibiotics for selection included Chloramphenicol at 10 µg-ml^{-1} , Rifampicin at 100 µg-ml^{-1}, Tetracycline at 10 µg-ml^{-1} , Erythromycin at 10µg-ml^{-1} , Spectinomycin at 500 µg-ml^{-1} and Streptomycin at 500 µg ml^{-1}. Transformation of *E. faecalis* was done as described previously (28).

Transposon mutagenesis:

A mutant transposon library was constructed in *E. faecalis* strains FA2-2 and OG1RF using the transposon mutagenesis vector pCAM45 (27). Electrocompetent *E. faecalis* were prepared as described previously (29). Briefly, pCAM45 (1 µg) was electroporated into *E. faecalis* strains (2.5 kV, 200 Ohms, 25 µF) in 0.2 cm electroporation cuvettes. Cells were allowed to recover for 2 hours at 30°C in 1 ml SGM17MC recovery medium before plating on THB containing erythromycin (50 µg-ml^{-1}) and kanamycin (2000 µg-ml^{-1}). Plates were incubated for 48-72 hours and resulting colonies were grown in THB broth supplemented with erythromycin at 20 µg-ml^{-1}. Following overnight growth at 30°C, cells were diluted 1:100 into fresh THB and incubated for 2 ½ hours at 30°C and then shifted to 42 °C for 2 hours. After the 42°C incubation step, cells were serially diluted and plated onto pre-warmed THB plates containing 20 µg-ml^{-1} erythromycin and incubated at 42°C overnight to obtain the transposon mutant library.

Screen for inhibitory activity against V583:

To identify OG1RF and FA2-2 mutants that no longer possessed the inhibitory activity against V583, a growth inhibition bioassay was performed. Briefly, 250 µl of an overnight culture of V583 was suspended in 7.5 ml of THB soft agar. The soft agar suspension was overlaid onto THB agar containing either the OG1RF or FA2-2 transposon mutants plated at a density of 200-300 colonies per plate. Colonies that failed to exhibit inhibitory activity against V583 were picked onto a THB agar plate containing rifampicin at 100 µg ml^{-1} and fusidic acid at 50 µg ml^{-1} to select for either OG1RF or FA2-2 derivatives for further characterization.

Mapping of transposon integration sites:

To map the location of the transposon in the mutants identified in the V583 killing screen, we sequenced the flanking DNA by amplifying the region surrounding the transposon insertion via PCR with a primer specific to one end of the Himar 1 transposon (VT01) and an arbitrary primer (VT02) with a constant region at the 5′ end. In a second round of PCR, a specific nested primer (VT04) to the Himar 1 transposon was used with a primer specific to the constant region of the arbitrary primer (VT03). The resulting product was sequenced by using a third nested primer (VT05) specific to the transposon as described previously (30). Briefly, a colony from the transposon mutant was suspended in 15 µl of ddH_2O. Two microliters of the suspension was added to 48 µl of a PCR master mix containing 5 µl of 10 X Taq Buffer, 1 µl of 50 mM $MgCl_2$, 4 µl dNTP's at 2.5 mM, 1 µl primer VT01, 1 µl primer VT02, 35.8 µl of ddH_2O, and 0.2 µl of Taq polymerase (1 unit). Following the 1st round of PCR, products were diluted 1:25 and 5 µl of the diluted suspension was added to 45 µl of master mix containing: 5 µl of 10 X Taq Buffer, 1 µl of 50 mM $MgCl_2$, 4 µl dNTP's at 2.5 mM, 1 µl primer VT04, 1 µl primer VT03, 35.8 µl of ddH_2O, and 0.2 µl of Taq polymerase (1 unit). To sequence the resulting PCR products, primer VT05 was used.

Construction of *E. faecalis pptAB* in-frame deletion mutant:

In frame deletion of *pptAB* was performed using a plasmid derived from pLT06 (25). Briefly, flanking regions (~1Kb) from both the 5' and 3' ends of the *pptAB* ABC transporter were PCR amplified by using the primers listed in Table 2-3. For the construction of the pSV02 plasmid (*pptAB* deletion) the primers PptABP1 and PptABP2 were used to amplify the region 5' to the translation start site of *pptA* on the V583 genome. Primers PptABP3 and PptABP4 were used to

Primer Name	Sequence Information
PptAB-P1	5’ GTGTGTCAGGTAATTCAGCC 3’
PptAB-P2	5’ CATTTCACCAGACTTGACGTC 3’
PptAB-P3	5’ TTCTGGGTGCCTTAATTGTAG 3’
PptAB-P4	5’ TTGGTGATGACGAACAATCTC 3’
PptAB-Up	5’ GAAAGAGTCTCAGGCATAG 3’
PptAB-Down	5’ AAACGACCTTTACCTGAACC 3’
VT01	5’ TTAAGATACTGCACTATCAACACACTC 3’
VT02	5’ GGCCAC GCGTCGACTAGTACNNNNNNNNNNGTAAT 3’
VT03	5’ GGCCACGCGTCGACTAGTAC 3’
VT04	5’ CTTCGATAT CAAGGGCGAATTCGC 3’
VT05	5’ CGAGGGGTATCGCTCTTGAAGGG 3’

Table 2-3: List of oligonucleotides used in this study.

amplify the region 3’ to the translation stop site of *pptB*. The PptABP1 and PptABP2 region contained EcoRI and BamHI sites and PptABP3 and PptABP4 contained BamHI and SphI sites. Each product was cut with BamHI, religated and reamplified with primers PptABP1 and PptABP4 to obtain an amplicon deleted for the pptAB genes. This amplicon was digested with EcoRI and SphI and ligated into similarly digested pLT06. Insert and vector were ligated and electroporated into competent *E. coli* ElectroTen-Blue cells (Stratagene, La Jolla, CA). Constructs were screened by colony PCR and positive clones were further confirmed by restriction mapping and DNA sequencing. The plasmid construct, designated pSV02 containing the deletion construct for *pptAB*, was electroporated into electrocompetent *E. faecalis* FA2-2 or V583 cells (29). SV02 [V583Δ*pptAB*] and SV04 [FA2-2Δ*pptAB*] were generated following the protocol previously described (31). Mutants were confirmed by PCR using the primers PptABUp and PptABDown.

Complementation of *E. faecalis* FA2-2 and V583 in-frame *pptAB* deletion mutants:

The in-frame *pptAB* deletion mutant in FA2-2 and V583 were complemented by using a previously described pLT06 vector system based knock-in strategy (32). For the construction of the complementation vector pSV06, primers PptABP1 and PptABP4 were used to amplify a ~4Kb

region containing the *pptAB* gene and the ~1Kb flanking regions on either side, from the V583 genomic DNA. This product was then cut with EcoRI and SphI and ligated into pLT06 cut with the same enzymes. The remaining steps were done similar to the procedures described above. Strains possessing the complemented pptAB were designated SV06 [FA2-2Δ *pptAB*::*pptAB*] and SV18 [V583Δ *pptAB*::*pptAB*], respectively.

Cell aggregation Assay:

Clumping activity was assayed using a protocol modified from Mori et al. (33). *E. faecalis* OG1SSp cells containing the different pheromone responsive plasmids pCF10, pAM714 (A pAD1 plasmid derivative) and pAM378 (A pAM373 plasmid derivative) were used as the donor cells. *E. faecalis* FA2-2 wild-type, Δ*pptAB* [SV02], and complement strain [SV06] were used as the recipients. Briefly, the donor cells and the recipient cells were grown overnight at 37˚C in 2 ml of THB. 50 µl of each donor cell was added to 450 µl of either FA2-2, SV04, or SV06 and this mixture was added to 4.5 ml of fresh THB medium. The cultures were grown at 37˚C in a shaking incubator (~ 200 rpm) for 4 hours to visually compare the difference in clumping phenotype.

Transconjugation assay:

Transconjugation efficiency was calculated using a mating assay modified from Mori et al. (33). After incubating the donor and recipient cells together for 4 hours under shaking condition [200 rpm], the cell cultures from the different plasmid systems were serially diluted in sterile PBS and track-diluted (34) onto THB containing rifampicin 100 µg-ml^{-1} and tetracycline 15 µg-ml^{-1} (for pCF10 and pAM378 transconjugants) or THB containing rifampicin 100 µg-ml^{-1} and erythromycin 10 µg-ml^{-1} (for pAM714). The cultures were also track-diluted onto THB plates containing spectinomycin at 500 µg-ml^{-1}, and streptomycin at 500µg-ml^{-1} to select for donor cells in all three

plasmid systems. Transconjugation efficiency was measured as the ratio of transconjugants to donor cells. For the exogenous addition of cAM373 and cCF10, 1mM stock solution of chemically synthesized cAM373 was prepared in 50% acetonitrile solution and 1mM stock of cCF10 was prepared in dimethyl formamide and these were serially diluted to obtain the working concentrations. Working stocks were added to the transconjugation reactions as indicated in Figure 2-7.

Confocal Biofilm analysis:

Confocal laser scanning microscopy (CLSM) was performed on 1-day-old biofilms as described previously (21). *E. faecalis* strains SV02 and SV18 were transformed with pMV158GFP to generate SV20 and SV23, respectively, as both these strains expressed GFP constitutively. VT09 [V583(pMV158GFP)] (21) along with SV20 and SV23 were used for confocal microscopy analysis. Briefly, biofilms were grown on sterile glass coverslips placed in six-well tissue culture plates. 5ml of TSB containing tetracycline was added to the wells containing the coverslip for plasmid maintenance. After 24 h of growth, the biofilms were gently washed with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]) and stained with 1 μM SYTOX orange (Invitrogen) for 6 to 7 min. The coverslips were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using a Zeiss LSM 5 Pa laser scanning confocal microscope.

Growth Curve analysis:

Standard growth curve analysis was performed to ensure that SV04 [Δ*pptAB*] and SV06 [*pptAB* complement] did not exhibit growth defects relative to the parental FA2-2 strain. Briefly, FA2-2, SV04 and SV06 were grown in THB overnight at 37˚C. The overnight cultures of these strains

were inoculated into 50ml tubes containing sterile THB at a 1:100 ratio and were incubated at 37 ˚C. Samples from each tube were aseptically withdrawn every hour and the optical density was measured at a wavelength of 600nm through stationary phase growth (~ 8 hours).

Skim milk plate Assay:

Skim milk plate assay was performed by spotting 5 µl of an overnight culture of FA2-2, OG1RF, OG1RF::ef0689a and OG1RF::ef0689b onto a THB plate containing 1.5% skim milk. The plate was incubated overnight at 37 ˚C and the zone of clearance was observed.

RESULTS:

Transposon screen identified mutants with altered activity at inhibiting V583:

With the goal of identifying factors that contributed to the pheromone-mediated killing of V583, a transposon mutagenesis screen was performed to identify genes whose protein products contribute to the inhibition of *E. faecalis* V583. A recent study by Gilmore *et al.* (PNAS submitted) identified the peptide pheromone, cOB1, as being responsible for the growth inhibition of strain V583. We anticipated that mutants discovered in such a screen would identify factors important in the synthesis of the cOB1 peptide pheromone. To address possible strain differences that might contribute to pheromone-mediated killing, we subjected strains *E. faecalis* strains FA2-2 and OG1RF to transposon mutagenesis using the Himar transposon in plasmid pCAM45 (27). Previous studies with OG1RF demonstrated a role for GelE in the turnover of peptide pheromones (35), and strain FA2-2 possesses a deletion that encompasses most of the *fsr* quorum sensing system and renders it less proteolytic than OG1RF (36). Transposon mutants from both strains were screened

for the ability to inhibit the growth of strain V583. In total, ~ 30,000 mutants were screened from both libraries, and 10 unique transposon insertions localizing to 7 predicted gene products were identified as displaying defects in peptide-mediated killing of V583 (Fig. 2-1 and Table 2-4).

Transposon mutant library background	Gene disrupted (No. of transposon hits, Unique Hits)	Predicted function
FA2-2	***ef0688*** **(2, 1)**	**ATP-Binding Protein**
FA2-2	***ef2470*** **(2, 1)**	**Hypothetical Protein**
FA2-2	***ef0252*** **(11, 2)**	**N-acetylmuramoyl-L-alanine amidase**
FA2-2	***ef2556*** **(1, 1)**	**Putative Cation Transporter**
FA2-2	***ef62_1081*****(3, 2)***	**Conserved Hypothetical Protein**
OG1RF	***ef0689*** **(2, 2)**	**EcsB Family ABC Transporter**
OG1RF	***ef1723*** **(1, 1)**	**Lipoprotein Signal Peptidase**

* Gene locus only found in the annotated *E. faecalis* 62 genome, all the other gene loci are reported based on sequence similarity to V583 gene designations for the genes disrupted in FA2-2 and OG1RF.

Table 2-4: Transposon gene disruption targets.

Predictably, we identified a transposon insertion in the OG1RF library that localized to EF1723, a predicted lipoprotein signal peptidase (LspA), thought to be responsible for cleaving lipoproteins at the conserved cysteine in the lipobox domain (37). Additional mutants were identified with insertions in EF0252, a predicted CHAP-domain containing amidase, a cation transporter (EF2556) as well as two predicted hypothetical proteins (EF2470 and EF62_1081). Our attention was focused on insertions that were confined to a predicted ABC transporter, as independent transposon insertions localized to either the putative ATP-binding cassette (EF0688) or the

predicted permease (EF0689) of the ABC transporter in both the FA2-2 and OG1RF transposon libraries.

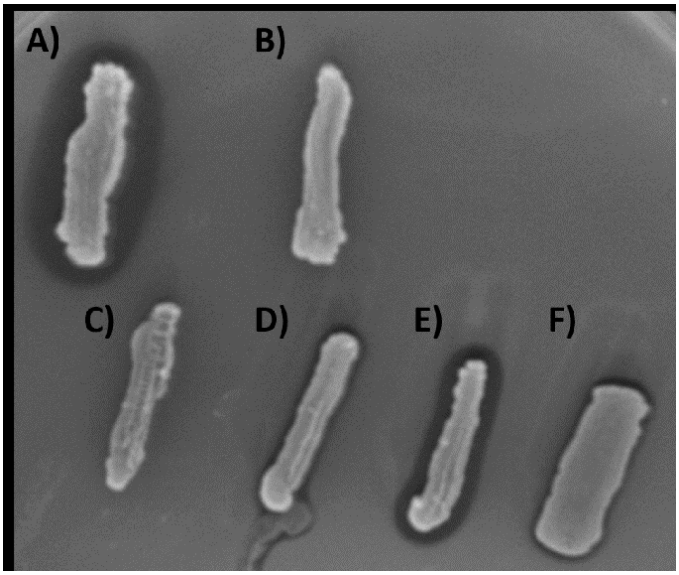


Figure 2-1: Growth Inhibition Assay. A) FA2-2 B) FA2-2::*ef0688* C) OG1RF::*ef0689a* D) OG1RF::*ef1723*(*lspA*) E) OG1RF F) OG1RF::*ef0689b*. Strains were streaked onto THB agar and grown overnight at 37°C, and then the indicator strain V583 was added in a soft agar overlay. Zones of clearance around the strains are indicative of growth inhibition of V583.

EF0688 shares 62% sequence identity (82% sequence similarity) with the *Bacillus subtilis* EcsA, a predicted ATP-binding cassette containing protein. EF0689 shares 30% sequence identity (52% sequence similarity) with the *B. subtilis* EcsB, a predicted ABC transporter permease. In *B. subtilis*, EcsAB has been shown to play an important role in exoenzyme secretion (E), competence (C) and sporulation (S) (16).

Disruption of the permease encoded by EF0689 does not alter exoprotease production in *E. faecalis*:

E. faecalis is not known to be naturally competent and lacks the sporulation machinery. We tested whether this transporter was involved in exoenzyme secretion in *E. faecalis*, as an *ecsAB* mutant in *Staphylococcus aureus* also resulted in changes to its secretome (38). Since strain OG1RF possesses a functional Fsr system and actively secretes the only known exoproteases (gelatinase and serine protease) in a quorum-dependent manner, we examined the proteolytic activity of the parental strain and the two independent transposon insertion mutants in the *ecsB* homolog, *ef0689*, which we now refer to as *pptB*.

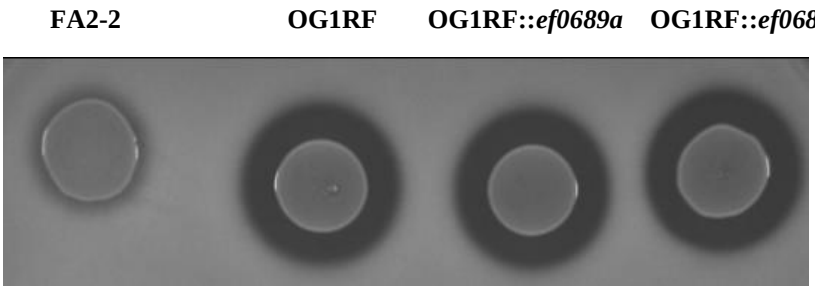


Figure 2-2: Milk Protease Assay. 5µl of FA2-2; OG1RF; OG1RF::*ef0689a*; OG1RF::*ef0689b* overnight cultures were spotted onto THB agar medium containing 1.5% skim milk. The plate was incubated overnight at 37˚C. Zones of clearance around the spotted cultures are indicative of protease activity.

As noted in figure 2-2, there was no apparent change in the level of proteolytic activity between the strains, suggesting that the ABC transporter does not affect exoprotease secretion in *E. faecalis.* In contrast, the known Fsr-deficient strain FA2-2 produces basal protease activity on skim milk agar. As previously stated, gelatinase has been shown to cleave peptide pheromones and this

activity reduces the mating efficiency between donor and recipient strains (35). In addition, gelatinase is also responsible for the proteolytic activation of the major autolysin, AtlA, and this leads to targeted lysis of bystander cells in a process termed fratricide (39). To both confirm the transposon mutant phenotype as well as generate a markerless mutation, we generated an in-frame deletion of both *ef0688* (*pptA*) and *ef0689* (*pptB*) in the FA2-2 strain background to minimize the contribution of gelatinase in subsequent assays. This mutant was designated FA2-2Δ*pptAB.*

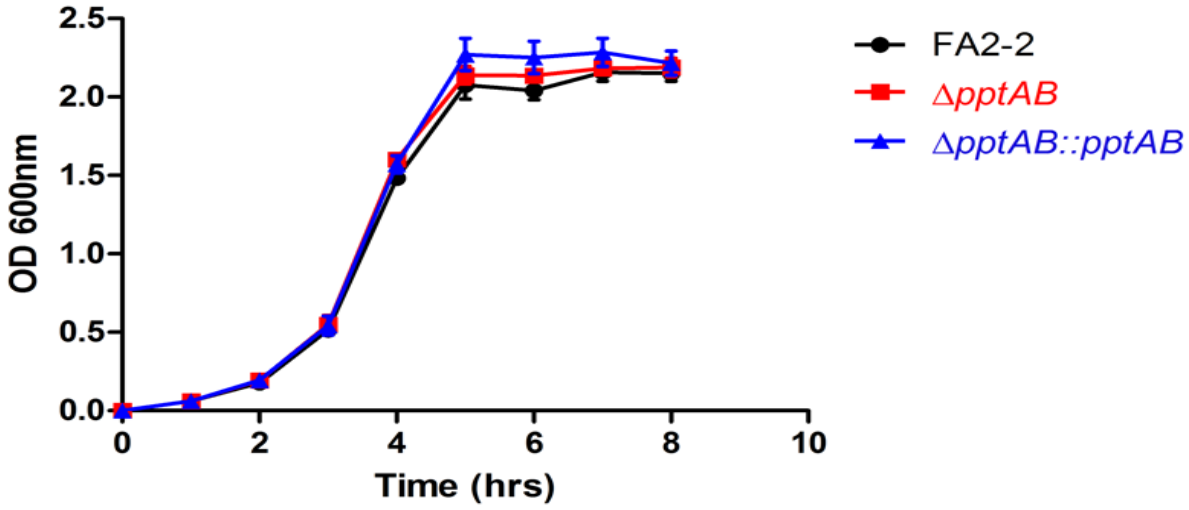


Figure 2-3: Bacterial growth curves. *E. faecalis* strains FA2-2, FA2-2Δ*pptAB* and FA2-2Δ*pptAB*::*pptAB* were grown under static conditions in THB broth at 37°C and samples were analyzed by optical density absorbance (OD_{600}).

Additionally, we created a complemented strain in which *pptAB* was genetically restored to its native locus, using a gene knock-in strategy (32), and this strain was designated FA2-2Δ*pptAB*::*pptAB*. We compared the growth of the FA2-2 parental strain along with the *pptAB*

deletion mutant and the complement in Todd-Hewitt broth and found that the growth for all three strains were nearly identical throughout the growth cycle (Fig. 2-3)

cOB1 is not detected in the culture supernatant of the *pptAB* deletion mutant:

To confirm that the lack of inhibition of V583 by the FA2-2Δ*pptAB* strain was due to the absence of secreted cOB1, we analyzed the culture supernatants from both FA2-2 and the *pptAB* deletion mutant for the presence of cOB1.

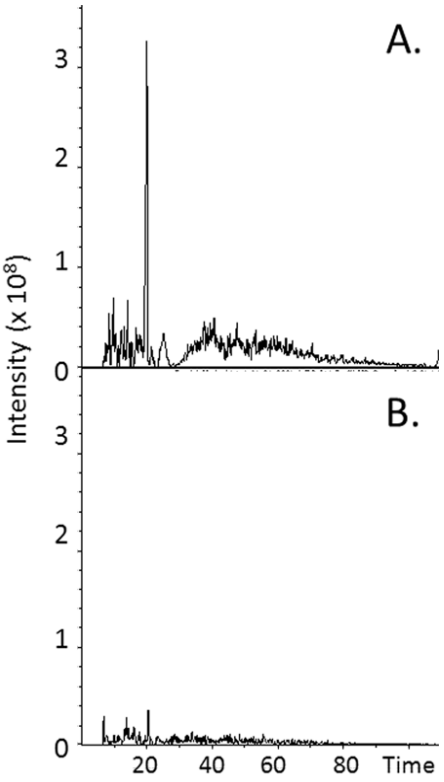


Figure 2-4: Analysis of the supernatant to identify c0B1. FA2-2 (A) and FA2-2Δ*pptAB* (B) were grown in chemically defined medium (CDM) overnight. Spent culture supernatants were analyzed by High Pressure Liquid Chromatography (HPLC). The peak present in panel A shows the relative amount of cOB1 eluted between 17-19 minutes. The time scale is in minutes. Experiment performed by Dr. John M. Tomich.

Strains were grown in complete defined medium (CDM) (40) to reduce the complexity of detecting a short hydrophobic peptide in rich lab medium. Spent culture supernatants from overnight cultures were harvested for HPLC analysis to identify the presence of cOB1. As expected, we were unable to detect cOB1 in the supernatant from the *pptAB* mutant, whereas detectable cOB1 signal was observed in the parental FA2-2 (Fig. 2-4).

The Δ*pptAB* mutant fails to aggregate in clumping assays with donor cells possessing a pheromone responsive plasmid:

The inability to detect cOB1 in the supernatant of a *pptAB* mutant suggested that the PptAB ABC transporter might be a global pheromone exporter. A hallmark of pheromone-mediated conjugation in broth matings is the formation of cellular aggregates or "clumps", and is indicative of a co-aggregation event between donor and recipient cells, recently reviewed by Clewell et al. (9). Plasmid-bearing donor cells respond to the presence of peptide pheromones produced by the recipient to induce the expression of conjugation machinery that also includes aggregation substance. Clumping occurs when aggregation substance on the donor cell binds to binding substance on the recipient to facilitate mating bridge formation and subsequent transfer of the plasmid from donor to recipient. The cell aggregation assay was performed using FA2-2, FA2-2Δ*pptAB*, and the complement strain FA2-2Δ*pptAB*::*pptAB* as recipient cells and OG1SSp (pCF10) as the donor cell. The transconjugation mixture containing the parental strain FA2-2 and the complement strain showed visible clumping with the OG1SSp (pCF10) donor while Δ*pptAB* mutant failed to induce detectable clumping (Fig. 2-5), suggesting that the peptide pheromone transporter is also essential for the secretion of other peptide sex pheromones, in addition to cOB1.

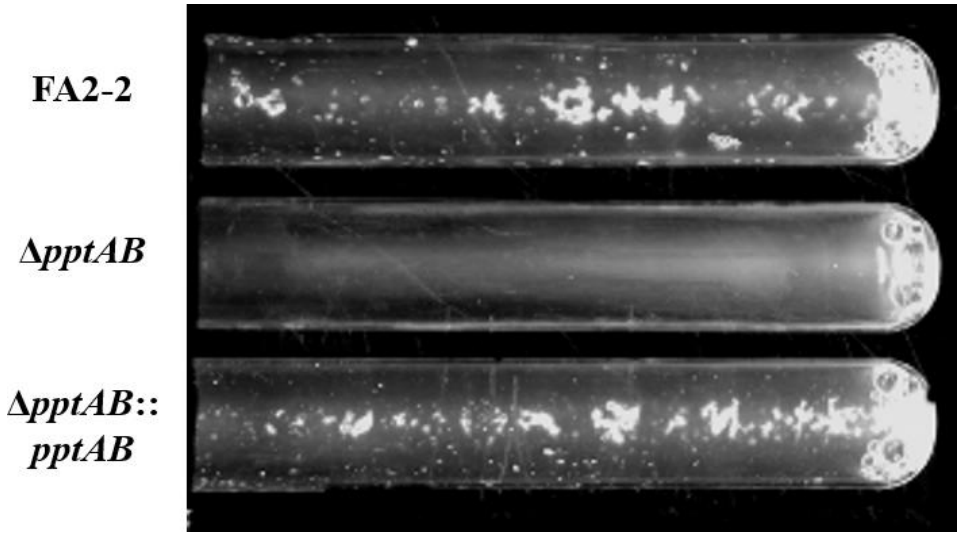


Figure 2-5: Clumping assay using FA2-2, FA2-2Δ*pptAB,* FA2-2Δ*pptAB::pptAB* as recipients. The OG1SSp donor cells harbored the pCF10 plasmid. After the donors and recipients were mixed and incubated for 4hrs under rigorous shaking conditions (200 rpm), the tubes were placed horizontally to visualize the extent of cell clumping and photographed.

Mating efficiency is significantly attenuated by deletion of the PptAB transporter:

Since the *pptAB* mutant showed no visible clumping in the cell aggregation assay, we examined whether the mating efficiency between the donor and recipient cells was affected in a broth mating assay and extended the observations to 3 unique and well-characterized pheromone responsive plasmid systems (pCF10, pAD1 and pAM373). The broth mating assay (33) involved the OG1SSp donor cell harboring either pCF10, pAM714 (23) and pAM378 (24) and recipient cells: FA2-2, FA2-2Δ*pptAB*, FA2-2Δ*pptAB*::*pptAB,* and as a control FA2-2Δ*eep* (20). The cells from each transconjugation mixture were serially diluted and plated onto medium containing antibiotics to select for transconjugants and onto to separate medium to select only for donors in order to calculate the transconjugation efficiency [# of transconjugants / # of donors]. The results of the assay are shown in Figure 2-6 and Table 2-5.

Cell Strain	OG1SSp(pCF10)[§]	OG1SSp(pAM714)[§]	OG1SSp(pAM378)[§]
FA2-2	$5.3X10^{-1} \pm 3.6X10^{-1}$	$2.3X10^{-1} \pm 1.2X10^{-1}$	$3.1X10^{-1} \pm 1.3X10^{-1}$
FA2-2Δ*pptAB*	$3.7X10^{-7} \pm 0.8X10^{-7}$	$1.0X10^{-6} \pm 0.2X10^{-6}$	$1.2X10^{-6} \pm 0.3X10^{-6}$
FA2-2Δ*pptAB*::*pptAB*	$9.3X10^{-1} \pm 7.5X10^{-1}$	$0.4X10^{-1} \pm 0.3X10^{-1}$	$1.2X10^{-1} \pm 0.2X10^{-1}$
FA2-2Δ*eep*	$5.0X10^{-4} \pm 4.5X10^{-4}$	$1.3X10^{-3} \pm 0.2X10^{-3}$	$8.6X10^{-1} \pm 8.0X10^{-1}$

Table 2-5: Transconjugation efficiency.

We observed that in all three plasmid systems, the mating efficiency of the Δ*pptAB* mutant was significantly impaired, displaying a 5-6 log reduction in mating efficiency compared to the parental. The complemented strain was restored to wild-type levels in all 3 systems, whereas the *eep* mutant exhibited a 2-3 log reduction as compared to the wild-type with plasmids pCF10 and pAM714. In contrast, the *eep* mutant did not exhibit a significant change from wild-type levels with the pAM378 plasmid, which is consistent with a previous report (14) that demonstrated a role for Eep in the generation of the cCF10 and cAD1 peptide pheromones, but not cAM373.

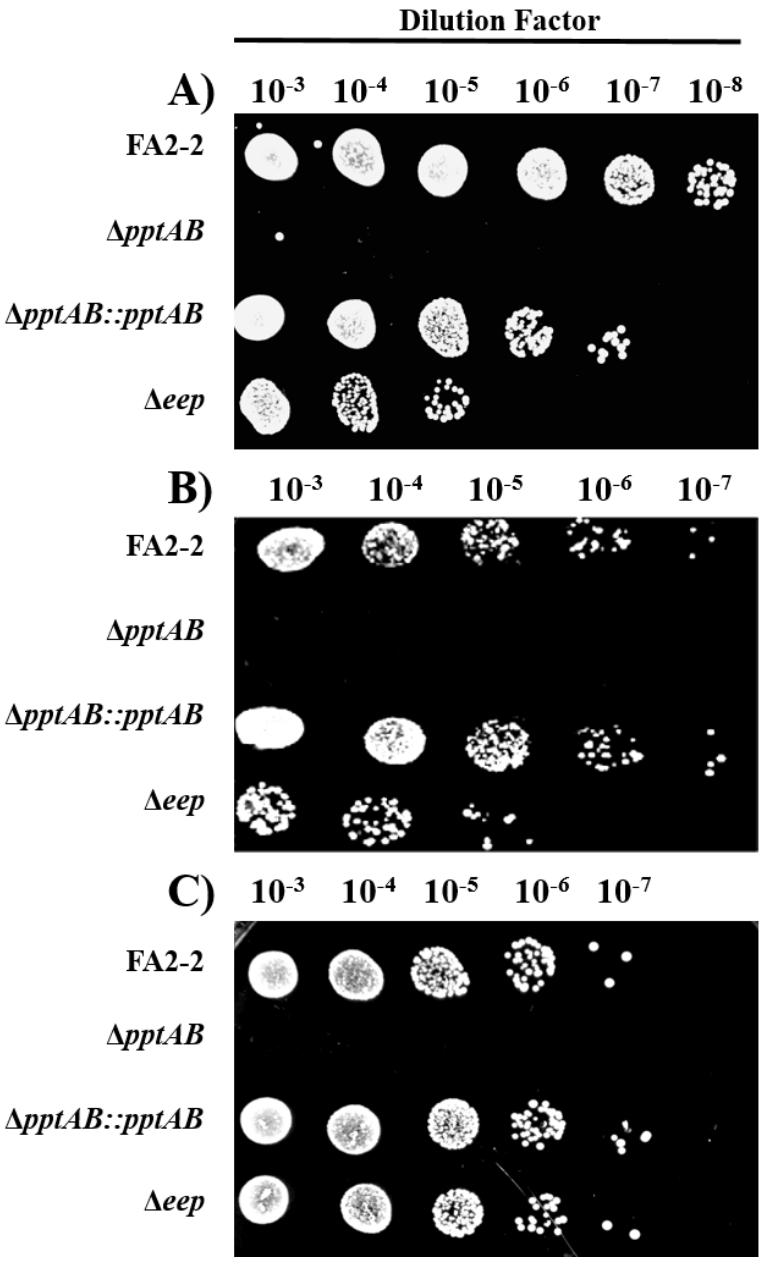


Figure 2-6: Recipient strains FA2-2, FA2-2Δ*pptAB*, FA2-2Δ*pptAB::pptAB*, and FA2-2Δ*eep* were mixed with the donor strain OG1SSp possessing the indicated pheromone responsive plasmid: A) pCF10 B) pAM714 and C) pAM378. After mixing, donors and recipients were incubated for 4hrs under rigorous shaking conditions. Following a series of 10-fold dilutions, 5µl of diluted culture was spotted onto plates with the appropriate antibiotics to select for transconjugants. Plates were incubated at 37°C overnight and photographed.

Exogenous Addition of Purified Sex Pheromone Complements for the Absence of the Transporter:

To determine whether the *pptAB* mutant was defective in cell aggregation assays and mating assays solely due to its inability to secrete peptide sex pheromones, we sought to complement the *pptAB* mutant phenotypes using synthetic pheromones.

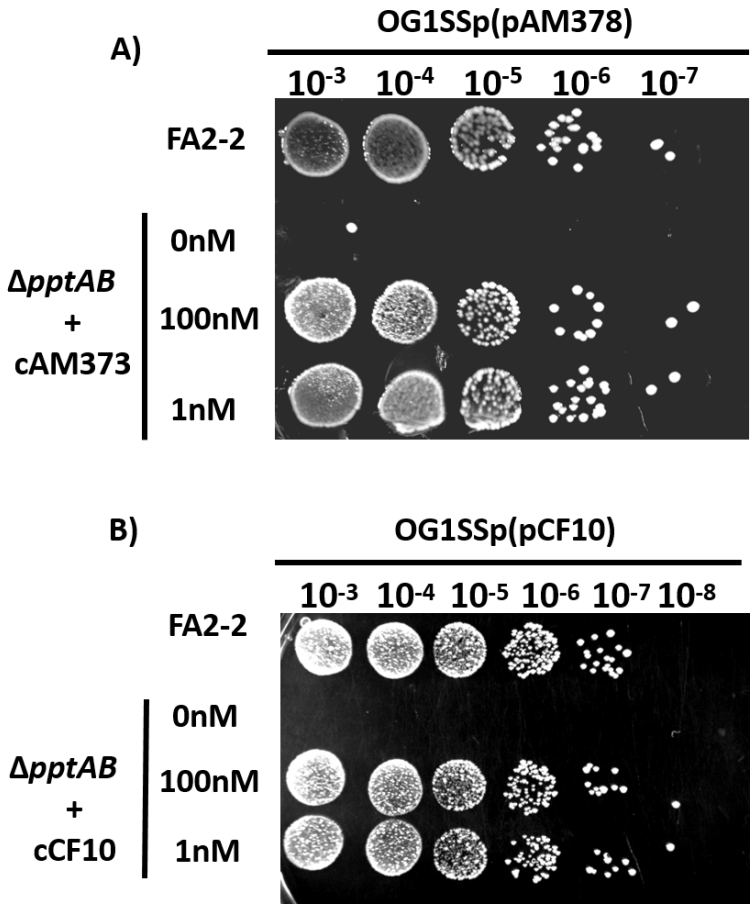


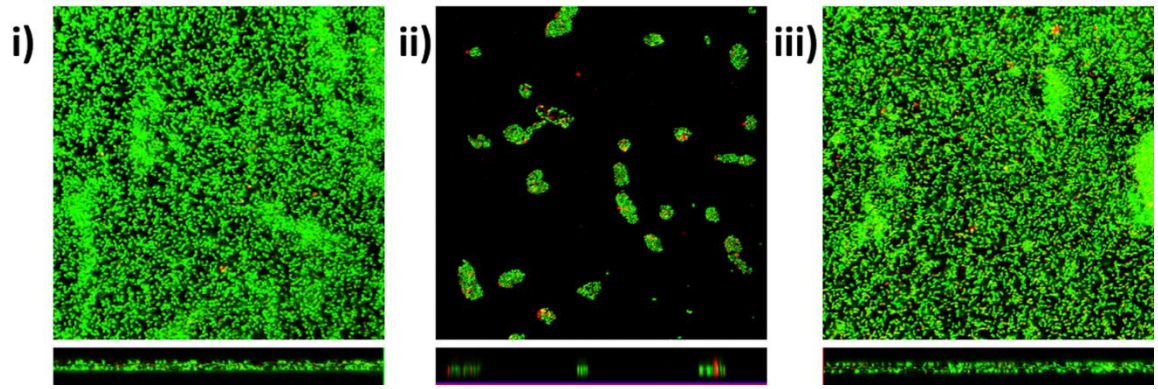
Figure 2-7: Exogenous addition of purified A) cAM373 and B) cCF10 restores mating defect of the *pptAB* mutant. Purified cAM373 and cCF10 at the concentrations indicated was added to the transconjugation mixture in which FA2-2Δ*pptAB* was used as a recipient. OG1SSp(pAM378) and OG1SSp(pCF10) were used as donors. FA2-2 parental strain was also used as a control recipient strain. 10 µl of all reactions were serially diluted and spotted on THB agar plates containing rifampicin at 10 µg ml^{-1} and tetracycline at 10 µg ml^{-1} to select for transconjugants.

Cell aggregation assays and mating assays were performed as described above wherein OG1SSp (pAM378) and OG1SSp (pCF10) were used as the donors and FA2-2 or FA2-2Δ*pptAB* was used as the recipient. Synthetic cAM373 and cCF10 were exogenously added to the corresponding reaction tubes containing FA2-2Δ*pptAB* recipient cells at varying concentration (1000 nM, 100nM and 1nM) and clumping was monitored. After 4 hours the cultures were plated to determine the mating efficiency. The results of these assays are shown in Figure 2-7. The exogenous addition of purified cAM373 or cCF10 restored the clumping and mating defects of FA2-2Δ*pptAB* recipient cells in the cognate pheromone responsive plasmid systems demonstrating that the inability of the transporter mutant to actively secrete pheromones is the basis for the observed mating defects in this mutant.

Deletion of the Peptide Pheromone Transporter affects biofilm formation:

Biofilm formation is a coordinated process that generally involves cell-cell communication. Since peptide pheromones are involved in direct cell-cell signaling, we hypothesized that the deletion mutant lacking the peptide pheromone transporter would display altered biofilm architecture compared to the parental strain. To test this hypothesis, we engineered the clinical isolate *E. faecalis* V583 to delete *pptAB* as previous studies showed that it was a better biofilm former than FA2-2 (21, 41). We also complemented the V583Δ*pptAB* mutant by restoring the *pptAB* genes back to their native locus using the gene knock-in strategy (32). To monitor biofilm development, plasmid pMV158GFP was introduced into each strain to provide constitutively expressed GFP (26). Confocal image analysis was performed on 1-day biofilms formed by V583, V583Δ*pptAB*, and the complemented strain V583Δ*pptAB*::*pptAB*. The results are shown in Figure 2-8(A)-2-8(B).

A)



B)

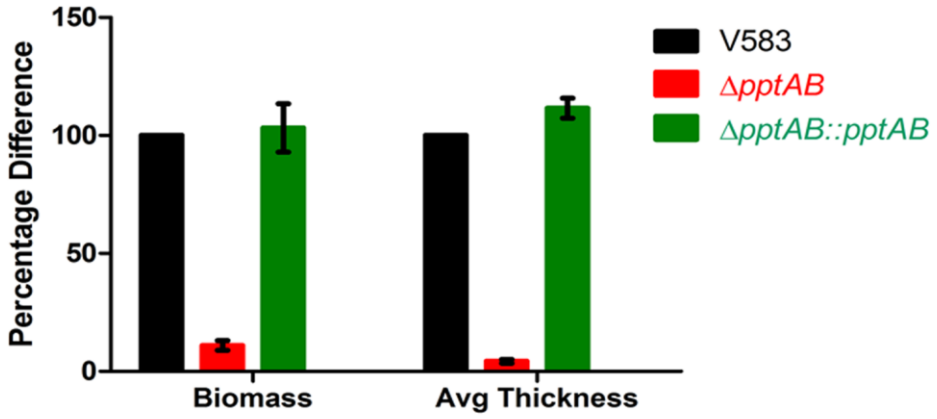


Figure 2-8: Confocal microscopy biofilm analysis was performed on 1-day old biofilms (A) i) V583 ii) V583Δ*pptAB* and iii) V583Δ*pptAB*::*pptAB*. All the strains contain the plasmid pMV158GFP, which provides constitutive expression of GFP. (B) Comstat image analysis of biofilms depicted in panel A.

Consistent with our hypothesis, the *pptAB* transporter mutant displayed a non-confluent biofilm phenotype compared to the parental and complement strains. By COMSTAT analysis (42) (Fig. 2-8(B)), the average biofilm thickness and overall biomass were reduced by ~ 90% in the *pptAB* mutant compared to V583 and the complement.

DISCUSSION:

In this study we have shown that the EcsAB homolog in *E. faecalis*, which we have renamed PptAB to reflect its apparent global role in peptide pheromone secretion, is important in both the well-known cell-cell communication events in plasmid conjugation, as well as biofilm development. The initial studies involving EcsAB postulated that this transporter might be involved in clearing the membrane from the accumulation of peptide signals, but the nature of the transported substrate was unknown (16, 38). Here we have shown for the first time that an important substrate of the ABC transporter is the peptide signals used in pheromone mating in *E. faecalis*. The pathway by which lipoproteins are processed leading to the production and release of pheromones is illustrated in Fig. 2-9. It is known that peptide sex pheromones in *E. faecalis* are derived from lipoprotein precursors (43, 44). The lipoprotein precursors are thought to be secreted in Gram-positive bacteria through a Sec-dependent pathway (45). It is also known that the prolipoprotein diacylglyceryl-transferase (Lgt) is important in lipoprotein membrane anchorage in *E. faecalis* (46) and the lipoprotein signal peptidase (LspA), which is a type II signal peptidase is important for N-terminal processing of lipoproteins (47). In the present study, our transposon screen confirmed a role for LspA in the production of cOB1. An et al. (14) showed that in addition to these upstream processing events of the lipoprotein precursor, Eep also enhanced recovery of the native pheromone. Our data extend this model further to show that the secretion of the peptide pheromones is dependent on active transport mediated by PptAB.

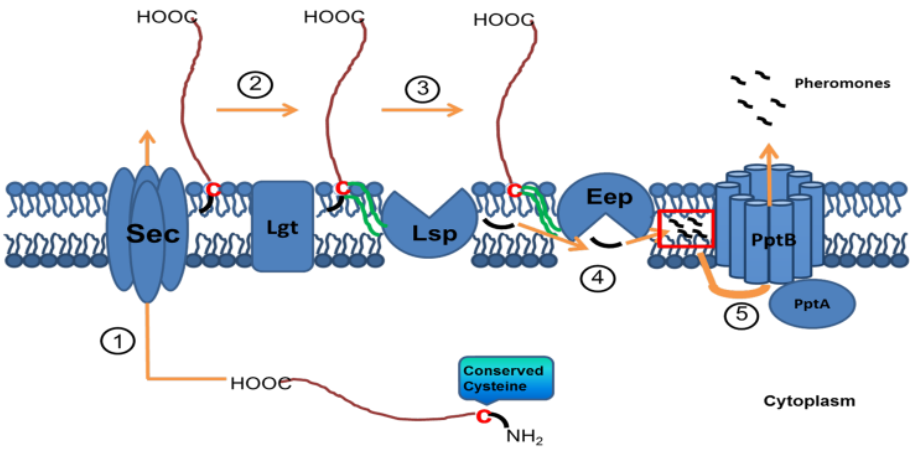


Figure 2-9: Mechanism of peptide sex pheromone production and secretion in *Enterococcus faecalis*. **1)** Lipoprotein produced in the cytoplasm is exported out through the secretory apparatus. **2)** Lgt diacylates the lipoprotein at the conserved cysteine (Indicated by C) and anchors it to the membrane. **3)** Lsp, a signal peptidase-2 cleaves the N-terminal overhang from the conserved cysteine. **4)** Eep, a membrane embedded zinc-metalloprotease further degrades the N-terminal overhang to produce pheromones. **5)** PptAB, the peptide transporter actively transports these pheromones outside the cell.

This is supported by two important observations 1) the addition of exogenous peptide restores the mating deficiency when the *pptAB* mutant is used as the recipient and 2) the peptide pheromone transporter mutant can function as a donor and efficiently transfer a pheromone responsive plasmid to a recipient suggesting that the inability to secrete peptide sex pheromones does not affect this mutant's ability to function as a pheromone responsive donor (Table 2-6). It is important to note that an *eep* deletion mutant shows only a partial attenuation in mating efficiency compared to the *pptAB* deletion mutant. This could possibly be due to the presence of a secondary protease that might be compensating for the activity of Eep, as was observed with LspA and Eep in *Streptococcus uberis* (48). The other possibility is that the peptide pheromone transporter might still be exporting the pre-pheromones with less efficiency compared to the processed pheromone,

and that these pre-pheromones may retain biological activity. Confirming these hypotheses is the current focus of ongoing studies in the lab.

Cell Strain (Donor→Recipient)	pCF10[§]	pAM714[§]	pAM378[§]
FA2-2→OG1SSp	$1.33X10^{-3} \pm 2.06X10^{-3}$	$6.82\ X10^{-4} \pm 1.99X10^{-4}$	$8.71\ X10^{-4} \pm 5.5X10^{-4}$
FA2-2Δ*pptAB*→ OG1SSp	$1.21X10^{-2} \pm 0.95X10^{-2}$	$8.12X10^{-2} \pm 5.98X10^{-2}$	$5.39X10^{-3} \pm 1.34X10^{-3}$

Table 2-6: Transconjugation efficiency with FA2-2Δ*pptAB* as a donor.

A recent study showed that peptide pheromones mediate biofilm formation in *Streptococcus pyogenes* by interacting with the Rgg family of transcriptional regulators (49). A study by Shelburne et al. (50) identified the secretion signal sequence from the *S. pyogenes* protein Vfr as a peptide pheromone that coordinates the activity of another Rgg family member, the RopB repressor. Recent work by Serror's group (51) identified an Rgg-homolog in *E. faecalis*, which they termed ElrR, as it was shown to positively regulate the expression of the enterococcal leucine-rich protein ElrA, but whether ElrR also regulates biofilm development is not presently known. It is possible that unidentified peptide pheromones of *E. faecalis* regulate biofilm formation, but whether those sequences derive from lipoprotein signal sequences, other secretion signal sequences or additional SHP-like sequences awaits further study to determine if PptAB is directly involved in the secretion of these peptides. Deciphering the link with PptAB and biofilm formation will be valuable in understanding the role of cell-cell communication in *E. faecalis* biofilm

development. Bioinformatic analysis revealed that the peptide pheromone transporter is highly conserved across the pathogenic Firmicutes (Fig. 2-10). Pheromone mediated transfer of genetic material is a hallmark of enterococcal biology allowing the acquisition of a variety of antibiotic resistance traits, making them a more persistent pathogen in the hospital setting (52), which may also disseminate resistance to other problematic pathogens. Studies have shown that clinical isolates of *Staphylococcus aureus* obtained vancomycin resistance gene via the conjugative transposon Tn1546 present in the co-infecting *E. faecalis* isolate (53, 54). This is attributed to the fact that *S. aureus* produces a pheromone, derived from a lipoprotein signal sequence, which is known to mimic the enterococcal pheromone cAM373, enabling exchange of genetic information between these two species (5). Recently, a genetic determinant in *Streptococcus gordonii* encoding a predicted lipoprotein was also identified as a precursor for a peptide pheromone resembling cAM373, which enables intergeneric DNA transfer between *E. faecalis* and *S. gordonii* (55). It is also interesting to note that peptide signaling has been employed by other gram-positives to perform a variety of cellular functions including competence, sporulation and biofilm development. In *B. subtilis*, Phr pentapeptides have been shown to be important for competence and sporulation initiation (56). In naturally transformable *Streptococcus mutans*, SigX-regulated genes are involved in the competence cascade, and SigX is regulated by ComR. ComR is a transcriptional regulator of the Rgg family, which is known to bind peptides derived from ComS (XIP). The XIP peptides are thought to be exported through an unidentified membrane transporter (57). More recently, Chang et al. showed that SHP pheromones, which affect virulence in *S. pyogenes* by binding Rgg, are processed by Eep, and are transported outside the cell by an unknown transporter (49).

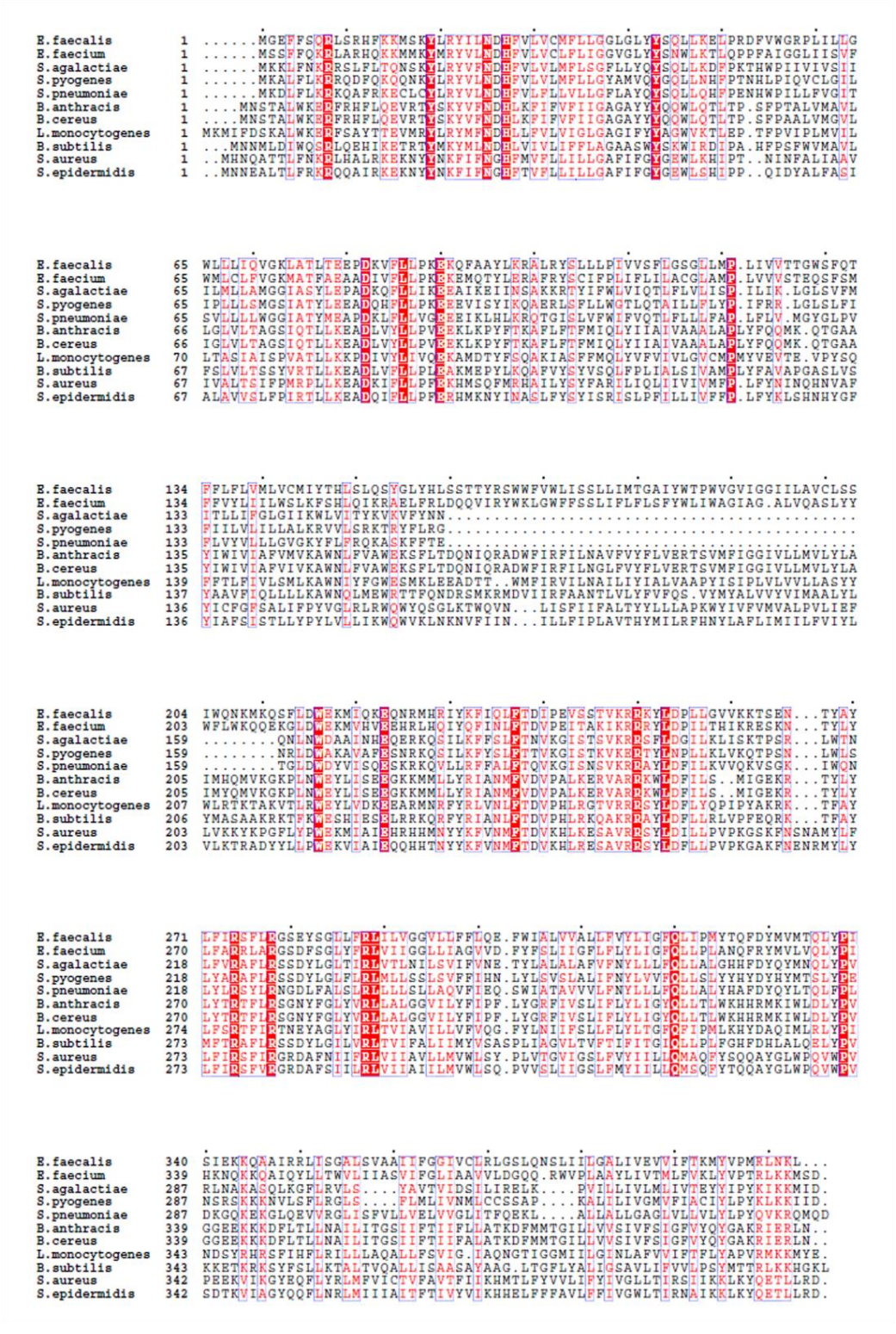


Figure 2-10: Multiple sequence alignment of PptB (EcsB) homologs from various pathogenic *Firmicutes*. Amino acids highlighted in red are conserved across all the above bacterial species.

Phr, XIP and SHP pheromones are hydrophobic in nature, as are the peptide pheromones that mediate conjugation in *E. faecalis*. Based on the observations we report in this study and the conservation of the PptAB (EcsAB) ABC transporter among pathogenic gram-positives, it is coherent to speculate that this transporter might be serving the same important peptide secretory function in these pathogenic species as in *E. faecalis*. Further characterization of this conserved transporter in these pathogenic bacteria is warranted.

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CHAPTER 3

DISCOVERY OF A SECOND OLIGOPEPTIDE PERMEASE COMPLEX IN *Enterococcus faecalis*

ABSTRACT:

Exchange of genetic information in enterococci occurs through the process of conjugation wherein a donor cell harboring a pheromone responsive plasmid responds to short (7-8 amino acid) predominantly hydrophobic peptide pheromones produced by the recipient cell. The oligopeptide permease (*opp*) operon encodes for proteins that aid in the import of these pheromones into the donor cell (1). Using a bacterial killing assay dependent on the presence of the sex pheromone cOB1, we show that a V583Δ*opp1* mutant only displays partial resistance to the inhibitory peptide pheromone cOB1 compared to the wild-type. A database search of the V583 genome identified a previously uncharacterized operon predicted to encode proteins with functions similar to oligopeptide permeases. Deletion of this second operon, which we termed *opp2*, in V583 also conferred partial resistance to cOB1. In contrast, the double mutant strain (Δ*opp1*Δ*opp2*) was completely resistant to cOB1 suggesting that the two Opp complexes are functionally redundant. Individual *opp* mutants exhibited only a minor decrease in mating efficiency, whereas the double Opp- mutant strain was highly attenuated as a donor in transconjugation assays involving distinct pheromone responsive plasmid systems. Confocal image analysis on 1-day biofilms revealed a significant decrease in the ability of the double Opp- mutant to form confluent biofilms compared to the wild-type suggesting the importance of peptide pheromone transport in biofilm formation. The *opp* double mutant and a deletion mutant (*pptAB*) for a previously reported ABC transporter involved in peptide pheromone secretion were also significantly attenuated in a *Caenorhabditis elegans* model of infection compared to the wild-type, indicative of a possible role played by these peptide transporters in virulence.

INTRODUCTION:

Enterococcus faecalis is an opportunistic pathogen that has emerged among the vanguard of multi-drug resistant microorganisms. Peptide pheromones play critical roles in cell-cell communication by contributing to the development of increased virulence and antibiotic resistance in *Enterococcus faecalis* (2-6) through induction of horizontal gene transfer (7). This is predominantly due to the fact that pheromone responsive plasmids which aid the donor cells in responding to the pheromones secreted by the recipient cells, are known to harbor antibiotic resistance genes as well as known virulence factors including the cytolysin and aggregation substance (8, 9). Recent data provide additional evidence that pheromone mediated transfer of antibiotic resistance also occurs at an inter-species level (10, 11). *Staphylococcus aureus* (11) and *Streptococcus gordonii* (10) have been shown to produce pheromones to which *E. faecalis* responds and this results in the transfer of the pheromone responsive plasmid pAM373 to these recipients. A number of factors are involved in the process of conjugation and the functional role of many of the plasmid-encoded gene products in *E. faecalis* have been delineated (12-14).

A study by Leonard *et al.* (1) showed that *E. faecalis* donor cells harboring the pheromone responsive plasmid pCF10 respond to the recipient cells by actively importing the cognate peptide sex pheromone cCF10 via the oligopeptide permease (Opp) complex (EF_0907 - EF_0912). The Opp complex consists of five proteins, including two transmembrane proteins (OppB and OppC), two ATP binding proteins (OppD and OppF), and a cell membrane anchored lipoprotein (OppA) which binds to pheromones with high affinity to direct the pheromone into the donor cell. It is also important to note that the pheromone responsive plasmids encode OppA homologs (PrgZ or TraC) that bind to their cognate pheromones with much higher affinity compared to OppA and hijack the chromosomally encoded Opp system to import these pheromones into the cell (1, 15, 16). We took

advantage of the fact that the peptide sex pheromone cOB1 inhibits the growth of *E. faecalis* strain V583 to determine whether a V583 strain lacking the aforementioned Opp complex was susceptible to the inhibitory effect of this pheromone (M. S. Gilmore, M. Rauch, M. Ramsey, P. Himes, S. Varahan, F. Lebreton, and L. E. Hancock, submitted for publication) (17). Interestingly, V583Δ*opp1* was only partially resistant to cOB1-mediated growth inhibition. This led us to hypothesize that *E. faecalis* might possess an additional oligopeptide permease complex. Careful examination of the *E. faecalis* V583 genome resulted in the identification of a second oligopeptide permease complex (EF_3106 - EF_3110). It was therefore of interest to further examine the contribution of both Opp systems to pheromone mating as well as biofilm development in *E. faecalis*. Our data show that the deletion of both of the *E. faecalis* oligopeptide permease complexes results in the failure of this bacterium to respond as a donor to three distinct peptide sex pheromones (cCF10, cAD1 and cAM373), attenuates biofilm development *in-vitro* and reduces virulence in a *C. elegans* infection model.

MATERIALS AND METHODS:

Bacterial Strains, Plasmids & Growth Conditions:

Pertinent bacterial strains and plasmids used in the current study are listed in Table 3-1 and Table 3-2. *E. faecalis* strains were cultured in Todd-Hewitt broth (THB) and grown at 37˚C unless otherwise indicated. *Escherichia coli* ElectroTen-Blue (Stratagene, La Jolla, CA) was used for maintenance and propagation of plasmid constructs. ElectroTen-Blue clones were cultured aerobically in Luria-Bertani broth at 37˚C. Antibiotics for selection included chloramphenicol at 10 μg ml^{-1} for *E. coli* and chloramphenicol at 15 μg ml^{-1}, spectinomycin at 500 μg ml^{-1},

Strain	Genotype or description	Reference
V583	Parental Strain	(18)
OG1RF	Parental Strain Rif^r Fus^r	(19)
OG1SSp	Parental Strain $Spec^r$ $Strep^r$	(20)
SV02	V583Δ*pptAB*	(17)
KS22	OG1RFΔ*gelE*	(21)
SV26	V583Δ*opp1*	This Study
SV27	V583Δ*opp2*	This Study
SV28	V583Δ*oppΔopp2*	This Study
VT01	V583(pMV158GFP) GFP^+ Tet^r	(22)
SV29	V583Δ*opp1*(pMV158GFP) GFP^+ Tet^r	This Study
SV30	V583Δ*opp2*(pMV158GFP) GFP^+ Tet^r	This Study
SV31	V583Δ*oppΔopp2* (pMV158GFP) GFP^+ Tet^r	This Study
SV74	OG1RFΔ*gelE*Δ*opp1*	This Study
SV91	OG1RFΔ*gelE*Δ*opp2*	This Study
SV92	OG1RFΔ*gelE*Δ*opp1*Δ*opp2*	This Study
SV75	OG1RFΔ*gelE*(pCF10)$^£$ Tet^r	This Study
SV76	OG1RFΔ*gelE*(pAM714)$^£$ Erm^r	This Study
SV77	OG1RFΔ*gelE*(pAM378)$^£$ Tet^r	This Study
SV78	OG1RFΔ*gelE*Δ*opp1*(pCF10)$^£$ Tet^r	This Study
SV79	OG1RFΔ*gelE*Δ*opp1*(pAM714)$^£$ Erm^r	This Study
SV80	OG1RFΔ*gelE*Δ*opp1*(pAM378)$^£$ Tet^r	This Study
SV93	OG1RFΔ*gelE*Δ*opp2*(pCF10)$^£$ Tet^r	This Study
SV94	OG1RFΔ*gelE*Δ*opp2*(pAM714)$^£$ Erm^r	This Study
SV95	OG1RFΔ*gelE*Δ*opp2*(pAM378)$^£$ Tet^r	This Study
SV96	OG1RFΔ*gelE*Δ*opp1*Δ*opp2* (pCF10)$^£$ Tet^r	This Study
SV97	OG1RFΔ*gelE*Δ*opp1*Δ*opp2* (pAM714)$^£$ Erm^r	This Study
SV98	OG1RFΔ*gelE*Δ*opp1*Δ*opp2* (pAM378)$^£$ Tet^r	This Study

Table 3-1: Bacterial strains used in this study.

streptomycin at 500 µg ml^{-1} , rifampicin at 50 µg ml^{-1} , tetracycline at 15 µg ml^{-1} and erythromycin at 10 µg ml^{-1} for *E. faecalis*. Transformation of plasmids into *E. faecalis* strains was done as described previously (23).

Plasmid	Description	Reference
pLT06	Deletion vector; chloramphenicol resistance	(24)
pSV12	pLT06 containing engineered *opp* deletion (~2-kb EcoRI/PstI fragment)- Deletion of *opp1* in V583 and OG1RF	This Study
pSV13	pLT06 containing engineered *opp2* deletion (~2-kb EcoRI/PstI fragment)- Deletion of *opp2* in V583	This Study
pCJK218	Modified pLT06 containing altered *pheS*	(25)
pSV31	pCJK218 containing engineered *opp2* deletion (~2-kb EcoRI/PstI fragment)- Deletion of *opp2* in OG1RF	This Study
pMV158GFP	*gfp* containing plasmid	(26)
pCF10[£]	Pheromone responsive plasmid harboring tetracycline resistance	(27)
pAM714[£]	Pheromone responsive plasmid harboring erythromycin resistance, Derivative of pAD1[£]	(28)
pAM378[£]	Pheromone responsive plasmid harboring Tetracycline resistance, Derivative of pAM373[£]	(29)

Table 3-2: Bacterial strains used in this study.

Construction of *E. faecalis* in-frame deletion mutants:

In frame deletions of the genes encoding the Opp1 and Opp2 complexes as well as the double deletion mutant of both *opp1* and *opp2* in *E. faecalis* strains V583 and KS22 [OG1RFΔ*gelE*] were completed using plasmids derived from pLT06 (24), an *E. coli*-enterococcal temperature-sensitive

cloning vector that possesses selectable and counterselectable markers that aid in the selection of markerless mutants containing the targeted deletions. The primers used for all the deletions are listed in Table 3. Flanking regions (~1Kb) from both the 5' and 3' ends of *opp1* and *opp2* complex genes were PCR amplified by using the primers listed in Table 3-3. For the construction of the

Primer	**Sequence (5'-3')**
Opp1P1	GAGAGAATTCCAAGACTCGTACCTAGAATC
Opp1P2	CTCTGGATCCGCTTGAGCTATCTGACGTTC
Opp1P3	GAGAGGATCCCTGAAGACGAAGTTGCAATG
Opp1P4	CTCTCTGCAGGATCTCCTCTGACTCAATAC
Opp1Up	GCTGGCAATCGCATAGTTTC
Opp1Down	TCGTTTAGACAAGCCACGTTC
Opp1-Internal	ACCGTCTGACCATTTTGCATC
Opp2P1	GAGAGAATTCCTTCGAATTGTTAGCAGCAG
Opp2P2	CTCTGGATCCATTTCCTTGCATCCATACGCA
Opp2P3	GAGAGGATCCGTCACAGCAGACTCACGG
Opp2P4	CTCTCTGCAGACCTTTCCAGACGTTGCATC
Opp2Up	CCTGTCATGGGAGAACCAG
Opp2Down	GTTAGCTAGTGCTGCTGCAAC
Opp2-Internal	AACCAGATTCTCCGACGATTGC
OG1RF-Opp2P3	GAGAGGATCCGTTGATACGAAAGATAATCCATGG
OG1RF-Opp2P4	CTCTCTGCAGCCAATGAGTTCACCATTCATG
OG1RF-Opp2Down	CCTGTTCAGCTAACTTCTTCG

The underlined sequences denote restriction sites that were added to the template-directed sequences to facilitate cloning.

Table 3-3: Primers used in this study.

pSV12 plasmid (*opp1* deletion) the primers Opp1P1 and Opp2P2 were used to amplify the ~1Kb regions flanking the 5' end of *opp1* on the V583 and KS22 genomes respectively. Primers OppP3 and OppP4 were used to amplify the ~1Kb region flanking the 3' end of the *opp1* region in both strains. The OppP1 and OppP2 primers contained EcoRI and BamHI sites, respectively and OppP3

and OppP4 primers contained BamHI and PstI sites, respectively. Each product was digested with the respective restriction enzymes, and ligated to pLT06 cut with EcoRI and PstI prior to electroporation into *E. coli* ElectroTen-Blue cells. Confirmation of the appropriate clones was performed by restriction digest and sequence analysis. An analogous approach was used for the construction of pSV13 (*opp2* deletion in V583) and pSV31 (*opp2* deletion in KS22 [OG1RFΔ*gelE*]). We were unable to use pSV13 construct for the deletion of *opp2* in OG1RF since the *opp2* flanking region in V583 is significantly different from that of OG1RF due to the presence of an IS element in V583. pSV13 was generated using pLT06, whereas pSV31 was generated using pCJK218 (a pLT06 based vector with an altered *pheS* synthetic sequence to reduce the likelihood of homologous recombination at that locus) (25). Purified plasmids from *E. coli* were electroporated into electrocompetent V583 and KS22 [OG1RFΔ*gelE*] cells. SV26 [V583Δ*opp1*], SV27 [V583Δ*opp2*], SV74 [KS22Δ*opp1*], and SV91 [KS22Δ*opp2*] were generated following the protocol as previously described (24). To create the double deletion of *opp1* and *opp2* in the V583 strain background, electrocompetent SV26 cells were transformed with pSV13, and the deletion mutant was designated as SV28 [V583Δ*opp1*Δ*opp2*]. To create the double deletion of *opp1* and *opp2* in the strain KS22, electrocompetent SV74 cells were transformed with pSV31, and the deletion mutant was designated as SV92 [KS22Δ*opp1*Δ*opp2*].

Growth Curve Analysis:

Standard growth curve analysis was performed to ensure that SV26 [V583Δ*opp1*], SV27 [V583Δ*opp2*] and SV28 [V583Δ*opp1*Δ*opp2*] did not exhibit growth defects relative to the parental V583 strain when grown in enriched medium (Tryptic Soy Broth). Briefly, V583, SV26 [V583Δ*opp1*], SV27 [V583Δ*opp2*] and SV28 [V583Δ*opp1*Δ*opp2*] were grown in TSB overnight at 37˚C. The overnight cultures of these strains were inoculated into a 96-well plate containing

TSB at a 1:100 ratio and were incubated at 37 ˚C. Optical density was measured at a wavelength of 600 nm using the Infinite M200 Pro plate reader [Tecan US, Inc; Morrisville, North Carolina].

Cell aggregation Assay:

Clumping activity was assayed using a protocol modified from Mori et al. (30). *E. faecalis* OG1RF, KS22 [OG1RFΔ*gelE*], SV74 [KS22Δ*opp1*], SV91 [KS22Δ*opp2*] and SV92 [KS22Δ*opp1*Δ*opp2*] cells containing the pheromone responsive plasmids pCF10, pAM714 (A pAD1 plasmid derivative encoding erythromycin resistance) and pAM378 (A pAM373 plasmid derivative encoding tetracycline resistance) were used as the donor cells. *E. faecalis* OG1SSp was used as the recipient. Briefly, the donor cells and the recipient cells were grown overnight at 37˚C in 2 ml of THB. 50 µl of each donor cell was added to 450 µl of OG1SSp and this mixture was added to 4.5 ml of fresh THB medium. The cultures were grown at 37˚C in a shaking incubator (~ 200 rpm) for 4 hours to visually compare the difference in clumping phenotype.

Transconjugation assay:

Transconjugation efficiency was calculated using a mating assay modified from Mori et al. (30). After incubating the donor and recipient cells together for 4 hours under shaking conditions [200 rpm], the cell cultures from the different plasmid systems were serially diluted in sterile PBS and track-diluted (31) onto THB containing streptomycin 500 µg-ml^{-1} and tetracycline 15 µg-ml^{-1} (for pCF10 and pAM378 transconjugants) or THB containing streptomycin 500 µg-ml^{-1} and erythromycin 10 µg-ml^{-1} (for pAM714). The cultures were also track-diluted onto THB plates containing rifampicin at 100 µg-ml^{-1} to select for donor cells in all three plasmid systems. Transconjugation efficiency was measured as the ratio of transconjugants to donor cells.

Confocal Biofilm Analysis:

Confocal laser scanning microscopy (CLSM) was performed on 1-day-old biofilms as described previously (22). *E. faecalis* strains SV26 [V583Δ*opp1*], SV27 [V583Δ*opp2*] and SV28 [V583Δ*opp1*Δ*opp2*] were transformed with pMV158GFP to generate SV29, SV30 and SV31, respectively, and these strains expressed GFP constitutively. VT09 [V583(pMV158GFP)] (22) along with SV29 [V583Δ*opp1*(pMV158GFP)], SV30 [V583Δ*opp2*(pMV158GFP)] and SV31 [V583Δ*opp1*Δ*opp2*(pMV158GFP)] were used for confocal microscopy analysis. Briefly, biofilms were grown on sterile glass coverslips placed in six-well tissue culture plates. Tetracycline was added to the TSB medium for plasmid maintenance. After 24 h of growth, the biofilms were gently washed with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4 [pH 7.4]) and stained with 1 μM SYTOX orange (Invitrogen) for 6 to 7 min. The coverslips were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using a Zeiss LSM 5 Pa laser scanning confocal microscope.

Assay for *C. elegans* killing by *E. faecalis*:

Bacterial strains were grown on brain heart infusion (BHI) agar medium in 35-mm tissue-culture plates. Lawns of V583, V583Δ*pptAB*, V583Δ*opp1*, V583Δ*opp2* and V583Δ*opp1*Δ*opp2* were grown as follows: 2 ml of BHI was inoculated with a single colony of the appropriate strain and grown at 37°C for 4–5 hr, and 35 μl of the culture was spread on each plate. The plates were incubated at 37°C overnight. *C. elegans* strain N2 was maintained and propagated on *Escherichia coli* strain OP50 with standard techniques. Between 10 and 15 *C. elegans* L4 or young adult hermaphrodites were transferred from a lawn of *Escherichia coli* OP50 to a lawn of the bacterium to be tested, incubated at 25°C, and examined at ~24-hr intervals with a dissecting microscope for viability. Worms were considered dead when they did not respond to touch with a platinum wire

pick. Each experimental condition was tested in duplicate or triplicate. Nematode survival was plotted with the Kaplan–Meier method using Graphpad Prism 5.0 statistical software (San Diego, CA). The Graphpad Prism software package was also used for testing equality of survival (Log-Rank (Mantle-Cox test)).

RESULTS:

Discovery of a second oligopeptide permease complex in *E. faecalis*:

A homology search of the *E. faecalis* V583 genome with genes encoding for components of the Opp1 complex [EF_0907 to EF_0912] first described by Leonard et al. (1) identified 5 additional open reading frames (ORF's) encompassing genes EF_3106 and EF_3110. The genes of this putative second *opp* operon along with the sequence identity shared with their Opp1 complex counterparts are depicted in figure 3-1.

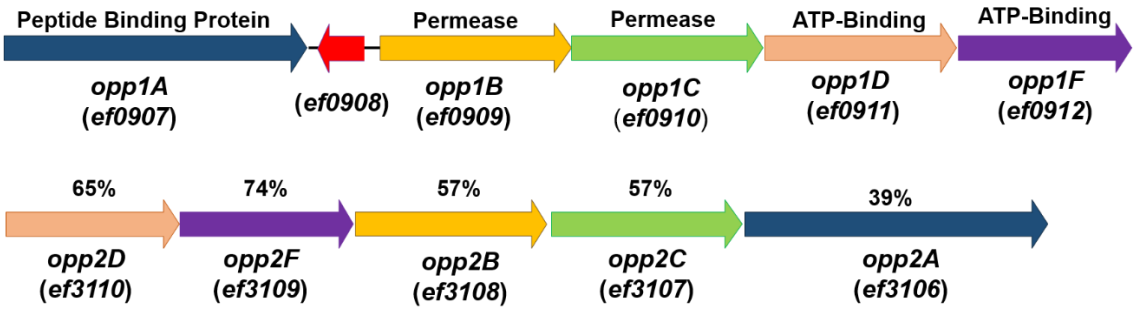

Figure 3-1: Genetic organization of *opp1* and *opp2* complexes in strain V583. All the corresponding homologs in each complex are color coded with the same color. The percentage values above the *opp2* complex indicate amino acid sequence identity of the *opp2* complex genes to their corresponding homologs in the *opp1* complex. *ef_0908* is an ORF present in the opp1 complex and is not present in the *opp2* complex.

Opp1A, the pheromone binding lipoprotein, shares 39% sequence identity with Opp2A. Opp1B and Opp1C, predicted membrane permeases, share 57% sequence identity with their Opp2B and Opp2C counterparts. Opp1D and Opp1F, predicted ATP-binding proteins, share 65-74% sequence identity with Opp2D and Opp2F. Interestingly, the small divergently transcribed and uncharacterized gene, *ef_0908,* located between the genes *opp1A* (*ef_0907*) and *opp1B* (*ef_0909*) is absent in the second Opp complex. Apart from the identified *opp2* genes, there were no additional Opp-related genes present in the V583 genome.

Oligopeptide permeases are essential for the import of peptide sex pheromone cOB1:

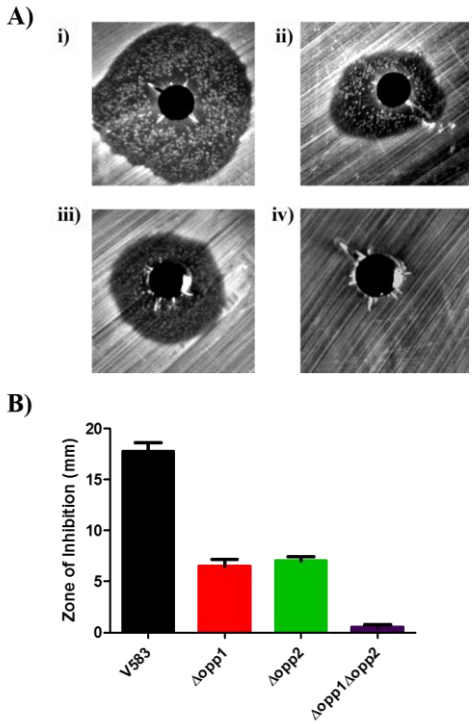

Figure 3-2: cOB1 spot assay. A) *E. faecalis* strains i)V583 ii)V583Δ*opp1* iii)V583Δ*opp2* and iv)V583Δ*opp1*Δ*opp2* were grown overnight at 37°C in THB and were spread on a plate. 100nM purified cOB1 peptide was spotted into the well in the middle of each plate and these were incubated overnight at 37°C and the plates were photographed to observe the growth inhibition. B) Zones of inhibition were quantified and the diameter of the zones are represented in the graph.

A recent study (M. S. Gilmore, M. Rauch, M. Ramsey, P. Himes, S. Varahan, F. Lebreton, and L. E. Hancock, submitted for publication) has demonstrated that commensal *E. faecalis* inhibit the growth of strain V583, a multi-drug resistant VRE prototype of the MLST ST6 lineage (32). The inhibition of V583 was due to the secretion of the peptide sex pheromone cOB1 by commensal strains. We wanted to test whether cOB1 was imported into V583 *via* either of the two oligopeptide permeases. Growth of V583 was substantially inhibited by the presence of cOB1, while V583Δ*opp1* and V583Δ*opp2* displayed partial resistance to the inhibitory effect of cOB1 (Fig 3-2A, 3-2B). Interestingly, V583Δ*opp1*Δ*opp2* was completely resistant to the inhibition by cOB1 suggesting that in the absence of the Opp complexes, cOB1 is unable to exert its inhibitory effects on the growth of V583 (Fig 3-2A, 3-2B).

Cell aggregation is impaired when *opp* complexes are deleted:

It is known that cells that harbor pheromone responsive plasmids encode for an OppA homolog that binds to the cognate pheromone with higher affinity (1). The plasmid encoded OppA homolog, typified by PrgZ and TraC, recruits the chromosomally encoded oligopeptide permease complex to import the cognate pheromones (16). The uptake of pheromones results in disruption of the PrgX repressor function leading to increased expression of the proteins involved in conjugative mating (33). We hypothesized that along with the *E. faecalis* Opp1 complex, that the Opp2 complex also contributes to the uptake of peptide sex pheromones and that their deletion in tandem would result in the attenuation of peptide uptake, leading to reduction in plasmid transfer. To assess cell aggregation and plasmid conjugation, we constructed the *opp* complex deletions in the plasmid-free OG1RF strain background, as strain V583 possesses three plasmid systems, two of which are pheromone-responsive and may interfere with data interpretation (34). In addition,

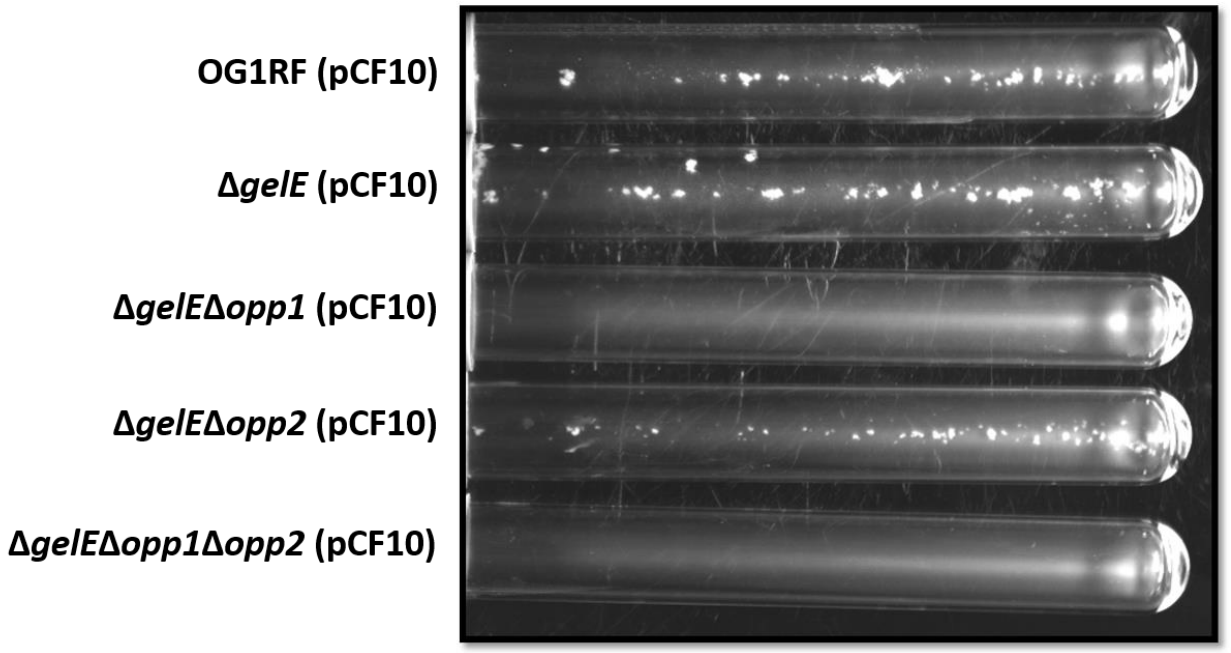

Figure 3-3: Cell aggregation assay. OG1RF, OG1RFΔ*gelE*, OG1RFΔ*gelE*Δ*opp1*, OG1RFΔ*gelE* Δ*opp2* and OG1RFΔ*gelE*Δ*opp1*Δ*opp2* harboring pCF10 were the donors and OG1SSp was used as the recipient. After the donors and recipients were mixed and incubated at 37°C for 4hrs under rigorous shaking conditions (200 rpm), the tubes were placed horizontally to visualize the extent of cell clumping and photographed.

Waters *et al.* (35) showed that the *E. faecalis* secreted zinc metalloprotease, gelatinase, is able to cleave peptide pheromones, which results in a partial reduction in plasmid transfer efficiency (36). We mobilized 3 distinct pheromones responsive plasmids pCF10, pAM714 (pAD1 derivative) and pAM378 (pAM373 derivative) into each strain background (Table 3-1). These plasmid harboring strains were used as the donor with OG1SSp as the recipient. The donor and recipients were mixed at a ratio of 1:10 and the extent of cell aggregation was evaluated after 4 hours. The results of this assay using the pCF10 system are shown in Figure 3-3. As expected when both OG1RF and OG1RFΔ*gelE* containing pCF10 were used as the donor, there was visible clumping, suggesting that pheromone mediated induction of conjugation machinery had occurred. However when

OG1RFΔ*gelE*Δ*opp1* harboring pCF10 was used as the donor there was no visible clumping suggesting that the Opp1 complex is the major contributor to the uptake of the cCF10 pheromone (Fig. 3-3). Interestingly the deletion of the *opp2* operon in OG1RFΔ*gelE* harboring pCF10 resulted in smaller aggregates compared to the parental strain suggesting that the Opp2 complex may partially contribute to the overall clumping response. Consistent with observations for the *opp1* deletion, a pCF10 donor mutant lacking both Opp systems (Δ*opp1*Δ*opp2*) showed no signs of visible clumping in response to a cCF10 producing recipient. Similar results were seen when this assay was performed using donor cells harboring pAM714 and pAM378 plasmid systems (data not shown).

Oligopeptide permease complex mutants exhibit attenuated transconjugation efficiency:

Since the deletion of the *opp* complexes in donor cells harboring the pheromone responsive plasmids pCF10, pAM714 and pAM378 resulted in an attenuation in cell aggregation, we wanted to determine whether these *opp* deletion mutants were impaired in their ability to detect peptide pheromones and transfer the pheromone responsive plasmids they harbor to the recipient population. The protocol for this assay is very similar to the protocol described for cell aggregation assay. After the donor cells (OG1RF, OG1RFΔ*gelE*, OG1RFΔ*gelE*Δ*opp*, OG1RFΔ*gelE*Δ*opp2* and OG1RFΔ*gelE*Δ*opp*Δ*opp2*) harboring pCF10, pAM714 or pAM378 were grown along with the recipient OG1SSp cells to induce broth mating, the cultures (Transconjugants and Donors) were track diluted onto THB plates containing spectinomycin-tetracycline or spectinomycin-erythromycin to select for transconjugants. The transconjugation efficiency for these reactions is shown in Table 3-4. Consistent with a previous report (36), the *gelE* deletion mutant was slightly

better in mating efficiency as the absence of gelatinase enhanced mating efficiency by ~ 0.5 $\log_{10}$, efficiency of OG1RFΔ*gelE*Δ*opp1* was attenuated by 2 logs in all 3 pheromone responsive plasmids

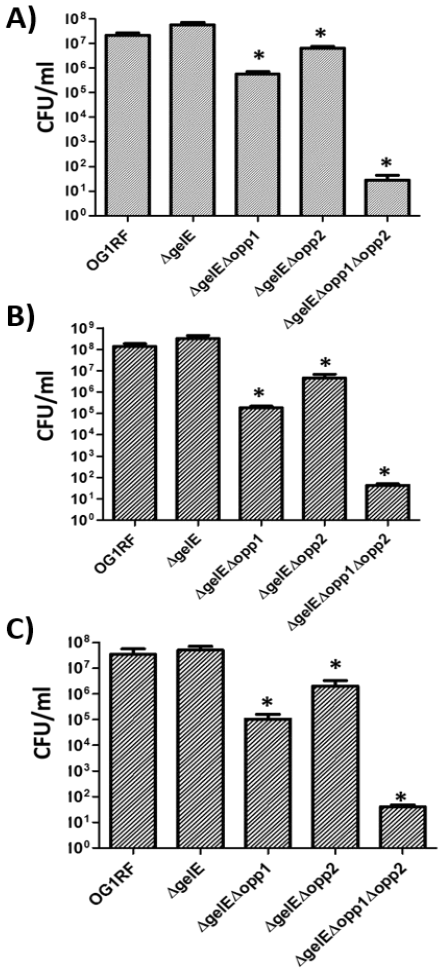

Figure 3-4: Transconjugation assay performed using OG1RF, OG1RFΔ*gelE*, OG1RFΔ*gelE*Δ*opp1*, OG1RFΔ*gelE*Δ*opp2* and OG1RFΔ*gelE* Δ*opp1*Δ*opp2* harboring A) pCF10 B) pAM714 and C) pAM378 as the donors and OG1SSp as the recipient. CFU/ml represent the number of colony forming units of the transconjugants. *, significant difference ($P < 0.05$) relative to wild-type OG1RF. Experiments were performed in triplicate, and error bars represent standard errors of the means (SEM).

systems compared to OG1RF and OG1RFΔ*gelE* donor cells suggesting that the Opp1 complex is playing a major role in the import of cCF10, cAD1 & cAM373 peptide pheromones (Fig. 3-4).

Strains	pCF10 system	pAM714 system	pAM378 system
OG1RF	$2.979 \times 10^{-1} \pm 0.642 \times 10^{-1}$	$2.963 \times 10^{-1} \pm 1.213 \times 10^{-1}$	$1.801 \times 10^{-1} \pm 0.834 \times 10^{-1}$
OG1RFΔ*gelE*	$5.965 \times 10^{-1} \pm 2.186 \times 10^{-1}$	$7.109 \times 10^{-1} \pm 1.389 \times 10^{-1}$	$6.245 \times 10^{-1} \pm 1.291 \times 10^{-1}$
OG1RFΔ*gelE*Δ*opp1*	$4.221 \times 10^{-4} \pm 2.732 \times 10^{-4}$	$3.923 \times 10^{-4} \pm 1.067 \times 10^{-4}$	$2.743 \times 10^{-4} \pm 1.412 \times 10^{-4}$
OG1RFΔ*gelE*Δ*opp2*	$2.502 \times 10^{-2} \pm 1.279 \times 10^{-2}$	$1.701 \times 10^{-2} \pm 1.025 \times 10^{-2}$	$5.638 \times 10^{-2} \pm 2.742 \times 10^{-2}$
OG1RFΔ*gelE*Δ*opp1*Δ*opp2*	$3.723 \times 10^{-6} \pm 0.889 \times 10^{-6}$	$3.4123 \times 10^{-6} \pm 1.027 \times 10^{-6}$	$5.621 \times 10^{-6} \pm 2.324 \times 10^{-6}$

Table 3-4: Transconjugation Efficiency: No. of Transconjugants/ No. of Donors

Interestingly, OG1RFΔ*gelE*Δ*opp2* displayed a very modest attenuation (~1.0 log attenuation compared to OG1RF and OG1RFΔ*gelE* donor cells) suggesting that the Opp2 complex may play a less significant role in the import of the mating peptides compared to the Opp1 system under the conditions tested. OG1RFΔ*gelE*Δ*opp1*Δ*opp2* however was highly attenuated as a donor and displayed a ~4-5 log attenuation in mating efficiency compared to OG1RF and OG1RFΔ*gelE* donor cells suggesting that the absence of both the oligopeptide permease complexes prevents the donor cells from responding efficiently to the recipient derived peptide pheromones (Fig. 3-4.; Table 3-4).

Deletion of Opp complexes results in biofilm defects:

Biofilm formation is a coordinated process that involves extensive cell-cell communication for the formation of highly ordered three dimensional structures. A growing number of studies have demonstrated the importance of peptide pheromones in biofilm development (37-39). Since the

Opp complexes contributed significantly to the import of peptide sex pheromones, we hypothesized that cells that lack these complexes will not be able to respond to important cell

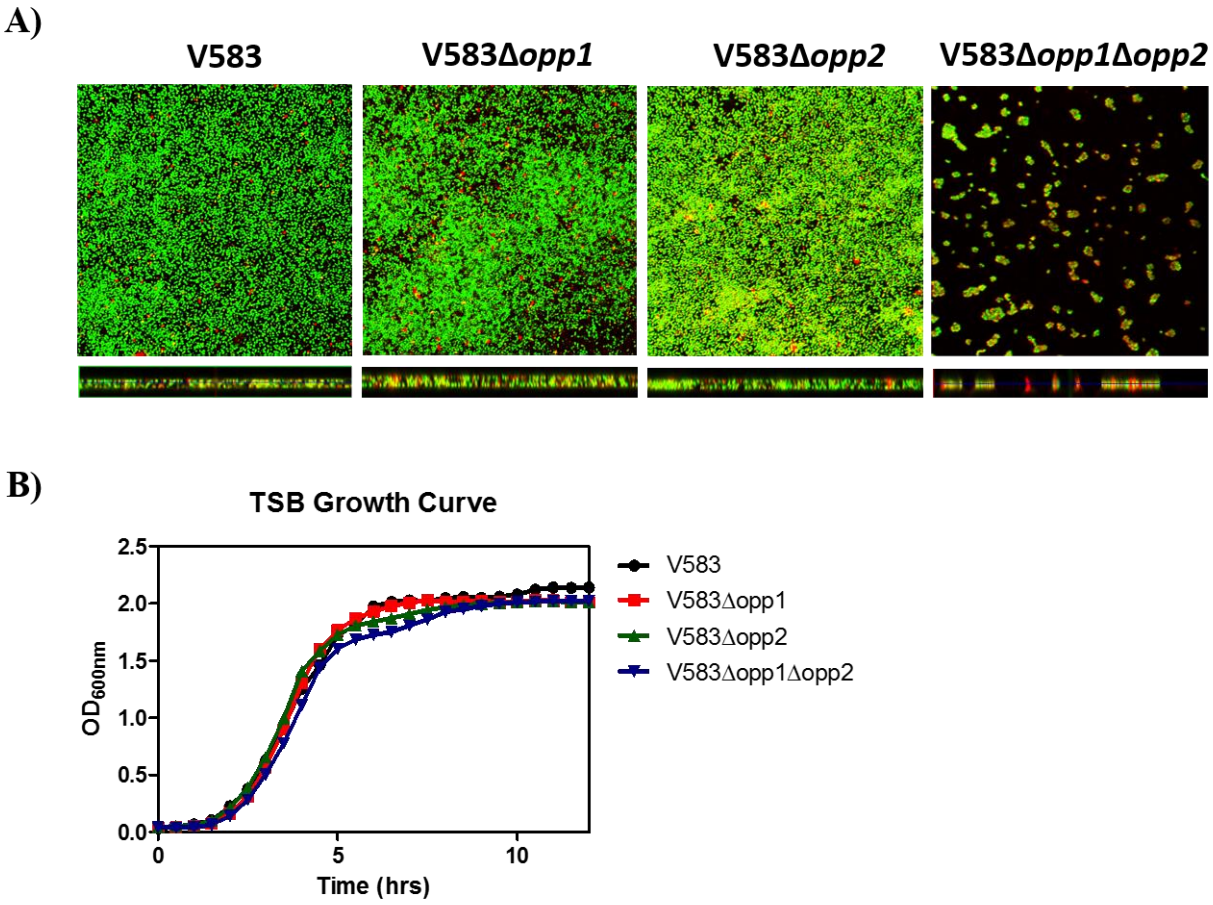

Figure 5: A) Confocal microscopy biofilm analysis was performed on 1-day old biofilms formed byV583, V583Δ*opp1*, V583Δ*opp2* and V583Δ*opp1*Δ*opp2*. All the strains contain the plasmid pMV158GFP, which provides constitutive expression of GFP. **B)** Bacterial growth curves. *E. faecalis* strains V583, V583Δ*opp1*, V583Δ*opp2* and V583Δ*opp1*Δ*opp2* were grown under static conditions in Tryptic Soy Broth (TSB) at 37°C and samples were analyzed by optical density absorbance (OD_{600}).

signaling peptide pheromones during biofilm formation and will exhibit biofilm defects. To test this hypothesis, we tested the *opp* deletion mutants in the V583 genetic background that also constitutively express GFP using the plasmid pMV158-GFP (26). The resulting strains

V583Δ*opp1*(pMV158GFP), V583Δ*opp2*(pMV158GFP), V583Δ*opp1*Δ*opp2*(pMV158GFP) along with wild-type control V583(pMV158GFP) were used in the confocal biofilm assays. After 24hrs, V583Δ*opp1*(pMV158GFP) and V583Δ*opp2*(pMV158GFP) formed comparable biofilms to that of V583(pMV158GFP), whereas V583Δ*opp1*Δ*opp2* (pMV158GFP) displayed a significant biofilm defect compared to the wild-type suggesting that the *E. faecalis* is unable to form confluent biofilms in the absence of both the Opp complexes (Fig. 3-5A). The biomass and average thickness of these biofilms were quantified by COMSTAT analysis (40) and the double *opp* mutant showed a 4.8-fold reduction in biomass and a 3.6-fold reduction in average biofilm thickness compared to the parental strain V583, whereas the individual opp deletions were not significantly different from the parental strain (Table 3-5).

Strains	**Biomass ($\mu m^3/ \mu m^2$)**	**Average Thickness (μm)**
V583	2.07 ± 0.783	2.67 ± 0.237
V583 Δ *opp1*	2.34 ± 0.334	2.73 ± 0.642
V583 Δ *opp2*	2.15 ± 0.248	2.45 ± 0.317
V583 Δ *opp1* Δ *opp2*	0.43 ± 0.169	0.73 ± 0.133

Table 3-5: COMSTAT analysis of 1-day old biofilms

Several studies have shown that the deletion of oligopeptide permeases affects the ability of those mutant microorganisms to grow in enriched media like TSB (41), peptide rich media (42, 43) or in *in-vivo* abscesses (41). We wanted to determine whether the biofilm defect was attributed to poor growth in TSB (biofilm medium). V583, V583Δ*opp1*, V583Δ*opp2* and V583Δ*opp1*Δ*opp2* were grown overnight in TSB at 37°C and were back diluted 1:100 in fresh TSB and growth was

monitored until late stationary phase (12 hrs.). None of the 3 deletion mutants displayed any significant growth defect compared to the wild-type under these growth conditions (Fig. 3-5B).

The transport of the toxic peptide bialaphos requires the Opp complex in several gram-positive species including *Bacillus subtilis*, *Streptococcus pyogenes* and *Listeria monocytogenes* as the deletion of the *opp* complex genes in these bacteria renders the cells refractory to the toxic effects of bialaphos, a tripeptide antibiotic that is known to enter cells via oligopeptide permeases (44-46). However, when we tested the sensitivity of V583, V583Δ*opp1*, V583Δ*opp1* and V583Δ*opp1*Δ*opp1* mutant strains against this toxic peptide, we did not observe any phenotypic differences even when bialaphos was used at 1-10 mM concentrations, suggesting that *E. faecalis* may have an altered intracellular target, rendering them resistant to bialaphos independent of a functional oligopeptide permease.

Deletion of peptide pheromones transporters results in attenuated virulence in a *C. elegans* infection model:

We recently showed that deletion of the peptide pheromone transporter (PptAB) that is essential for the secretion (export) of the peptide sex pheromones in *E. faecalis* results in a biofilm defect (17). Since the deletion of both the *opp* complexes also resulted in attenuated biofilm formation, we wanted to determine whether the absence of these peptide transporters affected the virulence of *E. faecalis* due to the inability of the transporter mutants to form biofilms. We used the *C. elegans* model of infection to address these questions since this model has been used by others to identify virulence factors in *E. faecalis* (47, 48). We tested V583, V583Δ*pptAB*, V583Δ*opp*, V583Δ*opp2* and V583Δ*opp*Δ*opp2* in this model and the results are shown in figure 6. While the

V583Δ*opp2* strain phenocopied the wild-type strain V583, the V583Δ*opp1* was significantly attenuated in *C. elegans* killing compared to V583 ($P < 0.05$; P=0.021), Log-Rank (Mantle-Cox test)) (Fig. 3-6A).

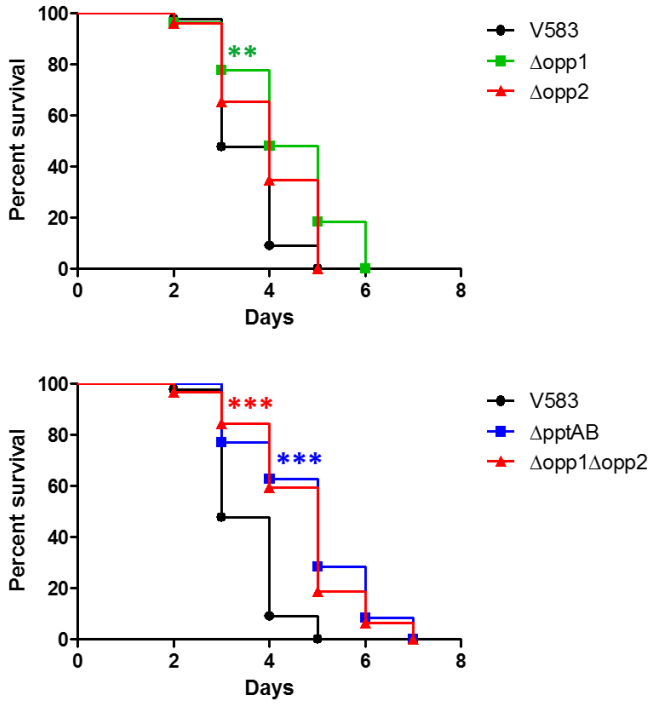

Figure 6: *C. elegans* killing assay. V583, V583Δ*pptAB*, V583Δ*opp1*, V583Δ*opp2* and V583Δ*opp1*Δ*opp2* were spotted on BHI agar and incubated at 37°C to obtain bacterial lawns. *C. elegans* strain N2 worms were transferred to these plates and were monitored for survival every 24 hrs. Survival curves were plotted using GraphPad Prism (San Diego, CA) and statistical analysis was done using Log-Rank (Mantle-Cox) test. ** and ***, significant difference ($P < 0.05$ and $P < 0.005$) relative to wild-type V583. Experiments were performed in triplicates.

Interestingly, V583Δ*pptAB* and V583Δ*opp1*Δ*opp2* were highly attenuated in the *C. elegans* killing assay compared to the wild-type strain V583 ($P < 0.01$; P = 0.0009), Log-Rank (Mantle-Cox test)) (Fig. 3-6B).

DISCUSSION:

In this study, we have identified and characterized a previously unidentified oligopeptide permease complex in the core genome of *E. faecalis* strain V583. Oligopeptide permeases (Opp) are an important class of membrane transporters that are known to play distinct roles in bacteria. They are predominantly involved in the uptake of short peptides for nutrient acquisition, but are also emerging as important mediators of gene regulation by contributing to the import of peptide pheromones (39, 49, 50). In this study, we have demonstrated that a second, previously uncharacterized Opp complex is encoded by the V583 core genome (EF_3110-EF_3107). We took advantage of the fact that the cOB1 peptide sex pheromone inhibits the growth of V583. Using synthetic cOB1 pheromone, we showed that only the deletion of both the *opp* complexes renders *E. faecalis* V583 refractory to the inhibitory effects of cOB1. This suggests that in the absence of one Opp complex, the other could be playing a compensatory role. We have also shown using 3 independent pheromone responsive plasmid systems that the donor cells respond very poorly to the peptide pheromones secreted by recipients in the absence of both the Opp complexes, which is indicative of overlapping roles played by these chromosomally encoded oligopeptide permeases.

Opps play an important role in the transport of peptide pheromones in several different gram-positive species. *B. subtilis* possesses two oligopeptide permease systems (Opp and App) that play overlapping roles in peptide transport (46) including the importation of Phr peptides (51) which in turn inhibit the activity of Rap phosphatases responsible for dephosphorylating Spo0F, a component of the phosphorelay involved in the phosphorylation of Spo0A, a master regulator in the sporulation response. Rap phosphatases also play a role in regulating competence development in *B. subtilis* and opp mutants also The activity of the neutral protease regulator, NprR, is also regulated by a small peptide termed NprX (46, 52, 53). In *Bacillus cereus*, the PapR peptide that

regulates gene expression via the PlcR transcriptional regulator is imported into the cell via an Opp complex and in *Streptococcus pyogenes*, the SHP peptides are imported via an Opp complex and these peptides regulate gene expression by interacting with the Rgg family of transcriptional regulators (39, 54). Interestingly, both *B. subtilis* and *S. pyogenes* are susceptible to the toxic tripeptide, bialaphos, and the deletion of the Opp complexes in these bacteria confers resistance to this peptide indicative of the involvement of the Opp complex in the transport of bialaphos (45, 46). However, the *E. faecalis* strain V583 did not display any susceptibility at 1-10 mM concentrations towards bialaphos and the deletion of the Opp complexes did not alter this phenotype suggesting that *E. faecalis* may be more inherently resistant to this tripeptide antibiotic through an unknown mechanism.

Increasing evidence in gram-positive bacteria suggests that short linear peptides are used as quorum-sensing signals and require oligopeptide permeases for uptake, and that such signals are perceived by gene regulators that bind the imported peptides (39, 54-61). A recent study by Chang *et al.* (39) showed that biofilm formation in *Streptococcus pyogenes* is regulated by Rgg transcriptional regulators that bind to short hydrophobic peptides (SHP) which are imported into the cells via the Opp complex. These short hydrophobic peptides are not derived from lipoprotein precursors unlike the well characterized enterococcal pheromones (cCF10, cAD1, cOB1) but are independent genes located adjacent to, but divergent from the cognate Rgg encoding genes. Our laboratory recently demonstrated that the deletion of the peptide pheromone transporter (PptAB) that secretes hydrophobic peptide pheromones in strain V583 resulted in attenuated biofilm defects (17). Interestingly, our data from this study indicate that the mutant strains that lack both the Opp systems are also unable to form confluent biofilms. Biofilm formation is an important pre-requisite for *E. faecalis* virulence since most infections caused by this bacterium are biofilm-associated (62,

63). When V583Δ*opp1*Δ*opp2* and V583Δ*pptAB* strains were tested in the *C. elegans* infection model along with the *opp* single deletions mutants, V583Δ*pptAB* and V583Δ*opp*Δ*opp2* were significantly attenuated in *C. elegans* killing compared to the wild-type.

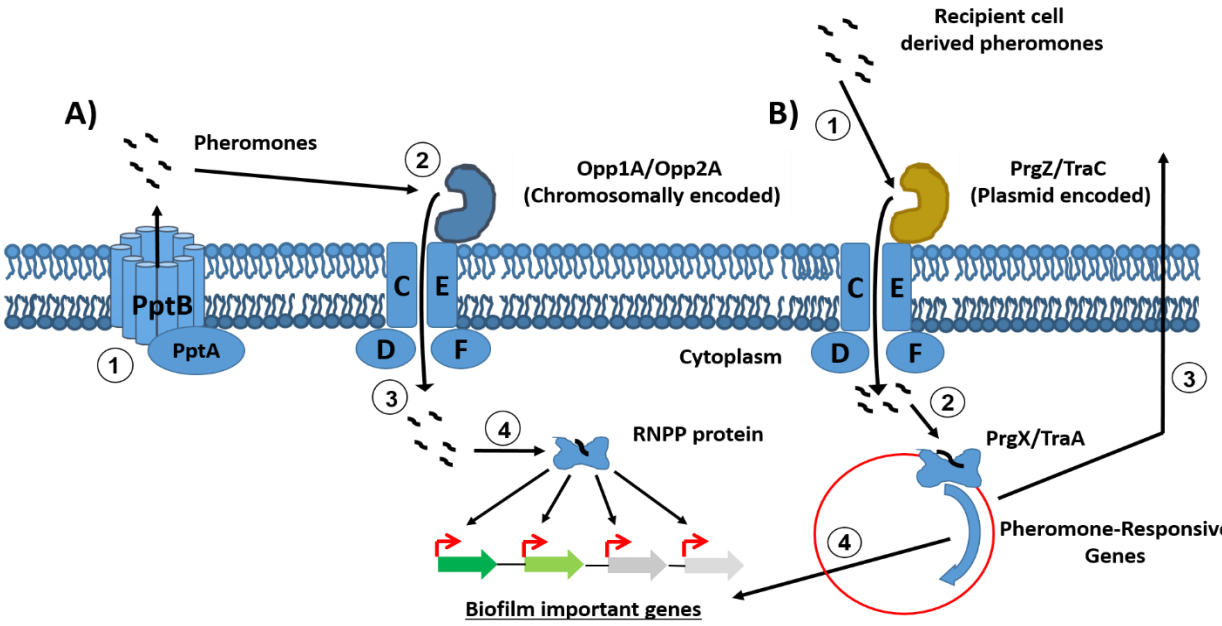

Figure 3-7: A) Pheromone-mediated biofilm regulation in *E. faecalis*. 1) Lipoprotein-derived peptide pheromones and/or peptides derived from putative short coding sequences (58) are exported via the Peptide Pheromone Transporter (PptAB) (17). 2) Secreted peptides are bound by OppA or its homologs with high affinity 3) and actively imported into the cell via the oligopeptide permease complex 4) where the peptide(s) interact with plasmid or chromosomally encoded RNPP family members in *E. faecalis* to regulate the expression of biofilm important genes. **B)** Involvement of Opp systems in pheromone mediated conjugation. 1) Peptide pheromones produced by recipient *E. faecalis* populations are sensed by the OppA homologs (PrgZ/TraC) encoded by the cognate pheromone-responsive plasmids and are imported into the donor cells via the chromosomally encoded oligopeptide permeases. 2) This alleviates the PrgX/TraA-mediated repression of conjugative functions resulting in activation of pheromone-responsive genes 3) with subsequent plasmid transfer to the recipient cells. 4) Aggregation substance, a pheromone-responsive gene product, is also known to contribute to ex-vivo biofilms (60) and endocarditis (61).

It is important to note that strains lacking the peptide pheromone transporter (PptAB) or both the Opp complexes (V583Δ*opp1*Δ*opp2*) exhibit biofilm defects and this could possibly explain the attenuated killing phenotype that these mutants exhibit in the *C. elegans* model. A possible model for pheromone-mediated regulation of biofilm formation in *E. faecalis* is depicted in Figure 7. It is possible that enterococcal pheromones (lipoprotein-derived and/or derived from putative short coding sequences (58) interact with RNPP protein family homologs (53, 59) present in *E. faecalis* to regulate the expression of genes involved in conjugal plasmid transfer as well as biofilm development. The RNPP family consists of Rap/NprR/PlcR/PrgX proteins that share an amino-terminal DNA binding domain along with a carboxy-terminal possessing tetratricopeptide repeats thought to be involved in binding small peptides (53). PrgX, a prototype-member of the RNPP family is encoded on the pCF10 pheromone-responsive plasmid in *E. faecalis* and represses conjugative functions including the intercellular adhesion, aggregation substance. Upon sensing the pheromone cCF10, repression by PrgX is alleviated and expression of aggregation substance allows for intercellular aggregation (13). Expression of aggregation substance is known to enhance colonization of explanted heart valves (60) as well as increasing vegetation size in a rabbit model of endocarditis (61). In addition to PrgX homologues encoded on the pheromone-responsive plasmids pTEF1 (EFA_0004) and pTEF2 (EFB_0005), *E. faecalis* V583 is also predicted to possess several chromosomally-encoded RNPP homologs [EF_0073, EF_1224, EF_1316, EF_1599 and EF_2687 (ElrR)] (59). Ibrahim *et al.* in 2007 identified several short hydrophobic peptide encoding genes in *E. faecalis* strain V583 using bioinformatic analysis and some of these genes are located adjacent to the genetic locus of the aforementioned RNPP family proteins (58). It is conceivable that these short hydrophobic peptides are transported by PptAB and then reimported by the Opp complexes to interact with these RNPP family proteins to regulate

gene expression in *E. faecalis*. Characterizing these *E. faecalis* RNPP homologs and identifying a role for their cognate peptides is the current focus of ongoing studies in the laboratory.

Recent studies have demonstrated a role for the oligopeptide permease complex in virulence in several pathogenic bacteria including *Mycobacterium tuberculosis*, *S. pyogenes* and *Borrelia burgdorferi* (62-64). To our knowledge, this is the first study examining the contribution of *E. faecalis* Opps in biofilm formation and virulence using the nematode infection model. Of the two Opps, Opp1 appeared to exhibit a greater contribution to pathogenesis in the nematode infection model, as well as pheromone uptake for conjugal mating. However, deletion of both Opps was required to achieve the strong attenuation in biofilm development. The question remains why *E. faecalis* acquired two Opp systems. There may be substrates to which one Opp is better tailored to import. It is noteworthy that recent transcriptome analyses comparing growth in serum and urine for host cues to infection identified the second Opp system [EF_3106-3110] as being significantly induced (4-6 fold) compared to growth in laboratory medium (65, 66), suggesting that Opp2 may have more relevancy in mammalian infection settings.

E. faecalis in recent years has surfaced as one of the leading causes of nosocomial infection due to its ability to survive antibiotic treatments and cause persistent infections in a host (57, 67). In the dawn of the post-antibiotic era, it is of paramount importance to identify novel candidate genes for targeted therapeutic intervention and oligopeptide permeases represent a viable target for rational drug design.

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CHAPTER 4

Eep CONFERS LYSOZYME RESISTANCE TO *Enterococcus faecalis* VIA THE ACTIVATION OF THE ECF SIGMA FACTOR, SigV.

ABSTRACT:

Enterococcus faecalis is a commensal bacterium found in the gastro-intestinal tract of most mammals including humans and is one of the leading causes of nosocomial infections. One of the hallmarks of *E. faecalis* pathogenesis is its unusual ability to tolerate high concentrations of lysozyme, which is an important innate immune component of the host. Previous studies have shown that the presence of lysozyme leads to the activation of SigV, an ECF sigma factor in *E. faecalis* and the deletion of *sigV* increases the susceptibility of the bacterium towards lysozyme. Here we describe the contribution of Eep, a membrane-bound zinc metalloprotease, to the activation of SigV under lysozome stress by its effects on the stability of the anti-sigma factor RsiV. We demonstrate that the Δ*eep* mutant phenocopies the Δ*sigV* mutant in lysozyme, heat, ethanol and acid stress susceptibility. We also show using an immunoblot analysis that in an *eep* deletion mutant, the anti-sigma factor RsiV is only partially degraded after lysozyme exposure, suggesting that RsiV is processed by unknown protease(s) prior to the action of Eep. An additional observation is that the deletion of *rsiV* which results in constitutive SigV expression leads to chaining of cells, suggesting that SigV might be involved in regulating cell-wall modifying enzymes important in cell wall turnover. We also demonstrate that in the absence of *eep* or *sigV*, enterococci bind significantly more lysozyme providing a plausible explanation for the increased sensitivity of these mutants towards lysozyme.

INTRODUCTION:

Enterococcus faecalis is a commensal organism present in the mammalian gastrointestinal system (1). Over the past few decades, *E. faecalis* has arisen as one of the leading causes of nosocomial infection (2). Its role as an opportunistic pathogen is strengthened by harboring mobile genetic elements, which are often responsible for conferring resistance to a broad range of antibiotics including vancomycin (3). In addition, *E. faecalis* is known to demonstrate a heightened ability to survive in the presence of environmental stress factors such as increased temperature, acidic pH, and oxidative stress (4). In addition to persistence in the presence of the aforementioned stress factors, previous studies have shown that *E. faecalis* is also highly resistant to lysozyme (5). This high-level resistance to lysozyme (> 62 mg/ml) is predominantly attributed to the ECF sigma factor SigV (5). ECF sigma factors are sequestered by membrane-bound anti-sigma factors and rendered inactive in the absence of a given external stress. Under stress-inducing conditions the anti-sigma factors are degraded by membrane and cytosolic proteases leading to the activation of ECF sigma factors in a process referred to as a regulated intramembrane proteolysis (RIP) (6).

RIP has been shown to play an important role in multiple transmembrane signaling processes associated with increased virulence and environmental fitness (7). In *Escherichia coli*, DegS, a site-1 protease and RseP, a site-2 protease, have been shown to degrade the anti-sigma factor RseA in response to environmental stress, thus mediating the release of SigE (8). A similar mechanism is displayed by *Bacillus subtilis* in which PrsW and RasP are the site-1 and site-2 proteases respectively (9). In *E. faecalis*, neither site-1 nor site-2 proteases involved in the processing of extracytoplasmic function (ECF) sigma factors have been identified to date. The release of SigV from RsiV is thought to require proteolytic cleavage of RsiV. A candidate site-2

protease was predicted to be Eep, as it possesses many of the characteristics of membrane localized site-2 proteases (10). We hypothesized that Eep would play an important role in the regulated intramembrane proteolysis of RsiV leading to the activation of SigV. Here we show that Eep is essential for the complete degradation of RsiV which in turn is essential for the activation of SigV. Furthermore, the deletion of *eep* retards the ability of the SigV regulon to respond to lysozyme and several other stresses, which likely explains the significant contribution that Eep plays during infection (11).

MATERIALS AND METHODS:

Bacterial Strains, Plasmids & Growth Conditions:

Pertinent bacterial strains and plasmids used in the current study are listed in Table 4-1 and Table 4-2. Strains were cultured in Todd-Hewitt broth (THB) and grown at 37˚C unless otherwise indicated. *Escherichia coli* ElectroTen-Blue (Stratagene, La Jolla, CA) and *E. faecalis* V583 were used for maintenance and propagation of plasmid constructs. ElectroTen-Blue clones were cultured aerobically in Luria-Bertani (LB) broth at 37˚C and *E. faecalis* V583 and FA2-2 derived strains were cultured in THB at 37˚C. Antibiotics for selection included chloramphenicol at 10 µg ml^{-1} and spectinomycin at 150 µg ml^{-1}, for *E. coli* and chloramphenicol at 15 µg ml^{-1} and spectinomycin at 500 µg ml^{-1}, for *E. faecalis*. Transformation of plasmids into *E. faecalis* was done as described previously (12).

Strain	Genotype or description	Reference
V583	Parental Strain	(48)
FA2-2	Parental Strain	(14)
SV03	V583Δ*eep*	This Study
SV05	FA2-2Δ*eep*	This Study
SV07	V583Δ*sigV*	This Study
SV08	SV03(pSV24) complement of *eep* mutant, Spec^r	This Study
SV17	SV03(pSV04) empty vector control, Spec^r	This Study
SV09	V583(pSV23) GFP-RsiV fusion, Spec^r	This Study
SV10	SV03(pSV23) GFP-RsiV fusion, Spec^r	This Study
SV11	V583(pSV14) *sigV* promoter fusion to *lacZ*, Spec^r	This Study
SV12	SV03(pSV14) *sigV* promoter fusion to *lacZ*, Spec^r	This Study
SV13	SV07(pSV14) *sigV* promoter fusion to *lacZ*, Spec^r	This Study
SV14	V583Δ*rsiV*	This Study
SV15	SV14(pSV14) *sigV* promoter fusion to *lacZ*, Spec^r	This Study
WM01	FA2-2Δ*sigV*	This Study
WM02	V583Δ*eep*Δ*rsiV*	This Study
VI50	V583Δ*pgdA*	This Study
VI60	V583(pVI16) *pgdA* promoter fusion to *lacZ*, Spec^r	This Study
VI61	SV03(pVI16) *pgdA* promoter fusion to *lacZ*, Spec^r	This Study
VI62	SV07(pVI16) *pgdA* promoter fusion to *lacZ*, Spec^r	This Study
SV16	SV14 (pVI16) *pgdA* promoter fusion to *lacZ*, Spec^r	This Study

Table 4-1: Bacterial strains used in this study

Plasmid	Description	Reference
pLT06	Deletion vector; chloramphenicol resistance	(13)
pSV03	pLT06 containing engineered *eep* deletion (~2-kb EcoRI/PstI fragment)	This Study
pSV15	pLT06 containing engineered *sigV* deletion (~2-kb EcoRI/PstI fragment)	This Study
pML28	pAT28 derivative containing the *aph* promoter	(18)
pSV17	pML28 derivative containing *rsiV* with an N-terminal flag tag	This Study
pSV23	pSV17 derivative containing a *gfp-rsiV* fusion under the constitutive *aph* promoter.	This Study
pTCVLac-Spec	Shuttle vector for promoter fusion studies, Erm^r, $Spec^r$	(16)
pKS12A	A derivative of pTCVLac-Spec in which the erythromycin methylase gene was removed by AflII digestion, $Spec^r$	This Study
pMV158GFP	*gfp* containing plasmid, Tet^r	(19)
pSV04	A derivative of pTCVLac-Spec in which the *lacZ* and erythromycin methylase genes were removed by SalI digestion, $Spec^r$	This Study
pSV24	pSV04 containing full length *eep* under the native *eep* promoter	This Study
pVI15	pLT06 containing engineered *pgdA* deletion (~2-kb EcoRI/PstI fragment)	This Study
pWM07	pLT06 containing engineered *rsiV* deletion (~2-kb BamHI/PstI fragment)	This Study
pSV14	pKS12A containing *sigV* promoter region (1234-bp EcoRI/BamHI fragment)	This Study
pVI16	pKS12A containing *pgdA* promoter region (1005-bp EcoRI/BamHI fragment)	This Study

Table 4-2: Plasmids used in this study.

Construction of *E. faecalis* in-frame deletion mutants:

Primer	Sequence (5'-3')
EepP1	GAGAGGATCCGACATCAATGACACGTTGCC
EepP2	CTCTTCTAGAGTTTTCATAGATGTCCTTCTTC
EepP3	GAGATCTAGAACTTGGAACGATATTCAACGC
EepP4	CTCTGCATGCCCACGAACCAAGACCATTAC
EepUp	TCTTTGGTGTACGGAGGACA
EepDown	GCATCTTCTTCTGTTGCTTC
Eep 5'	GAGAGAATTCACTTTCATGGTAGCACATGTC
Eep 3'	CTCTGGATCCCTGTTTCATTAAAACTCTCCTC
SigVP1	GAGAGAATTCGAGCAGATTCGGAACTTTGAG
SigVP2	CTCTGGATCCAACCATCGATTTCTGGAACCT
SigVP3	GAGAGGATCCCTCCGAAGTCTATTGAATTAGT
SigVP4	CTCTCTGCAGCAACTGACTTGGTTAGGTCAG
SigVUp	GTCACACATTGGCTTATAAGG
SigVDown	GCCACTTCTTCTTCGTTTCC
PgdAP1	GAGAGAATTCGCTTGATTTGCTTGCAGTGC
PgdAP2	CTCTGGATCCATGTCGCATACTTTCACTCCT
PgdAP3	GAGAGGATCCTAGAGCAACTCGGAGCAC
PgdAP4	CTCTCTGCAGGCCACCTTATGATCCAAGAG
PgdAUp	TCGCTTGGCTACTGTTGTGC
PgdADown	TTGCGAATACTCCTGAAGTAC
RsiVP1	GAGAGGATCCCGGTATCTGTTGTTAATGGTG
RsiVP2	CTTCTTCTAGACCGTTGGCAACGGTTGTTG
RsiVP3	GAGATCTAGATGATTCCTGATCAAGTCATTG
RsiVP4	CTCTCTGCAGCAATGACTTGGTCGTTGCTG
RsiVUp	CCGAGGAAGTCCTGCAAGG
RsiVDown	TCACTAATGGTAATGGTTGATC
RsiV5'	GACTACAAAGACGATGACGACAAGCATATGGAAGATTTTGTAAAAAGTG TG
RsiV3'	CTCTGCATGCGTCGCGTGTTTTTTACTGAGT
GFP5'	GAGAGGATCCAAGGAGGAAAAACATATGAGTA
GFP3'	CTCTATTAATTTTGTATAGTTCATCCATGCC
FLAGTAG	GAGAGGATCCAAGGAGGATTTATAGATGGATTATAAGGATCATGA TTATAAGGATCATGATATCGACTACAAAGACGATGACGACAAG

Table 4-3: Primers used in the study.

In frame deletions of *sigV, rsiV, pgdA* and *eep* individually and the double deletion mutant of *rsiV* and *eep* in *E. faecalis* were done using plasmids derived from pLT06 (13), an *E. coli* enterococcal temperature-sensitive cloning vector that possesses selectable and counterselectable markers that aid in the selection of mutants containing the targeted deletions. The primers used for all the deletions are listed in Table 4-3. Flanking regions (~1Kb) from both the 5' and 3' ends of *sigV, rsiV, pgdA* and *eep* were PCR amplified by using the primers listed in Table 4-3.

For the construction of the pSV03 plasmid (*eep* deletion) the primers EepP1 and EepP2 were used to amplify the ~1Kb region flanking the 5' end of *eep* on the V583 genome. Primers EepP3 and EepP4 were used to amplify the ~1Kb region flanking the 3' end of the *eep* region. The EepP1 and EepP2 primers contained EcoRI and XbaI sites, respectively and EepP3 and EepP4 primers contained XbaI and SphI sites, respectively. Each product was cut with the respective restriction enzymes, and ligated to pLT06 cut with EcoRI and SphI prior to electroporation into *E. coli* ElectroTen-Blue cells. Confirmation of the appropriate clones was performed by restriction digest and sequence analysis. An analogous approach was used for the construction of pSV15 (*sigV* deletion); pWM07 (*rsiV* deletion) and pVI15 (*pgdA* deletion). Purified plasmids from *E. coli* cells were electroporated into electrocompetent V583 cells. SV03 [V583Δ*eep*], SV07 [V583Δ*sigV*], SV14 [V583Δ*rsiV*], and VI50 [V583Δ*pgdA*] were generated following the protocol as previously described (13). The *eep* deletion allele was designed such that the first 2 and the last 7 codons remained (98% deleted). For *sigV,* the deletion allele consisted of the initial 6 and last 7 codons (92% deleted). The deletion for *rsiV* included the first 6 and last 7 codons (96% deleted), while the *pgdA* deletion allele possessed the first 3 and the last codon (99% deleted). To create the double deletion of *rsiV* and *eep,* electrocompetent SV03 cells were transformed with pWM07, and

the deletion mutant designated as WM02 [V583Δ*eep*Δ*rsiV*] was similarly created (13). To rule out strain differences associated with V583, we also created deletion mutants for *eep* and *sigV* in the FA2-2 (14) strain background. FA2-2 is a plasmid-free strain derived from the non-hemolytic, non-proteolytic clinical isolate JH2 (15). FA2-2 was transformed with pSV03 (Δ*eep*) and pSV15 (Δ*sigV*) and following plasmid integration and excision events, the deletion strains SV05 [FA2-2Δ*eep*] and WM01 [FA2-2Δ*sigV*] were created.

Complementation of *eep* deletion mutant:

An in-frame *eep* deletion in *E. faecalis* V583 was complemented with full length *eep* under the control of the native *eep* promoter region in a pSV04 vector background and was denoted as pSV24. The *eep* complement amplified from the V583 genome with primers Eep5' and Eep3' (Table 3) and subsequently inserted as an EcoRI/BamHI fragment into pSV04 cut with EcoRI/BamHI. To construct pSV04, plasmid pTCVLac-Spec (16) was digested with SalI and self-ligated to release the SalI fragment containing the *lacZ* gene. Plasmid pSV24 was transformed into SV03 [V583Δ*eep*], and phenotypic complementation was confirmed by a lysozyme minimum inhibitory concentration (MIC) assay.

MIC assay for determining lysozyme sensitivity:

The MIC of lysozyme against the strains V583, SV03 [V583Δ*eep*], SV07 [V583Δ*sigV*], SV14 [V583Δ*rsiV*], VI50 [V583Δ*pgdA*]; WM02 [V583Δ*eep*Δ*rsiV*], SV08 [SV03 with Eep complementation vector], along with FA2-2, and its isogenic derivatives SV05 [FA2-2Δ*eep*] and WM01 [FA2-2Δ*sigV*] were determined by two-fold serial dilution of a 250 mg/ml lysozyme stock in LB broth to achieve a series of lysozyme concentrations ranging from 0 mg/ml to 62.5mg/ml in a 96 well microtiter plate. Briefly, the strains were grown as standing cultures at 37°C in LB broth

overnight to reach stationary phase. These overnight cultures (~ 10^8 CFU/ml) were diluted 1:100 in fresh LB and then 100 µl was added to 100 µl of the LB containing serially diluted lysozyme, such that each well contained an initial inoculum of ~ 10^5 CFU. The plate was then incubated at 37˚C for 24 hours before the results were documented. SV08 [SV03 with *eep* complementation vector, pSV24] and SV17 [SV03 with empty vector pSV04] were grown overnight in the presence of 500 µg ml^{-1} spectinomycin for plasmid maintenance.

Settling and Chaining Assays:

V583, SV14 [V583Δ*rsiV*], SV03 [V583Δ*eep*], SV07 [V583Δ*sigV*] and WM02 [Δ*eep*Δ*rsiV*] were grown in THB overnight at 37°C with and without lysozyme at 1 mg/ml and photographed to observe the settling phenotype, which is noted by growth of the bacterium at the bottom of the test tube. The upper layer of the growth medium becomes transparent as cells settle and grow on the bottom of the tube. Liquid cultures from the respective strains were also Gram-stained and photographed to observe chaining (Nikon Eclipse 80i; 100X Oil Immersion Objective).

Miller assay using strains containing P_{sigV}-*lacZ* and P_{pgdA}-*lacZ* reporter fusion plasmids:

To investigate the transcriptional activity of known SigV-dependent promoters, we created *sigV* and *pgdA* promoter fusions to a *lacZ* reporter in plasmid pKS12A, a derivative of pTCV-Lac Spec (16) , in which a small AflII fragment containing the erythromycin methylase gene was deleted. pSV14 (P_{sigV}-*lacZ*) and pVI16 (P_{pgdA}-*lacZ*) were created by amplifying the promoter regions of *sig*V with primers SigVP1 and SigV2 and the *pgdA* promoter with primers PgdAP1 and PgdAP2, respectively. The promoter regions for both these plasmids were defined based on the known transcriptional start sites for both *sigV* and *pgdA* (4). These promoters contain the consensus SigV promoter recognition sequence (5' **TGAAAC**-N_{17}-**CGTC** 3'), and we included an additional ~ 1

kb region (1188 bp for *sigV* and 914 bp for *pgdA*) upstream of the transcriptional start site to provide additional genetic context for the promoter fusion studies. Primers were engineered with EcoRI and BamHI restriction sites to facilitate cloning into pKS12A. Resulting vectors pSV14 and pVI16 were transformed into V583, SV03 [V583Δ*eep*], SV07 [V583Δ*sigV*] and SV14 [V583Δ*rsiV*] strains. The new strains SV11 [V583 (pSV14)], SV12 [SV03 (pSV14)], SV13 [SV07 (pSV14)], SV15 [SV14 (pSV14), VI60 [V583 (pVI16)], VI61 [SV03 (pVI16)], VI62 [SV07 (pVI16)] and SV16 [SV14 (pVI16)] were grown overnight in THB containing 500 μg ml^{-1} of spectinomycin at 37°C for plasmid maintenance. The overnight cultures were diluted 1:100 in sterile THB containing spectinomycin and cultured to reach an OD_{600} of 0.5. At this point, cells were induced with lysozyme at a concentration of 1mg/ml for 30 minutes. The cultures were then processed to evaluate β-galactosidase activity according to the modified Miller assay as described (17). To establish a dose response curve to induction by lysozyme, SV11 [V583 (pSV14)] was exposed to increasing concentrations of lysozyme (0, 1, 10, 100 and 1000 μg/ml) for 30 minutes prior to assaying for Beta-galactosidase activity. All assays were repeated three times and statistical significance was determined using 1way-ANOVA.

Temperature, Ethanol and Acid Challenge Assays:

In order to determine the survival of V583, SV03, SV14, WM02 and SV07 under different stress conditions, the protocol described by Benachour *et.al* (4) was followed. The strains were grown in 5 ml THB medium at 37°C to an optical density OD_{600} of 0.5 (mid-exponential growth phase). Bacteria were harvested by centrifugation, resuspended in 5 ml of fresh medium, and then exposed to the following stresses: (i) for high-temperature heat shock, the cultures were transferred to 62°C (ii) for ethanol shock; ethanol was added to a final concentration of 22% (vol/vol) (iii) for acid shock, the pH was adjusted to 3.2 with 85% lactic acid. Cells were exposed to stress conditions

and surviving bacterial numbers were quantified by plate counting at 0, 1 and 2 hours after stress initiation. Assays were repeated three times and statistical significance was established using 2 way-ANOVA in the GraphPad 5 software package (Prism, San Diego, CA) Percent survival shown in the graphs represents the ratio of the number of viable cells after exposure to stress to the number of cells at time zero prior to challenge.

Coomassie Brilliant Blue (CBB) staining of whole cell lysates:

Whole cell extracts of V583, SV03 and SV07 were prepared by growing strains in THB to an OD_{600} of 0.7-0.8. Lysozyme at a concentration of 1mg/ml was added to the cultures for 2 hrs and the cultures were centrifuged and the pellet was washed twice with 1ml of TE Buffer (pH 8.0). These 1ml suspensions were lysed using a mini bead-beater (BioSpec Products, Bartlesville, OK) and the addition of a 500 µl volume of 0.1 mm zirconia beads with a speed setting set at 4800 rpm for 1 minute. After brief centrifugation to settle the beads, a 5X-SDS loading dye was added to the whole cell lysate and samples were boiled at 100°C for ten minutes. To normalize the amount of protein loaded onto the polyacrylamide gel, the whole cell lysates were also subjected to Bradford protein assay to determine protein amounts in each sample. The same amount of protein was loaded into each well and the samples were run at 200V for an hour and were then subjected to CBB staining. Whole cell lysates from cultures without lysozyme added and purified lysozyme protein were used as controls.

Immunoblotting to detect RsiV degradation:

Plasmid pSV23 expresses the GFP-RsiV fusion protein and was constructed in the following manner. The *rsiV* gene was amplified from V583 genome using primers RsiV5' and RsiV3', which contained NdeI and SphI sites respectively. In a second round of PCR, this product was amplified with an additional primer, designated FLAG-TAG and RsiV3', the resulting product contained an

introduced BamHI site and a Flag-tag encoding sequence at the 5' end. This BamHI and SphI digested product was cloned into similarly digested pML28 (A pAT28 derivative containing an *aph* promoter) (18) to create pSV17. We next swapped the Flag-tag for a GFP tag by digesting pSV17 with BamHI and NdeI. The GFP encoding region was amplified from pMV158GFP (19) using primers GFP-5'BamHI and GFP-3'AseI. This PCR product was digested with BamHI and AseI and ligated to BamHI and NdeI digested pSV17 to obtain pSV23. *E. faecalis* strains V583 and SV03 were transformed with pSV23 to create SV09 and SV10, respectively. Cultures of SV09 [V583 (pSV23)] and SV10 [SV03 (pSV23)] were grown overnight in THB at 37°C and diluted 1:100 into 10 ml of fresh THB. Cultures were grown to an OD_{600} of 0.7-0.8 at which point lysozyme was added to a concentration of 1 mg/ml and the cultures were incubated for an additional 2 hrs. SV09 and SV10 cultures to which no lysozyme was added were used as negative controls. All four cultures were centrifuged and the pellets were resuspended in 1ml TE buffer (pH 8.0). Protease inhibitors and EDTA were added to these suspensions and subjected to mini-bead beating as described. The lysates were analyzed on a 10% SDS-PAGE gel. Following electrophoresis, samples were electrotransferred to a PVDF membrane and blotted with Rabbit anti-GFP primary antibody (Cell Signaling Technology, Inc.) and anti-rabbit HRP conjugated 2° antibody (Sigma Aldrich).

RESULTS:

Deletion of *eep* renders *E. faecalis* more susceptible to lysozyme:

To test whether an *eep* deletion mutant showed increased susceptibility towards lysozyme when compared to the wild-type strain, we determined the lysozyme MIC for V583, SV03 and SV08 (*eep* complement). The results of this assay are shown in Table 4-4. The increased susceptibility of the *eep* mutant paralleled that of the *sigV* mutant, as both displayed MIC values at 5 mg/ml

compared to the > 62 mg/ml for V583. When the *eep* gene was complemented back into the *eep* deletion mutant using a low-copy plasmid, the complement strain behaved similar to the wild-type

Strain	MIC (mg/ml)
V583	> 62.5
FA2-2	> 62.5
SV03 [V583Δ*eep*]	5.0
SV07 [V583Δ*sigV*]	5.0
VI50 [V583Δ*pgdA*]	> 62.5
SV05 [FA2-2Δ*eep*]	5.0
WM01 [FA2-2Δ*sigV*]	5.0
SV08 [SV03 (pSV24)]	> 62.5
SV17 [SV03 (pSV04)]	5.0
WM02 [V583Δ*eep*Δ*rsiV*]	> 62.5
SV14 [V583Δ*rsiV*]	> 62.5

Table 4-4: Minimum Inhibitory Concentration (MIC) Assay.

suggesting that the phenotype we observed was Eep dependent. Consistent with previous observations, the *pgdA* deletion mutant did not show any change in lysozyme susceptibility compared to the wild-type (20) even though it is known to be regulated by SigV at the transcriptional level (4, 5). FA2-2 and mutants lacking *eep* and *sigV* in this genetic background were used as controls in this study to eliminate any strain bias in the lysozyme resistance mechanism. We observed a similar reduction in the MIC for the *eep* and *sigV* mutants in the FA2-2 strain background compared to FA2-2. Finally, as a proof of principle that Eep acts through

RsiV in the activation of SigV, we deleted *rsiV* in the *eep* mutant background, and showed that the lysozyme resistance level of this double mutant paralleled that of the wild-type (> 62.5 mg/ml).

Eep is essential for the expression of *sigV* at the transcriptional level:

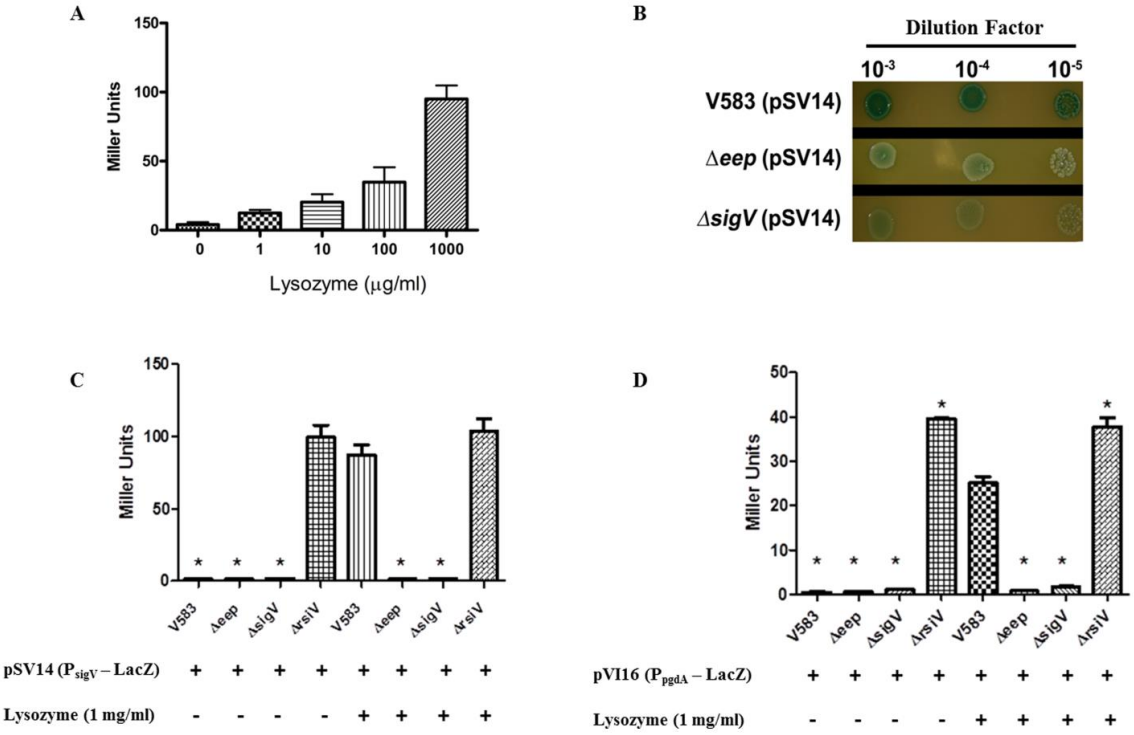

Figure 4-1: Qualitative and quantitative measurement of *sigV* promoter and *pgdA* promoter activity. (A) A *sigV-lacZ* reporter strain SV11 was subjected to Miller assay analysis after treatment with increasing concentrations of lysozyme. (B) Spot assay of *sigV-lacZ* reporter strains SV11, SV12 [Δ*eep*] and SV13 [Δ*sigV*] on THB plates containing 1 mg/ml lysozyme and 80 μg/ml X-gal. (C) Miller assay using LacZ reporter strains SV11, SV12, SV13, and SV15 [Δ*rsiV*] treated with and without 1 mg/ml of lysozyme. (D) Miller assay using *pgdA-lacZ* reporter strains VI60, VI61 [Δ*eep*], VI62 [Δ*sigV*] and SV16 [Δ*rsiV*] treated with and without 1 mg/ml of lysozyme. * indicates significant differences ($P < 0.001$) relative to wild-type V583 in the presence of lysozyme. Experiments were performed in triplicate and the error bars represent standard error of the mean (SEM).

It is known that SigV autoregulates its own expression (4). We constructed a *sigV* promoter fusion to *lacZ* to confirm these observations, and to ascertain the effect of both *sigV* and *eep* deletion on promoter activity. We confirmed that in wild-type cells containing the reporter plasmid (SV11) that the activity of the *sigV* promoter is induced by lysozyme in a dose dependent manner (Fig. 4-1A). In panel B of figure 4-1, we also demonstrated that promoter activity is dependent on a functional SigV as well as Eep for the response to lysozyme, as mutants in *eep* and *sigV* remain white in the presence of lysozyme, whereas the wild-type reporter strain turned blue on X-gal containing agar. Miller assays were performed with the reporter strains to quantify the amount of β-galactosidase protein produced in the wild-type compared to the mutants. SV11 produces ~80 fold more β-galactosidase activity when compared to the *eep* and *sigV* mutants upon lysozyme induction (Fig. 4-1C). We also analyzed reporter strains with the *pgdA* promoter fusion to *lacZ*, as expression of *pgdA* was previously shown to be SigV-dependent (4). Similar results were observed with the Miller assay (Fig. 4-1D) in these reporter strains as both the *eep* and *sigV* mutants displayed reduced promoter activity compared to the parental strain. Consistent with its known role as an anti-sigma factor, the deletion of *rsiV* resulted in constitutive expression of both SigV dependent promoters in the absence of lysozyme induction (Fig. 4-1C and 4-1D).

Eep confers resistance to other biological stressors:

Benachour *et al.* (4) showed that a *sigV* deletion mutant is attenuated when subjected to high temperature, low pH and ethanol stress conditions compared to a wild-type. Having established a link between Eep and SigV phenotypes for lysozyme resistance, we reasoned that other biological stresses known to impact a *sigV* mutant might also negatively affect an *eep* mutant compared to

its isogenic parent. We therefore tested the *eep* and *sigV* mutants against a variety of biological stresses (heat, low pH and ethanol stress) and found that the *eep* mutant phenocopied a *sigV* mutant

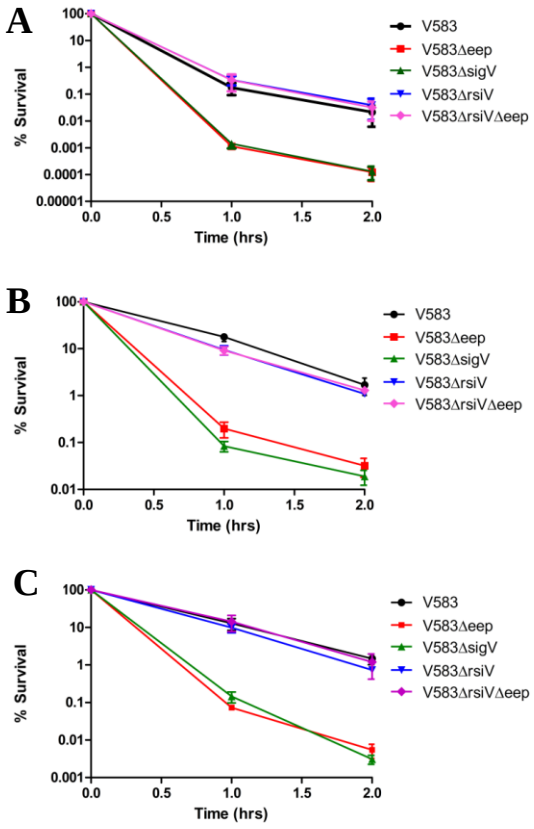

Figure 4-2: V583, SV03 [V583Δ*eep*], SV14 [V583Δ*rsiV*], WM02 [V583Δ*rsiV*Δ*eep*] and SV07 [V583Δ*sigV*] strains subjected to different stresses. (A) Strains were subjected to a temperature of 62˚C. (B) Strains were subjected to a pH of 3.2 using 85% lactic acid. (C) Strains were subjected to 22% ethanol treatment. Strains subjected to lactic acid stress and ethanol stress were incubated at 37 ˚C. Samples were drawn every hour for 2 hours and were serially diluted in sterile PBS (pH 7.4) and plated onto THB agar. V583Δ*eep* and V583Δ*sigV* were significantly attenuated compared to wild-type (*P <0.001*). Experiments were performed in triplicate and the error bars represent standard error of the mean (SEM).

in these biological aspects. Both mutants displayed near 2-log reductions against these stresses compared to V583 alone (Fig. 4-2). To confirm that the deletion of *rsiV* in the *eep* deletion strain

restores the stress tolerance to wild-type levels, the strain harboring a double deletion of *rsiV* and *eep* was also tested against the aforementioned biological stresses. This strain showed tolerance to these stresses similar to the wild-type. As a control, *rsiV* deletion mutant was also used in this study. This suggests that deletion of Eep has a direct effect on the ability of cells to adapt to stress and that this phenotype could be rescued by the constitutive expression of SigV.

Eep is essential for complete processing of RsiV:

Since the strain that lacked *eep* phenocopied the strain that lacked *sigV* in all the phenotypes tested, our next goal was to determine the exact role of Eep in the regulated intramembrane proteolysis pathway leading to release of SigV. To confirm that Eep is indeed the site-2 protease, we constructed plasmid system wherein GFP was fused to RsiV and introduced into either the parental strain V583 or its isogenic *eep* mutant [SV03].

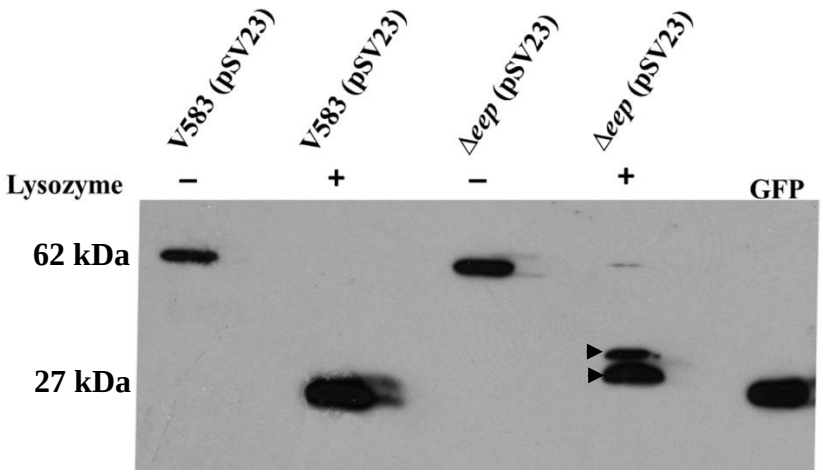

Figure 4-3: Immunoblot analysis with Anti GFP antibodies. A native GFP cell lysate was used as a control. The unprocessed GFP-RsiV translational fusion is predicted to migrate at 62 kDa, whereas the fully processed GFP-RsiV migrates at the native GFP position (27 kDa). Arrows depict incompletely processed GFP-RsiV in the *eep* mutant exposed to lysozyme.

Figure 4-3 demonstrates the result of this experiment wherein the plasmid bearing strains SV09 and SV10 [Δ*eep*] expressed the ~ 62 kDa GFP-RsiV fusion proteins in the absence of lysozyme stress. When lysozyme was added, RsiV in the V583 background was completely degraded by membrane and cytosolic proteases involved in RIP leaving only the ~27 kDa GFP protein. The presence of the GFP protein after SigV activation is likely attributed to the fact that GFP lacks the recognition domain that is required for Clp protease degradation of target substrates (21). However, accumulation of two partially processed GFP-RsiV products predicted to migrate between 34 and 38 kDa was observed when SV10 was treated with lysozyme.

***eep* and *sigV* mutants bind more lysozyme compared to the wild-type:**

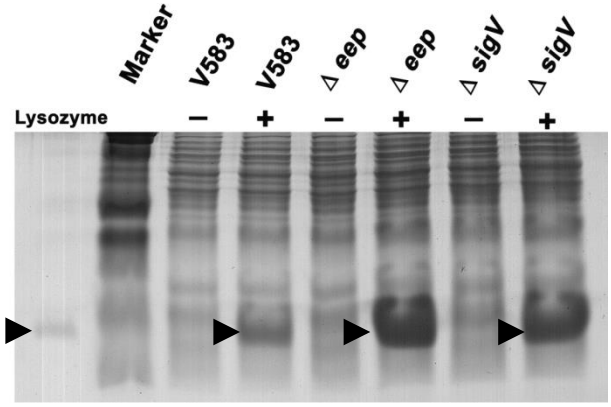

Figure 4-4: SDS-PAGE and Coomassie Brilliant Blue staining assay. V583, SV03 [Δ*eep*] and SV07 [Δ*sigV*] were grown to an OD_{600} of 0.8 in the presence and absence of 1 mg/ml lysozyme. Normalized lysates were loaded onto a 12% polyacrylamide gel and stained with Coomassie brilliant blue dye. Purified lysozyme (1 μg) was added to the left of the marker lane as a control. Arrows indicate bands corresponding to lysozyme.

Enhanced susceptibility of SV03 [Δ*eep*] and SV07 [Δ*sigV*] towards lysozyme led us to believe that these strains might be lacking some crucial mechanism that reduces the accumulation of lysozyme on the cell surface. The results depicted in figure 4-4 demonstrate that the *sigV* and *eep* mutants bind more lysozyme compared to the wild-type. Mass-spectrometry analysis was performed to confirm that the protein band that was visualized primarily consisted of lysozyme (data not shown). This data suggests that the hyper-susceptibility of SV03 and SV07 towards lysozyme is likely due to the enhanced accumulation of lysozyme on the cell surface of these mutants.

Constitutive expression of SigV results in a chaining phenotype:

Interestingly, when the gene encoding for RsiV was deleted in the wild-type and V583 Δ*eep* genetic backgrounds, a settling phenotype of the overnight culture was observed in both strains. Figure 4-5 demonstrates this settling phenotype. Gram-stain revealed significant chaining in strains lacking RsiV compared to the wild-type, suggesting that appropriate cell wall remodeling is compromised by constitutive expression of SigV. The chaining phenotype of the *rsiV* mutant is similar to that observed for an *atlA* mutant (22), suggesting that the cell wall modifications that render *E. faecalis* more resistant to lysozyme also perturb the activity of the major autolysin.

Lysozyme induction results in a chaining phenotype:

Because of the chaining phenotype observed in the *rsiV* mutant, we reasoned that exposure to lysozyme would induce a chaining phenotype that was SigV-dependent. To test this prediction,

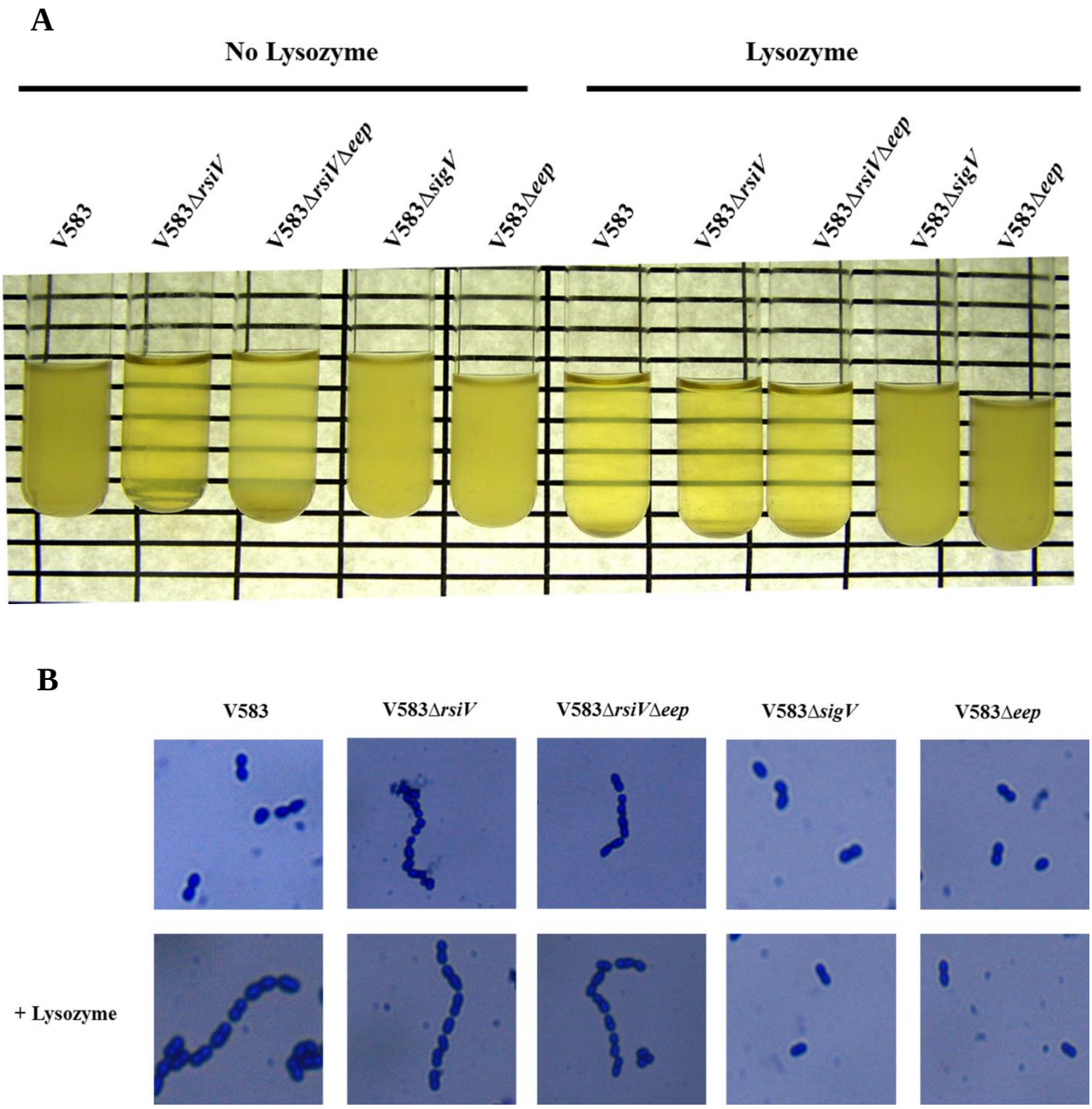

Figure 4-5: Chaining and settling phenotypes. A) Strains indicated were grown in the presence or absence of 1 mg/ml lysozyme in THB overnight at 37˚C and photographed to depict the settling of the culture on the bottom of the tube. B) Gram-staining was performed on each culture to observe cell chaining.

we exposed V583, SV03 [Δ*eep*], SV07 [Δ*sigV*], SV14 [Δ*rsiV*] and WM02 [Δ*eep*Δ*rsiV*] to 1 mg/ml lysozyme and examined the cultures for a settling phenotype. As predicted, a settling phenotype was observed in the wild-type when induced with lysozyme and chaining was confirmed by Gram staining (Fig. 4-5). In contrast, the *eep* and *sigV* mutants did not settle or chain in the presence of lysozyme. The strains containing an rsiV deletion: SV14 [V583Δ*rsiV*] and WM02 [V583Δ*rsiV* Δ*eep*] continued to chain and settle under lysozyme exposure. This suggests that activation of SigV leads to the chaining phenotype, and that genes under SigV control are also likely regulating the activity of the endogenous autolysins of *E. faecalis*.

DISCUSSION:

In this study, we have shown that Eep is essential for the activation of SigV which is an ECF sigma factor that contributes to lysozyme resistance in *E. faecalis* via the degradation of the anti-sigma factor RsiV (5). Eep belongs to a family of membrane embedded zinc metalloproteases (23). Previous studies have shown that Eep is involved in the processing of peptide pheromones (24-26). To the best of our knowledge, this is the first study that demonstrates an additional role for Eep in the regulated intramembrane proteolysis of the anti-sigma factor RsiV leading to the activation of SigV. An *eep* deletion mutant phenocopied a *sigV* deletion mutant, and was greater than 10-fold more susceptible to lysozyme when compared to the wild-type. Promoter fusion studies demonstrate that the *sigV* promoter is inactive in the absence of Eep. Our hypothesis that Eep is directly involved in the degradation of RsiV was strengthened by the observation that the resistance to lysozyme was restored to wild-type levels in the double mutant (Δ*rsi*VΔ*eep*) strain.

In addition, western blot analysis revealed that Eep is required for the complete degradation of RsiV. It has been shown that the activation of other ECF sigma factors (6), in the presence of a specific stress, requires the action of membrane proteases, and the data from this current study confirms the rationale that Eep is one such membrane protease. Of note, we were able to detect two intermediate processed forms of GFP-RsiV in the *eep* mutant background. This observation is consistent with the requirement for multiple RsiV processing events prior to Eep cleavage. In the activation of the *B. subtilis* SigW (ECF sigma factor), PrsW, a known site-1 protease cleaves RsiW (9, 27). Upon cleavage by PrsW, further processing of RsiW occurs by an as yet to be identified trimming protease activity (28), and this cleavage event prepares RsiW for further processing by the known site-2 protease RasP.

Data from a Coomassie-stained SDS-PAGE indicates that mutants lacking *eep* and *sigV* bind more lysozyme compared to the wild-type. Previous research has shown that two cell wall modifying enzymes, O-Acetyltransferase (OatA) and the D-alanylation complex (DltA-D) confer resistance to lysozyme in *B. subtilis,* and that these genes are directly regulated by SigV (29). However, in *E. faecalis* it has been shown previously that individual deletion of these genes did not significantly affect the lysozyme susceptibility of *E. faecalis*, nor where the *oatA* and *dltA-D* genes shown to be regulated in a SigV-dependent fashion (5). In our hands, we found that single deletions of both *oatA* and *dltA-D* in the V583 background did not alter the lysozyme resistance of these strains, as these mutants still showed growth up to 62.5 mg/ml lysozyme (data not shown). We also examined a double deletion of both *oatA* and *dltA-D* in the V583 background and found that this strain was only marginally reduced in lysozyme resistance to ~ 32 mg/ml (data not shown), which is in contrast to what was observed by Le Jeune (5), wherein a double mutant of *oatA* and *dlt* was as sensitive to lysozyme as the *sigV* mutant in the JH2-2 genetic background. Whether this difference

in lysozyme susceptibility can be accounted for by strain differences awaits additional study. It is however noteworthy that strain JH2-2 was derived from the parent strain JH2 by nitrosoguanidine mutagenesis (15), and the exposure to the mutagenizing agent may account for these aberrant strain differences. In contrast, the deletion of *sigV* rendered *E. faecalis* much more sensitive to lysozyme suggesting that SigV-dependent gene products are involved in modifying the enterococcal cell wall in a manner independent of peptidoglycan O-acetylation or teichoic acid D-alanylation. Furthermore, a study by Hébert (30) showed that a mutant in the gene encoding peptidoglycan deacetylase (*pgdA*) did not contribute to lysozyme resistance, despite the fact that this gene has been shown to be regulated in a SigV-dependent manner in *E. faecalis* (5).

Our present studies also confirmed the absence of a link between PgdA and lysozyme resistance in *E. faecalis* V583. PgdA does however contribute to lysozyme susceptibility in *Streptococcus pneumoniae* and virulence in animal models of infection (31-33), and recent evidence suggests that PgdA is linked to virulence in *E. faecalis* as a mutant in *pgdA* was attenuated in a *Galleria mellonella* infection model (20). What role PgdA might be playing in a mammalian host during infection remains to be elucidated. What is also clear from the present study and additional cited literature is that SigV contributes to lysozyme resistance in a unique manner and identifying the SigV regulon will be of paramount importance in understanding the unusual lysozyme resistance strategies employed by *E. faecalis*. Lysozyme is a naturally secreted antimicrobial agent, and is considered to be part of the innate immune system. It is secreted by a wide array of organisms in body fluids such as tears, mucus, and saliva (34-37). A study by Frank (11) showed that Eep is essential for the pathogenesis of *E. faecalis* in a rabbit endocarditis model. It has also been shown that the concentration of lactic acid is particularly high in the heart (38). One possible explanation as to why the virulence of an *eep* deletion mutant was highly attenuated in the endocarditis model

is that strains that lack *eep* are more susceptible to lactic acid stress as shown in the present study. Another plausible explanation is that strains lacking *eep* are more susceptible to lysozyme compared to wild-type controls, and are hence cleared more efficiently by components of the innate immune system (39, 40). The same idea could potentially be applied to a urinary tract infection (UTI) model due to the fact that it has been shown that the body responds to urinary tract infection by secreting an increased amount of lysozyme (41-43).

It is noteworthy that a *sigV* mutant of *E. faecalis* JH2-2 was shown to be attenuated by ~ 1.5-2 logs in bladder and kidney colonization compared to JH2-2 alone and an *eep* mutant of *E. faecalis* OG1RF is attenuated in a catheter associated UTI model (5, 44). Collectively, the available information to date suggests that the virulence associated with Eep is likely due to its effects on SigV activation. It will be of interest to establish a link with SigV in endocarditis. It is also important to note that lysozyme has been shown to be an important component of the innate immune defense system in the gastro-intestinal tract (45). Since *E. faecalis* is a commensal bacterium which predominantly resides in the gastro-intestinal tract of mammals, it is possible that this bacterium has unique mechanisms that confer high levels of lysozyme resistance giving it a competitive edge in the gastro-intestinal tract consortium. Figure 4-6 depicts the pathway by which SigV is released from the anti-sigma-factor in the presence of lysozyme. Having evidence that supports the rationale that Eep is a site-2 protease in the regulated intramembrane proteolysis pathway, we postulate that both a site-1 and trimming protease act upstream of Eep cleavage to initiate the response to lysozyme. The biochemical analysis of the GFP-RsiV fusion in an *eep* background exposed to lysozyme suggests the existence of such proteases, as noted by the intermediate forms of GFP-RsiV that accumulate in the immunoblot (Fig. 4-3). Numerous studies

have shown a requirement for site-1 proteases in initiating activation of ECF sigma factors (6), and future studies will be aimed at the identification of such a protease.

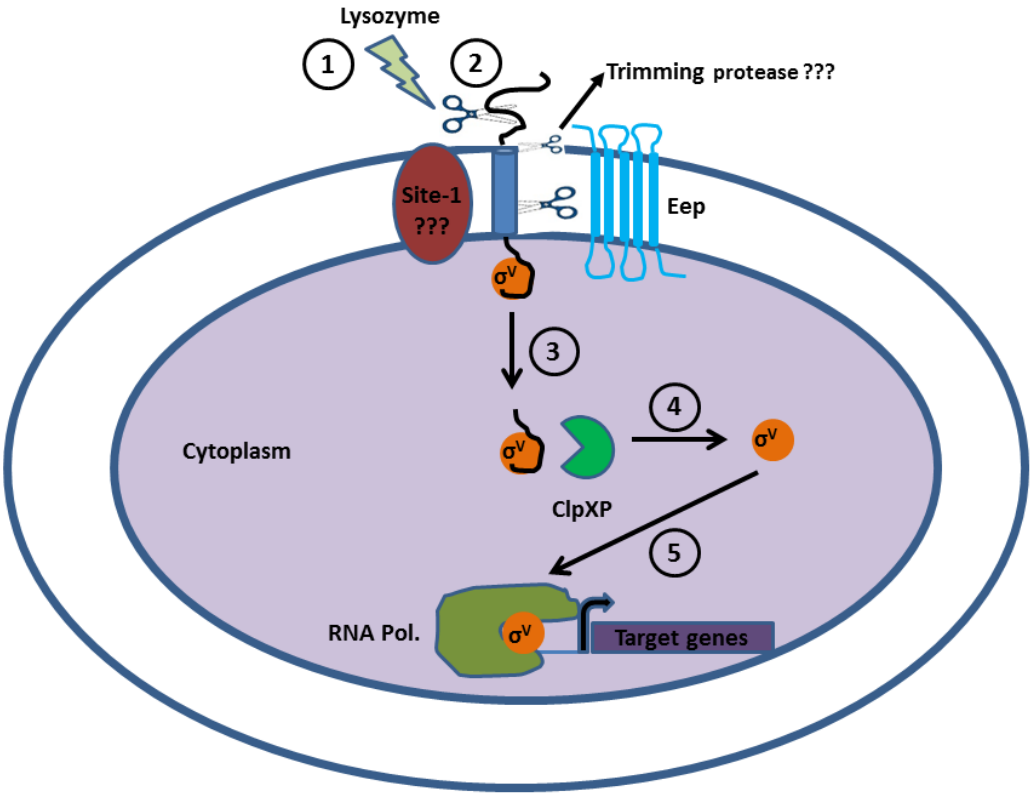

Figure 4-6: A model for the regulated intramembrane proteolysis (RIP) of RsiV. A series of proteolytic events leads to the release and activation of SigV from its anti-sigma factor RsiV. 1) *E. faecalis* perceives a stress signal, which in this case is lysozyme. 2) This leads to the cleavage of RsiV by the putative site-1 protease. Based on data from figure 3 and reference (28), *E. faecalis* and other Gram-positives such as *B. subtilis* possess an additional putative trimming protease activity that prepares the processed RsiV for further proteolytic targeting by Eep 3) Eep degrades the site-1 protease processed and trimmed RsiV, leading to the release of SigV into the cytoplasm. 4) ClpXP cytoplasmic protease further degrades RsiV to release active SigV. 5) SigV initiates the binding of the RNA polymerase upstream of specific genes that confer lysozyme resistance.

Following Eep cleavage, the model predicts that RsiV is further degraded by the cytosolic protease complex ClpXP, as this has been shown to be essential in the activation of SigW, a known ECF sigma factor in Bacillus subtilis (46). Recent work by Ellermeier's group established that SigV activation in Bacillus appears to uniquely hone to lysozyme cues (47), as other cell wall and membrane acting agents failed to induce SigV activation. Work by LeJeune (5) showed that nisin does not induce SigV activation in E. faecalis and the sigV mutant displays wild-type resistance to nisin, consistent with lysozyme being a key driver in SigV activation. An unusual finding from our present study is that both SigV and Eep also contribute to heat, low pH and ethanol stress tolerance. Attempts to use those conditions to induce SigV activation did not result in detectable β-galactosidase activity (data not shown), likely because the reporter protein might have been denatured under the tested conditions. Conversely, data presented by Benanchour (4) corroborate the fact that heat, low pH and ethanol stress fail to induce SigV activation as northern blots failed to show an increase in sigV transcript levels following exposure to these stress inducers. Western immunoblot analysis of SV09 gh2[V583 (pSV23)] lysates subjected to heat, low pH and ethanol stresses failed to induce the degradation of RsiV (data not shown). This could be attributed to the fact that the aforementioned stresses do not strongly induce the sigV regulon but resistance towards these stresses could still be SigV-dependent. We speculate that a basal level of SigV is required for activating genes required for tolerance to these stress conditions, and that Eep must also contribute to basal levels of SigV activation. It will be of interest to determine whether the transcriptional profile in the wild-type and an isogenic sigV mutant differ even in the absence of lysozyme induction.

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CHAPTER 5

DISCUSSION

DISCUSSION:

Enterococci are currently considered to be the second leading cause of nosocomial infections in the United States (1). A multitude of factors are thought to contribute to its emergence as a multi-drug resistant pathogen. Firstly, its ability to tolerate the bactericidal action of a wide variety of antibiotics via several distinct mechanisms combined with the capability to acquire and disseminate these antibiotic resistance traits offers it a competitive advantage over other bacteria including several nosocomial pathogens (2-4). Secondly, enterococci are innately resistant to a wide variety of biotic and abiotic stresses which enables them to colonize atypical host environments (5-9). Finally, its ability to cause persistent infections in a host via the formation of biofilms on host tissues as well as artificial implant devices (e.g. catheters) may contribute to its recent rise as a hospital-adapted pathogen (10, 11).

In the current study we attempted to dissect the role of three enterococcal membrane proteins peptide pheromone transporter (PptAB), oligopeptide permease complex (Opp1 & Opp2) and enhanced expression of pheromone protein (Eep) in cell-cell communication and stress adaptation. We took advantage of the fact that non-pathogenic *E. faecalis* strains like FA2-2 inhibit the growth of the multidrug resistant hospital lineage strains like V583 by secreting the peptide sex pheromone cOB1. A comprehensive transposon mutagenesis screen to identify the genes essential for this novel peptide killing activity led to the identification of an ABC transporter (PptAB) that was essential for the secretion of peptide sex pheromone, cOB1. We also demonstrated that this transporter (PptAB) is essential for the secretion of other well characterized peptide sex pheromones including cCF10, cAD1 and cAM373, suggestive of the global role played by this transporter in the secretion of lipoprotein derived peptide sex pheromones (**Chapter 2**) (12).

Interestingly, the deletion of *pptAB* in V583 resulted in a severe biofilm defect indicative of the potential roles played by peptide pheromones in coordinating this complex and crucial multicellular event.

Peptide pheromones play a pivotal role as signaling molecules in a multitude of gram-positive bacteria (14, 40). Often times, these linear hydrophobic peptides are proteolytically processed and secreted outside the cell (12), and re-imported back into the cell via the oligopeptide permease complex (13, 14). Leonard *et al.* identified a chromosomally encoded oligopeptide permease complex (Opp1) that contributes for the import of the cCF10 peptide sex pheromone in *E. faecalis* (15). We recently identified a second chromosomally encoded oligopeptide permease complex (Opp2) in *E. faecalis* that along with Opp1 also contributes to the import of peptide sex pheromones (**Chapter 3**) (Varahan *et al.*, Submitted-Journal of Bacteriology). We demonstrated using 3 distinct pheromone responsive plasmids systems (pCF10, pAD1 and pAM373) that both the Opp complexes contribute to the transport of peptide sex pheromones into the *E. faecalis* cells. Cells that do not possess both the Opp complexes (double *opp* deletion mutant) are unresponsive to recipient cell derived peptide sex pheromones. We also showed that deletion of both the *opp* complexes in tandem in strain V583 resulted in a severe biofilm defect which corroborates our previous finding that peptide pheromone transport is essential for *E. faecalis* biofilm formation (12). It is possible that *E. faecalis* is employing pheromone mediated cell signaling to coordinate the development of its complex three-dimensional biofilms.

In **Chapter 4**, we identified the additional roles played by the membrane embedded zinc metalloprotease Eep in the stress resistance pathways of *E. faecalis* (16). Enhanced Expression of Pheromone protein (Eep) was discovered as a protein that is essential for the processing of lipoprotein derived peptide sex pheromones (17). Eep is a prototypic member of the membrane

embedded site-2 proteases and has an intramembrane active site. It also possess the characteristic PDZ domain which is present in one of the external protein loops (18, 19). We recently demonstrated that Eep is essential for the activation of the extracytoplasmic function (ECF) sigma factor, SigV, which confers high level lysozyme resistance to *E. faecalis* (6, 16). Under normal conditions, SigV is inactivated by being sequestered to the membrane by the anti-sigma factor, RsiV. In the presence of lysozyme RsiV is thought to undergo a conformational change which results in its cleavage by both Site-1 and Site-2 proteases. We demonstrated that the cleavage of RsiV by Eep (Site-2 protease) is essential for the complete degradation of RsiV and subsequent activation of SigV (16). We also showed that Eep, along with the ECF sigma factor SigV is essential for *E. faecalis* to tolerate other biological stresses like high temperature, low pH and ethanol stress (16, 20).

Interestingly, we demonstrated that the deletion of the genes encoding the peptide pheromone transporter (PptAB) renders *E. faecalis* susceptible to lysozyme. A *pptAB* deletion mutant displayed a 10-fold increased sensitivity to lysozyme compared to the wild-type strain (Fig. 5-1A). We took advantage of the fact that SigV binds to its own promoter and activates its own expression and constructed a luciferase report plasmid pKS313, wherein the *sigV* promoter was fused to the *luxABCDE* cassette ($P_{sigV\text{-}luxABCDE}$). *E. faecalis* V583 harboring pKS313 generated detectable light signal only in the presence of 1 mg/ml lysozyme while the *pptAB* deletion strain harboring pKS313 failed to generate light signal even in the presence of lysozyme suggesting that SigV is constitutively inactivated in a *pptAB* deletion mutant (Fig. 5-1B). In 2008, Heinrich *et al.* showed that the deletion of the *pptAB* homolog (*ecsAB* in *B. subtilis*) resulted in the inactivation of the Site-2 protease RasP, which cleaves the anti-sigma factor RsiW and this in turn resulted in the constitutive inactivation of the ECF sigma factor SigW (21). The authors in this study were able

to partially complement the *ecsAB* deletion phenotypes by overexpressing RasP. We wanted to determine whether the overexpression of Eep in a *pptAB* deletion mutant would restore its lysozyme resistance to wild-type levels. The Eep complementation vector pSV24 (pEep) (16) was electroporated into Δ*pptAB* and as a control V583 and Δ*pptAB* harboring the vector pKS12 (16)

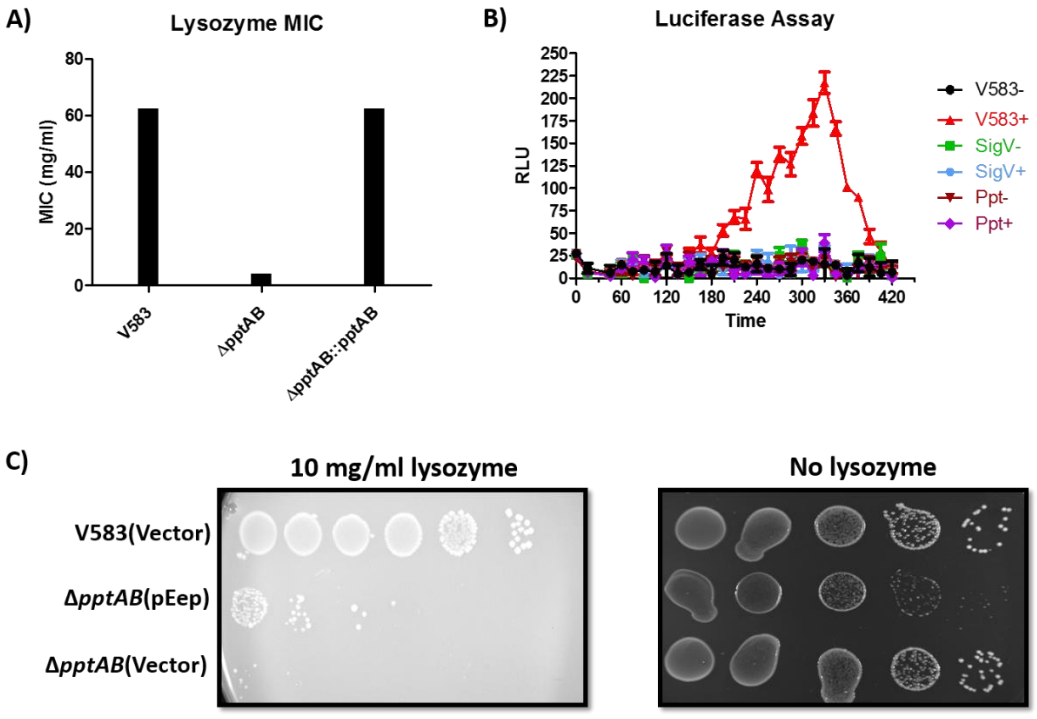

Figure 5-1: Deletion of *pptAB* results in increased lysozyme sensitivity**.** A) Lysozyme minimum inhibitory concentration (MIC) assay performed using the wild-type (V583), pptAB deletion mutant and the complement strain. B) Luciferase reporter assay was performed using V583(pKS313), Δ*pptAB*(pKS313) and Δ*sigV*(pKS313) in the presence (+) and absence (-) of 1 mg/ml lysozyme. RLU refers to relative light units. C) Complementation of Δ*pptAB* lysozyme defect using an *eep* complementation vector (pEep). As a control, V583 and Δ*pptAB* harboring the empty vector pKS12 were used. Plates with and without the lysozyme contained spectinomycin (500 µg/ml) for the maintenance of the plasmids.

backbone vector were also generated and these were spotted onto plates either containing 10 mg/ml lysozyme or no lysozyme. The results are shown in Figure 5-1C. The overexpression of *eep* in the *pptAB* deletion strain using the Eep complementation vector pEep (16) resulted in a modest complementation of the lysozyme sensitivity suggesting that the membrane activity of Eep might be compromised in a mutant strain lacking *pptAB.* Although the exact reason as to why SigV does not get activated in a *pptAB* mutant in the presence of lysozyme remains to be elucidated, it is possible that apart from its role in peptide pheromone secretion, PptAB might also be serving a more broader role of clearing the protein-based membrane debris present after protein processing in *E. faecalis*. Deletion of this transporter could result in the accumulation of peptide fragments that clog the membrane, resulting in the inability of membrane-embedded proteins (e.g. Eep) from functioning properly. Further studies are warranted to validate this hypothesis.

In the dawn of post-antibiotic era it is important to identify important genes that are essential for the pathogenesis of multi-drug resistant bacteria like *E. faecalis* since these could serve as potential therapeutic targets. In this work we have identified distinct roles played by 3 different membrane proteins in the biology of *E. faecalis*. It is interesting to note that the membrane protein Eep has been shown to play an important role in the enterococcal rabbit model of endocarditis (22) and the enterococcal murine model of catheter associated urinary tract infection (23). It is possible that lysozyme sensitivity associated with an *eep* deletion mutant (16) contributes to this phenotype since lysozyme is considered as a critical innate immune component of the host. Further studies are warranted to determine the exact role played by this membrane protein in the pathogenesis of *E. faecalis* in a host. Most infections caused by *E. faecalis* are biofilm associated indicative of the importance of this lifestyle during host adaptation. Several factors have already been shown to be critical players in *E. faecalis* biofilm development. These include the Fsr quorum sensing system

(24), the proteases (GelE and SprE) regulated by the Fsr system (25), the alternate sigma factor – RpoN (26), the extracellular polysaccharide of *E. faecalis* – Epa (27) and the endocarditis and biofilm-associated pilus – Ebp (28). We have recently demonstrated the involvement of peptide pheromone transporters during biofilm formation in *E. faecalis*. Deletion of the peptide pheromone transporter (PptAB) and both the oligopeptide permease (Opp) complexes in tandem results in a biofilm defect suggestive of a possible role played by linear peptide pheromones in *E. faecalis* biofilm development.

Peptide pheromones have been shown to play an important role in the biofilm development of several pathogenic microorganisms (24, 29, 30). In *E. faecalis*, the Fsr quorum sensing system results in the production of the cyclic peptide pheromone GBAP which in-turn regulates biofilm development by affecting the expression of the proteases GelE and SprE (31). However, the contribution of linear peptide pheromones (lipoprotein derived and genome encoded) in the regulation of *E. faecalis* biofilm development still remains to be elucidated. Recently, Chang *et al.* showed that two linear peptide pheromones (SHP2 and SHP3) regulate biofilm formation in *S. pyogenes* by interacting with the RNPP (Rap, NprR, PrgX, PlcR) family transcriptional regulators. Rgg2 and Rgg3 (32). The authors also showed that the processing of this peptide was Eep dependent and this peptide was imported back into the cell via an Opp complex. Taking into account the aforementioned similarities between the *E. faecalis* and *S. pyogenes* linear peptide pheromone signaling and the contribution of PptAB and Opp complexes in *E. faecalis* biofilm development (12), we hypothesized that biofilm formation in this bacterium could be regulated by RNPP family proteins. Bioinformatic analysis of the V583 genome resulted in the identification of several RNPP homologs amongst which 5 are chromosomally encoded (EF0073, EF1224, EF1316, EF1599 and ElrR (EF2687)) and 2 are encoded by the pheromone responsive plasmids,

pTEF1 and pTEF2, and closely resemble PrgX-like regulatory proteins (EFA0004 and EFB0005) (33). Ibrahim *et al.* in 2007 identified several short hydrophobic peptide encoding genes in *E. faecalis* strain V583 using bionformatic analysis and some of these genes are located adjacent to the genetic locus of the aforementioned RNPP family proteins (34). To explore the possibility that one of these RNPP homologs plays a role biofilm formation, we decided to test whether EF0073 plays a role in *E. faecalis* biofilm formation due to the significant sequence similarity and structural homology it shares with other RNPP family proteins (33).

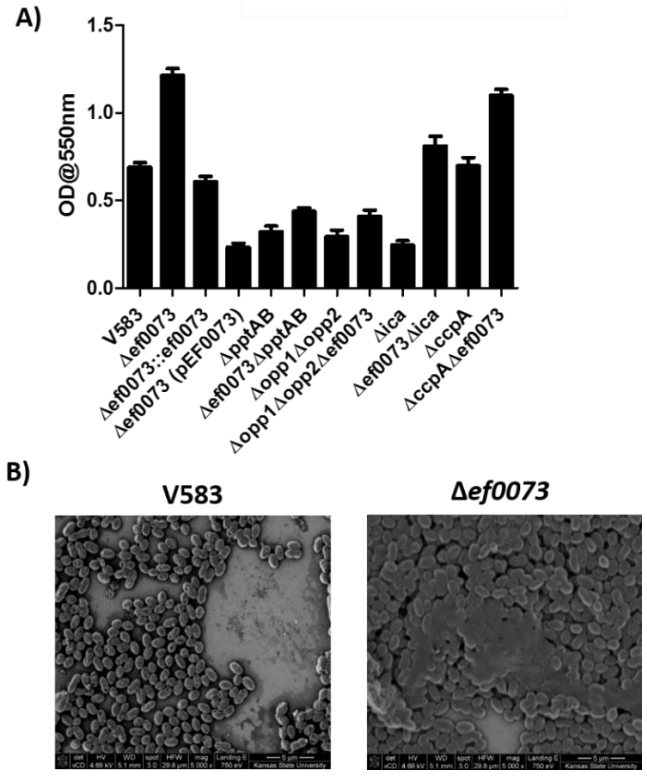

Figure 5-2: Deletion of *ef0073* results in hyper biofilm formation**.** A) 96 well plate biofilm assay using the V583, Δ*ef0073*, and its single copy genomic integration (Δ*ef0073*::*ef0073*) and multiple copy plasmid (Δ*ef0073*(pEF0073)) complementation strains B) Scanning electron microscopy analysis of V583 and Δ*ef0073* one day old static biofilms (see Appendix: Materials & Methods).

When we generated an *ef0073* deletion mutant strain, it formed significantly more biofilms compared to the wild-type (Fig 5-2A) in a plate biofilm assay (see Appendix: Materials & Methods) and we were able to corroborate this observation using scanning electron microscopy (SEM) analysis wherein there was a significant difference between the biofilms formed by the wild-type and the mutant (see Appendix: Materials & Methods) (Fig 5-2B). This phenotype was complementable when a single copy of this gene was integrated back into the genome of the *ef0073* deletion strain at an ectopic locus (Fig 5-2A). However, when *ef0073* was overexpressed in the mutant strain using a high copy plasmid, biofilm formation was attenuated significantly suggesting that EF0073 might be serving as a repressor of biofilm formation in *E. faecalis*. Since RNPP family proteins in other bacteria have been known to act as repressors (14), it is possible that EF0073 is one such protein. The fact that two genes that potentially encode for peptide pheromones are located adjacent to *ef0073* in the V583 genome further strengthens our hypothesis that EF0073 is potentially regulating biofilm formation by interacting with either of these peptides.

SEM analysis of the V583Δ*ef0073* biofilms also revealed the presence of an abundant exo-polymer matrix which was much less abundant in the wild-type V583 biofilms (Fig 5-2B). We hypothesized that EF0073 might be repressing the expression of genes that are involved in the synthesis of a major exo-polysaccharide and that the deletion of *ef0073* results in the over production of this exo-polymer matrix which in turn contributes to the hyper-biofilm phenotype. We wanted to determine whether EF0073 regulated the synthesis of the well characterized *E. faecalis* capsular polysaccharide (35) and a putative polysaccharide locus [EF_0883-EF_0891], which shares some sequence similarity with IcaA, part of the synthetic machinery for the intracellular adhesion polysaccharide found in *S. aureus* and *S. epidermidis* (36). We took advantage of a previously described capsular polysaccharide extraction/detection protocol (see Appendix: Materials &

Methods) (35) and extracted the exopolymer carbohydrate content of V583 and V583Δ*ef0073*. V583Δ*cpsC* was used as a negative control in this assay since it is known that this mutant is defective in synthesizing the capsular polysaccharide (35). We also layered the *cpsC* gene deletion in the V583Δ*ef0073* strain background to generate V583Δ*ef0073*Δ*cpsC* and we analyzed the carbohydrate content of this double mutant to determine whether the deletion of *ef0073* altered the synthetic pathway of the capsular polysaccharide. The cell wall carbohydrate content of V583, V583Δ*ef0073*, V583Δ*cpsC* and V583Δ*ef0073*Δ*cpsC* were detected following electrophoresis on a PAGE gel with the cationic dye, Stains-All (3,3′-dimethyl-9-methyl-4,5,4′5′-dibenzothiacarbocyanine). The results of this analysis are shown in figure 5-3.

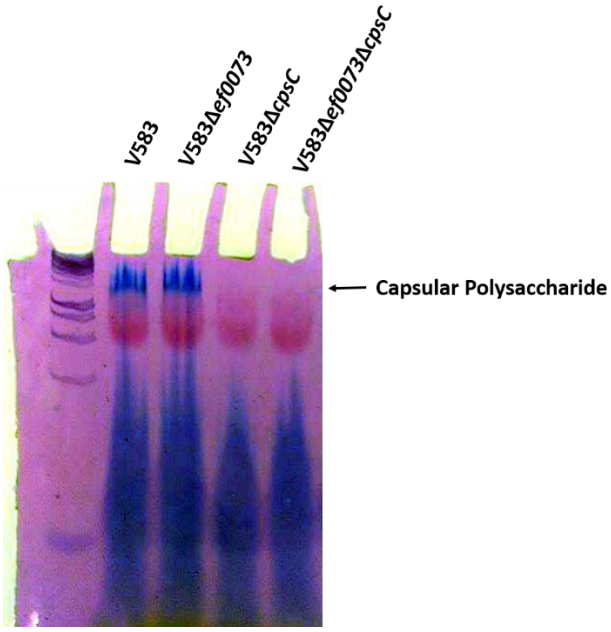

Figure 5-3: Detection of cell wall carbohydrate polymer species among V583, V583Δ*ef0073*, V583Δ*cpsC* and V583Δ*ef0073*Δ*cpsC* by electrophoresis through a 10% polyacrylamide gel with Stains-All detection (see Appendix: Materials & Methods).

Deletion of *ef0073* did not affect the production of the capsular polysaccharide suggesting that EF0073 is not regulating the synthesis of the *E. faecalis* capsular polysaccharide. When *cpsC* was deleted in V583Δ*ef0073* strain, the resulting double mutant strain did not make any capsular polysaccharide suggesting that capsule production in V583Δ*ef0073* was still dependent on the *cps* pathway. Since the deletion of the *ica* operon resulted in a severe biofilm defect in *S. aureus* (36), we wanted to determine whether the *EF_0883-EF_0891* operon in *E. faecalis* contributed to the hyper-biofilm phenotype exhibited by V583Δ*ef0073* . To assess this, we generated a deletion mutant in V583 and V583Δ*ef0073* strain backgrounds and tested these mutants in a plate biofilm assay (see Appendix: Materials & Methods). The deletion of *ef_0883-ef_0891* (Ica homolog) in V583 resulted in a severe biofilm defect suggestive of an important role played by this operon in *E. faecalis* biofilm formation. Biofilm formation by V583Δ*ef0073*Δ*ica* was also significantly reduced compared to V583Δ*ef0073* indicative of a possible role played by this operon in the hyper-biofilm phenotype exhibited by V583Δ*ef0073*. Analysis of the promoter upstream of *ef0883* that might possibly drive expression of the operon both in V583 and the *ef0073* mutant, however, failed to reveal significant differences in promoter activity under normal growth conditions (data not shown). Whether EF0073 contributes to the regulation of the *ef_0883-ef_0891* (Ica homolog) operon under biofilm-specific conditions remains to be elucidated and will likely be a component of future study.

Apart from causing persistent biofilm-associated infections like endocarditis and catheter associated urinary tract infections, *E. faecalis* also has the ability to disseminate a wide variety of antibiotic resistance genes not only amongst itself but to other pathogenic species including *S. aureus*. Studies have shown that clinical isolates of *S. aureus* obtained the vancomycin resistance genes via the conjugative transposon Tn1546 present in the co-infecting *E. faecalis* isolate (37,

38). This is attributed to the fact that *S. aureus* produces a pheromone, derived from a lipoprotein signal sequence that is known to mimic the enterococcal pheromone cAM373, enabling exchange of genetic information between these two species. Recently, a genetic determinant in *Streptococcus gordonii* encoding a predicted lipoprotein was also identified as a precursor for a peptide pheromone resembling cAM373, which enables intergeneric DNA transfer between *E. faecalis* and this dental pathogen (39).

In this dissertation, we have identified genes encoding membrane proteins that have overlapping roles not only in *E. faecalis* stress adaptation and biofilm formation but also in the transfer of pheromone-responsive plasmids (conjugation) which often results in the dissemination of antibiotic resistance genes and even virulence traits (40). Eep and PptAB have overlapping roles not only in cell-cell communication but also in lysozyme stress adaptation (12, 16, 17). Considering the fact that both the proteins are predicted to be membrane embedded, it is possible that Eep and PptAB interact to form a complex. Further studies are warranted to determine the exact nature of this proposed interaction. It is also possible that Eep and PptAB along with Opp complexes might be important for enterococcal virulence in a host based on the roles they seem to be playing in biofilm development, lysozyme resistance and transfer of pheromone responsive plasmids. Testing mutants that lack *eep*, *pptAB* or the *opp* complexes in relevant vertebrate host models is warranted to determine whether these enterococcal membrane proteins contribute to pathogenesis in a mammalian host.

Summary and Future Directions:

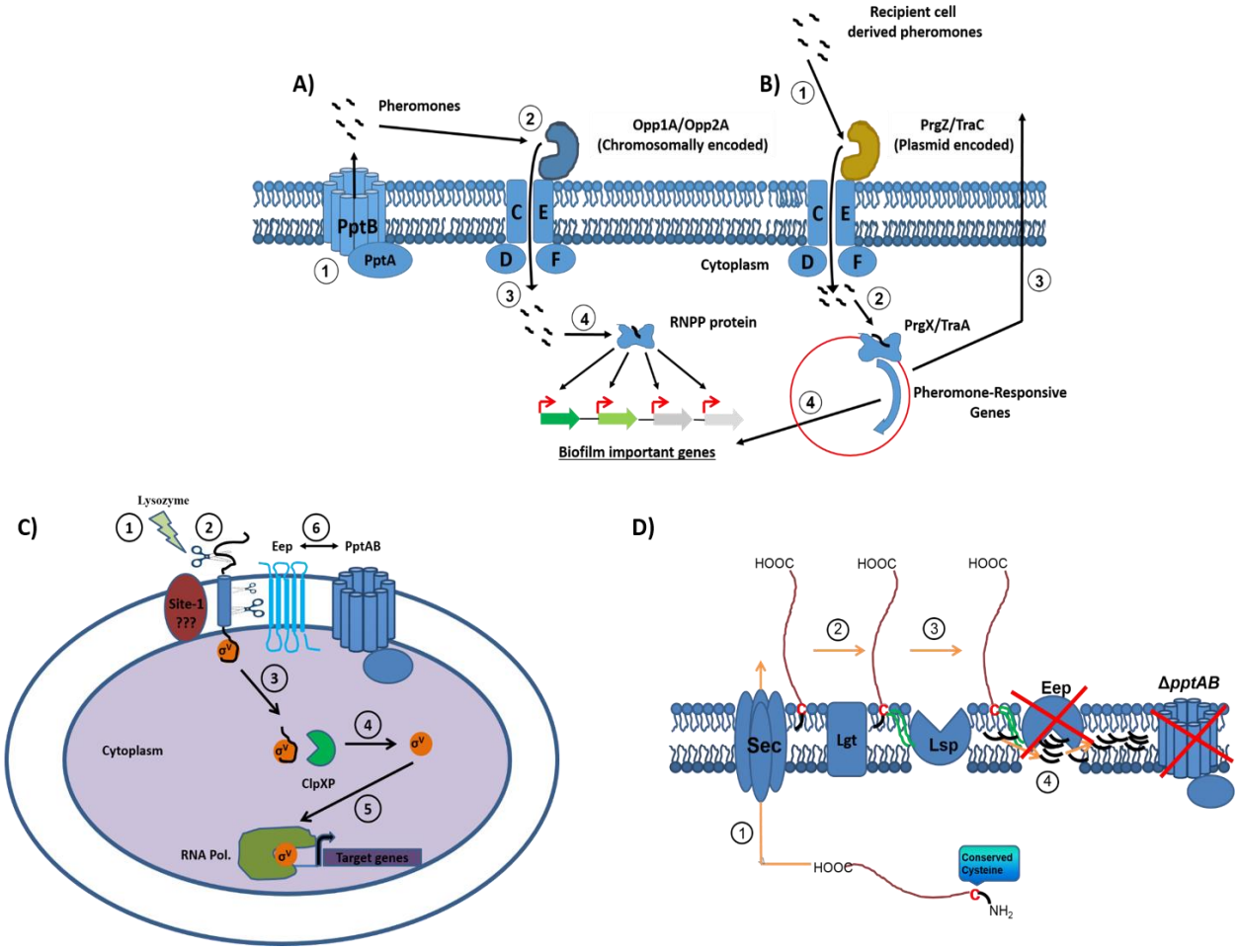

Figure 5-4: A/B) Peptide pheromone mediated biofilm regulation in *E. faecalis*. C) Activation of the extracytoplasmic function sigma factor, SigV, *via* the regulated intramembrane proteolysis pathway. D) "Vacuum cleaner model" hypothesis.

In this dissertation, we have identified the transporters that are essential for the import and export of lipoprotein derived peptide sex pheromones in *E. faecalis*. We have shown that the deletion of these peptide transporters results in a biofilm defect which is indicative of a role played by peptide signaling in regulation of biofilm development (12) (Varahan *et al.*, Submitted-Journal of

Bacteriology). A possible mechanism by which peptide pheromones could regulate biofilm formation in *E. faecalis* is depicted in figure 5-4A-B.

RNPP family protein mediated biofilm regulation (Fig 5-4A). 1) Lipoprotein-derived peptide pheromones and/or peptides derived from putative short coding sequences (34) are exported via the Peptide Pheromone Transporter (PptAB) (41). 2) Secreted peptides are bound by OppA or its homologs with high affinity 3) and actively imported into the cell via either of the oligopeptide permease complexes 4) where the peptide(s) interact with plasmid or chromosomally encoded RNPP family members in *E. faecalis* to regulate the expression of biofilm important genes. Pheromone-responsive plasmid mediated biofilm regulation (Fig 5-4B). 1) Peptide pheromones produced by recipient *E. faecalis* populations are sensed by the OppA homologs (PrgZ/TraC) encoded by the cognate pheromone-responsive plasmids and are imported into the donor cells via the chromosomally encoded oligopeptide permeases. 2) This alleviates the PrgX/TraA-mediated repression of conjugative functions resulting in activation of pheromone-responsive genes 3) with subsequent plasmid transfer to the recipient cells. 4) Aggregation substance, a pheromone-responsive gene product, is also known to contribute to ex-vivo biofilms (42) and endocarditis (43).

Identifying the specific RNPP family involved in the regulation of biofilm formation is warranted as this will provide a mechanism by which biofilm formation is regulated by peptide signaling. To elucidate the role played by pheromone-responsive plasmids (pTEF1 and pTEF2) in the virulence of *E. faecalis* V583, strain V19 which is V583 devoid of all its pheromone-responsive plasmids (pTEF1 and pTEF2) should be tested in relevant vertebrate host models along with V583 to determine the overall contribution that these plasmids play in enterococcal pathogenesis.

We also show that the deletion of the peptide pheromone transporter (PptAB) results in 10-fold reduction in lysozyme resistance compared to V583 (Fig 5-1A). Lysozyme resistance in *E. faecalis* is regulated by an extracytoplasmic function sigma factor called SigV which is activated only in the presence of lysozyme via regulated intramembrane proteolysis (Fig 5-4C). 1) *E. faecalis* perceives a stress signal, which in this case is lysozyme. 2) This leads to the cleavage of RsiV by the putative site-1 protease. Based on data from reference (44), *E. faecalis* and other gram-positives such as *B. subtilis* possess an additional putative trimming protease activity that prepares the processed RsiV for further proteolytic targeting by Eep 3) Eep degrades the site-1 protease processed and trimmed RsiV, leading to the release of SigV into the cytoplasm. 4) ClpXP or related cytoplasmic proteases further degrade RsiV to release active SigV. 5) SigV initiates the binding of the RNA polymerase upstream of specific genes that confer lysozyme resistance. 6) As membrane proteins, Eep and PptAB might be forming a complex and the deletion of *pptAB* might affect the proper functioning of Eep and this could explain the lysozyme resistance defect exhibited by a *pptAB* mutant.

Another interesting possibility is that PptAB serves as a "vacuum cleaner" clearing peptide debris from the membrane of *E. faecalis* and that the deletion of *pptAB* might result in the accumulation of lipoprotein derived peptide sex pheromones and possibly other peptide debris and this might interfere with the intramembrane active site of Eep resulting in the lack of activation of SigV under lysozyme stress (Fig 5-4D). We hypothesized that cells lacking PptAB would possess a wider cell membrane compared to wild-type V583 due to the accumulation of peptide pheromones and other peptide debris. We performed transmission electron microscopy (TEM) analysis to visualize the cell membrane of V583 and V583*ΔpptAB* and the results are shown below (Fig 5-5). The membrane thickness of V583*ΔpptAB* was significantly higher than that of the wild-type suggesting

that V583*ΔpptAB* might be accumulating more peptide debris compared to wild-type V583. Further studies are warranted to confirm this hypothesis.

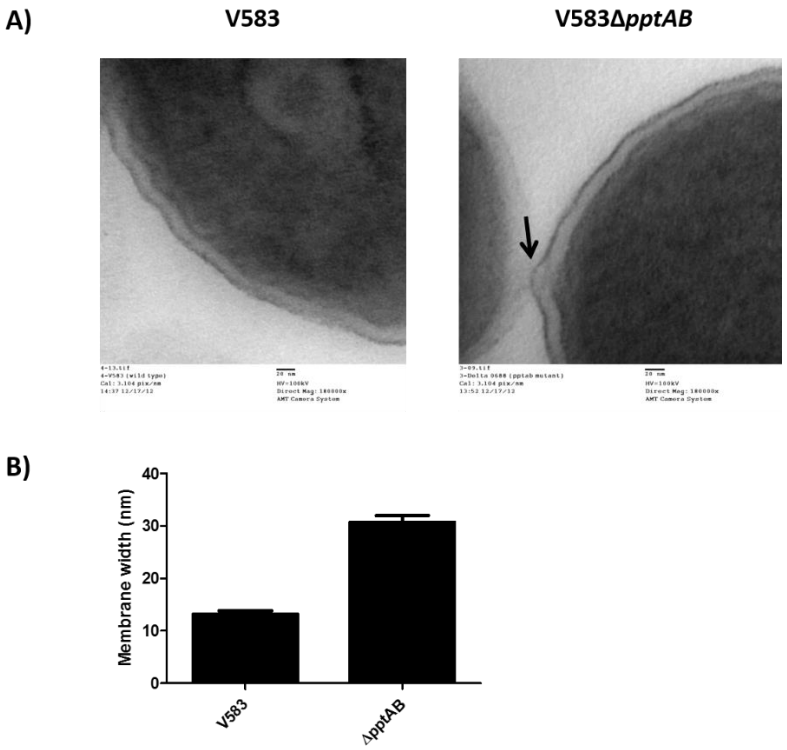

Figure 5-5: A) TEM analysis looking at the cell membranes of V583 and V583Δ*pptAB*. Black arrow indicates a region of V583Δ*pptAB* that appears to significantly wider that V583. B) Quantification of membrane thickness using Image J. Membrane width was measured in nanometers (nm) (see Appendix: Materials & Methods).

There is a growing appreciation for the roles played by linear peptide pheromones in the biology of several clinically relevant gram-positive pathogens. Opp complexes, Eep and PptAB are broadly conserved amongst all pathogenic *Firmicutes* and novel therapeutics that inhibit the normal functioning of these proteins in *E. faecalis* could very well serve as potent anti-infectives against

E. faecalis as well as other pathogenic bacteria. In the growing era of antibiotic resistance and the lack of industrial commitment to the discovery of new treatment options for drug-resistant infections, it is paramount that we focus efforts on the discovery of new paradigms for treating bacterial infections. One such strategy would be to target the aforementioned proteins (Eep, PptAB and the Opp complexes) to discover novel compounds that block their function and thus block dissemination of drug-resistance, prevent biofilm development and dampen the organism's ability to cope with the host immune system.

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APPENDIX

APPENDIX

MATERIALS AND METHODS:

Plate Biofilm Assay:

Biofilm formation on polystyrene was quantified with the crystal violet staining method as previously described (1). Each assay was performed in pentaplicates and repeated twice. All the strains tested in the plate biofilm assay were grown overnight in tryptic soy broth (TSB) at 37°C. V583Δ*ef0073* (pEF0073) was grown in TSB with spectinomycin at a concentration of 500 µg/ml for maintenance of complementation plasmid. These cultures were back diluted (1:100) in fresh TSB and seeded into the 96-well plate and were incubated at 37°C for 24 hours. Biofilms were quantified by crystal violet staining after these 24hr biofilms were thoroughly washed with phosphate buffered saline (pH 7.4) to remove planktonic bacteria.

Scanning Electron Microscopy:

V583 and V583Δ*ef0073* biofilms were grown for 24 hrs in glass coverslips using tryptic soy broth (TSB) at 37°C. Biofilms were immersion fixed in a fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) for 1-2 hrs at 4°C. The fixative completely immersed the entire biofilm. V583 and V583Δ*ef0073* biofilms were then gently wash 3 x 5 minutes using distilled water. Cover slips containing the biofilms were dried under a vacuum of approximately 10^{-3}torr for 1hr prior to mounting on aluminum stubs with carbon adhesive tape. Biofilms were imaged in a Nova NanoSEM 430 (FEI Company) using a low voltage high contrast

backscatter detector (vCD) under high vacuum using beam deceleration with an incident beam of 4.69kV and a landing energy of 750V.

Purification and Characterization of Capsular Polysaccharide:

E. faecalis strains V583, V583Δ*ef0073*, V583Δ*cpsC* and V583Δ*ef0073*Δ*cpsC* were grown at 37°C without aeration overnight Todd–Hewitt broth supplemented with 1.0% glucose. For purification of cell wall polysaccharides, the method of Hancock *et al.* (2) was used. After enzymatic digestion of the enterococcal cell wall, carbohydrates were precipitated by the addition of ethanol to a final concentration of 75%. The precipitable material was collected by centrifugation (22,000 × *g*, 20 min), washed with 75% ethanol, air-dried, and redissolved in 50 mM Tris·HCl (pH 8.0), 150 mM NaCl. Samples were analyzed by electrophoresis through 10% polyacrylamide (33:1) in Tris-borate buffer (0.2 M Tris-base/0.2 M boric acid/20 mM EDTA, pH 8.3), with detection of polysaccharides using the cationic dye Stains-All (3,3′-dimethyl-9-methyl-4,5,4′5′-dibenzothiacarbocyanine). Gels were stained for a minimum of 6 h in the dark and destained in distilled water before photography.

Transmission Electron Microscopy:

E. faecalis strains V583 andV583Δ*pptAB* were grown as 10ml cultures at 37°C without aeration overnight Todd–Hewitt broth. Cells were centrifuged and the pellets were fixed in 1ml of fixative solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer 7.2-7.4 for 16 hrs at 4°C with constant rotation. Samples were washed 3 times for 5 minutes in 1ml of 0.1 M sodium cacodylate buffer 7.2-7.4 at room temperature with constant rotation. Samples were fixed with 1-2% (Initial culture volume) osmium tetroxide in 0.1 M sodium cacodylate buffer 7.2-7.4 at room temperature with constant rotation for 2 hrs. Samples were

washed again 3 times for 5 minutes in 1ml of 0.1 M sodium cacodylate buffer 7.2-7.4 at room temperature with constant rotation. Samples were dehydrated using a series of acetone washes (washed 3 times for 5 minutes with 1ml acetone) with ascending concentrations of acetone (50%, 60%, 70%, 80%, 90%, 95% and 100%) at room temperature. The tubes were wrapped in silver foil to protect from light. Following the dehydration using acetone, samples were infiltrated with EMBED 812/Araldite resin at room temperature for 24-48 hrs. Finally, the resin containing the samples were polymerized by drying it in an oven at 60°C for 24-48 hrs. Cross sections of the polymerized resin containing the samples were made for visualization using a transmission electron microscope.

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