NANYANG
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**CHARACTERIZING THE RELATIONSHIP BETWEEN
SORTASE A-DEPENDENT SUBSTRATES AND THE
CONSEQUENCES OF *SRTA* DELETION
IN *ENTEROCOCCUS FAECALIS***

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**Characterizing the relationship between Sortase A-
dependent substrates and the consequences
of *srtA* deletion in *Enterococcus faecalis***

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Abbreviations

% – percent

°C – degree Celsius

µg – microgram

µm – micrometre

Ace – adhesin of collagen from Enterococci

A. oris – *Actinomyces oris*

AS – aggregation substance

Atn (AtIA) – autolysin

B. subtilis – *Bacillus subtilis*

BHI – Brain Heart Infusion

bp – *base pair*

BSA - bovine serum albumin

C200 – cysteine 200

CAUTI – catheter associated urinary tract infections

CIRCE – controlling inverted repeat of chaperone expression

Cm – chloramphenicol

CWSS – cell wall sorting signal

E. faecalis – *Enterococcus faecalis*

E. faecium – *Enterococcus faecium*

Ebp – endocarditis and biofilm associated pili

Enterococcus spp – *Enterococcus species*

Erm – Erythromycin

Abbreviations

Esp – enterococcal surface protein

Fus – fusidic acid

gDNA – genomic DNA

GlcNAc – N-acetylglucosamine HGT – horizontal gene transfer

HMWL – high-molecular-weight ladder

HRP – horseradish peroxidase

IEM – immunogold electron microscopy

IFM – immunofluorescence microscopy

Kan – kanamycin

kDa – kiloDalton

L. lactis – *Lactococcus lactis*

L. monocytogenes – *Listeria monocytogenes*

LTA - lipoteichoic acid

M. tuberculosis – *Mycobacterium tuberculosis*

min – minutes

MRS – multiple restriction site

MSCRAMM – microbial surface component recognizing adhesive matrix molecules

MurNAc *N* – acetylmuramic acid

MV – membrane vesicles

MW – molecular weight

n/a – not applicable

O/N - overnight

OD – optical density

P. aeruginosa – *Pseudomonas aeruginosa*

Abbreviations

PBPs – penicillin binding proteins

PBS – phosphate-buffered saline

PFA – paraformaldehyde

PG – peptidoglycan

PMNs - human neutrophils

Rif – rifampicin

RS – restriction site

RT – room temperature

RT-qPCR – real-time quantitative polymerase chain reaction

S. aureus – *Staphylococcus aureus*

S. intermedius – *Streptococcus intermedius*

S. mutans – *Streptococcus mutans*

S. pyogenes – *Streptococcus pyogenes*

s/n – supernatant

Spa – staphylococcal protein A

SrtA – Sortase A

SrtC – Sortase C

Tc – transconjugants

TCA – Trichloroacetic acid

TCS – two component system

TEM – transmission electron microscopy

TLR – Toll-like receptor

Tn – transposon

TSB – Tryptic soya broth

UDP-NM – Uridine diphosphate-N-acetylmuramic acid

Abbreviations

UTI – urinary tract infections

WT – wild type OG1RF

Abstract

Enterococcus faecalis is a gastrointestinal commensal bacterium that has emerged as an important opportunistic pathogen that is a serious public health threat due to its prevalent antibiotic resistance. A variety of virulence factors contributes to the pathogenesis of Enterococci, including Sortase A (SrtA) and several SrtA substrates including endocarditis and biofilm associated pili (Ebp), adhesin of collagen from Enterococci (Ace), and aggregation substance (AS). Understanding the expression relationship between SrtA and its substrates, as well as the expression relationship and any cross-regulation between SrtA substrates, is crucial to effectively target bacterial adhesion for the reduction of virulence in the future.

In this thesis, we showed that SrtA substrate expression does not depend on the presence of SrtA, and that Ebp accumulate in the plasma membrane in $\Delta srtA$ resulting in increased piliation. We showed that DnaK is important for Ebp biogenesis and that *dnaK* transcription is higher in $\Delta srtA$, suggesting that DnaK may have a role in processing additional SrtA substrates. We explored the possibility that accumulation of unprocessed substrates in $\Delta srtA$ would result in increased vesiculation, which is associated with membrane stress in other organisms, but instead detected a similar number of membrane vesicles (MV) between WT and $\Delta srtA$. However, as a result of increased membrane-associated Ebp, MV from $\Delta srtA$ were enriched in Ebp. Analysis of MV protein composition and high resolution imaging of MV led us to hypothesize several mechanisms for MV formation via explosive cell lysis, through cell-wall modifying enzymes or from microdomains. Lastly, we addressed the expression relationships between Ebp, AS, and Ace. When Ebp and AS are expressed on the same cells, Ebp prevent AS-mediated clumping and decreases the frequency of horizontal gene transfer between AS-expressing cells. We also observed temperature-dependent differential expression of Ebp and Ace, suggesting that the two adhesins might be inversely regulated by environmental cues. Taken together these findings

Abstract

enhance our understanding of SrtA, Ebp, and cell-envelope biogenesis in *E. faecalis*, and provide new insight into the expression relationship between multiple enterococcal adhesins.

1. Chapter 1. Introduction

Enterococcus spp. are members of the Gram-positive lactic acid bacteria. The most common human-associated Enterococci, *Enterococcus faecalis* and *Enterococcus faecium*, are commensal bacteria of the human gut. Enterococcal colonization occurs during the first few weeks after birth (Hufnagel et al., 2007; Mackie et al., 1999). Some strains of Enterococci have been used as probiotics as they compete with pathogenic bacteria for niche and nutrients (Nes et al., 2014). Despite the benefits conferred by Enterococcal colonization, it has also emerged as an important opportunistic pathogen. *E. faecalis* (**Figure 1.1**) and *E. faecium* both cause disease in humans, but *E. faecalis* is the major isolate from clinical specimens within the *Enterococcus* species (Gilmore, 2002).

Enterococci can cause bacteraemia, endocarditis, urinary tract infections (UTI), catheter associated urinary tract infections (CAUTI), wound infection, and meningitis (Vu and Carvalho, 2011). Infections by this opportunistic pathogen have been aggravated by the development of multiple antibiotic resistances. Different *E. faecalis* strains carry various conjugation plasmids and the majority of resistance genes are located on these plasmids (Clewell et al., 2014). Another way to confer Enterococci with antibiotic resistance is via composite transposons (Tn). Examples of widespread Tn are Tn5405, which encodes aminoglycoside and streptothricin resistance (Clewell et al., 2014; Lester et al., 2004), Tn1547

with vancomycin resistance genes, and elements that encode resistance to high levels of gentamicin (Clewett et al., 2014; Quintiliani Jr and Courvalin, 1996). Therefore, resistance may rapidly spread within enterococcal populations by plasmid conjugation or via mobile elements so that commensal strains are also at risk of acquiring resistance to antibiotics (Netherwood et al., 1999). Current treatment for multi-drug resistant enterococcal infection are “last resort” antibiotics vancomycin and daptomycin; however, resistance to both antibiotics has emerged rapidly (Miller et al., 2014; Miller et al., 2016). Therefore, Enterococci have developed resistance to virtually all antimicrobials currently used in clinical practice (Miller et al., 2014).

Several virulence factors contribute to the pathogenicity of *E. faecalis* including genes encoding autolysin, haemolysin, gelatinase, and metalloprotease (Frank et al., 2012; Guiton et al., 2009; Thurlow et al., 2010). In addition, a large number of microbial surface component recognizing adhesive matrix molecules (MSCRAMM) are encoded in *E. faecalis* (Sillanpaa et al., 2004). Characterized MSCRAMMS include enterococcal surface protein (Esp), aggregation substance (AS), adhesin of collagen from Enterococci (Ace), and endocarditis and biofilm associated pili (Ebp) (Nallapareddy et al., 2006; Sarteringen et al., 2000; Toledo-Arana et al., 2001). All of these adhesins are covalently attached to the cell wall by sortase enzymes. Thus, sortases themselves are also recognized as important virulence factors (Maresso and Schneewind, 2008).

1.1. Sortase A

Sortase enzymes are transpeptidases that catalyse the covalent attachment of substrate proteins to the cell wall. Sortases are conserved within nearly all Gram-positive bacteria and sequence homologues are also found in several Gram-negative bacteria and archaea (Pallen et al., 2001). Although the function of sortases outside Gram-positive bacteria has not been described, sortases in Gram-positive microorganisms are well-studied. The “housekeeping” sortase A (SrtA) and pilin-assembling Sortase C (SrtC) are present in *E. faecalis* (Marraffini et al., 2006).

SrtA is considered a “housekeeping” sortase because it covalently attaches substrate proteins to the growing cell wall. Cell wall synthesis has been extensively studied and general representation of the process on the example of *Staphylococcus aureus* is presented in **Figure 1.2**.

Figure 1.2 Peptidoglycan synthesis in Gram-positive bacteria based on the example of *S. aureus*.

(adapted from (Marraffini et al., 2006)).

The major cytoplasmic precursor for cell wall synthesis is Park nucleotide (UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala) which is synthesized by addition of L-stereoisomer amino acids and D-stereoisomer amino acids to Uridine diphosphate-N-acetylmuramic acid (UDP-NM) (Marraffini et al., 2006). After synthesis, Park nucleotide is attached to the undecaprenol pyrophosphate in the plasma membrane via a phosphodiester bond, forming Lipid I. Next, Lipid I is transformed to Lipid II by addition of N-acetylglucosamine (GlcNAc) to the NM

and addition of pentaglycine tail to L-Lysine. Lipid II is then translocated across the plasma membrane via specialized protein machinery and is processed by penicillin binding proteins (PBPs) (van Dam et al., 2007). PBPs, enzymes involved in the final stage of cell wall synthesis, catalyse three reactions. First, Lipid II undergoes transglycosylation, which results in formation of glycan strands MurNAc-GlcNAc. The second reaction is transpeptidation, when the 4th alanine of one Lipid II precursor is connected to the pentaglycine of another precursor molecule (Izaki et al., 1966). The last reaction catalysed by PBP is carboxypeptidation, where D-Ala is hydrolysed, which results in formation of mature peptidoglycan (Marraffini et al., 2006).

SrtA recognizes substrate proteins via recognition of a C-terminal cell wall sorting signal (CWSS) within the substrate. The CWSS for SrtA substrates includes an LPXTG motif (leucine, proline, X = any amino acid, threonine and glycine) followed by a trans-membrane domain and a positively charged cytoplasmic tail (Paterson and Mitchell, 2004). SrtA catalysis consists of two stages. In the first stage, SrtA cleaves the substrate between the T and G amino acids of the CWSS and forms covalent bond between the active site of sortase and the threonine at the C-terminal end of the substrate. Next, this acyl-enzyme ester undergoes a nucleophilic attack by Lipid II, and the protein becomes attached to lipid II of the growing cell wall (Perry et al., 2002; Suree et al., 2009). Importantly, Lipid II, but not mature peptidoglycan, serves as the attachment site and *in vivo* peptidoglycan substrate for sortase-catalysed surface protein anchoring. Based on homology between SrtA in *S. aureus* and *E. faecalis*, we can predict that SrtA in *E. faecalis* strain is embedded within the cell membrane and exhibits type 2 topology wherein the N-terminus is located inside the cytoplasm, whereas the active site and C-terminus of the protein are located on the external side of the plasma membrane (Marraffini et al., 2006). The N-terminus includes a hydrophobic region with a non-cleavable signal peptide which directs SrtA to the general secretion machinery and acts as a stop signal for translocation. The active site of the enzyme can also be predicted by sequence and structural alignment and includes histidine 138, cysteine 200 (C200) and arginine 208. The role of these three active site residues was

previously described for SrtA in *S. aureus* (Bentley et al., 2008). Indeed, recent studies in *E. faecalis* showed that when the catalytic residue C200 was mutated to alanine, the enzyme lost its activity, while C200A mutation did not affect the protein expression levels (Nielsen et al., 2013). When SrtA is inactive, substrates, which are translocated at discrete foci, are not incorporated into the cell wall and are retained in a single spot on the membrane when *srtA* is deleted (Kline et al., 2009; Nielsen et al., 2013).

SrtA is localized at distinct membrane foci in a number of Gram-positive bacteria: in *Streptococcus mutans*, *Streptococcus pyogenes*, as well as in *E. faecalis* (Hu et al., 2008; Kandaswamy et al., 2013a; Kline et al., 2009; Raz and Fischetti, 2008). By immunogold electron microscopy (IEM) it was shown that, independent of growth phase, the majority of *E. faecalis* cells have single SrtA foci. Discrete foci are mostly found near nascent septum, followed by the pole region and less frequently in the midcell of *E. faecalis* (**Figure 1.3**) (Kline et al., 2009).

SrtA has been well-studied and several extensive reviews described SrtA activity and contributions to bacterial biology (Marraffini et al., 2006; Paterson and Mitchell, 2004). However, despite the well know mechanism of action, regulation

of SrtA and regulation relationships with its substrate have never been reported. In *E. faecalis* $\Delta srtA$ cellular localization of AS and Ebp changes, where in $\Delta srtA$ Ebp remained in the plasma membrane and both Ebp and AS were enriched in a single foci in the plasma membrane when *srtA* was deleted (Kline et al., 2009; Nielsen et al., 2013). These findings suggested that in the absence of SrtA no other protein is able to attach the SrtA substrates to the cell wall and substrates go “off-pathway” accumulating in the plasma membrane instead of being attached to the cell wall. Moreover, there are more than 20 predicted SrtA-dependent adhesins identified in *E. faecalis* strain V583 based on the presence of a CWSS (Sillanpaa et al., 2004). Therefore, we predicted that deletion of *srtA* would result in accumulation of unprocessed substrates on the cell membrane that may in turn alter expression of those substrates (perhaps by negative feedback regulation) or trigger another cellular response to deal with unprocessed substrates. We asked how the cell responds to *srtA* deletion and what will happen to the expression profile of SrtA substrates in **Chapter 5** of this thesis. As a representative example of what happens to SrtA substrates in $\Delta srtA$ we investigated Ebp biogenesis in $\Delta srtA$ background in **Chapter 3**.

1.2. Sortase A substrates

SrtA in *E. faecalis* is an important virulence factor that contributes to biofilm formation and infection (Guiton et al., 2010; Guiton et al., 2009; Jonsson et al., 2002; Kemp et al., 2007a; Lévesque et al., 2005). In all Gram-positive bacteria examined, deletion of SrtA results in attenuation of virulence, presumably because adhesins or other sortase-dependent virulence factors are no longer attached to the cell wall (Maresso and Schneewind, 2008). Understanding the role of SrtA in the regulation of its cognate substrates may reveal new antimicrobial targets which will directly affect adhesion or host interactions leading to decreased pathogenicity. If, as we predict, the absence of SrtA results in downregulation of SrtA substrates, then identifying the regulator that feeds back to reduce substrate transcription could be a suitable target for increased transcription to reduce expression of multiple adhesins at the same time. Alternatively, if a common positive regulator of multiple adhesins is present, then

this regulator can be targeted by antimicrobials. In Enterococci, SrtA is responsible for anchoring described virulence factors including Esp, AS, Ebp, and Ace to the cell wall (Hendrickx et al., 2009).

1.2.1. Endocarditis and biofilm associated pili

Ebp is an important factor involved in both biofilm formation and endocarditis in *E. faecalis* (Nallapareddy et al., 2006; Nielsen et al., 2013). Ebp are high molecular weight polymers (>200 kDa) and the length of the pilus may reach 10 μm (**Figure 1.4**) (Sillanpaa et al., 2004).

Ebp consist of 3 subunits – EbpA, EbpB and EbpC transcribed in a single operon (Nallapareddy et al., 2006). EbpC is the main subunit of the pilus, with EbpB found at the base and EbpA on the tip (**Figure 1.5**) (Nielsen et al., 2013). EbpA mediates attachment to host fibrinogen and collagen and plays an important role in UTI, CAUTI, and endocarditis (Flores-Mireles et al., 2014; Nallapareddy et al., 2011a; Nallapareddy et al., 2006). Mutations in the EbpA adhesive tip prevent Ebp-associated biofilm formation *in vitro* as well as CAUTI in mice (Flores-Mireles et al., 2014; Nallapareddy et al., 2006; Nielsen et al., 2012).

Pilin subunits are polymerized by SrtC, which is encoded downstream of the *ebpABC*. Upon assembly, the pilus is attached to the cell wall by a housekeeping SrtA encoded 1.5 mega base pairs downstream in the genome (Kline et al., 2009).

Expression of Ebp is phase variable and pilus expression can be induced by exposure to serum, glucose, or bicarbonate (Bourgogne et al., 2010; Kline et al., 2009; Nallapareddy et al., 2011a; Nallapareddy et al., 2006). Since displayed surface proteins of bacteria are recognized by immune system and may trigger immune response with subsequent clearance, bacteria evolved strategy to rapidly alter displayed proteins. Therefore, phase variation of surface proteins is beneficial to bacteria, allowing bacteria to diminish the effect of acquired immunity by changing epitopes (Deitsch et al., 2009). Since enterococcal pili are immunogenic, the presence of pili on a small population of cells and the ability to alter pili expression may be advantageous to *E. faecalis*, allowing a subpopulation to persist during infections without stimulating an immune response or Ebp null population to avoid immune clearance (Nallapareddy et al., 2006).

Although the mechanism of variable Ebp expression is unknown, the main Ebp transcriptional regulator EbpR was identified in an early microarray screen for Fsr quorum-sensing system regulated genes (Bourgogne et al., 2007). *ebpR* is encoded upstream of the *ebpABC* and is transcribed in the opposite direction (**Figure 1.5A**). An *ebpR* deletion decreases *ebpA*, *ebpB* and *ebpC* transcription levels 200, 100, 80 times respectively and the number of piliated cells in the population drops from 36% to 1%. On the other hand, when *ebpR* is complemented from a plasmid, piliation increases but does not reach 100%, suggesting that presence of EbpR *per se* in a cell is important but not sufficient for pilus expression and other unidentified regulatory factors might be involved. Moreover *rnjB*, a putative RNase J2, is also important for *ebp* transcription, where *rnjB* deletion resulted in significant decrease of *ebpABC* mRNA (Gao et al., 2010a). The exact mechanism of regulation is unknown, but RNase J2 was proposed to regulate and stabilize *ebpABC* transcripts, degrade small inhibitory RNA or influence *ebpABC* promoter activity directly or indirectly (Gao et al., 2010a).

1.2.2. Aggregation substance

AS is a 137 kDa protein encoded by *prgB* on the pCF10 plasmid that contributes to biofilm formation, cellular aggregation, plasmid transfer, and increased virulence in endocarditis models (Bhatty et al., 2015; Chuang-Smith et al., 2010b; Hirt et al., 2002; Schlievert et al., 1998; Waters and Dunny, 2001). AS is anchored to the cell wall by SrtA via LPKTG anchor motif and in Δ *srtA* pheromone induced cells fail to clump likely due to erroneous AS placement on the surface (Kline et al., 2009; Kristich et al., 2005). AS facilitates adherence of *E. faecalis* to renal tubular cells, intestinal epithelial cells, as well as binding to and survival in human neutrophils (PMNs) (Chuang et al., 2009; Rakita et al., 1999; Süßmuth et al., 2000). The pCF10 plasmid encodes tetracycline resistance and is responsive to a chromosomally encoded peptide (LVTLVFV)

called cCF10. *E. faecalis* encodes *ccF*, the genetic determinant for the cCF10, within its core genome, however plasmid free cells lack an efficient pheromone intake system, therefore pheromone primarily remains in the secreted fraction (Antipporta and Dunny, 2002; Leonard et al., 1996). cCF10 is secreted as a pre-pheromone and processed by Eep protease to form a functional pheromone (An et al., 1999; Mori et al., 1988). Upon secretion, cCF10 binds to plasmid-encoded PrgZ which is a lipid-anchored protein with high affinity to cCF10 and is delivered into the cell (Leonard et al., 1996). Once in the cytoplasm, cCF10 binds to the pCF10-encoded repressor PrgX allowing transcription of conjugation genes *prgB*, *pcfC* and *pcfG*. Expression of these genes facilitates cell-to-cell contact and exchange of genetic material (**Figure 1.6**) (Bae et al., 2000; Chatterjee et al., 2013).

In the absence of the cCF10 pheromone, expression of AS and most of the pCF10 plasmid-encoded genes is inhibited by a small plasmid-encoded peptide iCF10 that competes with cCF10 for binding to PrgX and stabilizes the repression of PrgX on the *prgQ* operon (Nakayama et al., 1994). This repression prevents self-induction and autocrine pheromone signalling (**Figure 1.6**) (Chatterjee et al., 2013; Shi et al., 2005).

Alternatively, expression of AS can be induced by albumin/lipid complexes in the bloodstream that sequester or degrade iCF10, resulting in the activation of autocrine pheromone signalling (Chandler et al., 2005). Expression of AS is variable within a population even at saturating levels of pheromone as observed by emission scanning electron microscopy (**Figure 1.7**) (Olmsted et al., 1993).

1.2.3. Adhesin to collagen

Ace is 71 kDa protein that contributes to endocarditis and UTI (Nallapareddy et al., 2011a; Roh et al., 2014; Singh et al., 2010). Ace has a conserved collagen binding domain and facilitate adhesion to Collagen type IV, laminin, collagen type I and dentin (Kowalski et al., 2006; Nallapareddy et al., 2000). Ace expression is induced by various environmental cues including serum, urine, increased temperature (46°C), and bile salts (Lebreton et al., 2009; Nallapareddy and Murray, 2006; Shepard and Gilmore, 2002; Xiao et al., 1998). Transcription of *ace* also increases in response to specific host matrix factors such as type IV and I collagen but not in the presence of fibrinogen or bovine serum albumin (BSA) (Nallapareddy et al., 2006). Transcriptionally, *ace* is regulated by the two component system (TCS) GrvRS, which stands for global regulator of virulence, formerly known as EtaRS (enterococcal two-component system a) (Roh et al., 2014; Teng et al., 2002). Deletion of *grvR* results in reduced collagen binding and biofilm formation (Roh et al., 2014). The same *grvR* mutant is also less virulent in mouse peritonitis model (Roh et al., 2014). In addition to transcriptional regulation, Ace is also regulated post-translationally by Fsr and the catabolite control protein A (CcpA). The Fsr quorum sensing system controls expression of gelatinase (GelE), which cleaves Ace from the cell surface during late stationary phase resulting in a decreased surface display of Ace (Pinkston et al., 2011). In a Δ *ccpA* mutant, transcription of Ace is unaffected, yet less Ace can be detected by immunoblot independent of gelatinase cleavage (Gao et al.,

2013). The mechanism for Ace regulation by CcpA is unclear, although CcpA is thought to affect Ace protein production rather than cellular processes of secretion and sorting, as the amount of Ace universally decreases in all cellular fractions of *ccpA* mutant. (Gao et al., 2013).

E. faecalis possess a number of adhesins, but whether any inter- or cross-regulation is present between them is not known. In clinical *E. faecalis* isolate mRNA levels of the adhesins *esp*, *ace* and *asa* (for AS) are similarly affected by environmental cues found in urine and serum; however, neither the mechanism underlying this regulation nor the cues themselves are known (Shepard and Gilmore, 2002). Evidence supporting the notion of inter-related regulation of adhesin expression within a single organism can be found in *E. coli*, where expression of pyelonephritis-associated pili (Pap-pili) and type I fimbriae is mutually exclusive, and the PapB positive regulator of *pap* genes represses expression of type I fimbrial genes (Holden et al., 2001; Xia et al., 2000). Similarly, in *Pseudomonas aeruginosa*, flagellar expression, important for motility, in response to cyclic-di-GMP and cAMP is cross-regulated with Type 4 pilus expression, important for a sessile growth (Kazmierczak et al., 2015).

In *E. faecalis*, some factors that regulate expression of the individual adhesins AS, Ebp and Ace have been described, but a relationship or coordination between adhesin expression has yet to be addressed. In the current work, we examine whether regulation of any of these three adhesins affects the expression of another.

1.3. Stress response

Cellular processes within bacteria are highly regulated. One consequence of certain types of cellular stress, including oxidative, temperature or chemical stress, is protein misfolding, malfunctioning, or aggregation (Cabisco et al., 2010; Mogk et al., 2003). Prokaryotes have evolved systems to sense and repair misfolded proteins, where transcription of the repair proteins increases rapidly upon increase in a number of the misfolded proteins (Arsene et al., 2000). One

of the well-characterized and conserved stress-response chaperones are heat-shock proteins Hsp70 and its cofactor Hsp10, also known as DnaK and DnaJ respectively in bacteria (Genevaux et al., 2007). Under normal conditions, these two proteins are involved in folding of de-novo synthesized proteins, but under stress conditions they prevent protein aggregation and assist in protein re-folding (Feldman and Frydman, 2000). The DnaK and DnaJ chaperones function in a complex in which DnaJ recognizes exposed alpha helices (hydrophobic areas) on newly synthesized or misfolded proteins, binds them, and recruits DnaK to the complex for further processing (Kampinga and Craig, 2010). DnaJ recognizes the DnaK ATPase domain which allows the two chaperones to form a functional complex, where DnaJ is released from the DnaJ-DnaK complex after DnaK docking (Gässler et al., 1998). Under stress conditions, DnaK remains in complex with the misfolded protein to prevent the protein from aggregation (Richter et al., 2010). Therefore, DnaK-DnaJ functions to fold newly synthesized proteins under normal conditions and prevent the unfolded protein from aggregation under stress conditions.

In *E.coli*, transcriptional regulation of chaperones is governed by sigma factor σ^{32} (RpoH) (Guisbert et al., 2004). DnaK binds directly to RpoH and inhibits RpoH dependent-transcription *in vitro*, however under stress conditions, DnaK forms complex with a misfolded protein and releases RpoH that in turn induces transcription of heat-shock genes. *E. faecalis* lacks RpoH but is able to similarly upregulate the DnaK under stress (Boutibonnes et al., 1993; Flahaut et al., 1996b). In Gram-positive bacteria, including *E. faecalis*, the two transcriptional repressors HrcA and CtsR control chaperone expression by binding to the palindromic operator sequence CIRCE (for “controlling inverted repeat of chaperone expression”) within the promoter of target genes (Chastanet et al., 2003; Derré et al., 1999; Laport et al., 2004; Zuber and Schumann, 1994). DnaK also facilitates protein targeting to the membrane by working together with SecA, SecB and GroESL and is important for protein secretion (Castanié-Cornet et al., 2014).

In the Gram-positive bacteria *Streptococcus intermedius*, *S. aureus*, and *Lactococcus lactis*, DnaK is crucial under stress conditions and contributes to basic cellular processes. (Koch et al., 1998; Singh et al., 2007b; Tomoyasu et al., 2012). In *S. intermedius* the role of DnaK was tested for its role in regulation of virulence genes encoding the major virulence factor ILY that is essential for the invasion and cytotoxicity to human cells and stress-inducible ClpP peptidase. No differences in expression of the two virulence factors were observed between *S. intermedius* WT and *dnaK* null mutant, suggesting the general role of DnaK in stress response, rather than virulence in particular. However, in some Gram-negative bacteria such as *Salmonella typhiurium*, *Yersinia pestis* and *P. aeruginosa*, the chaperone-protease ClpXP and DnaK have been linked to bacterial pathogenesis (Borrero-de Acuña et al., 2015; Jackson et al., 2004; Yamamoto et al., 2001). In light of role of DnaK in virulence factor biogenesis, DnaK is now recognized as a potential drug target (Chiappori et al., 2015). In *E. faecalis* various stresses including heat shock, exposure to bile salts, and detergent universally induce expression of DnaK and GroEL (Flahaut et al., 1996a; Rince et al., 2000). In *E. faecalis* V583, 4 out of 9 TCS are induced by at least 1 of the stresses tested, where Err10 (EtaR of EtaRS TCS) is a negative regulator of DnaK and GroEL and an *err10* mutant showed higher survival rates at high temperature and in bile salts (Le Breton et al., 2003). Examination of the transcriptional response to bile salts and detergent revealed changes in genes encoding proteins involved in membrane composition and remodelling were expressed at higher levels under these stress conditions.

Here we hypothesized that accumulation of unprocessed substrates on the membrane in $\Delta srtA$ evoke membrane stress where cellular chaperones might be induced to deal with accumulated SrtA substrates (Kline et al., 2009).

1.4. Membrane vesicles in Gram-positive bacteria

Both Gram-positive and Gram-negative bacteria secrete MV into the environment. In Gram-negative bacteria, increased vesiculation has been linked to stress responses such as increased temperature where activation of stress

response proteins or increased membrane fluidity results in increased vesiculation (Katsui et al., 1982). The SigmaE stress response pathway also affects vesiculation, where in response to accumulated unfolded proteins, SigmaE activates chaperone gene expression, resulting in higher protein content on the membrane, where building pressure facilitates vesicle release (McBroom and Kuehn, 2007; Raivio, 2005).

In Gram-positive bacteria, MV have been detected in *S. aureus*, *Bacillus subtilis*, *Bacillus anthracis*, *S. pyogenes*, *Streptococcus pneumoniae*, *S. mutans*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* (Biagini et al., 2015; Gurung et al., 2011; Jiang et al., 2014; Lee et al., 2009; Lee et al., 2013b; Liao et al., 2014; Olaya-Abril et al., 2014; Rath et al., 2013; Resch et al., 2016; Rivera et al., 2010; Thay et al., 2013). The MV composition in *S. aureus*, *B. anthracis*, *L. monocytogenes* and *S. pyogenes* includes virulence factors such as toxins, adhesins, proteolysins, and tissue destructing enzymes (Gurung et al., 2011; Lee et al., 2009; Lee et al., 2013a; Resch et al., 2016; Rivera et al., 2010).

MV can also trigger host immune responses. MV from *S. pneumoniae* are immunogenic and elicit protective immune response upon immunization (Olaya-Abril et al., 2014). MV from *S. aureus* are enriched in α -toxin that contribute to HeLa cell cytotoxicity and are also taken up by the HEp-2 cells inducing HEp-2 cell death (Gurung et al., 2011; Thay et al., 2013). In Mycobacteria, MV can trigger inflammatory responses in mice in a Toll-like receptor (TLR)-2 dependent manner, due to interaction between TLR-2 and lipoproteins on the MV (Prados-Rosales et al., 2011). In contrast, lipoprotein-enriched MV from *S. pyogenes* fail to activate TLR-2, suggesting that lipoproteins organized in these membrane vesicular structures do not act as TLR-2 agonists (Biagini et al., 2015).

While several mechanisms that trigger outer membrane vesicle (OMV) formation in Gram-negative bacteria have been proposed, the mechanism of MV formation in Gram-positive bacteria is largely unknown (Kuehn and Kesty, 2005). In Gram-positive bacteria, MV biogenesis can be regulated genetically. In *L. monocytogenes*, RNA polymerase sigma factor σ B plays a role in MV

biogenesis (Lee et al., 2013b). Similarly, MV biogenesis in *M. tuberculosis* is affected by VirA (vesiculogenesis and immune response regulator) and vesiculation increases in VirA deficient mutant (Rath et al., 2013). Recently in *S. pyogenes* the TCS CovRS was shown to negatively impact vesiculation (Resch et al., 2016)

Apart from genetically regulated vesiculation, MV can also be produced actively during the cell wall remodelling in response to a secretion signal, or passively during the daughter cell separation in the areas of thinned cell wall (Brown et al., 2015). MV from *S. aureus* carry functional cell-wall modifying enzymes that partially support release of the MV themselves, where active cell-wall modifying enzymes partially digest the cell wall to create the pores for facilitate release of MV in the environment (Brown et al., 2015; Lee et al., 2009).

Snap-shots of MV formation have been captured by TEM and SEM in several Gram-positive bacteria. In *S. pneumoniae*, MV were observed within the cell by TEM (Olaya-Abril et al., 2014). In *B. subtilis*, MV release was observed by TEM and MV-like structures were found on the surface of the bacteria by SEM (Liao et al., 2014). Similarly, vesicle-like blebs were observed on the surface of *M. tuberculosis* by TEM and SEM (Prados-Rosales et al., 2011). Recent work in *P. aeruginosa* described a role for explosive cell lysis in MV formation and eDNA release, which contribute to biofilm formation (Turnbull et al., 2016). A prophage endolysin, encoded within the R- and F-pyocin gene cluster, was essential for the explosive cell lysis event, where expression of endolysin is mediated by RecA during the SOS stress response (Turnbull et al., 2016). It is yet unclear whether explosive cell lysis is a programmed process by an altruistic cell or just a direct consequence of the release of phage particles. However, since during cell lysis public goods are released in the environment, together with MV, that can either contribute to biofilm formation or create a pool of mobile genomic elements, then MV biogenesis via explosive cell lysis might be a universal process in bacterial communities (Domingues and Nielsen, 2017; Turnbull et al., 2016).

To date, MV in Enterococci have not been studied. However, we and others observed abundance of the SrtA substrate Ebp in the supernatant of *E. faecalis* cultures (Kline et al., 2009; Nielsen et al., 2013). In $\Delta srtA$, unprocessed SrtA substrates accumulate in the membrane, and in Gram-negative bacteria proteins accumulation in the outer membrane triggered MV formation (McBroom and Kuehn, 2007). Thus we hypothesized that in *E. faecalis* pili might be released in MV-dependent manner in $\Delta srtA$ and that accumulation of SrtA substrates on the membrane might enhance MV biogenesis.

1.5. Introduction summary

Adhesion is a crucial step in bacterial attachment to host tissues and important for the development of infection. The Enterococcal adhesion network consists of multiple players: SrtA, SrtC, known adhesins: Ace, Ebp, AS, Esp and regulators of the above (Bourgogne et al., 2007; Flores-Mireles et al., 2014; Heikens et al., 2011a; Heikens et al., 2011b; Kristich et al., 2005; Nielsen et al., 2013; Olmsted et al., 1991; Rakita et al., 1999; Roh et al., 2014; Shepard and Gilmore, 2002; Sillanpaa et al., 2004).

While the regulation and role of individual adhesins in specific diseases have been addressed, no studies have examined the interrelationship between enterococcal adhesin expression or the role of SrtA in the regulation of adhesin expression. Moreover while SrtA inhibitors are being developed to abolish SrtA function, little is known on how bacteria will respond to *srtA* loss-of-function and what will happen to the many SrtA substrates in a SrtA deficient cell (Cascioferro et al., 2015; Guo et al., 2015).

In order to fill these gaps in knowledge, this thesis addresses the following questions:

1] What is the fate of SrtA-dependent substrates (using the model system of Ebp) in the absence of SrtA and what is the overall cellular response to *srtA* deletion?

and

2] Are known SrtA-dependent adhesins in *E. faecalis* differentially expressed and regulated within the population?

1] The role of SrtA in the virulence and in the attachment of multiple LPXTG-containing proteins has been well characterized in Gram-positive bacteria (Cossart and Jonquières, 2000; LeMieux et al., 2008; Lévesque et al., 2005; Marraffini et al., 2006; Raz and Fischetti, 2008; Wu et al., 2014b). However, the response to *srtA* deletion seems to vary between the Gram-positive organisms. In *S. pyogenes*, SrtA depleted cells show aberrant morphology (Raz et al., 2015). In $\Delta srtA$ *S. aureus* three tested SrtA substrates – staphylococcal protein A (Spa), fibronectin-binding protein A (FnbA) and clumping factor A (ClfA) – were mislocalized and isolated from cytoplasm and plasma membrane compared to cell wall in WT *S. aureus* (Mazmanian et al., 2000). In the same study localization of Spa was tested by IFM and no Spa was detected on the surface of a *srtA* mutant. (Mazmanian et al., 2000). In *Actinomyces oris*, deletion of *srtA* is lethal due to toxic accumulation of surface glycoprotein in the plasma membrane (Wu et al., 2014a). While in *E. faecalis*, $\Delta srtA$ cells were reported to have increased piliation (Nielsen et al., 2013). This finding implies a regulatory feedback from deletion of SrtA on the expression of Ebp in *E. faecalis*. We hypothesized that deletion of *srtA* and subsequent perturbation in the composition of the plasma membrane and the cell wall may trigger cellular feedback on the expression of SrtA substrates and in particular on Ebp. To test this hypothesis in **Chapter 3** we addressed the effect of $\Delta srtA$ on Ebp biogenesis in *E. faecalis*. Since we detected increased piliation in $\Delta srtA$ both in the plasma membrane and supernatant that was independent of *ebp* transcription and couldn't be explained by reduced turnover of pili from the cell wall, in **Chapter 4** we hypothesized that accumulation of multiple substrates (or at least Ebp and Ace examined here) in the plasma membrane triggers membrane vesicle formation resulting in a release of Ebp in the supernatant. Lastly, since *ebp* transcription was unchanged in a *srtA* mutant, we examined whether other predicted SrtA substrates were also transcribed at the same levels as in WT and

accumulated in the membrane of *srtA* mutant cells. And if this was the case, then how does the cell respond to membrane perturbations caused by the accumulation of multiple off-pathway substrates in the plasma membrane? We hypothesized that bacteria experience stress and might possess systems crucial for dealing with unprocessed sortase substrates. Therefore, the cellular transcriptional response to *srtA* deletion is addressed in **Chapter 5**.

2] While SrtA is responsible for the attachment of the Ace, Ebp and AS and other putative substrates to the cell wall, the relationship between substrate expressions remains unclear. From Gram-negative systems, we know that certain adhesins are differentially regulated to allow bacteria optimized adherence in different environments or to switch from sessile or mobile mode. In *E. coli* Pap pili and Type I fimbriae are differentially regulated, similar to flagella and pili in *P. aeruginosa* (Kazmierczak et al., 2015; Xia et al., 2000).

In *E. faecalis*, regulation of Ace, Ebp and AS has been studied, however, it is not known whether the three adhesins have common regulators, are co-expressed/differentially expressed within the population, or have independent regulators responsive to various environmental cues. Moreover, since expression of two of the adhesins – Ebp and AS – was reported to be variable on cells within a population, we wondered whether different population subsets express different adhesin repertoires. To test this, we examined the expression profile of Ebp and AS upon AS-induction in **Chapter 5**. Lastly, to test if Ace and Ebp have common regulators, we tested how known regulators and environmental cues for one of the adhesins affect regulation of another adhesin in **Chapter 6**.

2. Chapter 2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in the **Table 2.1**.

Table 2.1 Bacterial strains and plasmids

Strains	Characteristics	Resistance, concentration	Fraction Location (5'-3') of Tn Insertion in gene	Source
OG1RF (WT)	human oral isolate OG1	Rifampicin (Rif) and fusidic acid (Fus), 25 µg/ml	not applicable (n/a)	(Dunny et al., 1978)
OG1RF Δ <i>srtA</i>	Contains in-frame-deletion of <i>srtA</i> gene	Rif, Fus, 25 µg/ml	n/a	(Kline et al., 2009)
OG1SS	Derivative of OG1	Streptomycin (Str), 500 µg/ml	n/a	(Franke and Clewell, 1981)
OG1X	Derivative of OG1	Str, 500 µg/ml	n/a	(Dunny et al., 1979)
OG1SS Δ <i>srtA</i>	Contains in-frame-deletion of <i>srtA</i> gene	Str, 500 µg/ml	n/a	(Kline et al., 2009)
OG1X Δ <i>srtA</i>	Contains in-frame-deletion of <i>srtA</i> gene	Str, 500 µg/ml	n/a	(Kline et al., 2009)
OG1X Δ <i>srtC</i>	Contains in-frame-deletion of <i>srtC</i> gene	Str, 500 µg/ml	n/a	(Kline, Kau et al. 2009)
OG1RF Δ <i>srtC</i>	Contains in-frame-deletion of <i>srtC</i> gene	Rif, Fus, 25 µg/ml	n/a	(Kemp et al., 2007a)
OG1RF Δ <i>atlA</i>	Contains in-	Rif, Fus, 25 µg/ml	n/a	(Guiton et

	frame-deletion of <i>atlA</i> gene			al., 2009)
OG1RF Δ <i>ebpA</i> <i>BC</i> Δ <i>srtC</i>	Contains in-frame-deletion of <i>ebpABC</i> and <i>srtC</i> gene	Rif, Fus, 25 μ g/ml	n/a	(Nielsen et al., 2013)
OG1RF <i>ebpR</i> ::Tn	<i>ebpR</i> transposon mutant	Rif, Fus, 25 μ g/ml; chloramphenicol (Cm) 10 μ g/ml	0.23	(Kristich et al., 2008)
OG1RF <i>ace</i> ::Tn	<i>ace</i> transposon mutant	Rif, Fus, 25 μ g/ml; Cm, 10 μ g/ml	0.33	(Kristich et al., 2008)
OG1RF <i>grvR</i> ::Tn	Response regulator of two-component system TCS	Rif, Fus, 25 μ g/ml; Cm, 10 μ g/ml	0.87	(Kristich et al., 2008)
OG1RF <i>grvS</i> ::Tn	Histidine kinase of GrvRS TCS	Rif, Fus, 25 μ g/ml; Cm, 10 μ g/ml	0.07	(Kristich et al., 2008)
OG1RF <i>dnaK</i> ::Tn	<i>dnaK</i> transposon mutant	Rif, Fus, 25 μ g/ml; Cm, 10 μ g/ml	0.51	(Kristich et al., 2008)
<i>E. coli</i> DH5 α	<i>E. coli</i> competent cells		n/a	
plasmids				
pAK1		Kan, 500 μ g/ml	n/a	(Kline et al., 2009)
pAK1-SrtA	encodes <i>srtA</i> under control of the native <i>srtA</i> promoter	Kan, 500 μ g/ml	n/a	(Kline et al., 2009)
pAK1-SrtAC200A	encodes <i>srtA</i> containing a point mutation at the cysteine within the active site, under control of the native <i>srtA</i> promoter	Kan, 500 μ g/ml	n/a	(Kline et al., 2009)
pAK1 - DnaK (<i>pdnaK</i>)	encodes <i>dnaK</i> under <i>srtA</i>	Kan: 50 μ g/ml (<i>E. coli</i>);	n/a	this study

	promoter	500 µg/ml (<i>E. faecalis</i>)		
pCF10	cCF10 pheromone responsive plasmid, encodes aggregation substance and conjugation apparatus	Tetracycline (Tet), 15 µg/ml	n/a	(Mori et al., 1988)
pGCP213	Temperature-sensitive plasmid for generation of deletions	Erythromycin (Erm): 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	n/a	(Nielsen et al., 2012)

For all experiments bacteria were streaked from a 50% glycerol stock stored at -80°C onto the agar plate and grown overnight at 37°C. The next day, a single colony was inoculated in a fresh liquid media and grown overnight before subculture to obtain log phase bacteria. Subculture was performed by 1:10 dilution with fresh media, grown to optical density at a wavelength of 600 nm (OD_{600})=0.45-0.6. *E. faecalis* strains were grown in Brain Heart Infusion (BHI broth; BD Difco, USA) or BHI plus 1.5% agar (BD Difco, USA) *E.coli* was grown in Luria-Bertani broth or agar (BD Difco). Antibiotics were added to culture the strains with the plasmid in the liquid or agar media in concentrations listed in **Table 2.1**.

2.2. Molecular techniques

Table 2.2 Primers used in the study

Name of the primer	Sequence (5`-3`)	Description
<i>dnaK</i> _F	TATTATTATAGGAATTCAATAC AGATTGGAGAT	anneals to 5' end of <i>dnaK</i> gene, carries EcoRI restriction site (RS) (underlined)
<i>dnaK</i> _R	CCTAAAATCAATTGTGGATCC TTATTTGTC	anneals to 3' end of <i>dnaK</i> gene, carries BamHI RS (underlined)
pAK1_F	TATCCGTGTCGTTCTGTCCA	forward pAK1 primer, binds upstream of multiple restriction site (MRS) on the pAK1 plasmid
pAK1_R	GCCTAAAGACAAGCCACCTG	reverse pAK1 primer, binds downstream of MRS on the pAK1 plasmid
M13_F	GTAAAACGACGGCCAGTG	forward pGCP213 primer, binds upstream of pGCP213 MRS
M13_R	CAGGAAACAGCTATGAC	reverse pGCP213 primer, binds downstream of pGCP213 MRS
<i>dnaK</i> _KO_F1 (EcoRV)	GTGGTCGCATGATATCGTTAG ATTT	upstream fragment to knock out <i>dnaK</i> , anneals to 5' end 1000 bp upstream of <i>dnaK</i> gene
<i>srtA</i> _F	TCGTACGCCGTTAGCAAGTT	anneals to 5' end within <i>srtA</i> gene
<i>srtA</i> _R	TTCATCACCGCTTCTGTGCT	anneals to 3' end within <i>srtA</i> gene
<i>prgB</i> _F	GCCAACAGAAGTTGCACCAG	anneals to 5' end within <i>prgB</i> (AS) gene
<i>prgB</i> _R	CGCATGGCCACCTTTATTCG	anneals to 3' end within <i>prgB</i> (AS) gene

2.2.1. pAK1-DnaK construction

dnaK gene was amplified using genomic OG1RF DNA (gDNA) as a template, with *dnaK_F* and *dnaK_R* primers (**Table 2.2**). The PCR product was purified and digested, along with the pAK1 plasmid, with EcoRI_HF and BamHI-HF restriction enzymes (New England Biolabs, UK) for 30 min at 37°C. Digested product were gel purified (Qiagen, USA) and ligated at a 1:3 (vector:insert) molar ratio, transformed into electro competent *E. coli* DH5 α , and plated on selective LB + kan 50 μ g/ml plate. Clones were screened with pAK1 primers (pAK1_F/R, **Table 2.2**) for the correct insert size. PCR-positive clones were sequenced prior transformation into OG1RF *dnaK::Tn*, where clones grown on the BHI + kan 500 μ g/ml plate were further screened for the presence of pAK1-DnaK plasmid.

2.2.2. OG1RF_*dnaK::Tn* Δ *srtA* construction

Temperature sensitive plasmid pGCP213 that carries ligated fragments of approximately 800 bp upstream and downstream of *srtA* gene (Kline et al., 2009) was transformed into OG1RF_*dnaK::Tn* electro competent cells. After transformation successful clones on BHI + erm 25 μ g/ml plate were passaged as described before first at 30°C with erm, then at 42°C to integrate the plasmid in the genome and then again at 30°C without the erm to excise the plasmid (Nielsen et al., 2012). Clones that lost the plasmid with double cross-overed events were screened for the *srtA* deletion using SrtA_F/R primers (**Table 2.2**) followed by immunoblot with SrtA antiserum.

2.3. Trichloroacetic acid precipitation

Proteins were trichloroacetic acid (TCA) precipitated according to the protocol of California institute of technology (California Institute of Technology, 2001). Briefly, cells were grown to mid-log growth phase in BHI and volume of cells

corresponding to $OD_{600}=0.6$ was then transferred to a new 1.5 ml Eppendorf tube. Cells were centrifuged at $14,000 \times g$ for 2 min and supernatant was collected and filtered through $0.22 \mu m$ filter to remove any remaining cells. Proteins from the cell-free supernatants were precipitated by adding 100% TCA to a final concentration of 20%. Samples were vortexed and incubated on ice for 10 min. Precipitated proteins were sedimented by centrifugation at $14,000 \times g$ for 5 min and the pellet was next washed with cold acetone twice at $14,000 \times g$ for 5 min. After drying over the $95^{\circ}C$ heat block, proteins was resuspended in 1x SDS in 1X phosphate-buffered saline (PBS) with a final volume of 100 μl and boiled for 10 min at $95^{\circ}C$. 10 μl of TCA precipitated proteins from the supernatant was loaded on the gel for immunoblot analysis.

2.4. Silver staining

10 μl of protein samples were run on 4-12% NuPAGE® Bis-Tris mini gel (ThermoFisher Scientific, USA) according to the manufacturer's protocol and silver stained using The Rockefeller University protocol (The Rockefeller University, 2016). Briefly gel was first fixed in 50% methanol + 5% acetic acid for 20 min, washed with 50% methanol and then water for 10 min each. Next gel was sensitized with 0.02% thiosulfate for 1 min, followed by 2 x 1 min washes with water. Silver reaction was performed for 20 min with 0.1% silver nitrate with 0.08% formalin. After that the gel was washed 2 x 1 min with water and developed using fresh 2% sodium carbonate with 0.04% formalin solution. Reaction was stopped by incubation with 5% acetic acid for 10 min with a final 10 min wash with water.

2.5. Mating assay

To construct OG1RF pCF10 and OG1RF $\Delta ebpABC\Delta srtC$ pCF10 we performed conjugation between donor OG1SS pCF10 (str^+ , tet^+) and recipient OG1RF and OG1RF $\Delta ebpABC\Delta srtC$ (both rif^+). Briefly two recipient strains were grown overnight in BHI broth. OG1SS pCF10 was grown overnight in BHI + tet 15 $\mu g/mL$. The overnight culture of OG1SS pCF10 was diluted 1:10 in fresh media

and the cCF10 peptide LVTLVFV (custom order from Axil Scientific, Singapore) was added to a final concentration of 0.12 ng/mL to induce AS expression (adapted from (Antiporta and Dunny, 2002)). cCF10 induction was carried out for 1 hour with shaking at 200 rpm at 37°C. Simultaneously, OG1RF or OG1RFΔ*ebpABCΔsrtC* were subcultured 1:10 in fresh BHI media and 4.5 mL of this diluted culture was mixed with 0.5 ml of 1 hour-cCF10-induced OG1SS pCF10. Conjugation was allowed to proceed for 30 min at 37°C, with shaking at 200 rpm at 37°C. 50 uL of mated cultures were plated on selective BHI plates (rif⁺, tet⁺) to select for transfer of tetracycline resistant pCF10 plasmid to OG1RF and OG1RFΔ*ebpABCΔsrtC* strains. None of the parent strains grow on these plates as OG1RF is only resistant to rif, but not to tet and OG1SS pCF10 is resistant to str and tet (tet resistance is carried on the plasmid). Clones were screened by PCR for the presence of pCF10 plasmid using the *prgB* F/R primers listed in **Table 2.2.**

2.6. Membrane vesicle isolation

2.6.1. Crude MV fraction preparation

A single colony was grown overnight in BHI broth. The resulting overnight culture was diluted with a warm fresh BHI to OD₆₀₀= 0.1 and grown to mid-log phase (OD₆₀₀=0.5-0.6, approx. 1 hour 40 min). 500 ml of mid-log phase cells were harvested using a Beckman centrifuge and JA 12.650 at 7,000 rpm for 15 min at 4°C. Supernatants were then filtered through 0.45 µm vacuum filter and concentrated to 70 ml using a VIVAFLOW 100,000 MWCO Hydrosart (Sartorius, Germany). Concentrated supernatants were then ultracentrifuged at 160, 000 x g for 3 hours at 4°C using a Beckman Ti 45 rotor (rotor was pre-chilled at 4°C overnight) in ultracentrifuge (Beckman, Germany). Supernatants were completely removed and the pellet was resuspended in 50 ul of chilled PBS and stored at – 80°C until further analysis.

2.6.2. Vesicles purification in a density gradient

Crude MV fractions isolated as above were fractionated in OptiPrep gradient created similarly to the OptiPrep gradient described earlier (Sjöström et al., 2015). Shortly, crude MV fractions were adjusted with chilled PBS to a final volume of 600 μ l and layered over an OptiPrep density gradient in a 13 ml tube in the following order from bottom to top: 1.5 mL 45%, 1.8 mL 35%, 1.8 mL 30%, 1.8 mL 25%, 1.8 mL 20%, 1.5 mL, 1.8 mL 10%, sample– 600 μ l). The gradient was then ultracentrifuged for 3 hours at 160,000 $\times$ g at 4°C using a Beckman SW 41 rotor in Beckman Ultracentrifuge (Beckman, USA).

After centrifugation fractions from the gradient of 12.6 mL equal fractions of 600 μ l were removed from the top (21 fractions in total). Based on the experimental data presented in section 4.2, MV migrate to the area of 35% OptiPrep that corresponds to fractions 17-18 and boundary fractions 16 and 19. Therefore, fractions 16-19 were combined and washed with PBS, and again centrifuged at 160, 000 $\times$ g for 3 hours, 4°C using Beckman Ti 45 rotor in Beckman Ultracentrifuge (Beckman, USA). The resulting pellet was resuspended in 50 μ l of chilled PBS and stored at – 80°C until further analysis.

2.6.3. Transmission electron microscope (TEM) analysis

For negative staining, 4 μ l of samples was added on glow-discharged carbon-coated copper grids (NTU, Singapore). After 1 min, the sample was blotted with a filter paper and 4 μ l of 1% uranyl acetate was added onto the grid. After 1 min, excess uranyl acetate was removed. Samples were visualized using a T12 120kV TEM (Fei, USA).

2.6.4. Nanosight analysis

Purified MV samples were analyzed with NanoSight NS300 (Malvern Instruments, UK) according to a manufacture's protocol. Briefly, the system was

first washed 3 times with 1 mL sterile water. Next the sample was resuspended in 1:10 ratio in 1xPBS and loaded into the chamber with sterile syringe ensured no bubbles were trapped in the installed tubing. All samples were captured under the same settings and concentration and size were calculated automatically by NanoSight NS300 Control Software and further recalculated based on the dilution factor and expressed as number of particles per colony-forming unit (Malvern Instruments, UK).

2.6.5. Atomic force microscopy (AFM)

10 μ l vesicle suspension was placed on freshly cleaved ruby red mica disks (Goodfellow Cambridge Ltd, UK), incubated for 5 min at room temperature (RT), and washed 3 times with 1 ml Milli-Q water. Samples were blotted dry before placement into a desiccator for a minimum of 2 hours. Images were collected on a Nanoscope V AFM (Bruker software) using ScanAsyst in air with ScanAsyst cantilevers. Images were collected at a scan rate of approximately 0.9-1 Hz.

2.6.6. *Proteomics*

Purified MV samples were boiled with 1X NuPAGE® LDS sample buffer (Invitrogen, USA) and 0.1 M dithiothreitol (DTT) at 95°C for 10 min. Samples were run on 10% NuPAGE® Bis-Tris mini gel (ThermoFisher Scientific, USA) and run in 1x MOPS SDS running buffer in XCellSureLock®Mini-Cell for 10 min at 150V. Gel lane with silver stained proteins was cut from the gel and sent to the Harvard Medical School Taplin Mass Spectrometry facility for mass spectrometry (MS) analysis (Harvard, USA).

For the prediction of cellular localization FASTA sequences of identified proteins (NCBI accession: NC_017316.1) were run through two independent software that predict subcellular localization of Gram-positive bacterial proteins and topology of the protein (Shen and Chou, 2009; Tusnady and Simon, 2001).

2.7. Generation of polyclonal antisera

Recombinant protein fragments were design, expressed and purified using Protein Production Platform (NTU, Singapore) with a technology and workflow of the former Biotechnology unit at the Structural Genomics Consortium at Karolinska Institutet in Stockholm, Sweden (Karolinska Institutet, 2017). Briefly, the targets (truncated DNA of the protein of interest as listed in Table 2.3), were amplified using a multi-construct approach and cloned in pNIC28-Bsa4 with N-terminal His-Tag followed by a TEV protease cleavage site. The resultant plasmid was then transformed to BL21 (DE3) *E. coli* and recombinant protein was expressed following overnight induction with Isopropyl β -D-1-thiogalactopyranoside. Cells were lysed and recombinant protein was purified by IMAC affinity utilizing the His-tag followed by a size exclusion chromatography. The purity of the recombinant protein was accessed by SDS-PAGE and mass by MS. Polyclonal antisera were generated commercially (SABio, Singapore) by immunization of hosts (listed in Table 2.3) with purified recombinant proteins. Specificities of the immune sera were confirmed by a lack of signal on Western blots of cell lysates from the appropriate deletion or transposon mutants.

Table 2.3 Recombinant proteins used to generate polyclonal antisera.

Target Protein	Size (amino acids)	Nucleotides within the target protein gene used to produce the recombinant protein	Host to generate antiserum
SrtA	82	1-245	Mouse
SrtC	61	53-234	Mouse
AS	184	550-1100	Rabbit
EbpC	209	1-627	Guinea pig
SecA	126	2-381	Rabbit
Ace	220	30-691	Rabbit

2.8. Immunoblot analysis

A single colony was selected from agar plates, inoculated into liquid media, and grown overnight. Overnight cultures were then diluted 1:10 in a fresh media in the presence of antibiotic where appropriate and grown until the mid-log phase with $OD_{600}=0.45-0.6$ unless otherwise stated. Samples were normalized to $OD_{600}=0.6$ and centrifuged at $12\,000 \times g$ for 5 min to obtain a cell pellet. The cell pellet was resuspended in 65 μ L of 10 mg/ml of lysozyme in Christie lysozyme buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA, 0.75M sucrose) and incubated at 37°C for 1 hour. After lysozyme treatment, 75 μ L of sample was added to 25 μ L of 4X NuPAGE[®] LDS sample buffer (Invitrogen, USA). Samples were heated at 95°C for 15 min and stored at -20°C until analysis.

For immunoblot analysis, 10 μ L of each sample was loaded onto a 4-12% gradient NuPAGE[®] Bis-Tris mini gel for SecA, SrtA, SrtC and AS immunoblot or 3-8% gradient NuPAGE[®] Bis-Tris mini gel for EbpC and Ace immunoblot and run in 1xMOPS (for 4-12% gel) or tris-acetate (for 3-8% gel) SDS running buffer, respectively, in XCellSureLock[®] Mini-Cell for 50 min at 200V. Proteins from the gel were then transferred to a membrane using the iBlot[™] Dry Blotting System protocol provided by Life Technologies (Invitrogen, USA) for 14 min for subsequent Ebp immunoblot or for 7 min for all the other proteins listed in **Table 2.3**. The membrane was then blocked with 3% bovine serum albumin (BSA) in phosphate buffer saline with 0.05% Tween20 (PBS-T) for one hour, shaking at RT. After blocking, the membrane was washed with PBS-T 3 times for 5 min each time, with shaking. The membrane was then incubated overnight at 4°C with primary antibodies (Table 2.3) at the concentrations described in **Table 2.4**. All antibodies were diluted in PBS-T + 3% BSA. The next day, the membrane was washed with PBS-T 3 times for 10 min each time with shaking and incubated with appropriate IgG secondary horseradish peroxidase (HRP)-conjugated antibodies (all Thermo Scientific, Singapore) at RT, in the dark for 1 hour with shaking. Following secondary antibody incubation, the membrane was washed with PBS-T 3 times for 10 min with shaking in the dark. Finally, SuperSignal West Femto or Pico Chemiluminescent Substrate (Thermo

Scientific, Singapore) was used to detect the presence of HRP on the immunoblot.

Table 2.4 Antibodies used for immunoblot

Protein of interest	Primary antibody concentration; host	Secondary antibody concentration	Substrate, dilution
SrtA	1:1000; Mouse	1:5000	Femto, 1:1
SrtC	1:200; Mouse	1:1250	Femto, 1:1
AS	1:1000; Rabbit	1:5000	Femto, 1:1
EbpC	1:3000; Guinea pig	1:6000	Femto, 1:1:8(water)
SecA	1:3000; Rabbit	1:6000	Pico, 1:1:8(water)
Ace	1:3000 Rabbit	1:6000	Femto, 1:1:8(water)

2.9. Immunofluorescence microscopy (IFM)

A single colony was inoculated into 5 ml of BHI broth with a selective antibiotic where appropriate and grown overnight at 37°C. Overnight cultures were subcultured in a fresh medium 1:10 and grown to mid-log phase ($OD_{600}=0.45-0.6$) at 37°C unless otherwise stated. The cells were then normalized to $OD_{600}=1.0$, centrifuged for 2 min at 14,000 x g and the cell pellet washed in 1 ml of PBS. Following washing, cells were fixed in 4% paraformaldehyde (PFA) diluted in PBS for 20 min at 4°C. Simultaneously, Poly-L-Lysine pre-coated slides (Polysciences Inc., USA) were washed with ethanol, then water, and then dried in a hybridization oven at 46°C. After fixation, cells were washed with 1 ml PBS, resuspended in 1 ml and diluted 1:4 ratio in PBS. 20 µl of resuspended cells was applied to the pre-treated poly-L-lysine slides and dried in the hybridization oven at 46°C for 10 min. When dried, cells were blocked with 20 µl of PBS +2% BSA, for 20 min at RT, washed 3 times with PBS and again dried in a hybridization oven. 20 µl of primary antibody was then added to the samples and slides were placed at 4°C overnight. Primary antibody (**Table 2.3**) was diluted in PBS+2%

BSA in concentrations listed in **Table 2.5**. After primary antibody treatment, samples were washed 3 times with PBS. 20 μ l of fluorescently conjugated secondary antibody Alexa Fluor 488 (Invitrogen Inc., USA) was then added and incubated in the dark for 1 hour at room temperature. Secondary antibody dilutions are also listed in **Table 2.5**. After secondary antibody incubation, slides were washed 3 times with PBS, dried in the dark, and mounted with VectaShield mounting media. The slides were visualized 30 min after mounting or stored at 4°C in the dark prior visualization on an inverted Epi-fluorescence microscope (Zeiss Axio observer Z1, Germany), using 100X oil immersion objective. Phase contrast, fluorescent, and merged field images were captured using AxioVision software under the same settings. For Super resolution imaging by Zeiss Elyra PS.1 (Zeiss, Germany). DNA was additionally stained with Hoechst 33342 dye (Thermofisher, USA) at a final concentration of 1 μ g/ml for 20 min at RT in the dark.

Table 2.5 Antibodies and corresponding concentrations used for IFM

Antigen	Primary antibody; host	Secondary antibody
EbpC	1:500; guinea pig	1:1000 Alexa Fluor 568 goat anti-guinea pig
Ace	1:500; rabbit	1:500 Alexa Fluor 488 goat anti-rabbit IgG
AS	1:200; rabbit	1:500 Alexa Fluor 488 goat anti-rabbit IgG

2.10.RNA Extraction

3.6 ml of mid-log phase cells ($OD_{600}=0.45-0.6$) were harvested at 12,000 x g for 2 min. RNA was isolated using UltraClean® Microbial RNA isolation Kit (MoBio Laboratories Inc, USA) according to manufacturer's protocol. After RNA extraction, DNase treatment was performed using Turbo DNA-free™ kit following rigorous DNA treatment procedure with incubation time of 1 hour at 37°C (Life Technologies, USA), followed by purification using Agencourt®RNAClean XP® Purification beads (Beckman Coulter, USA), all according to the manufacturer's instructions. The quality and integrity of the RNA was determined using the

TapeStation™ according to manufacturer's protocol (Agilent Technologies Inc, USA). RNA and DNA concentrations were measured with Qubit® 2.0 Fluorometer (Invitrogen, USA). RNA samples with RNA integrity number (RIN) above 7.0 and DNA contamination below 5% were stored at -80°C prior to subsequent analysis.

2.11. RT-qPCR

500 ng of isolated RNA was converted to complementary DNA (cDNA) using SuperScript® III First-Strand Synthesis SuperMix Kit (Invitrogen, USA). Following cDNA synthesis, 5 ng of cDNA per well was used in RT-qPCR with KAPA SYBR® FAST qPCR Kit MasterMix (2X) (KAPA Biosystems, USA) in StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). No amplification was observed for no-template control in qPCR reaction (Ct values above 35). To compare the differences of the target genes, the $\Delta\Delta C_T$ method was used (Livak and Schmittgen, 2001). Prior the $\Delta\Delta C_T$ analysis qPCR data was validated by running a standard curve for each gene as described in Applied Biosystems User Bulletin No.2 (P/N 4303859) and elsewhere (Livak and Schmittgen, 2001). The housekeeping gene gyrase A (*gyrA*) was used as an endogenous control in this study (Bourgogne et al., 2007; Lebreton et al., 2009). Primers used in the study are listed in **Table 2.6** and were generated using NCBI primer design software to amplify PCR products of similar size of 80-105 bp.

Table 2.6 Primers used for RT-qPCR

Gene	Primer	Sequence (5'-3')
<i>gyrA</i>	<i>gyrA_F</i>	TGTTTCGTCGGGATGTGAGTG
	<i>gyrA_R</i>	GGTACGCCTTTTTCGATGGC
<i>ebpC</i>	<i>ebpC_F</i>	CGGTCATACCGACGACCAAA
	<i>ebpC_R</i>	TGTCACATCGCCATCGACTT
<i>ebpR</i>	<i>ebpR_F</i>	GGCAAAAACGTCAACGACCA
	<i>ebpR_R</i>	TCGAGCAAAAGAAGAGCGACT
<i>dnaK</i>	<i>dnaK_F</i>	AACCCAGAAGGTAACCGCAC
	<i>dnaK_R</i>	GACGTTTTGCTACTTCGCCC

2.12.RNA-sequencing

Ribosomal rRNA from the total isolated RNA was removed using the Ribo-Zero™ Magnetic Kit (Bacteria) (Epicenter, USA). mRNA libraries and subsequential reads mapping was performed by SCELSE sequencing facility (NTU, Singapore), where libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) according to the manufacturer's protocol. RNA sequencing reads were mapped to the *E. faecalis* OG1RF reference genome (NCBI accession: NC_017316.1) using BWA (v0.5.9) with default parameters (Li and Durbin, 2009; Nagalakshmi et al., 2010). RNA-sequencing data analysis was performed by Swaine Chen (A*Star, Singapore). Sequencing reads mapping to predicted open reading frames (ORFs) were quantified using HTSeq. Counts for ribosomal and transfer RNA sequences (based on the Genbank annotation) were filtered out of the data set. Differential expression analyses were performed in R (version 2.15.1) using the Bioconductor package edgeR which provides statistical methods for identification of differentially expressed gene, according to the author's recommendations (Robinson et al., 2010). Significantly differentially expressed genes were determined using a P value and false discovery rate (FDR) cut off of 0.05.

2.13. Horizontal gene transfer (HGT) assay

OG1SS pCF10 was grown overnight in BHI with tet 15 µg/ml and then diluted to OD₆₀₀=0.1 in a 5 ml of a fresh medium in the presence of 0.12 ng/ml of cCF10 peptide. After 2 hours of shaking at 200 rpm at 37°C, visible clumps formed on the bottom of the tube. The top 2 ml represented unclumped (supernatant fraction) and 1 ml from the bottom represented the clumped fraction. EDTA at a final concentration of 0.05 M was added to each fraction and vortexed for 15 sec to separate clumped cells (Chuang-Smith et al., 2010a)). Cells were normalized to the same OD₆₀₀= 1 and washed with PBS. Donor cells (from the supernatant or clumped cells) were mixed with recipient OG1RFΔ*ebpABC*Δ*srtC* cells in a ratio of 1:10 (donor: recipient) and grown statically at 37°C. After 30 min and 120 min 1 ml of cells was vortexed for 15 sec, serially diluted and plated on selective plates to quantify donor OG1SS pCF10 cells on BHI+Str(25)+Tet(15) plates and transconjugants (Tc) OG1RFΔ*ebpABC*Δ*srtC* pCF10 on BHI +Rif (25)+Tet (15) plates. HGT rates were assessed as number of Tcs per donor cell (Tc/Donor) as described previously (Bhatty et al., 2015). Similarly OG1RF pCF10 and OG1RFΔ*ebpABC*Δ*srtC* were grown in BHI with tet 15 µg/ml overnight and diluted to OD₆₀₀=0.1 in fresh media the next day in the presence of 0.12 ng/ml of cCF10. Cells were induced for 2 hours at 200 rpm at 37°C, vortexed with EDTA with a final concentration of 0.05 M, normalized to the same OD₆₀₀= 1 and washed with PBS. Washed donor cells were mixed with OG1XΔ*srtC* (str⁺, polymerized pili-negative) recipient cells in a ratio of 1:10 (donor:recipient) and grown statically at 37°C. After 30 min and 120 min 1 ml of cells was vortexed for 15 sec, serially diluted and plated on selective plates to quantify donor OG1RFpCF10 or OG1RFΔ*ebpABC*Δ*srtC* pCF10 cells on BHI+Rif(25)+Tet(15) plates and transconjugants (Tc) OG1X Δ*srtC* pCF10 on BHI +Str (500)+Tet (15) plates. HGT rates were assessed as number of Tcs per donor cell (Tc/Donor) as described previously (Bhatty et al., 2015).

2.14. Crystal violet biofilm assay

Crystal violet biofilm assays were performed in a 96 wells plate (Thermofisher, USA) as described previously (Bhatty et al., 2015) with the following modifications. Overnight cultures were normalized to $OD_{600}=0.7$, washed and resuspended in 1 ml PBS. 200 μ l of normalized culture was added to 5 ml of Tryptic soya broth (TSB) containing 0.25% of glucose with or without 10 ng/ml of cCF10. 200 μ l of vortexed cells were seeded into 96-well plates in triplicate and grown at 37°C for 24 hours. Wells were then washed 2 times with PBS and stained with 0.1% crystal violet solution (prepared from 1% aqueous crystal violet solution, Sigma-Aldrich, Germany) and incubated for 30 min at 4°C. Wells were then washed three times with PBS and blot dried. 200 μ L of ethanol:acetone (80:20) solution was added to each well to solubilize crystal violet and incubated for 45 min at RT. After incubation, OD_{595} readings were taken using a spectrophotometer (UVmini-1240, Shimadzu, Japan).

2.15. Statistical analysis

All experiments were performed at least in three biological replicates and the mean value was calculated. All graphs indicate standard deviation from independent experiments. Statistical analysis was performed by unpaired t-test using GraphPad (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; **** $p<0.0001$, ns: $p>0.05$).

3. Chapter 3. The role of SrtA in the expression and fate of Ebp

SrtA is a conserved enzyme within Gram-positive bacteria. The function of sortase and its enzymatic activity have been characterized, and the crystal structure has been determined (Paterson and Mitchell, 2004). Because *srtA* mutants in a variety of species display attenuated virulence in various animal models of infection, the enzyme is also an attractive antibiotic target (Cascioferro et al., 2015). However, we know little of how the cell responds to the loss of the functional enzyme and why exactly bacteria are less virulent. It has been widely assumed that attenuation in the absence of *srtA* is associated with SrtA substrate virulence factors not being attached to the cell wall and therefore not being presented on bacterial surface. In *A. oris*, SrtA is essential and in the absence of the enzyme, one of the SrtA substrates – a surface glycoprotein -- accumulates in the plasma membrane and is toxic to the cell (Wu et al., 2014b). In *S. pyogenes*, deletion of *srtA* alters cell morphology and compromises membrane integrity (Raz et al., 2015). In *E. faecalis*, SrtA is not essential, although in the absence of SrtA, at least two substrates are affected – Ebp is retained on the protoplast and AS accumulates focally (Kline et al., 2009; Nielsen et al., 2013). We predicted that accumulation of unprocessed substrates in the plasma membrane may in turn alter expression of those substrates (perhaps by negative feedback regulation). We address this hypothesis using Ebp as a model substrate in the $\Delta srtA$ background.

3.1. Increased Ebp piliation in $\Delta srtA$ populations

Ebp are expressed on only a subset of *E. faecalis* cells in any given population. In the absence of SrtA, pili are not attached to the cell wall, but accumulate in the plasma membrane and are also present in the supernatant (Kline et al., 2009; Nielsen et al., 2013). Increased levels of Ebp from whole cell lysates are detected by immunoblot in $\Delta srtA$, suggesting that unanchored Ebp that went “off-

pathway” upon *srtA* deletion have increased expression levels (Nielsen et al., 2013). To further understand changes in Ebp expression in $\Delta srtA$, we quantified the number of pilus-expressing cells in wild type (WT) and $\Delta srtA$. By performing immunofluorescence microscopy (IFM) of whole cells stained with antiserum raised against EbpC, we observed a higher number of Ebp-expressing cells in the *srtA* mutant (**Figure 3.1A**) increasing from 37% of piliated cells in WT to 51% in $\Delta srtA$. Relevant controls and image presentation of Ebp-staining of WT and $\Delta srtA$ are presented in **Appendix A Figure 10.1**. We confirmed by immunoblot that the *srtA* mutant population displayed increased levels of Ebp (which appear as pilus high-molecular-weight ladder (HMWL) of polymerized pili of different lengths) as compared to WT (**Figure 3.1B**) and (Nielsen et al., 2013)). We used SecA to control for equal protein loading. In order to determine if increased piliation is specific to OG1RF, we extended our analysis to two related, but genetically different strains of OG1X $\Delta srtA$ and OG1SS $\Delta srtA$ and observed a similar increase in piliation (21% in WT OG1X to 66% in OG1X $\Delta srtA$ and 13% in WT OG1SS to 35% in OG1SS $\Delta srtA$) as observed in OG1RF $\Delta srtA$ (**Figure 3.1A**), suggesting that increased piliation in the absence of *srtA* is not limited to strain OG1RF.

To test if increased piliation of $\Delta srtA$ was due to the loss of SrtA transpeptidase activity we complemented OG1RF $\Delta srtA$ with *srtA* expressed from a plasmid under control of the native *srtA* promoter, and also tested a complementation construct in which the SrtA active site catalytic cysteine residue C200 is changed to alanine C200A, where proteins levels of both WT SrtA and C200A SrtA remain similar (Nielsen et al., 2013). We observed that complementation with WT *srtA* expression restored piliation to WT levels (from 57% in $\Delta srtA$ to 36% in complement), but the catalytically inactive mutant did not. That is, piliation remained high in the catalytically inactive *srtA* complemented strain at 70%, similar to the empty vector control, showing that increased piliation is a result of a loss of SrtA transpeptidase activity (**Figure 3.2**).

Figure 3.2 Increased piliation in $\Delta srtA$ is dependent on the loss of SrtA enzymatic activity.

IFM with EbpC antiserum on WT, $\Delta srtA$, $\Delta srtA$ complemented with an empty vector (pEmpty), wild type *srtA* under native promoter (pSrtA_{wt}) or *srtA* with point mutation in the active site (pSrtA_{C200A}) where % of EbpC+ cells within a population was quantified. Mean values from 3 independent experiments are shown. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad **** $p < 0.0001$, n.s.: $p > 0.05$.

Based on the increased Ebp levels in the *srtA* mutant strains observed by immunoblot, we hypothesized that *ebpC* expression is upregulated in the absence of SrtA. To test this hypothesis we performed RT-qPCR on *ebpC* transcripts from cultures grown in the same manner as the immunoblot experiments, and detected similar *ebpC* transcription levels between WT and $\Delta srtA$ (**Figure 3.3**).

Figure 3.3 *ebpC* transcription is the same in WT and $\Delta srtA$.

RT-qPCR analysis of *ebpC* expression in the OG1RF and $\Delta srtA$. RT-qPCR was performed in biological triplicate and analysed by the $\Delta\Delta Ct$ method, using *gyrA* as a housekeeping gene. Fold change indicates the change in *ebpC* transcription, compared to control (WT). Statistical analysis was performed by the unpaired t-test using GraphPad n.s.: $p > 0.05$.

Since we observed more Ebp present in $\Delta srtA$, despite the similar *ebpC* transcription levels, we next hypothesized that the increased pilus expression may be due to differences in cellular localization of pili in the wild type and $\Delta srtA$. In WT cells, polymerized pili are attached to the cell wall by SrtA, while in $\Delta srtA$ no other enzyme is able to substitute for SrtA function and polymerized pili are retained in the plasma membrane (Nielsen et al., 2013). To further confirm that in $\Delta srtA$, the SrtA-dependent substrates Ebp as well as Ace accumulate in the plasma membrane we performed cellular fractionation by separating cell wall from the protoplast (**Figure 3.4**) and tested the two fractions for the presence of Ebp and Ace. We removed the cell wall with lysozyme in lysis buffer following centrifugation, obtained a pelleted fraction that contained protoplast, and collected supernatant that represented the cell wall fraction. To test the specificity of the Ebp antibody, we included $\Delta srtC$ that is unable to polymerize pili and in which EbpC pilin subunits accumulate in the plasma membrane. We observed thick band of accumulated EbpC around 65 kDa and an absence of HMWL that confirmed that HMWL is indeed polymerized pili (**Figure 3.4**). To

control for cell lysis and purity of the two fractions, we performed immunoblot with cytoplasmic SecA and did not observe any signal in the cell wall fraction (**Figure 3.4**). We observed that both Ebp and Ace accumulated in the plasma membrane in the absence of SrtA and similar to Ebp, Ace expression levels per cell are higher in $\Delta srtA$ compare to WT (**Figure 3.4**).

Knowing that pili remain in the plasma membrane in $\Delta srtA$, we hypothesized that the increased piliation we observed in $\Delta srtA$ is a result of different fate of cell-wall associated pili and plasma membrane associated pili.

Figure 3.4 Pili and Ace are retained in the plasma membrane in $\Delta srtA$ cells.

(A) Anti-EbpC immunoblot of protoplast and cell wall fraction of WT, $\Delta srtA$ and $\Delta srtC$. The cell wall was removed by treating whole cells in lysis buffer with lysozyme and cells were then separated by centrifugation – protoplast in the pellet, cell wall fraction in the supernatant. In $\Delta srtA$, polymerized Ebp and unpolymerized EbpC monomers in $\Delta srtC$ are not attached to the cell wall and accumulate in the plasma membrane. SecA serves as a loading control and controls for absence of cell lysis during the sample preparation. (B) Same WT and $\Delta srtA$ samples as in (A) run with Ace antiserum. Ace accumulates in the plasma membrane in $\Delta srtA$ and is attached to the cell wall in WT. The predicted MW of Ace is 74 kDa, but the soluble protein runs higher.

3.2. Increased Ebp piliation in Δatn populations

During cell growth and division, fragments of peptidoglycan are released from the cell into the supernatant during normal peptidoglycan turnover (Reith and Mayer, 2011). Since pili are covalently attached to the cell wall via SrtA, we reasoned that pili should similarly be released from WT cells during normal growth by murein hydrolases, but in $\Delta srtA$ cells pili are plasma membrane-attached and therefore would not be released (or shed) along with cell wall during normal growth. We speculated that this plasma membrane retention may therefore give rise to the increased Ebp levels observed in $\Delta srtA$. In *E. faecalis*, one of the major murein hydrolases involved in normal cell wall turnover, particularly during daughter cell separation during cell division and release of peptidoglycan (PG) fragments in the environment, is autolysin (Atn also known as AtlA) (Eckert et al., 2006; Mesnage et al., 2008). To test our pilus “shedding hypothesis”, i.e. normal cell wall-associated pilus shedding does not occur in $\Delta srtA$ leading to increased pilus expression levels on the cells, we asked whether we would see similar increased pilus expression in the Δatn strain, which also has reduced PG turnover rates compare to WT (Mesnage et al., 2008).

When we performed an EbpC immunostaining of WT and Δatn cells, we observed significantly more EbpC-stained cells on the chained Δatn cells (31% in WT vs 74% in Δatn , similar to higher (57%) numbers of Ebp fluorescing cells in

srtA mutant cells (**Figure 3.5**). These results are consistent with the notion that during normal cell growth and cell wall turnover, pili are shed into the supernatant along with the cell wall fragments, and since in $\Delta srtA$ pili are not attached to the cell, they can not be shed with PG fragments that may result in the increased piliation of $\Delta srtA$ cells. However since piliation in Δatn is significantly higher than in $\Delta srtA$ (74% vs 57%) that suggested that decreased shedding is not the only reason for increased piliation of *srtA* population.

Figure 3.5 Increased piliation in Δatn .

IFM with EbpC antiserum where % of EbpC+ cells within a population was quantified. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad **** $p < 0.0001$.

If pili are indeed shed in the supernatant during cell wall turnover, we would expect to observe more pili in the supernatant of the WT, as compared to $\Delta srtA$. To test the amount of pili in the supernatant of WT and $\Delta srtA$, we collected and TCA precipitated supernatants from same number of cells and tested them for Ebp (**Figure 3.6**). However, contrary to our hypothesis, we observed even more pili in the supernatant of $\Delta srtA$ compared to WT (**Figure 3.6** and (Kline et al., 2009)). Interestingly, only HMWL without Ebp monomers or dimers were detected in the supernatant, suggesting that only fully polymerized pili are being

released in the supernatant. To ensure that the supernatants were precipitated from an equal number of cells, we performed side to side EbpC and SecA immunoblot on the whole cell lysate of the pelleted cells that remained after the supernatant was collected. Increased piliation within the $\Delta srtA$ cells and in the supernatant suggested that increased piliation in $\Delta srtA$ cannot be only explained by decreased cell turnover and pilus release from the cells.

Figure 3.6 More pili are present in the supernatant of $\Delta srtA$.

(A) Representative immunoblot with EbpC antiserum on TCA precipitated supernatants from the same number of mid-log grown cells in BHI. (B) EbpC and SecA immunoblots on the whole cell lysates from the samples with collected supernatants for (A).

3.3. Discussion

We started this chapter by asking the question of why piliation increases in $\Delta srtA$, as observed previously by us and others (Kline et al., 2009; Nielsen et al., 2013). During our research we discovered the following novel points:

- 1) An increase in piliation from 31% in WT to 57% in $\Delta srtA$ is independent of *ebpC* transcription, which remains unchanged in both strains.
- 2) The total amount of pili (within the cells and in the supernatant) of $\Delta srtA$ is higher compared to total amount of pili in WT.
- 2) Ace, similar to Ebp, accumulates in the plasma membrane in $\Delta srtA$.
- 3) Piliation in Δatn increases from 31% in WT to 74%, likely due to the lack of PG turnover and shedding.

While we observed plasma membrane accumulation of the two SrtA-substrates Ebp and Ace in *E. faecalis* in $\Delta srtA$, such retention of SrtA substrates is not universal in other Gram-positives. In contrast to *E. faecalis*, in *S. pneumoniae* two of SrtA substrates - β -galactosidase and neuraminidase A - are released into the supernatant in the absence of SrtA and are not retained within the cell (Kharat and Tomasz, 2003). In another Gram-positive bacterium, *A. oris*, *srtA* mutation is lethal to the bacterial cells, since accumulation of an unanchored SrtA-dependent surface glycoprotein is toxic to the cell (Wu et al., 2014b). Hence, the mechanism to deal with all unprocessed SrtA substrates or only with some of the substrates may vary between Gram-positive bacteria. We do not exclude the possibility that, while two of the SrtA substrates accumulate in the plasma membrane, some of them may not be retained in the plasma membrane and instead are being secreted in the environment. While cell surface expressed proteins may remain anchored on the membrane as lipoprotein, non-covalently associated with cell-wall fragments, or attached to teichoic acid and lipoteichoic acid, covalent attachment of proteins containing a cell wall sorting sequence inclusive of LPXTG motif in Gram-positive bacteria is performed by conserved sortases enzymes (Brinster et al., 2007; Cossart and Jonquières, 2000). Only two sortases - SrtA and SrtC - are encoded in *E. faecalis*, where SrtC is specific

to pilus biogenesis, and is only capable of polymerizing Ebp subunits, and does not attach polymerized pili to the cell wall (Dramsi et al., 2005; Kemp et al., 2007a; Nielsen et al., 2013). Similarly, in *S. aureus*, in the absence of *srtA*, three of the tested sortase-dependent substrates remained uncleaved at the sorting signal (Mazmanian et al., 2000). Therefore, it is unlikely that in *E. faecalis* SrtA-dependent substrates can be covalently attached to the cell wall by SrtC or by any other means. We hypothesized that the absence of Ebp on the cell wall or the accumulation of Ebp in the plasma membrane may feedback through cell-wall bound or plasma membrane bound proteins to alter transcription of *ebp* that results in the observed increased piliation of $\Delta srtA$. However, unchanged *ebpC* transcription in $\Delta srtA$ suggested that increased piliation in the plasma membrane *per se* does not signal to reduce *ebpABC* transcription of pili that went “off-pathway”. Although the exact mechanism to control *ebpABC* expression is unknown, *ebpABC* transcription increases in response to environmental cues and is regulated by EbpR (Bourgogne et al., 2010; Bourgogne et al., 2007). Apparently, accumulation of the protein in the plasma membrane, whether it is Ebp or other SrtA substrates, is not the cue recognized by *ebpABC*-transcription controlling machinery.

Since *ebpC* transcription is unchanged between the two strains, we hypothesized that Ebp biogenesis is unchanged in $\Delta srtA$, and increased piliation was a result of different fates of cell wall-attached proteins and plasma membrane-bound proteins. During the bacterial life cycle, various cell wall hydrolases modify the cell wall that results in fragments of the cell wall together with proteins that are covalently attached to peptidoglycan to be released in the supernatant. In *S. aureus*, a cross-wall murein hydrolase contributes to the release of one of its SrtA substrates SpA (Becker et al., 2014). Similarly, we hypothesized that pili attached to the cell wall in WT *E. faecalis* are shed into the environment by enterococcal hydrolases, but that piliation increases in $\Delta srtA$ because pili are retained in the plasma membrane and therefore cannot be removed by the cell wall hydrolases. To test this, we utilized a well-characterized *atn* mutant that has reduced PG turnover (Mesnage et al., 2008). In agreement with our shedding hypothesis, we detected more pili-expressing cells in Δatn by

immunoblot and IFM (74% vs 31% in WT). To further identify whether increased piliation is dependent on the reduced-PG-turnover in Δatn , we may first perform qPCR to check that *ebp* transcription is unchanged and quantify the levels of PG-associated Ebp in the supernatants of the WT cells (similar to that done for protein A in *S. aureus* (Becker et al., 2014)). Since other proteases and cell-surface hydrolases are present in the genome of OG1RF, we are interested to test if they are also important for the release of Ebp in the supernatant and whether a specific hydrolase acts on Ebp. However, increased piliation of $\Delta srtA$ cannot be explained only due to lack of shedding, since if it was the only reason for increased piliation in $\Delta srtA$, we would expect to see similar piliation between $\Delta srtA$ and Δatn , while the later has significantly higher pilated rates (74% vs 57 in $\Delta srtA$). Moreover we would detect less pili in the supernatant from $\Delta srtA$ compared to WT. However, we observed even more pili in the supernatant from $\Delta srtA$. Therefore, although *ebpC* transcription is the same between the two strains, we detected more Ebp in $\Delta srtA$ in both cell-associated and in the supernatant fractions.

The following questions arose as a result of experimental work in **Chapter 3**.

1) Why more pili are present in all fractions examined in $\Delta srtA$ compared to WT while *ebpC* transcription is unchanged?

Increased piliation might be explained by several hypotheses: first expression of genes involved in cellular metabolism and Ebp biogenesis is higher, resulting in higher rates of Ebp translation and assembly (partially addressed in **Chapter 5**). To test this we can compare Ebp levels in the supernatant and on the cells at different stages of growth, including early exponential and late stationary phase where bacteria are less metabolically active. If piliation is universally higher throughout the different growth stages, then a second possibility for the increased piliation is different stability of Ebp in the supernatant, where pili in the supernatant of $\Delta srtA$ are more stable. In order to test this hypothesis, we would test stability of Ebp in the supernatants of both WT and $\Delta srtA$. This could be achieved by using a previously described Pulse and Western method (Kim et al., 2009). In this method supernatants are first subjected to an excess amount of a

protease for a short amount of time and after reaction is quenched samples are subjected to immunoblot to quantify protein of interest. Lastly, despite similar transcription of *ebp* in both WT and $\Delta srtA$, $\Delta srtA$ cells may provide translational feedback specific to Ebp, where Ebp are being translated at higher levels. To test this hypothesis we may inhibit translation by adding inhibitory concentrations of cm that binds to the 50S ribosome and inhibits peptidyl transferase. Therefore comparing Ebp levels post inhibition in whole cells and supernatants between the two strains; we can test whether increased Ebp levels in $\Delta srtA$ are result of increased translation in $\Delta srtA$.

However, Ebp stability and other factors involved in Ebp biogenesis may not explain the presence of pili in the supernatant of $\Delta srtA$. Therefore, the next unanswered question is:

2) How are unanchored pili being released in the supernatant in $\Delta srtA$ if not through cell-wall shedding?

In Gram-negative bacteria, accumulation of proteins (either folded or unfolded) on the outer membrane and in the periplasm results in increased vesiculation (Kulp and Kuehn, 2010; McBroom and Kuehn, 2007). Therefore, one hypothesis to explain increased pili in the supernatant in $\Delta srtA$ is that the accumulation of pili in the plasma membrane in $\Delta srtA$ may trigger membrane vesicle formation. We examine this hypothesis in the next **Chapter 4** of this thesis.

3) Since at least two of the SrtA substrates – Ebp and Ace – accumulate in the plasma membrane, how does the cell respond to such a perturbation of plasma membrane composition?

To address this question we studied transcriptional response to *srtA* deletion in **Chapter 5** of this thesis.

4) Since Ebp are important for biofilm formation and enterococcal pathogenesis what remains unclear is why $\Delta srtA$ cells, that have increased piliation, are deficient in both biofilm formation and various infectious models (Guiton et al., 2010; Kemp et al., 2007b; Nallapareddy et al., 2006; Singh et al., 2007a). Contrary to Spa in *S. aureus* that is not detected on the cell surface (Mazmanian

et al., 2000), we observed that in *E. faecalis* $\Delta srtA$, Ebp is readily detected by IFM without removal of cell wall.

These questions are important to understand enterococcal pathogenies, and should be addressed in future studies in the laboratory.

4. Chapter 4. Membrane vesicle biogenesis in *E. faecalis*

MV are well recognized in Gram-negative bacteria there they are most often referred to as outer-membrane vesicles and are often produced under stress conditions. Since Gram-positive bacteria cell lack outer membrane and have rigid and thick peptidoglycan cell that was thought to be a thick barrier for MV release, MV in Gram-positives were mostly neglected for MV biogenesis until the first report in 2009 that characterized MV from *S. aureus* (Brown et al., 2015). Since then, MV have been reported in numerous Gram-positive bacteria but not in *E. faecalis*. Moreover, the mechanism of MV formation in Gram-positive bacteria remains largely unknown. Based on our observations in **Chapter 3** that SrtA substrates accumulate in the plasma membrane in $\Delta srtA$ and also that Ebp from the plasma membrane are released in the supernatant in unknown-manner, we hypothesized that a) accumulation of SrtA substrates in the plasma membrane triggers membrane stress, MV formation, and as a consequence b) Ebp in $\Delta srtA$ are being released in the supernatant via MV. We have tested both these hypotheses below.

4.1. Crude MV fraction of $\Delta srtA$ are enriched in Ebp

As we detected more pili in the supernatant of $\Delta srtA$, we hypothesized that they might be partially associated with MV, released from the pili-enriched plasma membranes either during normal MV biogenesis or via induced MV biogenesis caused by perturbation of plasma membrane as a result of accumulation of unprocessed SrtA substrates. First, we tested the hypothesis that pili in the supernatant from the $\Delta srtA$ are associated with MV. We collected supernatants from the same number of OG1RF wild type (WT) and $\Delta srtA$ cells, filtered them through 0.45 μm filters to remove intact cells, concentrated the supernatants via a 100 kDa membrane to 70 ml and centrifuged the concentrated supernatants at 160, 000 x g as described by others to collect the crude MV-containing

fraction (Gurung et al., 2011). We performed TCA precipitation on 2 ml aliquots from 70 ml concentrated supernatants and on 2 ml aliquots from the same supernatants after the centrifugation. From the same centrifuged samples, we resuspended the crude MV pellet in 100 μ l of PBS and used 20 μ l to prepare immunoblot samples.

We next performed immunoblot using anti-EbpC antiserum on TCA precipitated supernatants before and after the spin and on the crude MV fraction. We observed EbpC HMWL in the supernatant before and after the spin for both WT and $\Delta srtA$, the $\Delta srtA$ MV pellet had pili (**Figure 4.1**). This result suggested to us that in the $\Delta srtA$ but not in WT, pili might be partially associated with MV within the pellet. However, Ebp are also present in $\Delta srtA$ un-associated with MV as Ebp is detected in the supernatant after the ultracentrifugation that pelleted all MV from the sample. To examine whether any MV are produced by *E. faecalis* we performed TEM and observed spherical, bilayered structures within the size range of 50-400 nm, similar to previously reported MVs for various Gram-positive bacteria, in both of WT and $\Delta srtA$ samples (**Figure 4.2**) (Brown et al., 2015). However, crude fractions from both WT and $\Delta srtA$ also contained a similar background of cloudy material and filamentous structures that varied within the same grid or between different experiments.

Figure 4.1 Pili are present within the crude MV fraction of $\Delta srtA$.

Anti-EbpC immunoblot on TCA precipitated supernatants of WT and $\Delta srtA$ before and after the ultracentrifugation (1st and 3rd boxed lanes) and crude MV pellet after the centrifugation (middle boxed lanes).

We next quantified the number of MV in the crude fractions of WT and $\Delta srtA$ to address if vesiculation is affected by perturbation of the plasma membrane in $\Delta srtA$ where the composition associated with accumulation of SrtA substrates is altered. We performed nanoparticle tracking analysis (Nanosight) on supernatants, where the particles contained in the sample are visualized by

virtue of the light they scatter when illuminated by laser light. The light scattered by the particles is captured using a scientific digital camera and the motion of each particle is tracked from frame to frame by proprietary software. This rate of particle movement is related to a sphere equivalent hydrodynamic radius as calculated through the Stokes-Einstein equation (Wright, 2012). Using this approach, we prepared MV as described above for the crude fraction, and observed similar numbers and size-distribution of MV in both WT and $\Delta srtA$ (**Figure 4.3**). Taken together, our data suggested that MVs are produced by *E. faecalis* so pili detected in the supernatant of $\Delta srtA$ may indeed be associated with released MV, but that actual vesiculation is strain-independent. Additionally or alternatively, it was also possible that pili in the crude MV sample simply co-sediment with the MV.

Figure 4.2 Crude MV fractions of WT and $\Delta srtA$ contain MV.

Examples of MV observed by TEM from crude fractions from WT (**A**) and $\Delta srtA$ (**B**). Background (indicated with white arrows) composed of uncharacterized material of bacterial origin (lipid droplets, protein complexes, cell debris) or non-bacterial origin (stain, dirt). Scale bar is 100 nm.

4.2. MV purification and characterization

To more carefully address whether MV-associated pili are released in the supernatant of $\Delta srtA$ cultures, we isolated pure MV by gradient centrifugation out of the crude MV fractions of WT and $\Delta srtA$. We used a standard method of MV purification previously described using OptiPrep density gradients (Gurung et al., 2011; Sjöström et al., 2015). After centrifugation of 12.6 ml gradients, we collected 21 fractions of equal volume (600 μ l each) starting from the top of the tube (**Figure 4.4**).

In OptiPrep gradients, samples are separated based on size and density, where large and heavier (in density) particles, including MV, travel through the gradient faster compared to soluble proteins and protein complexes (Li and Donowitz, 2008). Since MVs contain various proteins of different molecular weight, we analyzed every other fraction on SDS-PAGE and performed silver staining to identify the fraction where proteins associated with MV migrated within the gradient. We observed most of the proteins within the 30-40% OptiPrep gradient (**Figure 4.4**), suggesting that MV of *E. faecalis* migrate to that area. We have noticed multiple prominent bands in various fractions and had previously sent one of those bands (at 70kDa) for MS and the best hit was the phage structural protein OG1RF_11061. We believe that these bands are phage tail structural proteins, since tails of different sizes were observed by TEM and therefore they would migrate to different densities within the gradient.

We are addressing phage involvement in **section 4.4**.

Figure 4.4 MV-associated proteins of WT migrate to the area of 30-35% OptiPrep density.

Every other fraction from 21 fractions from 12.6 ml gradient was loaded consequently and silver stained. On the right is a cartoon depicting the gradient with corresponding sample volume and % of OptiPrep loaded. Fractions were collected from the top of the tube as indicated.

In order to test if the proteins detected in fraction 17, where we observed the most protein, are MV-associated, we next analyzed a subset of fractions 21, 17, 11 and 1 by AFM (**Figure 4.5**). Samples from OptiPrep were directly deposited to the sample mounting disks and scanned by AFM that allows high resolution of the specimen on the surface. We observed elevated spherical blebs – MV in fraction 17 (**Figure 4.5**), similar to MV identified by AFM in *S. aureus* and *S. pyogenes*, but not in the other 3 analyzed fractions which contain minimal or no detectable protein (Resch et al., 2016; Thay et al., 2013). This suggests that the proteins detected by silver stain in fraction 17 are indeed MV-associated. Fractions 21 and 1 did not have any distinct structural features, while the cloudy blot in fraction 11 is thought to be the OptiPrep deposits (**Figure 4.5**).

Figure 4.5 MV of WT are enriched within fraction 17 corresponding to ~35% of OptiPrep density.

AFM on 4 fractions within the OptiPrep gradient as depicted on the cartoon (**Figure 4.4**). (**A**) Bottom of the gradient ~45% OptiPrep; (**B**) Spherical MV within ~35% OptiPrep. (**C**) ~25% OptiPrep; background is the OptiPrep medium. (**D**) Crude MV sample after the ultracentrifugation, no MV are observed, since they migrated to higher density (~35%) during the centrifugation. Scale bar – 1 μ m.

To address our initial question about whether pili are associated with MV in the supernatant of Δ *srtA*, we performed immunoblot with EbpC antiserum on the OptiPrep gradient fractions. Consistent with the absence of pili in the crude MV

fraction of WT *E. faecalis* (**Figure 4.1, middle lanes**) we detected no pili within the OptiPrep gradient for WT (**Figure 4.6A**). However, in $\Delta srtA$ fully assembled pili were enriched within the MV fractions 16-19 (~35 % OptiPrep density), but also remain on the top of the gradient (fractions 1-7, ~10-15% OptiPrep density), where larger protein complexes were retained (**Figure 4.6B**). Pili on the top of the gradient might be bundled pili that co-precipitated with MV from the supernatant during the first ultracentrifugation or some MV burst during the gradient centrifugation and released Ebp. Ebp that are not associated with MV do not migrate low in the gradient and remain within 10-20% of OptiPrep density. TEM with anti-EbpC sera is underway to further confirm the presence of Ebp associated with MV.

Figure 4.6 Ebp are associated with MV in $\Delta srtA$.

Anti-EbpC immunoblot on every other OptiPrep fractions from the gradient (as in cartoon **Figure 4.4**) from WT (**A**) and $\Delta srtA$ (**B**). Fully assembled pili are present within MV fractions 17-19 and on the top of the gradient.

To further test for the presence of pili within MV fractions of WT and $\Delta srtA$, we combined fractions 16-19 for each WT and $\Delta srtA$ and further purified MV from

OptiPrep by ultracentrifugation. We quantified number of MV in each fraction and immunoblotted these pure MV fractions with EbpC antiserum and similarly to **Figure 4.3** detected equal number of MV with enrichment of assembled Ebp in pure MV fraction from $\Delta srtA$ **Figure 4.7**.

From these experiments we conclude that in $\Delta srtA$, pili are present both in the supernatant associated with MV but are present in MV-free supernatant after the centrifugation, released by an unidentified mechanism. We concluded that accumulation of pili in $\Delta srtA$ does not augment MV formation; however, MV from the $\Delta srtA$ mutant are enriched in Ebp as a consequence of increased membrane-associated pili in this strain.

4.3. Analysis of purified membrane vesicles

Although pilus (or sortase substrate) accumulation in the plasma membrane does not induce MV release in the $\Delta srtA$ mutant, we nevertheless decided to probe the constituents of *E. faecalis* MV by mass spectrometry. We hypothesized that since one of the SrtA substrates – Ebp – is enriched within the MV of $\Delta srtA$ than maybe other putative or characterized SrtA substrates would be also present in abundance within $\Delta srtA$ MV samples. Moreover, as Ebp pili accumulate at the distinct foci on the membrane in the absence of $\Delta srtA$, and we showed that MV from $\Delta srtA$ were enriched with Ebp, we sought to address if MV might be formed from these distinct foci.

Purified MV were analyzed by microcapillary LC/MS/MS on gel-digested proteins on three biological replicates. Identification of peptides and proteins was performed by the data analysis pipeline of the Taplin Facility (Harvard Medical School). MS RAW data files were matched to OG1RF genome using the Sequest algorithm (Eng et al., 1994). The proteins that have at least 1 unique peptide and FDR of 0 were presented as HTML tables accessible via a web browser and are shown in **Table 4.1**. The protein profile between WT and $\Delta srtA$ MV was very similar; however, while more pili are present in pure MV fraction from $\Delta srtA$ by immunoblot (**Figure 4.7**), we couldn't detect EbpA, EbpB or EbpC (OG1RF_10869-10872) in either of the samples.

Table 4.1 Proteins identified in the purified MV fraction from WT and $\Delta srtA$.

Proteins that have at least 1 unique peptide and false discovery rate of 0 are listed in the alphabetic order for WT and $\Delta srtA$ pure MV fraction. Proteins identified in biological triplicate are indicated with ***, in any two replicates with **, with * proteins detected only in one of the biological replicates.

MV WT	MV $\Delta srtA$	Gene description	Localization
Aad***	Aad***	aldehyde-alcohol dehydrogenase	cytoplasmic
AceF*	AceF*	pyruvate dehydrogenase complex E2, dihydrolipoamide	plasma membrane

		acetyltransferase	
	AlsS*	acetolactate synthase	plasma membrane
ArgR3*	ArgR3*	ATP synthase F1 subcomplex subunit alpha	cytoplasmic
AtpA*		putative calcium-transporting ATPase	cytoplasmic
AtpA2***	AtpA2***	ATP synthase F1 subcomplex subunit alpha	plasma membrane
AtpC2*	AtpC2***	ATP synthase F1 sector epsilon subunit	cytoplasmic
AtpF3***	AtpF3**	ATP synthase F0 subunit B	plasma membrane
AtpD2*		ATP synthase F1 sector beta subunit	Cell membrane
AtpG*		ATP synthase F1 sector gamma subunit	cytoplasmic
clpX**		ATP-dependent Clp protease ATP-binding subunit	plasma membrane
DivIVA2*	DivIVA2*	cell division initiation protein DivIVA	cytoplasmic
DltD**		ATP synthase F1 sector epsilon subunit	plasma membrane
DnaA*	DnaA*	ATPase	cytoplasmic
DnaK*	DnaK**	chaperone DnaK	cytoplasmic
EcfA*		Energy-coupling factor transporter ATP-binding protein	cytoplasmic
Eno*	Eno**	enolase	plasma membrane
FabZ*	FabZ*	(3R)-hydroxymyristoyl-ACP dehydratase	cytoplasmic
FruA*	FruA**	PTS family fructose porter, IIABC component	plasma membrane
FtsH*	FtsH**	cell division protein	plasma

		FtsH	membrane
FtsZ*	FtsZ**	cell division protein FtsZ	cytoplasmic
Gap2***	Gap2***	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	cytoplasmic
	GatB**	glutamyl-tRNA(Gln) amidotransferase subunit B	cytoplasmic
GlnP**	GlnP**	D-alanyl-lipoteichoic acid biosynthesis protein DltD	plasma membrane
GlyA*		serine hydroxymethyltransferase	cytoplasmic
GroL*	GroL*	chaperonin <i>GroL</i>	cytoplasmic
Hup*	Hup*	DNA-binding protein HU	cytoplasmic
Idh***	Idh***	L-lactate dehydrogenase	cytoplasmic
	LepB*	signal peptidase I LepB	plasma membrane
	LuxS*	S-ribosylhomocysteine lyase	cytoplasmic
LytR**	LytR**	response regulator	cytoplasmic
Lyzl6***	Lyzl6***	cell wall lysis protein	cell wall
ManX2*	ManX2**	PTS system mannose transporter subunit IIAB	cytoplasmic
	MetQ**	ABC superfamily ATP binding cassette transporter, binding protein	plasma membrane
MscL*	MscL*	large conductance mechanosensitive channel protein	plasma membrane
MurB*		UDP-N-acetylmuramate dehydrogenase	cytoplasmic
Ndh3***	Ndh3***	NADH dehydrogenase	plasma membrane

NhaC*	NhaC*	Na ⁺ /H ⁺ antiporter NhaC	plasma membrane
	OG1RF_10021**	PTS system mannose/fructose/sorb ose transporter subunit IID	plasma membrane
	OG1RF_10069*	OG1RF_10069	plasma membrane
	OG1RF_10082***	M protein trans-acting positive regulator	cytoplasmic
OG1RF_10090*	OG1RF_10090*	putative lipoprotein	plasma membrane
OG1RF_10124***	OG1RF_10124***	ABC superfamily ATP binding cassette transporter, binding protein	plasma membrane
OG1RF_10125**	OG1RF_10125***	ABC superfamily ATP binding cassette transporter, binding protein	plasma membrane
OG1RF_10126**	OG1RF_10126***	ABC superfamily ATP binding cassette transporter, ABC protein	cytoplasmic
OG1RF_10131***	OG1RF_10131***	hypothetical protein	cytoplasmic
OG1RF_10281*	OG1RF_10281*	hypothetical protein	extracellular
OG1RF_10370**	OG1RF_10370**	hypothetical protein	plasma membrane
OG1RF_10421**		hypothetical protein	cytoplasmic
OG1RF_10479**	OG1RF_10479*	sodium/dicarboxylate symporter family protein	plasma membrane
	OG1RF_10667*	hypothetical protein	plasma membrane
OG1RF_10871**		cell wall surface anchor family protein	cell wall
	OG1RF_10875*	hypothetical protein	plasma membrane
OG1RF_11059*	OG1RF_11059*	phage tail protein	extracellular phage

OG1RF_11060**	OG1RF_11060***	phage structural protein	extracellular phage
OG1RF_11061**	OG1RF_11061***	hypothetical protein	extracellular phage
OG1RF_11094*	OG1RF_11094**	SPFH domain/band 7 family protein	plasma membrane
	OG1RF_11130**	pheromone cAM373 lipoprotein	plasma membrane
	OG1RF_11402*	hypothetical protein	plasma membrane
	OG1RF_11506*	putative lipoprotein	plasma membrane
OG1RF_11602*		putative calcium-transporting ATPase	plasma membrane
OG1RF_11679*	OG1RF_11679*	ABC superfamily ATP binding cassette transporter, binding protein	plasma membrane
	OG1RF_11697*	hypothetical protein	plasma membrane
	OG1RF_11698*	hypothetical protein	plasma membrane
OG1RF_11718**		hypothetical protein	extracellular
	OG1RF_11810*	amino acid permease	plasma membrane
OG1RF_11912*		hypothetical protein	cytoplasmic
OG1RF_11938***		fumarate reductase	cytoplasmic
OG1RF_11999*		P-ATPase superfamily P-type ATPase cadmium transporter	plasma membrane
OG1RF_12020*		hypothetical protein	plasma membrane
OG1RF_12067*		tellurite resistance protein	cytoplasmic
OG1RF_12203**		ABC superfamily ATP binding cassette transporter, substrate-binding protein	cytoplasmic
OG1RF_12384*	OG1RF_12384*	non-specific	plasma

		serine/threonine protein kinase	membrane
OG1RF_12417*	OG1RF_12417*	hypothetical protein	plasma membrane
OG1RF_12508*		thiamine biosynthesis lipoprotein	plasma membrane
OG1RF_12509***	OG1RF_12509**	pheromone cAD1 lipoprotein	plasma membrane
OppA**	OppA*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	plasma membrane
OppD*		ABC superfamily ATP binding cassette transporter, ABC protein	cytoplasmic
	Ohr*	organic hydroperoxide resistance protein	cytoplasmic
OpuAA*		glycine betaine/L-proline ABC superfamily ATP binding cassette transporter, ABC protein	cytoplasmic
Pbp1A***	Pbp1A***	penicillin-binding protein 1A	plasma membrane
PabC*		aminodeoxychorismate lyase	cytoplasmic
PepA2*		glutamyl aminopeptidase	cytoplasmic
PdhB*	PdhB**	pyruvate dehydrogenase complex E1 component beta subunit	cytoplasmic
PflB*		formate acetyltransferase	cytoplasmic
	PepA2**	glutamyl aminopeptidase	cytoplasmic
	PfkA*	6-phosphofructokinase	cytoplasmic

Pgk*	Pgk*	phosphoglycerate kinase	cytoplasmic
PrsA**	PrsA**	peptidyl-prolyl cis-trans isomerase	cytoplasmic
	PstS2	phosphate ABC transporter ATP-binding protein	extracellular
Pta*		phosphate acetyltransferase	plasma membrane
Pyk*		pyruvate kinase	cytoplasmic
	PyrG*	CTP synthase	plasma membrane
PyrH*	PyrH*	UMP kinase	cytoplasmic
	RplA*	50S ribosomal protein L1	cytoplasmic
RplB**	RplB**	50S ribosomal protein L2	cytoplasmic
	RplC*	50S ribosomal protein L3	cytoplasmic
RplD**	RplD**	50S ribosomal protein L4/L1 family protein	cytoplasmic
	RplE**	50S ribosomal protein L5	cytoplasmic
RplK**	RplK**	50S ribosomal protein L11	cytoplasmic
	RplM**	50S ribosomal protein L13	cytoplasmic
	RplN*	50S ribosomal protein L14	cytoplasmic
	RplP*	50S ribosomal protein L16	cytoplasmic
	RplQ**	50S ribosomal protein L17	cytoplasmic
RplS*	RplS*	50S ribosomal protein L19	cytoplasmic
	RplT*	50S ribosomal protein L20	cytoplasmic

	RplU*	50S ribosomal protein L21	cytoplasmic
	RplV*	50S ribosomal protein L22	cytoplasmic
RpoC*	RpoC*	DNA-directed RNA polymerase subunit beta prime	cytoplasmic
RpsB*	RpsB*	30S ribosomal protein S2	cytoplasmic
RpsC*	RpsC*	30S ribosomal protein S3	cytoplasmic
	RpsD*	30S ribosomal protein S4	cytoplasmic
	RpsE*	30S ribosomal protein S5	cytoplasmic
RpsI3*	RpsI3*	30S ribosomal protein S9	cytoplasmic
RpsJ***	RpsJ*	30S ribosomal protein S10	cytoplasmic
	RpsK*	30S ribosomal protein S11	cytoplasmic
RpsL*	RpsL*	30S ribosomal protein S12	cytoplasmic
	RpsR*	30S ribosomal protein S18	cytoplasmic
SecY*		preprotein translocase subunit SecY	plasma membrane
	Tig2**	peptidyl-prolyl isomerase	cytoplasmic
	TpiA*	triose-phosphate isomerase	cytoplasmic
TraC2*	TraC2*	peptide ABC superfamily ATP binding cassette transporter, binding protein	cytoplasmic
Tuf***	Tuf**	elongation factor EF1A	cytoplasmic
TyrS	TyrS*	tyrosine-tRNA ligase	cytoplasmic

Even though by MS we couldn't fully address whether SrtA substrates are enriched in MV from $\Delta srtA$, we examined cellular location of proteins within MV to gain some insights in MV biogenesis. To do that, we annotated each protein with a predicted cellular localization (**Table 4.1** last column) and plotted percentage distribution of proteins based on their cellular localization (**Figure 4.8**). We observed similar distribution between WT and $\Delta srtA$, with majority of proteins being cytoplasmic (56% and 59% respectively) followed by membrane proteins (35% and 34%) and the minority of protein identified were cell-wall associated or secreted proteins/phage proteins. The data suggested that MV from WT and $\Delta srtA$ were being formed in a similar manner where significant amount of cytoplasmic content was carried within MV.

Since we hypothesized that MV are formed similarly between the two strains we analysed the proteins found within MV to look for proteins that might be involved in MV formation. Abundance of few proteins may suggest the mechanism of MV formation in *E. faecalis*:

OG1RF_11059, OG1RF_11060, OG1RF_11061 - phage proteins

Lyzl6 - cell wall lysis protein

OG1RF_11718 - putative autolysin

OG1RF_11094 - flotillin

OG1RF_10090, OG1RF_11130, OG1RF_11506, OG1RF_12508,
OG1RF_12509 - lipoproteins

The role of the above proteins will be addressed in the following paragraph **4.4** and in the section **4.5 Discussion**.

During our MS analyses of proteins within MV pure fractions described above, we detected phage proteins (OG1RF_11059, OG1RF_11060, OG1RF_11061, **Table 4.1**). WT *E. faecalis* strain OG1RF encodes a cryptic phage02 that has been described as a part of core genome and is thought to devoid of lytic activity since major structural genes appear to deteriorate to pseudogenes what often coincides with a loss of function (Duerkop et al., 2014; McBride et al., 2007). While performing TEM on crude MV fractions, we also observed structures similar in size and appearance to phage tails (**Figure 4.9**). These tail-like structure resembled ~200 nm non-contractile tails of family *Siphoviridae* and phage02 is homologues to characterized *L. lactis* bacteriophage Tuc2009 from the same *Siphoviridae* family (McBride et al., 2007; Murphy et al., 2016).

Figure 4.9 Phage tails like structures are present in WT MV crude fraction.
Scale bar is 100 nm.

Recently, *P. aeruginosa* MV formation was shown as a result of explosive cells lysis mediated by a phage endolysin (Turnbull et al., 2016). In WT *E. faecalis*, the phage endolysin OG1RF_11063 is also present within the prophage operon. Similar to cell-associated MV produced during explosive cell lysis in *P. aeruginosa*, we also observed cell-associated MV in WT *E. faecalis* (**Figure 4.10**) (Turnbull et al., 2016). The observations that structures reminiscent of phage tail fibers are present in *E. faecalis* culture supernatants, and cell-associated MV can be seen, suggested to us that explosive cell lysis may occur in *E. faecalis* which may contribute to MV release.

4.4. Discussion

MV are universally produced in both Gram-positive and Gram-negative bacteria, however the mechanisms that govern MV formation in Gram-positives bacteria

including *E. faecalis* are largely unknown. We explored the possibility that vesiculation in *E. faecalis* is enhanced under perturbation of plasma membrane composition caused by accumulation of unprocessed SrtA substrates (such as “off pathway” pili and Ace presented in **Chapter 3** of this thesis) in $\Delta srtA$. However, nanoparticle tracking analyses of MV isolated from WT or $\Delta srtA$ showed no differences in MV numbers between the strains. Similarly, no differences in MV numbers between the WT and $\Delta srtA$ were observed for *S. mutans* (Liao et al., 2014). However, as a result of increased membrane-associated pili in $\Delta srtA$, MV of $\Delta srtA$ were also enriched with pili. Next, by MS we tested if other SrtA substrates were similarly enriched in MV of $\Delta srtA$, but due to technical issues we couldn't detect Ebp or any of SrtA substrates in either WT or $\Delta srtA$. This may be due to limitation of the MS technique: immunoblot is much more sensitive and can detect protein levels below 1 ng, whereas the typical detection range for whole lane MS is 5-10 ng. To increase the sensitivity of the assay, an alternative approach is to use in-solution digestion and scale up the protein sample to be visible by Coomassie blue staining for a more comprehensive analysis of all the proteins present in pure MV fraction of WT and $\Delta srtA$.

When we characterized MV proteins based on the cellular localization, we observed that majority of the protein in both WT and $\Delta srtA$ were cytoplasmic. Similar abundance of cytoplasmic proteins was described in *S. aureus* and *S. pneumoniae* (Lee et al., 2009; Olaya-Abril et al., 2014). We are not sure, whether detected cytoplasmic proteins are specifically contributed and secreted through MV, or whether cytoplasmic proteins were “trapped” in MV due to explosive cell lysis or MV biogenesis. Since in *S. pneumoniae* MV were shown to be enriched in specific lipids, compare to whole cell membrane suggested that MV biogenesis is a specific process and maybe cytoplasmic proteins also play an important role for MV function (Olaya-Abril et al., 2014).

Bacteria contain functional microdomains important for cell-to-cell signalling that harbour homologues of flotillin-1 found exclusively in lipid rafts in eukaryotes (López and Kolter, 2010). Microdomains were reported in *S. pyogenes*, *B.*

subtilis, *S. aureus* and *E. faecalis* (López and Kolter, 2010; Rosch et al., 2007) (Kandaswamy et al., 2013b). We detected flotillin (OG1RF_11094) in MV from both WT and $\Delta srtA$ suggesting that MV might be formed from membrane microdomains. Since flotillin-associated membrane microdomains are postulated to be enriched in a subset of cationic lipids we are currently performing lipidomics on the purified MV to address if MV consist of distinct lipid domains and/or are enriched in specific lipids as compared to the cell membrane lipids (Bramkamp and Lopez, 2015).

As reported in *P. aeruginosa*, one mechanism for MV formation is via cell-wall modifying phage endolysins, in which holins create pores in the inner membrane, allowing endolysins to translocate to the periplasm and degrade peptidoglycan resulting in an explosive cell lysis (Turnbull et al., 2016). As a result of explosive cell lysis bacterial membrane fragments self-anneal forming MV (Turnbull et al., 2016). We hypothesize that similarly in *E. faecalis*, some MV might be released via explosive cell lysis mediated by phage endolysin OG1RF_11063. We observed phage tails in MV fractions, suggestive that MV release might be coupled with phage tail release. Moreover, we detected phage proteins in both WT and $\Delta srtA$ suggesting that explosive cell lysis might be a general process resulting in MV formation in *E. faecalis*. To further test this hypothesis we will image live *E. faecalis* WT or endolysin mutant cells for explosive cell lysis as has been described for *P. aeruginosa* (Turnbull et al., 2016). We also plan to quantify the MV numbers from autolysin mutant as well as a whole phage operon deletion mutant. We predict to detect less MV from these mutants, compare to WT, since we hypothesis that at least partially MV are formed via explosive cell lysis. We are also performing TEM on phage deletion mutant to address if phage tail-like structures disappear.

Another possibility for MV biogenesis in *E. faecalis* is release via cell-wall modifying enzymes. We identified Lyl6, designated as cell wall lysis protein (NCBI database), in MV from both WT and $\Delta srtA$ by MS and this protein might contribute to MV release. To further test this hypothesis we will quantify MV from the *lyl6* mutant and also test this protein as MV biomarker. If Lyl6 is

responsible in MV formation we would detect enrichment of LysI6 within the MV compared to whole cell. Additionally we will then test whether LysI6 is enzymatically active and its localization – both within the cell and MV. We are generating antiserum to LysI6 to address its localization and test if this enzyme is universally present in MV from different enterococcal strains. Another putative cell wall lysis protein OG1RF_11718 was identified in the MV from WT, further supporting the hypothesis that MV partially are released during the cell-wall remodelling.

Interestingly, we detected 5 lipoproteins, where in *S. pyogenes* release of lipoproteins was described associated with lipoprotein-rich MV (Biagini et al., 2015). Lipoproteins have been described as virulence factors contributing to bacterial adhesion, membrane stability and the role in interaction with the host via TLR-2 receptors (Kovacs-Simon et al., 2011). Similarly in *E. faecalis* lipoprotein within MV may play an important role in bacteria-host interaction. Since MV are readily released *in vivo*, MV from Gram-positive bacteria were shown to interact with the host, eliciting immune response and causing death in some cases (Gurung et al., 2011; Jiang et al., 2014; Thay et al., 2013). Finally we are interested to address the role of MV in *E. faecalis* interaction with the host, to understand if MV contributes to *E. faecalis* pathogenesis. For these studies, we will utilize an NF- κ B reporter assay in macrophages model previously optimized in our lab to test the ability of MV to suppress or activate immune response via NF- κ B pathway (Tagore, 2016). Preliminary data suggests that MV from Δ *srtA*, but not from WT stimulate NF- κ B pathway, presumably because MV from Δ *srtA* are enriched in pili that are known to be immunogenic (Flores-Mireles et al., 2014; Nallapareddy et al., 2011b).

5. Chapter 5. Cellular response to *srtA* deletion

5.1. SrtA substrates expression does not require SrtA expression

SrtA attaches a number of proteins to the growing cell wall. Only two sortases – SrtA and SrtC are present in *E. faecalis* strain OG1RF, where SrtC polymerizes pili and SrtA is a housekeeping sortase responsible for the attachment of various proteins to the growing cell wall (Kemp et al., 2007a). In the absence of SrtA, SrtA substrates accumulate in the membrane that may create membrane stress and feedback on expression of SrtA substrates. Since in **Chapter 3** we observed that under planktonic condition, during mid-log growth phase Ebp and Ace accumulated in the plasma membrane while expression of *ebpC* remained unchanged, we wondered whether other SrtA substrates – characterized and putative similarly are being transcribed on the same levels in $\Delta srtA$. If true, we hypothesized that cell will experience membrane stress and/or trigger transcriptional response to deal with accumulation of unanchored proteins in the plasma membrane. To address this question we performed RNAseq on WT and $\Delta srtA$.

To address how SrtA substrates, both putative and characterized ones are being transcribed on the the same level in $\Delta srtA$, we first identified the putative SrtA substrates in OG1RF. To create a list of all of the putative sortase substrates in strain OG1RF (by Swaine Chen, personal communication) homologues of 33 putative SrtA substrates described for *E. faecalis* V583 were identified in OG1RF genome (**Table 5.1**, second column) and the list was supplemented with cell wall surface anchor family proteins annotated as such in NCBI database for *E. faecalis* OG1RF reference genome (NCBI accession: NC_017316.1) (**Table 5.1**) (Boekhorst et al., 2005). Based on these criteria, we identified 27 of putative sortase substrates in *E. faecalis* OG1RF. The differences in smaller number of

putative SrtA substrates in OG1RF vs V583 is likely due to a smaller genome size of OG1RF (2,739,625 bp vs 3,218,031) and also due to a large scale genomic variation between the two strains (Bourgogne et al., 2008).

Table 5.1 Putative and previously characterized SrtA substrates in OG1RF

Gene number	V583 homologue	Gene name	Annotation
OG1RF_10084	EF0062	-	cell wall surface anchor protein
OG1RF_10088	EF0089	-	Pectate lyase superfamily protein
OG1RF_10485	EF0750	-	cell wall surface anchor family protein
OG1RF_10490	-	-	cell wall surface anchor family protein
OG1RF_10508	EF0775	-	von Willebrand factor domain LPTXG domain protein
OG1RF_10766	EF1033	-	indole acetamide hydrolase
OG1RF_10785	EF1896	-	cell wall surface anchor family protein
OG1RF_10786	-	-	cell wall surface anchor family protein
OG1RF_10811	-	<i>cna</i>	collagen adhesion protein
OG1RF_10869	EF1091	<i>ebpA*</i> (Nallapareddy et al., 2006)	von Willebrand factor type A domain-containing protein
OG1RF_10870	EF1092	<i>ebpB*</i> (Nallapareddy et al., 2006)	cell wall surface anchor family protein
OG1RF_10871	EF1093	<i>ebpC*</i> (Nallapareddy et al., 2006)	cell wall surface anchor family protein
OG1RF_10878	EF1099	<i>ace</i> (Rich et al.,	collagen adhesin protein

		1999)	
OG1RF_11037	EF1269	-	cell wall surface anchor family protein
OG1RF_11531	EF1824	-	
OG1RF_11757	-	<i>ypdD</i>	cell wall surface anchor family antigen
OG1RF_11764	EF2224	-	cell wall surface anchor family protein
OG1RF_11897	EF2465	-	hypothetical protein
OG1RF_11924	EF2505	-	cell wall surface anchor family protein
OG1RF_12054	EF3187	-	cell wall surface anchor family protein
OG1RF_12251	-	-	cell wall surface anchor family protein
OG1RF_12268	EF2986	-	
OG1RF_12303	EF3023	-	family 8 polysaccharide lyase
OG1RF_12346	EF3076	-	cell wall surface anchor family protein
OG1RF_12451	-	-	cell wall surface anchor family protein
OG1RF_12506	EF3253	-	cell wall surface anchor family protein
OG1RF_12558	EF3314	-	cell wall surface anchor family protein
*EbpA, EbpB and EbpC are the three subunits of Ebp pili, but only EbpB was shown to be recognized by SrtA (Nielsen et al., 2013).			

With a list of all putative OG1RF substrates in hand, we next performed RNA-seq to determine whether any SrtA substrates were differentially expressed in wild type (WT) compared to a *srtA* deletion strain grown to mid-log phase in BHI media. Genes with significantly different abundance of mRNAs were determined using a P-value and false discovery rate (FDR) cutoff of 0.05. We observed no changes in mRNA abundance for any of the substrates listed in the **Table 5.1** (p-values > 0.05 between biological triplicates of WT and $\Delta srtA$). However, 3 out of

27 SrtA substrates listed in Table 1, namely OG1RF_10785, OG1RF_10786 and OG1RF_11897 were not expressed at all under the tested conditions (**Figure 5.1**), and where number of sequencing reads mapped against OG1RF genome for the three genes was not significantly different from number of background sequencing reads for *srtA* gene in $\Delta srtA$.

We observed 105 genes in total that had more abundant mRNAs when compared to WT (see **Appendix B** for a full list). First the 105 genes were assigned with Kyoto Encyclopedia of Genes and Genomes (KEGG) identification numbers (Damien Keogh, personal connection) (refer to **Appendix B**) (KEGG: Kyoto Encyclopedia of Genes and Genomes - GenomeNet, 2016). Next we mapped assigned genes by KEGG ID to the KEGG pathway maps for cellular and organismal functions and listed the pathways with the highest number of genes with more abundant mRNAs(**Figure 5.2**). Although we did not observe changes in SrtA substrate expression, we saw that the chaperones *dnaK* and *dnaJ* were significantly upregulated, 1.2 and 1.3 log₂-fold changes respectively in the $\Delta srtA$ mutant strain (**Figure 5.3**).

Figure 5.2 The top 8 KEGG pathways containing the highest number of the regulated genes.

105 genes in $\Delta srtA$ with higher mRNA levels were assigned KEGG ID number and KEGG pathways were annotated using KEGG mapper (KEGG: Kyoto Encyclopedia of Genes and Genomes - GenomeNet, 2016). The full list of all the pathways identified is available in **Appendix C**.

Figure 5.3 *dnaK* and *dnaJ* expression is higher in $\Delta srtA$ compared to WT.

RNAseq was performed on biological triplicate for each strain grown to mid-log phase in BHI. Data shown indicate the log2 fold change expression of *dnaK* and *dnaJ* relative to WT, where **** PValue < 0.0001 and FDR < 0.05

5.2. Role of DnaK in Ebp biogenesis

Two chaperones, DnaK and DnaJ, function in a complex where DnaJ is a cofactor of DnaK (Genevaux et al., 2007; Kampinga and Craig, 2010). DnaK is essential under stress conditions but also facilitates protein targeting to the membrane and is important for protein secretion (Castanié-Cornet et al., 2014). Moreover in *P. aeruginosa*, DnaK is essential for flagellum assembly and co-localizes with membrane proteins at the cell surface (Borrero-de Acuña et al., 2015). Since in $\Delta srtA$ piliation increases (**Chapter 3.**) and transcription of *dnaK* also increases, we hypothesised that DnaK may facilitate pilin subunits folding or assist in pili assembly at the membrane. To address the role of DnaK in Ebp biogenesis, we first validated the RNAseq data by RT-qPCR, using RNA derived from the same growth conditions as used for the RNAseq experiment, and again detected a significant increase in *dnaK* expression in $\Delta srtA$ (**Figure 5.4**).

Figure 5.4 *dnaK* expression is higher in $\Delta srtA$ compared to WT.

RT-qPCR on RNA isolated from mid-log cells grown in BHI. The experiment was performed in triplicate using the $\Delta\Delta C_t$ method to assess expressional changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *dnaK* expression in $\Delta srtA$ compared to WT. Statistical analysis was performed by the unpaired *t*-test using GraphPad * $p < 0.05$.

Therefore we predicted that in the absence of these chaperones, sortase substrates expression might be affected. In order to test this we next examined Ebp expression in strain in which *dnaK* and *dnaJ* are disrupted (*dnaK*::Tn and *dnaJ*::Tn (Kristich et al., 2008)) compared to WT. We performed immunofluorescence microscopy (IFM), staining pilus-expressing cells with α -EbpC antibody, and observed 43% and 48% fewer EbpC-expressing cells in *dnaK*::Tn and *dnaJ*::Tn respectively when compared to WT (**Figure 5.5A**). Since the two chaperones DnaK and DnaJ function together, we considered that piliation decreased in both transposon mutants due to the similar mechanism and continued on studying the role of DnaK in Ebp biogenesis. Similar to IFM data, by immunoblot we observed a significant decrease in EbpC protein levels in the *dnaK*::Tn and compared to WT, and this could be restored with *dnaK* complementation from a plasmid (**Figure 5.5B**). We observed that EbpC antibody nonspecifically reacts with a protein around 70 kDa which we think might be DnaK, since the band disappeared in *dnaK*::Tn and is more prominent in *pdnaK*, but also present in Δ ebpABC (similarly observed in **Figure 5.10B** and **Figure 10.2**).

Figure 5.5 Piliation decreases in *dnaK*::Tn and *dnaJ*::Tn compare to WT.

(A) IFM with EbpC antiserum. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad **** $p < 0.0001$. *** $p < 0.001$, n.s.: $p > 0.05$. (B) Anti-EbpC immunoblot on whole cell lysates of mid-log grown cells. The blot depicts general pattern observed in three independent experiments.

Since DnaK is a general cellular chaperone, we questioned whether the contribution of DnaK to pilus expression was direct, or whether it could be acting indirectly on proteins or enzymes that are “upstream” of polymerized Ebp cell surface expression. Therefore to address whether DnaK might be acting directly on Ebp biogenesis, we first analyzed the protein involved in Ebp biogenesis, namely SecA, SrtC and SrtA involved in Ebp secretion, polymerization, and attachment to the cell wall respectively for the presence of DnaK-binding domains. Since DnaK binds to hydrophobic regions of polypeptide, all of the tested proteins had multiple putative DnaK-binding regions (**Appendix D**). Next we performed immunoblots of cells grown to mid-log phase and observed that the levels of SecA and SrtA were unchanged in *dnaK*::Tn, but levels of SrtC were visibly lower compared to WT (**Figure 5.6A**).

Figure 5.6 SrtC levels are affected in *dnaK::Tn* while total protein content between the WT and *dnaK::Tn* is similar.

(A) Immunoblot shows a general pattern observed for the three strains in 3 independent experiments. **(B)** Coomassie blue staining on whole cell lysates. The thick band in lane 3 indicated with (*) is likely to be a DnaK (predicted MW of 70 kDa) that is overexpressed from the multicopy number plasmid.

Promoter mapping experiments have previously demonstrated that *srtC* has its own promoter, but can also be transcribed, albeit to lower levels, via read through from the upstream *ebpABC* promoter (Bourgogne et al., 2007; Nallapareddy et al., 2006). Decrease in SrtC levels in *dnaK::Tn* could have affected observed lower levels of Ebp, however since we do not see accumulation of EbpC in *dnaK::Tn* mutant but only in $\Delta srtC$ (**Figure 5.5B**) suggests that low levels of SrtC are still sufficient to polymerize all translated EbpC subunits.

Therefore, we sought to determine whether the decrease in both EbpC and SrtC expression that we observed (**Figure 5.6A**) were indicative of DnaK specificity

toward factors involved in Ebp biogenesis or whether there may be a more global effect on protein stability and expression in the *dnaK::Tn* mutant strain. To address whether there was a difference in overall protein content between WT and *dnaK::Tn*, we performed Coomassie blue staining on the protein samples derived from whole cell lysates. Similar to that reported in *S. intermedius*, overall protein content was similar between WT and *dnaK::Tn* (**Figure 5.6B**) (Tomoyasu et al., 2012).

Figure 5.7 *ebpC* transcription is reduced in *dnaK::Tn* independent of *ebpR* transcription.

RT-qPCR on RNA isolated from mid-log bacteria grown at 37°C. The experiment was performed in triplicate using the $\Delta\Delta C_t$ method to assess expressional changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *ebpC* (A) and *ebpR* (B) transcription in *dnaK::Tn* n compare to WT. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad **** $p < 0.0001$, n.s.: $p > 0.05$

In the complemented *dnaK::Tn* *pdnaK* strain, *dnaK* is expressed from the multicopy number plasmid that likely results in higher DnaK levels (**Figure 5.6B**, indicated with (*)), but levels of pilus expression between the WT and complementation strain are similar (**Figure 5.5**), suggesting that although the

presence of DnaK is important for Ebp biogenesis, more DnaK does not correlate with increased piliation. To further address the role of DnaK on Ebp biogenesis, we investigated whether Ebp transcription is lower in *dnaK::Tn*, and observed a reduction in *ebpC* transcription in *dnaK::Tn* (**Figure 5.7A**). Because the *ebpABC* is positively regulated by EbpR ((Bourgogne et al., 2007)), we wondered whether DnaK might act on EbpR protein expression to give rise to its effects on Ebp pilus expression, or whether *ebpR* transcription was also reduced, similar to the *ebpABC* in the *dnaK::Tn* strain. RT-qPCR analysis of *ebpR* in WT and *dnaK::Tn* showed that *ebpR* levels were unchanged (**Figure 5.7B**). We concluded, that reduced piliation in *dnaK::Tn* is a direct result of reduced transcription of *ebpC* and/or instability of the *ebpC* transcripts, rather than downregulation of its positive regulator *ebpR*. When we abolish *dnaK*, there is less Ebp pili (and also lower *ebpC* gene expression) and less SrtC proteins, but SrtA proteins remain unchanged. This suggests SrtA and DnaK have no direct interaction, but *dnaK* and *ebpC/srtC* gene expression seems to be directly correlated. To check the correlation between *dnaK* and *ebpABCsrtC* expression, we looked at *dnaK* expression in the $\Delta ebpABCsrtC$, where we predicted that *dnaK* transcription might be reduced. However we observe no differences in *dnaK* levels (**Figure 5.8**), suggesting that under WT condition *dnaK* might be already expressed on the basal level that is sufficient to maintain expression of *ebpABCsrtC* possibly along with other SrtA substrates. And only when Ebp accumulates in the plasma membrane and is not being anchored to the cell wall, more DnaK is needed to chaperone the pilus assembly machinery.

Figure 5.8 *dnaK* expression remains unchanged in $\Delta\textit{ebpABCsrtC}$.

RT-qPCR on RNA isolated from mid-log bacteria grown at 37°C. The experiment was performed in triplicate using the $\Delta\Delta\text{Ct}$ method to assess expressional changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *dnaK* compare to WT. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad, n.s.: $p > 0.05$

While performing IFM to address piliation in *dnaK::Tn*, we noticed that *dnaK::Tn* cells had an aberrant morphology that appeared elongated, chained with multiple septum compared to WT (**Figure 5.9A**). To quantify the described morphology changes observed in *dnaK::Tn*, we counted the number of cells with altered morphology (**Figure 5.9B**). This number was close to the number of pilus expressing cells (~ 33%, **Figure 5.5A**) in WT compared to 24% of the aberrant cells in *dnaK::Tn* (**Figure 5.9B**).

Similarly, altered morphology, including filamentous cells defective in cellular division were previously reported when *dnaK* was deleted in *E. coli*, where overproduction of FtsZ suppressed filamentation (Bukau and Walker, 1989).

Since the absence of *dnaK* results in the downregulation of pilin transcripts which may suggest the mutant cell is not equipped to handle normal pilus biogenesis we hypothesized that the morphological change we observed in *dnaK::Tn* cells may be caused by misfolded pili accumulated in the plasma membrane or within the cell that are not properly dealt with in the absence of DnaK, as this has been observed in our laboratory for other mutants associated with “off pathway” pilus biogenesis observed in $\Delta\textit{srtA}$ mutant backgrounds (Adeline Yong, personal communication). To test this, we generated strain *dnaK::Tn* $\Delta\textit{srtA}$, with the hypothesis that increasing pilus accumulation in the membrane in $\Delta\textit{srtA}$ (as discussed in **Chapter 3. Figure 3.1**) coupled with *dnaK::Tn* would give rise to even more severe morphology defects compared to *dnaK::Tn* alone. First we quantified the number of pilus-expressing cells in the single and double mutant strains and observed decrease in the number of piliated cell in the *dnaK::Tn* compared to WT from 33% to 19%. Similarly, we observed a decrease from 57%

piliation in $\Delta srtA$ to 33% in *dnaK::Tn* $\Delta srtA$ further detected by immunoblot with EbpC antiserum, where SecA was used as a loading control (**Figure 5.10**). These data suggested that the absence of *dnaK* in any multiple genetic backgrounds conferred a piliation defect.

Figure 5.9 Approximately 20% of cells in *dnaK::Tn* have aberrant morphology.

(A) Representative phase contrast image of mid-log cell of WT cells (top) and *dnaK::Tn* (bottom). Scale bar is 1 μ m. (B) Percent of cells within the population with aberrant morphology was calculated from the phase contrast images. Mean values are shown from 3 independent experiments in which at least 300 of cells were screened. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad * $p < 0.05$, ** $p < 0.01$, n.s.: $p > 0.05$.

Figure 5.10 Decreased piliation in both WT and $\Delta srtA$ in *dnaK::Tn* genetic background.

(A) IFM with EbpC antiserum where % of EbpC stained cells within a population was quantified. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad * $p < 0.05$, ** $p < 0.01$, n.s.: $p > 0.05$. (B) Immunoblot with EbpC antiserum (top) and loading control with SecA antiserum (bottom) on mid-log grown bacteria. Reduced HMWL in *dnaK::Tn* and in *dnaK::TnΔsrtA*. EbpC antiserum nonspecifically reacts with 70 kDa protein and with 50 kDa unidentified protein.

However, after quantitative analysis of morphology in *dnaK::TnΔsrtA* compared to *dnaK::Tn*, we saw no difference in the % of cells with aberrant morphology between the strains (Figure 5.9). Consistent with this, we performed IFM on EbpC stained WT, *dnaK::TnΔsrtA*, and *dnaK::Tn*, and observed pili on both

normal and aberrant cells (**Figure 5.11**). These data suggest that morphology defects associated with *dnaK*::Tn are independent of pili. We concluded that DnaK positively contributes to pilus biogenesis and normal diplococcal morphology, but impaired pili biogenesis in Ebp-expressing population in *dnaK*::Tn is not the reason for the aberrant morphology.

Figure 5.11 Piliation state in *dnaK::Tn* does not correlate with aberrant morphology.

(A) Representative images of IFM on anti-EbpC stained cells. Scale bar = 1 μ m. (B) Number of Ebp (+) cells within the aberrant cells based on IFM. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad ***p<0.001; n.s.: p>0.05.

5.3. Discussion

In **Chapter 3** we showed that increased piliation in $\Delta srtA$ was caused by accumulation of membrane-associated pili (as opposed to “normal” cell wall-associated) in absence of SrtA. We have also showed that *ebpC* is still being transcribed on the same levels in WT and $\Delta srtA$. Here we further explored the transcriptional response of the remaining SrtA substrates upon to *srtA* deletion. Similar to the unchanged transcription of *ebpC* in WT and $\Delta srtA$, we observed no transcriptional changes within 27 putative and characterized SrtA substrates. However, while levels of transcription varied between the substrates, three of 27 SrtA substrates were not transcribed in BHI during the mid-log growth phase. We know that Ebp expression is variable within a given population, but know little of how expression of the rest of the SrtA substrates may be coordinated within the population – whether all of them are expressed on all cells or if a subset of cells within the population expresses certain SrtA substrates. In the next 2 chapters, address the relationship between the 3 characterized SrtA substrates – Ace, Ebp and AS – as a first step in understanding the coordination between SrtA substrates in *E. faecalis*.

Despite the absence of a differential transcriptional response of SrtA substrates in $\Delta srtA$, we detected multiple cellular pathways with higher mRNA levels in the $\Delta srtA$ mutant background. It is unclear whether regulation of these pathways is caused by accumulation of unprocessed substrates on the bacterial membrane or by potential malfunctioning of unattached SrtA substrates that are not only adhesins. However, upregulation of stress response proteins DnaK and DnaJ suggest that $\Delta srtA$ cells experience stress which most likely derives from membrane stress associated with accumulated substrates.

The role of DnaK in stress responses has been investigated, but its role in general cellular processes is not well characterized. In *E. coli*, DnaK is important for proper cellular division (Bukau and Walker, 1989). Similarly in *S. intermedius*, *dnaK* mutants exhibit slower growth (Tomoyasu et al., 2012). Under stress conditions caused by high temperature or chemical stress, DnaK plays a crucial role in cellular survival. Here we have shown that DnaK is important for Ebp

biogenesis in BHI media at 37°C where levels of both *ebpC* transcripts and Ebp protein are reduced in *dnaK::Tn* compare to WT. One possible explanation for this finding is that in *dnaK::Tn*, the positive *ebpABC* regulator EbpR, while transcribed at the same rates as in WT, undergoes impaired translation in the absence of DnaK resulting in downregulation of the *ebpABC* operon. Alternatively, DnaK may directly facilitate Ebp pilin subunits targeting to the membrane or assist in stability of the SrtC-Ebp polymerizing complex, as SrtC levels are also reduced in *dnaK::Tn* compared to WT. Since RNase J is also important for *ebp* transcription, maybe DnaK influences expression/stability of RnjB, a positive *ebp* regulator. Moreover, DnaK was shown to activate transcriptional regulator of flagellar in *Salmonella enterica* and similarly in *E. faecalis* may work together with other proteins to trigger *ebp* expression (Takaya et al., 2006).

To further address the mechanism of decreased piliation in *dnaK::Tn*, we will utilize a bacterial two-hybrid system to test for direct interaction between EbpR, EbpC and SrtC with DnaK. We are also generating EbpR antiserum to test the stability of EbpR protein in the absence of DnaK. As an alternative approach to address interactions between Ebp subunits with DnaK, we are going to perform affinity co-purification of EbpC with its interaction partners to determine if DnaK is one of them. Moreover, as Ebp is an important virulence factor of *E. faecalis* that contributes to biofilm formation, UTI, and CAUTI, and since pilus biogenesis is impaired in *dnaK::Tn*, we will also test ability of *dnaK::Tn* to form biofilm and cause infection in vivo. Alternatively, since DnaK may work together with other cellular chaperones, Ebp biogenesis might be indirectly linked with DnaK (Patra et al., 2015). To test if other chaperones (GroES, GrpE, ClpB, ClpA) influence *ebpABCsrtC* expression we will look at *ebpC* expression in Tn mutants of the above. Moreover, even though we did not observe reduced transcription of positive *ebpR* regulator of *ebpABC* operon, we do not exclude the possibility that DnaK may affect folding and stability of EbpR. Once EbpR antibody is ready, we will test EbpR protein levels in *dnaK::Tn*. Ebp is only one of the possible SrtA substrates that is affected in *dnaK::Tn*. It is unclear whether DnaK is also important for the biogenesis of other characterized SrtA substrates such as

adhesin to collagen (Ace) and aggregation substance (AS). Because Ace expression is induced at 46°C, but *dnaK*::Tn didn't grow at this temperature overnight ($OD_{600} < 0.2$), AS may be a better candidate to test the role of DnaK in other SrtA substrates processing. To address these possibilities, we will transform AS-encoded pCF10 plasmid in *dnaK*::Tn and assess the AS expression profile upon pheromone induction in *dnaK*::Tn compared to WT. A more general approach to address the role of DnaK in multiple SrtA substrate expression and basic cellular processes is to perform protein profiling between WT and *dnaK*::Tn. Since *dnaK*::Tn cells display an aberrant morphology, independent of Ebp expression, protein profiling may reveal why cells have morphological defects and what the cell division proteins affected in *dnaK*::Tn might be. In *E. coli*, the FtsZ division protein is responsible for the filamentous phenotype observed in a $\Delta dnaK$ mutant strain. However, as FtsZ is important for septation and since we observed multiple septa in *dnaK*::Tn in WT, we hypothesize that not only FtsZ but multiple division proteins might be affected in DnaK. Finally, it is yet unclear how various cellular chaperones work together and what determines whether one chaperone can compensate for the loss of function of another cellular chaperone. By comparing the protein profile of a single chaperone mutant to a double chaperone mutant, we may be able to identify the protein(s) that is(are) chaperoned by either one or both of the two chaperones to further study the mechanism of chaperone's specificity.

In conclusion we have shown here that DnaK is not only important for general cellular processes such as cellular division in *E. faecalis*, but also plays an important role in Ebp biogenesis.

6. Chapter 6. Relationship between Ebp and AS expression

E. faecalis encodes multiple adhesins but whether or how they are co-regulated or co-expressed in a population or on a single cell is not well understood. Ebp are variably expressed within a population and the level of variation partially depends on environmental cues such as serum, glucose, or bicarbonate (Bourgogne et al., 2010; Kline et al., 2009; Nallapareddy et al., 2011a; Nallapareddy et al., 2006). AS is induced by pheromone but is also variably expressed within a population, based on SEM images, even at saturating pheromone levels (Olmsted et al., 1993). Both of these adhesins are important for biofilm formation, which suggests that they might be co-expressed within a population under biofilm conditions (Bhatty et al., 2015; Nallapareddy et al., 2006). However, based on the variable expression of the two adhesins, we hypothesized that the two adhesins may be expressed on different subsets of cells within the same population. Such differential expression might be beneficial to diverse adhesive capacities within a population and reduce immunogenic potential of a single cell. Moreover, we wondered whether expression of AS or Ebp affects function or expression profile of each other.

6.1. Presence of the pCF10 plasmid does not affect piliation levels in *E. faecalis*

AS is encoded on the pheromone inducible pCF10 plasmid and, hence, studies of AS expression must be performed in this genetic background. Therefore, before testing the hypothesis that AS and Ebp are present on different cells within the population, we first examined whether the presence of the pCF10 plasmid per se, even in the absence of pheromone induction, affected Ebp expression. To do this, we first created strain OG1RF pCF10 and then quantified piliation in the absence of pheromone. Under these conditions, we detected no difference between the strains, with each expressing a similar percentage of

pilus stained cells in the population, with a mean of 36% for both strains (**Fig. 6.1**), consistent with previous reports (Bourgogne et al., 2007; Nallapareddy et al., 2006). From this experiment, we concluded that presence of the pCF10 plasmid alone does not affect piliation levels in *E. faecalis* OG1RF and so this strain is suitable for examining the relationship between AS and Ebp expression.

Figure 6.1 OG1RF and OG1RF pCF10 have a similar percentage of pilus-expressing cells during exponential growth.

IFM was performed with EbpC antiserum and the % of EbpC+ cells within the population was quantified. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad (n.s.: $p > 0.05$).

6.2. AS and Ebp are co-expressed on the same cells

The variable expression of AS has been previously reported, but not quantified (Olmstead et al., 1993). Therefore, we quantified AS expression in the population under our growth conditions after cCF10 pheromone induction for differing lengths of time. After 30 min of pheromone induction (0.12 ng/ml) 82% of the cells expressed AS on the cell surface, and 95% of cells expressed AS by 90 min post-pheromone induction (**Figure 6.2**).

Figure 6.2 The majority of cells rapidly produce AS upon pheromone induction with a significant drop in AS-expressing cells in overnight cultures (O/N ~18 hours).

IFM was performed with AS antiserum and the % of AS+ cells within a population was quantified. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired t-test using GraphPad * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, n.s.: $p > 0.05$.

The numbers for AS-expressing cells were higher than we expected based on the earlier SEM work in which representative images of exponentially grown bacteria showed ~25% of cells lacked AS (Olmsted et al., 1993). The differences between the previous work and our own results may be due to the increased sensitivity of AS detection by IFM compared to SEM. After 18 hours of growth in the presence of cCF10, the inducing pheromone, the percentage of AS-expressing cells in the population dropped significantly to 4% (**Figure 6.2**). These observations are consistent with previous reports where AS transcription peaks between 30-60 min after addition of pheromone and returns to uninduced levels by the end of the second hour (Chandler et al., 2005; Chatterjee et al., 2013). Therefore, in the absence of pheromone, 30-40% of cells within the *E. faecalis* population express Ebp and none express AS. However, more than 80%

of cells express AS within 30 min of pheromone induction. To address the relationship between pheromone exposure and/or AS expression on Ebp expression, we performed Ebp immunostaining on pheromone induced cells. Since 80% of cells expressed AS within 30 min of cCF10 pheromone exposure, we hypothesized that if Ebp expression is cCF10- or AS- responsive, the Ebp expression profile may also be altered as early as 30 min post cCF10 exposure. To test this, we examined pilus expression within the population on induced and uninduced cells at 30, 60, and 120 min after cCF10 addition. However, we observed no significant difference in Ebp expression between pheromone-induced and uninduced cultures (**Figure 6.3**).

Figure 6.3 cCF10 pheromone exposure does not alter Ebp expression.

IFM was performed with EbpC antiserum and the % of EbpC+ cells within a population was quantified. Mean values are shown from 3 independent experiments in which at least 300 of cells were counted. Error bars represent the standard deviation. Statistical analysis was performed by the unpaired *t*-test using GraphPad * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, n.s.: $p > 0.05$.

However, independent of pheromone exposure we noticed a significant decrease in a number of pilated cells 30 min post overnight subculturing compared to the overnight culture itself, which represents the early log stage of growth and which

suggests that Ebp-expressing cells might grow slower earlier during the early growth stage or that over time cells accumulate pili on the surface, and upon division partially transfer pili to the daughter cells resulting in increased number of the piliated cells (**Figure 6.3, first two bars**). We are yet to examine any of these possibilities.

While we originally hypothesized that piliation and AS expression might not occur on the same cells, since pheromone-induced AS expression was rapid and extensive and did not affect variable Ebp piliation levels, we revised our hypothesis and predicted that AS and Ebp would be co-expressed on the same individual cells. Indeed, when we performed co-immunostaining with AS and EbpC antiserum of pheromone induced cells, we observed that the two adhesins were displayed on the same cells and co-localized at the same hemispherical areas of the cell (**Figure 6.4**).

6.3. Pheromone-induced cells physically separate based on the presence of pili

AS was originally described for its association with cellular aggregation (Dunny et al., 1978). While we did not observe a difference in Ebp expression between AS-expressing and AS-non-expressing populations, we noticed that not all cells in cCF10-induced cultures aggregated, but instead separated into clumped cells that settled at the bottom of the tube and supernatant cells that remained in suspension. Separation became obvious 1.5 - 2 hours post induction (**Figure 6.5**).

After 2 hours of pheromone exposure, most cells were expressing AS (**Figure 6.2**), but only a subset of cells expressed pili (**Figure 6.3**, **Figure 6.4**). Since Ebp

in *E. faecalis* also contribute to cellular aggregation (La Rosa et al., 2016), we hypothesized that the clumping we observed in pheromone-induced cultures might be pilus dependent, where pilus expressing cells facilitate AS-mediated clumping. To test this hypothesis we separated the clumped cells from the supernatant and performed IFM with Ebp antiserum. In contrast to our hypothesis, we observed fewer Ebp-expressing cells in the clumped pellet and more in the supernatant when compared to the overall induced culture where pellets and supernatants were not separated (**Figure 6.6A**).

We next performed RT-qPCR on the pellet cells and confirmed that *ebpC* is lower in the pellet cells compared to the overall population. Moreover, lower expression of *ebpC* in the pellet was likely caused by downregulation of positive transcriptional regulator *ebpR* that is also reduced in the pellet (**Figure 6.6B**). Given that Ebp expression levels within the overall population is unchanged upon cCF10 pheromone exposure (**Figure 6.3**), we concluded that Ebp-expressing cells physically segregate to the supernatant and are mostly excluded from the aggregate pellet. Based on this separation, we hypothesized that pilus-mediated steric hindrance could interfere with AS-mediated clumping.

(A) IFM was performed 2 hours post-induction with EbpC antiserum and the % of EbpC+ cells within a population was quantified. Mean values are shown from 3 independent experiments. Error bars represent the standard deviation. (B) RT-qPCR on RNA isolated from pheromone induced cells two hours post-induction. The experiment was performed in triplicate using the $\Delta\Delta C_t$ method to assess expression changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *ebpC* and *ebpR* transcription in the pellet cells, compared to control (overall). Statistical analysis was performed by unpaired *t*-test using GraphPad (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, n.s.: $p > 0.05$)

6.4. Ebp impede AS-facilitated horizontal gene transfer

Since AS-mediated clumping facilitates transfer of the pCF10 plasmid to recipient cells (Bhatty et al., 2015), we hypothesized that non-clumping supernatant cells, which express both AS and pili, would display a reduced ability to undergo AS-mediated plasmid transfer compared to the pellet cells. To test this, we assessed conjugation frequency using supernatant or pellet cells as donors during horizontal gene transfer (HGT). Two hours post pheromone-induction, we collected the top 2 ml of each culture, containing supernatant cells and bottom 1 ml of clumped cells containing the pellet. Cell numbers were normalized and mixed with OG1RF Δ *ebpABCsrtC* recipient cells lacking pCF10. We used Ebp deficient recipient cells to avoid pilus-mediated interference when incubated with the donor cells. Consistent with our hypothesis, after 30 min of mating, the mixture of low-piliated pellet donor cells and the Ebp null recipient cells yielded approximately 10 times more conjugants compared to the highly pilated supernatant donor cell mixture (**Figure 6.7A**).

To further test the hypothesis that pili interfere with AS-mediated HGT, we used either WT or an *ebpABC* null mutant as the donor strain and compared conjugation rates to polymerized-pili deficient OG1X Δ *srtC* (str resistant) recipient (**Figure 6.7B**). At 30 min post-mating, the *ebpABC* null strain was more efficient in plasmid transfer as compared to WT, while conjugation rates equalized after two hours. Similar to HGT experiments with supernatant or pellet donors, differences between the two strains were no longer apparent after 1 hour of mating. We believe this delayed equalization of recovered transconjugants

may be due to a second round of transfer of the plasmid from new donors to recipient cells, switch to “on” pilliation of originally “off” pilliation donor cells and/or enhanced cellular density that facilitated cell-to-cell contact.

Figure 6.7 Clumped (Ebp low) and Ebp null cells are more efficient in horizontal genetic plasmid transfer than supernatant (Ebp high) cells.

A. HGT rates from s/n and pellet cells to plasmid free recipient are expressed as the number of transconjugants (Tc's) per donor cell 30 and 60 min post mating. **B.** HGT rates from WT pCF10 and $\Delta ebpABCsrtC$ pCF10 donor cells to plasmid free recipient are expressed as a number of transconjugants per donor cell 30 and 120 min post mating. Error bars represent the standard deviation from 3 independent experiments. Statistical analysis was performed by unpaired *t*-test using GraphPad * $p < 0.05$, ** $p < 0.01$, n.s.: $p > 0.05$.

6.5. AS contributes to biofilm formation similar to Ebp.

AS and Ebp both contribute to biofilm formation (Bhatty et al., 2015; Nallapareddy et al., 2006) and cCF10 pheromone-induced bacteria form more robust biofilm compared to their uninduced counterpart (Bhatty et al., 2015). However, in that study, bacteria were induced with 10 ng/ml cCF10, which is 10 times higher than our working concentration, which may contribute to the artificial biofilm enhancement with prolonged cCF10 induction, where all cells would continuously express AS. In addition, the contribution of pili was not addressed in

that study (Bhatty et al., 2015). Therefore, we sought to address the role of AS in biofilm formation in the absence of biofilm-forming pili. We reasoned that this situation might be important during early stages of biofilm formation when AS has been shown to be important or under conditions when pili are not expressed (Chuang-Smith et al., 2010a). To test the role of AS in biofilm formation, independent of Ebp, we diluted overnight cultures of OG1RF pCF10 and OG1RF Δ *ebpABCsrtC* and induced them with pheromone (0.12 ng/ml) in a 96-well plate. 24 hours later we assessed biofilm formation by crystal violet staining and compared them to biofilms formed by uninduced bacteria. Similar to observations when a high concentration of cCF10 was used, we found that as little as 0.12 ng/ml was sufficient to increase biofilm biomass compared to the uninduced OG1RF pCF10 (**Figure 6.8**) (Bhatty et al., 2015). As reported previously, we also observed that non-piliated mutants were deficient for biofilm formation (**Figure 6.8**) (Nallapareddy et al., 2006). Interestingly, in the absence of pili, AS induction was sufficient to revert biofilm of the *ebpABC* null strain to levels similar to the uninduced (AS-) wild type strain (**Figure 6.8**). This observation suggests that AS is sufficient to promote “normal” biofilm formation in the absence of Ebp. Moreover, the two adhesins appear to act synergistically within the biofilm giving rise to enhanced biofilm formation compared to biofilms formed in the presence of either individual adhesin alone.

Figure 6.8 AS contributes to biofilm formation similar to Ebp.

Biofilm formed by induced and uninduced WT pCF10 and OG1RFΔ*ebpABCsrtC* pCF10 on plastic after 24 hours. Error bars represent the standard deviation from 3 independent experiments. Statistical analysis was performed by unpaired *t*-test using GraphPad * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, n.s.: $p > 0.05$

6.6. Discussion

In this chapter, we addressed the relationship between expression of two enterococcal adhesins AS and Ebp at the population level. We examined the hypothesis that either the abundance of pheromone alone or expression of AS in a response to pheromone may affect Ebp expression. Since pheromone is being produced by all cells and is also present both in donor and recipients, we were not certain whether it may also act on bacterial chromosome encoded genes and in particular *ebpABC* and *ebpR*. Previously expression of a subset of genome encoded genes was tested in response to pheromone, but no changes in gene expression were observed, however mostly genes involved in pheromone secretion and uptake were tested (Hirt et al., 2005). Here we show that cCF10 and rapid induction of AS does not impact Ebp expression. Instead, independent

of pheromone or AS expression, Ebp remains variably expressed on a subset of the population and is regulated by its pheromone-irresponsive positive EbpR regulator, where Ebp(-) cells have reduced *ebpR* transcription. Perhaps the lack of the influence of the cCF10 pheromone to Ebp is not surprising, since only a subset of enterococcal strains carry the pCF10 plasmid, selective pressure to drive cross-regulation between chromosomally-encoded and plasmid-encoded genes may not have arisen. Despite the lack of direct cross-regulation, we found that simultaneous expression of AS and Ebp results in altered clumping and HGT frequency. We show here that AS-mediated clumping is reduced in Ebp-expressing cells, which may be due to steric hindrance by protruded pili preventing AS from binding to its ligand, lipoteichoic acid (LTA), on neighbouring cells or by masking the LTA from AS preventing clumping. Notably, we can only detect the differences in HGT rates at early time points, where the differences disappear 2 hours post mating. We may contribute this to increased cell density, secondary plasmid transfer and possible switch between Ebp on/off cells. Under high cell density and under static biofilm settings plasmid copy number significantly increases when compared to planktonic cultures (Cook and Dunny, 2013). Recently Ebp was shown to positively contribute of conjugative pAM β 1 plasmid transfer when compared to *ebp* null counterpart (2.5×10^4 Tc/donor for WT vs 1.5×10^4 Tc/donor for $\Delta ebpABC$) (La Rosa et al., 2016). Detected differences might be accounted not only for the different nature of the plasmid, but also for a longer mating time (5 hours) and higher cell density, where early biofilm aggregates by Ebp are being formed that facilitated the plasmid transfer.

Maintaining a subset of unclumped cells after AS induction in the population may benefit the bacterial population by preserving the possibility of both AS- and Ebp-mediated attachment. For example, during catheter-associated urinary tract infection (CAUTI), Ebp(+) AS(+) cells would bind via the EbpA tip adhesin to fibrinogen that is released as part of an inflammatory cascade (Flores-Mireles et al., 2014), while Ebp(-) AS(+) cells may bind to PMNs where AS promotes intercellular survival of bacteria in PMNs (Rakita et al., 1999). Alternatively, Ebp(+) AS(+) cells may remain attached to the surface via Ebp, while Ebp(-) AS(+) cells clump to colonize another area within the same environment. Ebp

are highly immunogenic, so Ebp(-) AS(+) cells would enable escape from Ebp-mediated adaptive immunity-based clearance. One could propose that the opposite may also be true - that full-length polymerized Ebp may prevent AS-mediated clearance by masking the AS on the surface. However, since we readily detect AS on the surface of Ebp expressing cells (**Figure 6.4**), the data suggests that AS can be readily accessed by small proteins and antibodies and Ebp does not mask AS. Lastly, we show that Ebp interferes with AS-mediated plasmid transfer at early time points after pheromone-induction. With time, the differences in conjugation frequency disappear, likely due to secondary plasmid transfer events from primary transconjugants related to a switch in pilus expression and/or increased density and thus proximity of cells. For planktonic cells, Ebp may contribute to intake which may in turn contribute to genome stability and increased fitness within the population (Starikova et al., 2013).

Apart from clumping and HGT differences caused by co-expression of AS and Ebp at early time points, the two adhesins may also perform coordinated functions in biofilm settings and under higher cellular density under planktonic conditions. As both Ebp and AS contribute to biofilm formation, the situation when the two adhesins are expressed within the same environment may result in more robust biofilm. In such a case, either Ebp or AS can mediate initial attachment with further promotion and spread of biofilm on the surface. In support of this hypothesis, in preliminary IFM, we observed AS-mediated clumps interspersed together with Ebp(+) cells that in the host settings may accelerate biofilm formation when compared to Ebp(+)AS(-) or Ebp(-)AS(-) cells. Finally, the two populations expressing either AS only, or both AS and Ebp, may in some cases display different spatial localization within the biofilm to optimize biofilm adhesive potential, which can be tested by fluorescent-conjugation/staining of AS and Ebp within the biofilm. We propose that Ebp might be important for the initial attachment to the surface, creating the base and skeleton of the biofilm that is further enhanced or built upon by AS-clumped cells. In the next chapter, we seek to address relationship between Ebp and Ace.

7. Chapter 7. Relationship between Ebp and Ace expression

7.1. Ebp expression is reduced at 46°C compared to 37°C

As discussed in **Chapter 6**, Ebp is phase variable and EbpR is its main positive transcriptional regulator (Bourgogne et al., 2007). Ebp expression can be influenced by environmental cues such as serum, glucose, or bicarbonate, where only the latter was tested and shown to activate *ebpABC* transcription via upregulation of *ebpR* (Bourgogne et al., 2010; Kline et al., 2009; Nallapareddy et al., 2011a; Nallapareddy et al., 2006). Ace is another *E. faecalis* adhesin that can bind collagen type IV and I, laminin and dentin (Kowalski et al., 2006; Nallapareddy et al., 2000) and is implicated in endocarditis and UTI (Nallapareddy et al., 2011a; Singh et al., 2010). Ace is positively regulated by the two-component system GrvRS (Roh et al., 2014), where expression of Ace can be induced by serum, components of host extracellular matrix and stress conditions, including bile salts and high temperature of 46°C (Lebreton et al., 2009; Nallapareddy and Murray, 2006; Xiao et al., 1998). To extend our earlier work examining the possible interrelated expression of previously described *E. faecalis* adhesins, we first examined whether or how these known regulators and stress conditions may differentially affect expression of the two adhesins. We utilized transposon mutants of transcription regulators of *ace* and *ebpABC*, namely *ebpR*::Tn and *grvR*::Tn, to test if the known regulators are involved in cross-regulation (if any) of the two adhesins after growth at physiological 37°C and under stress conditions of 46°C. We also used $\Delta ebpABC$ and *ace*::Tn strains as controls and $\Delta srtA$ to test if at 37°C and 46°C the two adhesins will accumulate in the plasma membrane at both temperatures, similar to that observed at 37°C in **Chapter 3**. At 37°C we did not detect any differences in Ebp expression levels within the screened strains, except for higher Ebp levels in $\Delta srtA$ as observed previously (**Chapter 3, Figure 7.1**, top panel). Ebp expression was also similar in *grvRS*::Tn and *ace*::Tn, indicating that neither the absence of

GrvRS nor Ace affects Ebp expression at 37°C. Similarly, at 46°C, we did not observe any Ebp expression differences in *ace::Tn* and *grvS::Tn* compared to the wild type (WT), showing that Ace and the histidine kinase GrvS are not involved in Ebp biogenesis at 46°C.

Figure 7.1 Expression levels of Ebp and Ace at 37°C and 46°C for various mutant strains involved in biogenesis of the two adhesins.

Same whole lysate samples were immunoblotted with EbpC antiserum (top), Ace antiserum (middle), SecA antiserum (bottom) as a loading control.

As expected, we didn't detect Ebp in *ebpABC* and *ebpR* mutants that serve as negative controls for *ebpABC* expression at both 37°C and 46°C. However, we observed decreased Ebp expression at 46°C compared to 37°C; while the same amount of the protein was loaded as determined by SecA blotting (**Figure 7.1**).

In addition, we detected less Ebp at 46°C in *grvR::Tn* when compared to basal levels of Ebp in WT at 46°C (**Figure 7.1**). These two observations will be addressed later in this chapter, and they suggest that Ebp expression is reduced at 46°C and might be inversely regulated with Ace in a temperature-dependent manner and that GrvR might be involved in positive regulation of *ebpABC* at 46°C.

In contrast to Ebp expression, Ace was minimally expressed at 37°C (**Figure 7.1** middle panel), but enriched within $\Delta srtA$ mutant (similar to what has been discussed for accumulation of Ebp and Ace in **Figure 3.4**). We have also observed accumulation of Ace in $\Delta srtA$ at 46°C. This is consistent with the shedding hypothesis proposed in **Chapter 3**, in which Ace is retained on the membrane in a *srtA* mutant, rendering it inaccessible by gelatinase GelE that cleaves Ace from the cell wall at late growth phase and lack of cell wall shedding (Gao et al., 2013). As expected at 46°, we observed no Ace in the absence of GrvRS or in *ace::Tn* that serves as a negative control. Finally, at 46° no changes in Ace expression were observed in *ebpR::Tn* or $\Delta ebpABC$. Taken together, we conclude that Ace is not regulated via EbpR and is not influenced by presence of Ebp.

7.2. Differential Ebp and Ace expression with a temperature shift from 37°C to 46°C

As described above, we were surprised that Ebp expression was decreased at 46°C compared to 37°C, and this did not appear to be a general phenomenon since SecA levels were unchanged. In contrast to Ebp expression, Ace expression is higher at 46°C compared to 37°C (**Figure 7.1**). These observations suggest that under the stress of high temperature, Ebp and Ace are differentially expressed. To further characterize this phenomenon, we examined protein expression at smaller temperature increments of 30°C, 37°C, 42°C and 46°C. Expression of Ace and Ebp changes rapidly in an on/off switch between 42°C and 46°C (**Figure 7.2**).

Increased Ace expression levels at high temperature had been previously observed (Xiao et al., 1998), but temperature dependent expression has not been reported for Ebp. To better understand the mechanism by which Ebp expression levels fall with increasing temperature, we performed both IFM to examine if the fraction of piliated cells changes at 46°C and RT-qPCR to test if *ebpC* is transcriptionally downregulated at 46°C.

Figure 7.2 Temperature dependent shift in Ebp and Ace expression.

Immunoblot with anti-Ebp (top) and anti-Ace (middle) sera and anti-SecA sera at increasing temperatures. Cells were grown at the described temperatures overnight, subcultured into fresh media 1:10 and grown until mid-log for various times (~1 hour 40 min at 37°C; ~2 hour 20 minutes for 30°C and 42°C; 2 hours 40 minutes at 46°C) and later normalized to the same OD₆₀₀ = 0.6

Consistent with immunoblot data showing decreased expression of EbpC at 46°C (**Figure 7.1**), we observed significantly fewer EbpC expressing cells by IFM at 46°C as compared to 37°C (**Figure 7.3A**). Similarly, we observed reduced *ebpC* transcription by RT-qPCR at 46°C as compared to 37°C (**Figure 7.3B**). To

test whether *ebpC* transcription was decreased due to a downregulation of its positive transcriptional regulator *ebpR*, we performed RT-qPCR. However, we observed that *ebpR* is transcribed at the same levels at 46°C and 37°C (**Figure 7.3C**). Together these findings show that piliation decreases at 46°C due to reduced *ebpC* transcription in *ebpR*-transcription independent manner, although EbpR can be also regulated post-transcriptionally.

Figure 7.3 Decreased Ebp and *ebpC* transcription independent of *ebpR* transcriptional changes, at 46°C.

(A) IFM with EbpC antiserum. Error bars represent the standard deviation from 3 independent experiments. RT-qPCR on *ebpC* (B) and *ebpR* (C) on RNA isolated from mid-log bacteria grown at 37°C and 46°C. The experiment was performed in triplicate using $\Delta\Delta Ct$ method to assess expressional changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *ebpC* (B) and *ebpR* (C) transcription, compared to control (WT at 37°C). Statistical analysis was analyzed by unpaired *t*-test using GraphPad **** $p < 0.0001$, * $p < 0.05$, n.s.: $p > 0.05$.

7.3. GrvR negatively affects Ebp expression at 46°C, but not at 37°C

Based on experiments in which we observed decreased Ebp protein expression levels in *grvR::Tn* when compared to the wild type at 46°C but not at 37°C

(Figure 7.1), we hypothesized that *ebpC* might be transcriptionally downregulated at 46°C in *grvR::Tn* compared to WT at 46°C. To test this hypothesis, we grew the strains to mid-log phase as described earlier, performed IFM and RT-qPCR for pilus protein and gene expression, respectively, and detected both fewer piliated cells and decreased transcription of *ebpC* and *ebpR* in the *grvR::Tn* mutant strain as compared to WT (Figure 7.4).

Figure 7.4 Decreased expression of Ebp in *grvR::Tn* at 46°C by downregulation of *ebpC* and *ebpR*.

(A) IFM with EbpC antiserum. Error bars represent the standard deviation from 3 independent experiments. RT-qPCR on *ebpC* (B) and *ebpR* (C) on RNA isolated from mid-log bacteria grown at 46°C. The experiment was performed in triplicate using $\Delta\Delta Ct$ method to assess expressional changes with *gyrA* as a housekeeping gene. Fold change indicates the change in *ebpC* (B) and *ebpR* (C) transcription, compared to control (WT at 46°C). Statistical analysis was analyzed by unpaired *t*-test using GraphPad * $p < 0.05$, ** $p < 0.01$.

In order to establish whether GrvR controls *ebpC* expression via effects on the expression its positive regulator *ebpR*, we tested *ebpR* levels in *grvR::Tn* compared to WT type at 46°C. We observed that, in the absence of GrvR, *ebpR* expression is significantly lower than WT, which may explain the decreased *ebpC* transcription under stress conditions (Figure 7.4B). As GrvR is induced at

46°C (Le Breton et al., 2003), taken together our data suggests that GrvR may act to positively regulate *ebpR* transcription at 46°C, but not at 37°C.

7.4. Discussion

Previously, both Ebp and Ace were shown to be induced by the same environmental cue – serum, suggesting that similar regulation of the two adhesins may take place (Nallapareddy and Murray, 2006). However the adhesion properties of the two adhesins are different, where Ace is mainly responsible for adhesion to collagen, while Ebp contributes to adhesion to fibrinogen (Flores-Mireles et al., 2014; Nallapareddy et al., 2011a). Therefore, different substrate specificities may also suggest that the two can be regulated independently. In early attempts to understand the relationship between Ace and Ebp, the ability of *E. faecalis* to adhere to collagen in the absence of the individual Ace and Ebp adhesins compared to double mutant was tested. In that study, Ebp moderately contributed to adhesion to collagen, whereas adhesion was drastically reduced in Δace (Nallapareddy et al., 2011a). These results suggested that the two adhesins work synergistically and are expressed at the same time. However, the opposite experiment – testing Ebp adherence to fibrinogen has not been tested, so it is yet hard to conclude whether the two adhesins work synergistically in adherence to all host tissues, or just overlap in certain areas. In support of a substrate- or niche-specific synergy hypothesis (rather than broad-spectrum synergy), in a mouse UTI model, Δace and $\Delta ebpABC$ were as attenuated as $\Delta ace\Delta ebpABC$ when compared to WT, suggesting that both adhesins are important in experimental UTI, but may substitute for each other's function (Nallapareddy et al., 2011a).

Here we have tested whether known molecular factors or environmental cues involved in Ebp or Ace biogenesis may similarly or differentially regulate the two adhesins.

We report a significant decrease in Ebp levels at 46°C compared to 37°C, while expression of transcriptional regulator of *ebpABC*, *ebpR*, remained unchanged. In contrast to Ebp, expression of Ace increases with a temperature shift from

37°C to 46°C. While Ace is regulated by the GrvRS TCS under stress conditions and at high temperature, the mechanism by which *E. faecalis* negatively regulates Ebp in response to high temperature is unknown at this time. Disruption of GrvRS increases bacterial survival at high temperature (55°C), suggesting that this TCS may respond to temperature as an environmental cue (Teng et al., 2002). However, GrvRS is not the negative regulator of *ebpABC* at 46°C, since in *grvR::Tn* and *grvS::Tn* the levels of Ebp are not higher than in WT. Moreover, it appears that GrvR is a weak positive regulator of *ebpR* and *ebpC* at 46°C, since in *grvR::Tn* at 46°C (but not at 37°C) transcription of *ebp* is further reduced with concomitant reduction in Ebp piliation compare to WT at 46°C. While many potential TCS are encoded in OG1RF genome, only GrvRS was shown to be differentially regulated under stress of heat (55°C) and acidification and *grvRS* mutants to be attenuated in mouse peritonitis model (Teng et al., 2002). Therefore as for now, we do not consider other TCS as potential regulators of *ebp* expression at 46°C.

Systems involved in bacterial thermosensitive regulation may also play a role in Ebp expression at 46°C. For example, the activity of protein sensors, including transcriptional regulators, chaperones, and proteases may be affected by structural changes caused by temperature shifts that in turn influence expression of other proteins (Klinkert and Narberhaus, 2009). This has been observed for *L. monocytogenes* where the Shine-Dalgarno sequence of transcriptional regulator *prfA* melts at 37°C allowing translation to proceed (Giard et al., 2006). The role of PrfA homologue in *E. faecalis* Ers (for the enterococcal regulator of survival) in the expression of *ace* was tested (Cohen et al., 2013; Giard et al., 2006). No changes in Ace expression were observed in Δ *ers*, however an “Ers box” consisting of a specific sequence (AACATTTGTTG) found in the *ace* promoter is required for *ace* expression (Cohen et al., 2013; Lebreton et al., 2009; Riboulet-Bisson et al., 2008). However, we did not detect “Ers box” within promoter region of *ebpABC* to link Ers to downregulation of *ebpABC* at 46°C. In another Gram-positive bacterium, *S. pyogenes*, capsule synthesis increases at 37°C compared to 30°C, however the mechanism of capsule thermoregulation remains unknown (Kang et al., 2012). Alternatively, RNA-thermosensors undergo conformation

changes at increased temperature, allowing transcription of the target genes (Klinkert and Narberhaus, 2009). Therefore, with respect to Ebp regulation at 46°C, either *ebpABC* RNA or Ebp protein stability might be affected at 46°C resulting in reduced Ebp levels. Even though we did not observe transcriptional change in *ebpR*, the stability of EbpR protein might be affected resulting in reduced *ebp* transcription. To differentiate between these possibilities, we are testing EbpC and EbpR protein and *ebp* RNA stability at 46°C. To achieve this, we are blocking protein synthesis by cm in order to determine stability of Ebp and Ace at 46°C compared to 37°C and we are using a standard RNA half-life analysis method to address RNA stability. (Flahaut et al., 1996a; Selinger et al., 2003).

In **Chapter 5** we showed that DnaK is necessary for efficient Ebp biogenesis (**Figure 5.5**), where *ebpC* transcription is reduced and *ebpR* transcription is unchanged in *dnaK::Tn* (**Figure 5.7**). In this chapter we made similar observations at 46°C, in which *ebp* transcription is reduced but *ebpR* transcription is unchanged in WT (**Figure 7.3B,C**). Since DnaK is crucial for *E. faecalis* survival at 46°C, another explanation for decreased Ebp levels at 46°C is that DnaK is titrated out by heat-destabilized unfolded proteins at this temperature and is no longer available to facilitate Ebp biogenesis (El-Samad et al., 2005; Herman et al., 2003). To test if DnaK is a limiting factor in Ebp expression at 46°C, we will overexpress *dnaK* in WT cells, using *pdnaK*, a multicopy-number plasmid that expresses *dnaK* under control of the *srtA* promoter to test if increased levels of DnaK restore Ebp to levels observed at 37°C.

The second major finding we report in this chapter, in addition to differentially regulated expression of Ebp and Ace by temperature, is the observation of lower Ebp levels in *grvR::Tn* compared to WT at 46°C. The decrease in Ebp cannot be explained by EbpR instability at 46°C, but we need to further validate the dependence of reduced transcription of *ebpC* and *ebpR* on GrvR by complementing *grvR* back to *grvR::Tn* and testing whether this restores Ebp expression back to levels observed in WT at 46°C. The transcriptional regulator

GrvR is a part of the GrvRS TCS that is induced by stress conditions such as bile salt, low pH and high temperature and controls the expression of *ace* in response to these stress conditions in *E. faecalis* (Roh et al., 2014; Teng et al., 2002). In a mouse peritonitis model, the *grvRS* mutant was attenuated, whereas the Δace and $\Delta ebpA$ was not attenuated in the same model, suggesting that *grvRS* is involved in regulating additional virulence factors that are important during mammalian infection at 37°C (Roh et al., 2014; Singh et al., 2007a; Singh et al., 2010; Teng et al., 2002). However, Ebp is not regulated via *grvRS* at 37°C, since we did not detect any differences in Ebp in WT and *grvR::Tn* and *grvS::Tn* at 37°C in OG1RF. Here we show that GrvR might partially positively regulate Ebp expression at 46°C via *ebpR*, as we detect a reduction in Ebp when compared to 37°C in WT. However, as we did not observe the increase in Ebp similar to increase in Ace at 46°C for the WT, we cannot link GrvRS to co-regulation or cross regulation between Ebp and Ace. As discussed above, instability of *ebpC* RNA or protein may explain why Ebp levels are lower at higher temperature. However, as GrvRS is also induced and regulates Ace under the stress of bile salts, examining expression of Ebp under bile salts in WT and *grvR::Tn* and *grvS::Tn* may help determine whether GrvRS plays a role in regulation of the two adhesins in response to various stresses. An alternative condition in which the expression of Ace and Ebp could be examined is exposure to serum, since expression of both of the adhesins increases in the presence of serum.

In the host, bacteria encounter multiple stresses, and while 46°C is not likely to be one of them, other *in vivo* stress conditions that induce virulence factor expression via GrvRS include bile salts, serum, or change in pH. We are currently testing whether Ebp expression is similarly altered in response to bile salts, which may provide insights in relationship between Ace and Ebp and their cross regulation and the relevance of this during infection.

8. Chapter 8. Conclusion

In this thesis we addressed the two major questions: 1) what is the *E. faecalis* cellular response to the deletion of *srtA* and 2) how are Ebp-Ace and Ebp-AS are related?

Here we have shown that in $\Delta srtA$ all the putative SrtA substrates are still transcribed at the same level, and at least two of them – Ebp and Ace – accumulate in the plasma membrane resulted in a hyper piliated state for $\Delta srtA$ compared to WT. Interestingly, while we showed for the first time that *E. faecalis* produce MVs, accumulation of unanchored substrates in the plasma membrane does not contribute to MV formation since a similar number of MV are produced in WT and $\Delta srtA$. However, since piliation is higher in $\Delta srtA$ we observed more pili associated with pure MV. Since perturbation of the plasma membrane does not enhance MV biogenesis we were interested to address the composition of MV to gain some insights into MV biogenesis in *E. faecalis*. We observed that the majority of MV proteins are cytoplasmic, suggesting that a significant amount of cytoplasmic content is being packaged into and exported in MV. We do not yet know what triggers MV formation in Gram-positive bacteria and in *E. faecalis* in particular, but the presence of membrane domain-associated proteins such as flotillin and an abundance of cell-wall digesting enzymes may suggest that MV formation is a guided process and we hope that lipidomics on MV will further advance our understanding of MV biogenesis. In other bacteria, MV biogenesis is thought to be an active process involving of TCS, RNA polymerase sigma factor σB , and active cell wall remodelling, and/or via spontaneous membrane reannealing during the activated phage lytic cycle (Brown et al., 2015; Lee et al., 2013b; Resch et al., 2016). We favour the latter for a role in *E. faecalis* MV biogenesis because we observed an abundance of phage tail proteins by MS and the presence of putative assembled phage tails by TEM in MV fractions, which suggests that these tails were made within bacterial cell and subsequently released into supernatant. The traditional way phage tails would be released

from bacterial cell is via phage endolysin/holin induced cell lysis which would result in explosive cell lysis and spontaneous membrane reannealing to form MV as seen in *P. aeruginosa* (Turnbull et al., 2016). We cannot yet estimate the contribution of explosive cell lysis contributes to *E. faecalis* MV formation, but if it is involved, proteomics and lipidomics analysis may be more challenging, since “random” proteins and lipids are being captured. Another open and interesting question is the biological significance of MV for *E. faecalis*. Since tested strain *E. faecalis* OG1RF does not encode or produce toxins, it is unlikely that MV will trigger host cell death as shown for *S. aureus* (Thay et al., 2013). Instead, we think that MV might be important for cell to cell communication, biofilm formation, and interactions with the host immune system.

Despite the lack of involvement of MV in responding to SrtA substrate accumulation in the plasma membrane, the cell still has to respond to such a presumably large perturbation in a plasma membrane composition. And indeed we have shown that $\Delta srtA$ elicits a strong transcriptional response upregulating chaperone genes, PTS genes, ABC transporters and biosynthesis of antibiotics. A similar response is triggered in the presence of environmental stresses and suggests that $\Delta srtA$ experiences stress (Laport et al., 2004; McMahon et al., 2007; Peng et al., 2017). Moreover, transcriptional upregulation of *dnaK* in $\Delta srtA$ where all of the SrtA proteins are expressed on the same level and may “crowd” the membrane led us to investigate the role of DnaK in SrtA using the example of Ebp substrate biogenesis. We showed that DnaK is an important player in Ebp biogenesis. We summarize the major findings of **Chapters 3,4,5** in **Figure 8.1**.

SrtA is a tempting anti-virulence target since *srtA* mutants of multiple Gram-positive bacteria are attenuated in various infection models (Cossart and Jonquière, 2000; Kharat and Tomasz, 2003; Wu et al., 2014b). However the exact mechanism of reduced virulence of $\Delta srtA$ is unknown. It has generally been thought that *srtA* mutants are attenuated because they lack surface display of important substrate virulence factors. However, here we characterized the cellular response to *srtA* deletion and our data suggest that attenuated virulence may not be simply due to the improper display of adhesins, since piliation increases in $\Delta srtA$ population and fully-assembled pili protrude from the cell while retained on the cell membrane. Therefore, the attenuation of $\Delta srtA$ may also be due to the presence of a cellular stress response and/or secretion of Ebp-enriched MV that can provoke immune response and promote subsequent bacterial clearance. Further research on MV interaction with the host can provide new insights into enterococcal pathogenesis and will hopefully advance strategies to combat enterococcal infections. It is important to note that SrtA

inhibition is not toxic to the cell and should not provoke rapid antibiotic resistance; however, since $\Delta srtA$ experiences stress, multiple pathways including biosynthesis of antibiotics and ABC transporters, that contribute to antibiotic resistance are upregulated (McMahon et al., 2007). From the literature we know that previously stressed bacteria eventually gain higher antibiotic resistance (McMahon et al., 2007). Therefore, treating bacterial infection with non-lethal SrtA inhibitors may make cells more resistant towards subsequent eradication treatment.

Based on **Chapters 3,4,5** we conclude that SrtA itself does not affect transcription of its substrates, suggesting that transcription of substrates is independently regulated by other proteins. In the case of Ebp, EbpR and putative RNase J2 are known to be important for Ebp transcription, while pheromone cCF10 activates AS promoter on the pCF10 plasmid (Bourgogne et al., 2007; Chandler et al., 2005; Gao et al., 2010b). It remains unclear whether the known regulators of these SrtA substrate adhesins can cross-regulate other adhesins, similar to Gram-negative bacteria where surface proteins can be differentially regulated (Holden et al., 2006; Kazmierczak et al., 2015). Moreover, since both Ebp and AS are phase variable, we hypothesized that the two might be differentially expressed in the population. Our results suggest that the two adhesins are independently regulated, where Ebp variable expression is independent of pheromone or AS-expression. Despite the independent regulation of the two adhesins, when both are expressed on the surface cells Ebp interferes with AS-mediated clumping and HGT at early time points but both enhance biofilm formation. While Ebp and AS are simultaneously and independently produced within the population, we observed that at high temperature of 46°C Ebp and another SrtA-dependent substrate, Ace, are differentially regulated. Since *E. faecalis* is a robust bacterium that is able to survive at 60°C for 30 minutes, we think that reduced transcription of Ebp is likely to be a result of a stress response, rather than agglomeration and instability of general enterococcal proteins (Murray, 1990). However, it is yet unclear whether the two adhesins are always differentially expressed, or whether some condition like 46°C differentially regulates the two, while certain environmental cues such

as serum similarly enhances presence of both Ebp and Ace (Shepard and Gilmore, 2002). We summarized the main finding of **Chapters 6 and 7** in **Figure 8.2**.

Hence, knowing which adhesins are co-expressed under which conditions may be leveraged for niche-specific therapies. For example, biofilm formation facilitated by Ebp can be fully restored in the absence of Ebp by AS expression alone. Therefore, to effectively reduce biofilm formation under these conditions, both adhesins should be targeted. On the other hand, Ace and Ebp are inversely regulated by high temperature stress conditions. Understanding the molecular basis of this regulation may provide means to control expression of Ebp to reduce enterococcal virulence.

9. References

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10. Appendices

Appendix A

EbpC antiserum specifically reacts with Ebp presented on the cell surface of WT and $\Delta srtA$ (by IFM) (**Figure 10.1**) and with fully polymerized pili by immunoblot (**Figure 10.2**).

IFM staining with anti-EbpC antibody and Alexa Fluor 568 goat anti-guinea pig secondary antibody of WT,

Appendix B

Table 10.1 List of genes with more abundant mRNAs in $\Delta srtA$ compared to WT.

Genes with significantly different abundance of mRNAs were determined using a PValue and false discovery rate (FDR) cutoff of 0.05. The Log2FC represents the log2 change between biological triplicate of $\Delta srtA$ compared to WT.

gene	log ₂ FC	Gene	Annotation	KEGG ID
OG1RF_10029	1.0876	-	hypothetical protein	K14205
OG1RF_10057	4.3518	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	K15580
OG1RF_10074	2.6261	-	hypothetical protein	
OG1RF_10075	2.2718	-	hypothetical protein	
OG1RF_10098	1.9040	argR	arginine repressor	K03402
OG1RF_10099	5.2602	arcA	arginine deiminase	K01478
OG1RF_10100	5.4360	arcB	ornithine carbamoyltransferase	K00611
OG1RF_10101	4.6589	arcC	carbamate kinase	K00926
OG1RF_10102	3.9135	ntcA	global nitrogen regulator NtcA	
OG1RF_10103	4.1272	-	UIT3 family protein	
OG1RF_10107	1.6233	-	family 20 glycosyl hydrolase	K12373
OG1RF_10108	1.3817	-	endoribonuclease L-PSP	
OG1RF_10122	1.4629	deoC	deoxyribose-phosphate aldolase	K01619
OG1RF_10124	1.1938	-	ABC superfamily ATP binding cassette transporter, binding protein	K07335
OG1RF_10125	1.1205	-	ABC superfamily ATP binding cassette transporter, binding protein	K07335
OG1RF_10127	1.3193	-	ABC superfamily, ATP binding cassette transporter, membrane protein	K02057
OG1RF_10128	1.3588	-	ABC superfamily, ATP binding cassette transporter, membrane protein	K02057
OG1RF_10198	2.1160	aldA	aldehyde dehydrogenase	

OG1RF_10202	0.9585	-	polysaccharide biosynthesis family protein	K03328
OG1RF_10226	1.0121	fabF	3-oxoacyl-(acyl-carrier-protein) synthase II	K09458
OG1RF_10282	1.3959	-	vitamin-B12 independent methionine synthase	K00549
OG1RF_10296	4.1219	mtlA2	PTS family mannitol porter, EIICB component	K02799
OG1RF_10297	4.3439	mtlF2	PTS family fructose/mannitol porter component IIA	K02798
OG1RF_10298	3.7666	mtlD	mannitol-1-phosphate 5-dehydrogenase	K00009
OG1RF_10303	1.4790	-	ABC superfamily ATP binding cassette transporter, membrane protein	K02004
OG1RF_10354	1.3815	-	hypothetical protein	K06872
OG1RF_10394	1.9042	yebC	glucose-1-phosphate adenylyltransferase	
OG1RF_10395	2.3944	-	C_GCAxxG_C_C family protein	
OG1RF_10414	1.4639	algC	phosphomannomutase	
OG1RF_10415	1.5236	-	GNAT family acetyltransferase	
OG1RF_10417	1.1931	pbp2A	penicillin-binding protein 2A	K12555
OG1RF_10431	2.3467	fruK	1-phosphofructokinase	K00882
OG1RF_10432	2.3159	-	PTS family fructose porter component IIBC	K02769
OG1RF_10442	1.3691	-	putative lipoprotein	
OG1RF_10717	1.4357	argR3	arginine repressor	K03402
OG1RF_10751	1.5249	lacF	PTS family lactose/cellobiose porter component IIA	K02759
OG1RF_10752	1.4957	-	PTS family lactose/cellobiose (lac) porter component IIC	K02761
OG1RF_10753	1.5862	bglA2	6-phospho-beta-glucosidase	K01223
OG1RF_10778	1.4032	pfkA	6-phosphofructokinase	K00850
OG1RF_10838	1.7867	-	NRAMP family Mn ²⁺ /Fe ²⁺ transporter	K03322
OG1RF_10895	3.1800	-	glutamine ABC superfamily ATP binding cassette	K10040

			transporter, membrane protein	
OG1RF_10896	3.3847	-	amino acid ABC superfamily ATP binding cassette transporter, membrane protein	K10040
OG1RF_10897	3.4251	-	glutamine ABC superfamily ATP binding cassette transporter, binding protein	K10039
OG1RF_10898	3.5913	-	ABC superfamily ATP binding cassette transporter, ABC protein	K10041
OG1RF_11003	2.6443	-	ABC superfamily ATP binding cassette transporter, membrane protein	K17319
OG1RF_11004	3.5590	-	ABC superfamily ATP binding cassette transporter, membrane protein	K17320
OG1RF_11078	1.2322	dnaK	chaperone DnaK	K04043
OG1RF_11079	1.7802	-	hypothetical protein	
OG1RF_11080	1.3236	dnaJ	chaperone DnaJ	K03686
OG1RF_11146	1.0807	gldA	glycerol dehydrogenase	K00005
OG1RF_11182	2.3333	moaA	molybdenum cofactor biosynthesis protein A	K03639
OG1RF_11183	3.5380	-	MOSC domain-containing protein	
OG1RF_11184	2.9081	moaB	molybdenum cofactor biosynthesis protein B	
OG1RF_11185	2.6537	-	molybdopterin-binding domain protein	
OG1RF_11227	6.6353	clcB	L-alanine-DL-glutamate epimerase	
OG1RF_11228	5.2932	-	transglutaminase domain- containing protein	
OG1RF_11229	5.5184	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	K15580
OG1RF_11283	1.0833	aroC	chorismate synthase	K01736
OG1RF_11363	1.3888	-	abortive infection protein	K07052
OG1RF_11430	1.5433	pyrB	aspartate carbamoyltransferase	K00609
OG1RF_11431	1.7319	pyrP	NCS family uracil:cation	K02824

			symporter	
OG1RF_11432	1.7877	upp	uracil phosphoribosyltransferase	K02825
OG1RF_11534	1.6352	-	membrane protein	
OG1RF_11535	1.7554	-	WxL domain surface protein	
OG1RF_11537	1.4893	-	hypothetical protein	
OG1RF_11591	1.7097	glpO	glycerol-3-phosphate oxidase	K00105
OG1RF_11611	2.4201	-	phosphosugar isomerase	
OG1RF_11612	4.2416	-	phosphosugar-binding protein	
OG1RF_11613	4.9839	-	PTS system mannose/fructose/sorbose transporter subunit IID	K02796
OG1RF_11614	4.7834	-	PTS family mannose/fructose/sorbose porter component IIC	K02795
OG1RF_11615	4.1471	ulaB	PTS family ascorbate porter, IIB component	K02794
OG1RF_11616	4.0277	-	PTS system mannose/fructose/sorbose transporter subunit IIA	K02793
OG1RF_11753	1.3296	treB	PTS family trehalose porter, IIBC component	K02818
OG1RF_11771	2.7938	-	brp/Blh family beta-carotene 15,15'-monooxygenase	
OG1RF_11871	1.2692	-	hypothetical protein	
OG1RF_11872	1.4672	-	hypothetical protein	K07220
OG1RF_11873	1.5152	-	phosphate transporter	K03306
OG1RF_11893	1.4183	suhB	inositol-phosphate phosphatase	K01092
OG1RF_11894	1.2987	-	hypothetical protein	
OG1RF_11941	2.4103	gltA	glutamate synthase	K00266
OG1RF_11949	1.8984	-	putative cysteine desulfurase	
OG1RF_12135	1.4552	thiD2	phosphomethylpyrimidine kinase	K00941
OG1RF_12168	1.4450	-	integrating conjugative element protein	
OG1RF_12178	1.1093	accA	acetyl-coA carboxylase carboxyl transferase subunit	K01962

			alpha	
OG1RF_12235	1.8464	-	S1 family extracellular protease	K02799
OG1RF_12279	2.3477	allB	allantoinase	K01466
OG1RF_12350	1.2688	-	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	K15580
OG1RF_12366	3.2080	-	peptide ABC superfamily ATP binding cassette transporter, binding protein	K02035
OG1RF_12367	3.2311	-	ABC superfamily ATP binding cassette transporter, ABC protein	K02034
OG1RF_12368	2.7726	-	oligopeptide ABC superfamily ATP binding cassette transporter, membrane protein	K02033
OG1RF_12369	2.2879	-	ABC superfamily ATP binding cassette transporter, ABC protein	K02032
OG1RF_12370	2.0810	-	ABC superfamily ATP binding cassette transporter, ABC protein	K02031
OG1RF_12402	2.4109	sorC	PTS family mannose/fructose/sorbose porter component IIC	K02795
OG1RF_12403	2.7389	gldA2	putative glycerol dehydrogenase	K08317
OG1RF_12404	2.3585	-	D-isomer specific 2-hydroxyacid dehydrogenase	
OG1RF_12425	1.4251	trePP	glycosyl hydrolase	
OG1RF_12426	1.2086	yvdM	beta-phosphoglucomutase	K01838
OG1RF_12430	3.2334	-	hypothetical protein	
OG1RF_12456	1.0155	-	WxL domain surface protein	
OG1RF_12523	1.6139	-	hydantoinase/oxoprolinase	
OG1RF_12524	1.7661	-	hypothetical protein	K09703
OG1RF_12564	1.1026	citF	citrate (pro-3S)-lyase	K01643
OG1RF_12569	1.6303	gcdB	glutaconyl-CoA decarboxylase	K01572
OG1RF_12571	1.6551	-	hypothetical protein	

Appendix C

Full list of the KEGG pathways of genes with more abundant mRNA in OG1RF Δ srtA compared to WT. Obtained from KEGG-mapper – Search Pathway using ‘efi’ (for *Enterococcus faecalis*) as a reference organism and the KEGG IDs listed in **Appendix B**.

ko01100 Metabolic pathways (23)

ko:K00005 gldA; glycerol dehydrogenase [EC:1.1.1.6]

ko:K00266 gldD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13
1.4.1.14]

ko:K00549 metE; 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [EC:2.1.1.14]

ko:K00609 pyrB; aspartate carbamoyltransferase catalytic subunit [EC:2.1.3.2]

ko:K00611 OTC; ornithine carbamoyltransferase [EC:2.1.3.3]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko:K00941 thiD; hydroxymethylpyrimidine/phosphomethylpyrimidine kinase [EC:2.7.1.49 2.7.4.7]

ko:K01092 E3.1.3.25; myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]

ko:K01466 allB; allantoinase [EC:3.5.2.5]

ko:K01478 arcA; arginine deiminase [EC:3.5.3.6]

ko:K01572 oadB; oxaloacetate decarboxylase, beta subunit [EC:4.1.1.3]

ko:K01736 aroC; chorismate synthase [EC:4.2.3.5]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko:K02769 PTS-Fru-EIIB; PTS system, fructose-specific IIB component [EC:2.7.1.202]

ko:K02793 PTS-Man-EIIA; PTS system, mannose-specific IIA component [EC:2.7.1.191]

ko:K02794 PTS-Man-EIIB; PTS system, mannose-specific IIB component [EC:2.7.1.191]

ko:K02795 PTS-Man-EIIC; PTS system, mannose-specific IIC component

ko:K02796 PTS-Man-EIID; PTS system, mannose-specific IID component

ko:K02825 pyrR; pyrimidine operon attenuation protein / uracil phosphoribosyltransferase [EC:2.4.2.9]

ko:K03639 moaA; GTP 3',8-cyclase [EC:4.1.99.22]

ko:K09458 fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko01110 Biosynthesis of secondary metabolites (8)

ko:K00105 E1.1.3.21; alpha-glycerophosphate oxidase [EC:1.1.3.21]

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13
1.4.1.14]

ko:K00549 metE; 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [EC:2.1.1.14]

ko:K00611 OTC; ornithine carbamoyltransferase [EC:2.1.3.3]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K01478 arcA; arginine deiminase [EC:3.5.3.6]

ko:K01736 aroC; chorismate synthase [EC:4.2.3.5]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko01120 Microbial metabolism in diverse environments (6)

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13
1.4.1.14]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko:K01466 allB; allantoinase [EC:3.5.2.5]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko:K02769 PTS-Fru-EIIB; PTS system, fructose-specific IIB component [EC:2.7.1.202]

ko01130 Biosynthesis of antibiotics (6)

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13 1.4.1.14]

ko:K00611 OTC; ornithine carbamoyltransferase [EC:2.1.3.3]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K01478 arcA; arginine deiminase [EC:3.5.3.6]

ko:K01736 aroC; chorismate synthase [EC:4.2.3.5]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko01200 Carbon metabolism (3)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko01212 Fatty acid metabolism (2)

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko:K09458 fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]

ko01230 Biosynthesis of amino acids (5)

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13 1.4.1.14]

ko:K00549 metE; 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [EC:2.1.1.14]

ko:K00611 OTC; ornithine carbamoyltransferase [EC:2.1.3.3]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K01736 aroC; chorismate synthase [EC:4.2.3.5]

ko00010 Glycolysis / Gluconeogenesis (2)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K01223 E3.2.1.86B; 6-phospho-beta-glucosidase [EC:3.2.1.86]

ko00030 Pentose phosphate pathway (2)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K01619 deoC; deoxyribose-phosphate aldolase [EC:4.1.2.4]

ko00051 Fructose and mannose metabolism (10)

ko:K00009 mtlD; mannitol-1-phosphate 5-dehydrogenase [EC:1.1.1.17]

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K00882 fruK; 1-phosphofructokinase [EC:2.7.1.56]

ko:K02769 PTS-Fru-EIIB; PTS system, fructose-specific IIB component [EC:2.7.1.202]

ko:K02793 PTS-Man-EIIA; PTS system, mannose-specific IIA component [EC:2.7.1.191]

ko:K02794 PTS-Man-EIIB; PTS system, mannose-specific IIB component [EC:2.7.1.191]

ko:K02795 PTS-Man-EIIC; PTS system, mannose-specific IIC component

ko:K02796 PTS-Man-EIID; PTS system, mannose-specific IID component

ko:K02798 PTS-Mtl-EIIA; PTS system, mannitol-specific IIA component [EC:2.7.1.197]

ko:K02799 PTS-Mtl-EIIB; PTS system, mannitol-specific IIB component [EC:2.7.1.197]

ko00052 Galactose metabolism (1)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko00500 Starch and sucrose metabolism (5)

ko:K01223 E3.2.1.86B; 6-phospho-beta-glucosidase [EC:3.2.1.86]

ko:K01838 pgmB; beta-phosphoglucomutase [EC:5.4.2.6]

ko:K02759 PTS-Cel-EIIA; PTS system, cellobiose-specific IIA component [EC:2.7.1.196 2.7.1.205]

ko:K02761 PTS-Cel-EIIC; PTS system, cellobiose-specific IIC component

ko:K02818 PTS-Tre-EIIB; PTS system, trehalose-specific IIB component [EC:2.7.1.201]

ko00520 Amino sugar and nucleotide sugar metabolism (5)

ko:K02793 PTS-Man-EIIA; PTS system, mannose-specific IIA component [EC:2.7.1.191]

ko:K02794 PTS-Man-EIIB; PTS system, mannose-specific IIB component [EC:2.7.1.191]

ko:K02795 PTS-Man-EIIC; PTS system, mannose-specific IIC component

ko:K02796 PTS-Man-EIID; PTS system, mannose-specific IID component

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00620 Pyruvate metabolism (2)

ko:K01572 oadB; oxaloacetate decarboxylase, beta subunit [EC:4.1.1.3]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko00640 Propanoate metabolism (2)

ko:K00005 gldA; glycerol dehydrogenase [EC:1.1.1.6]

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko00562 Inositol phosphate metabolism (1)

ko:K01092 E3.1.3.25; myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]

ko00720 Carbon fixation pathways in prokaryotes (1)

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko00680 Methane metabolism (1)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko00910 Nitrogen metabolism (2)

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13
1.4.1.14]

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko00061 Fatty acid biosynthesis (2)

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha
[EC:6.4.1.2]

ko:K09458 fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]

ko00561 Glycerolipid metabolism (1)

ko:K00005 gltA; glycerol dehydrogenase [EC:1.1.1.6]

ko00564 Glycerophospholipid metabolism (1)

ko:K00105 E1.1.3.21; alpha-glycerophosphate oxidase [EC:1.1.3.21]

ko00230 Purine metabolism (2)

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko:K01466 allB; allantoinase [EC:3.5.2.5]

ko00240 Pyrimidine metabolism (2)

ko:K00609 pyrB; aspartate carbamoyltransferase catalytic subunit [EC:2.1.3.2]

ko:K02825 pyrR; pyrimidine operon attenuation protein / uracil
phosphoribosyltransferase [EC:2.4.2.9]

ko00250 Alanine, aspartate and glutamate metabolism (2)

ko:K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13
1.4.1.14]

ko:K00609 pyrB; aspartate carbamoyltransferase catalytic subunit [EC:2.1.3.2]

ko00270 Cysteine and methionine metabolism (1)

ko:K00549 metE; 5-methyltetrahydropteroyltriglutamate--homocysteine
methyltransferase [EC:2.1.1.14]

ko00220 Arginine biosynthesis (3)

ko:K00611 OTC; ornithine carbamoyltransferase [EC:2.1.3.3]

ko:K00926 arcC; carbamate kinase [EC:2.7.2.2]

ko:K01478 arcA; arginine deiminase [EC:3.5.3.6]

ko00400 Phenylalanine, tyrosine and tryptophan biosynthesis (1)

ko:K01736 aroC; chorismate synthase [EC:4.2.3.5]

ko00450 Selenocompound metabolism (1)

ko:K00549 metE; 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [EC:2.1.1.14]

ko00513 Various types of N-glycan biosynthesis (1)

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00531 Glycosaminoglycan degradation (1)

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00603 Glycosphingolipid biosynthesis - globo and isoglobo series (1)

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00604 Glycosphingolipid biosynthesis - ganglio series (1)

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00550 Peptidoglycan biosynthesis (1)

ko:K12555 pbp2A; penicillin-binding protein 2A [EC:2.4.1.129 2.3.2.-]

ko00511 Other glycan degradation (1)

ko:K12373 HEXA_B; hexosaminidase [EC:3.2.1.52]

ko00730 Thiamine metabolism (1)

ko:K00941 thiD; hydroxymethylpyrimidine/phosphomethylpyrimidine kinase [EC:2.7.1.49 2.7.4.7]

ko00780 Biotin metabolism (1)

ko:K09458 fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]

ko00790 Folate biosynthesis (1)

ko:K03639 moaA; GTP 3',8-cyclase [EC:4.1.99.22]

ko00253 Tetracycline biosynthesis (1)

ko:K01962 accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]

ko00521 Streptomycin biosynthesis (1)

ko:K01092 E3.1.3.25; myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]

ko04122 Sulfur relay system (1)

ko:K03639 moaA; GTP 3',8-cyclase [EC:4.1.99.22]

ko03018 RNA degradation (2)

ko:K00850 pfkA; 6-phosphofructokinase 1 [EC:2.7.1.11]

ko:K04043 dnaK; molecular chaperone DnaK

ko02010 ABC transporters (6)

ko:K10039 ABC.GLN1.S; putative glutamine transport system substrate-binding protein

ko:K10040 ABC.GLN1.P; putative glutamine transport system permease protein

ko:K10041 ABC.GLN1.A; putative glutamine transport system ATP-binding protein [EC:3.6.3.-]

ko:K15580 oppA; oligopeptide transport system substrate-binding protein

ko:K17319 lplB; putative aldouronate transport system permease protein

ko:K17320 lplC; putative aldouronate transport system permease protein

ko02060 Phosphotransferase system (PTS) (10)

ko:K02759 PTS-Cel-EIIA; PTS system, cellobiose-specific IIA component [EC:2.7.1.196 2.7.1.205]

ko:K02761 PTS-Cel-EIIC; PTS system, cellobiose-specific IIC component

ko:K02769 PTS-Fru-EIIB; PTS system, fructose-specific IIB component [EC:2.7.1.202]

ko:K02793 PTS-Man-EIIA; PTS system, mannose-specific IIA component
[EC:2.7.1.191]

ko:K02794 PTS-Man-EIIB; PTS system, mannose-specific IIB component
[EC:2.7.1.191]

ko:K02795 PTS-Man-EIIC; PTS system, mannose-specific IIC component

ko:K02796 PTS-Man-EIID; PTS system, mannose-specific IID component

ko:K02798 PTS-Mtl-EIIA; PTS system, mannitol-specific IIA component
[EC:2.7.1.197]

ko:K02799 PTS-Mtl-EIIB; PTS system, mannitol-specific IIB component
[EC:2.7.1.197]

ko:K02818 PTS-Tre-EIIB; PTS system, trehalose-specific IIB component
[EC:2.7.1.201]

ko02020 Two-component system (2)

ko:K01643 citF; citrate lyase subunit alpha / citrate CoA-transferase
[EC:2.8.3.10]

ko:K14205 mprF; phosphatidylglycerol lysyltransferase [EC:2.3.2.3]

Appendix D

Predicted DnaK binding domains of the proteins involved in Ebp biogenesis. FASTA sequence for the SrtC, EbpC, SrtA and SecA was downloaded from NCBI database, using the gene number as an enquiry. The sequence was analysed by online DnaK-binding site prediction software (Switch Laboratory, 2017)

SrtC: OG1RF_10872

```
>AEA93559.1 sortase [Enterococcus faecalis OG1RF]
MKSKKKRRIIDGFMILLIIIGIGAFAYPFVSDALNNYLDQQIIAHYQAKASQENTKEMAELQEKMEKKNQ
ELAKKGSNPGLDPFSETQKTTKKPKDSYFESHTIGVLTIPKINVRLPIFDKTNALLLEKGSSLLEGTSP
TGGANTHAVISGHRGLPQAKLFTDLPELKKGDEFYIEVNGKTLAYQVDQIKTVEPTDTKDLHIESGQDLV
TLLTCTPYMINSHRLLVRGHRIPYQPEKAAAGMKKVAQQQNLWTLTLLIACALIIISGFIIWYKRRKKT
RKPK
```

Entry name	Position	Sequence	Score
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	14-20	MILLII	16.1
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	15-21	ILLIIG	16.4
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	35-41	NNYLDQQ	11.1
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	113-119	NVRLPIF	14.2
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	124-130	ALLLEKG	12.9
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	160-166	KLFTDLP	12.0
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	173-179	EFYIEVN	14.1
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	208-214	DLVTLLT	11.4

OG1RF]			
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	222-228	SHRLLVR	12.0
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	223-229	HRLLVRG	14.0
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	250-256	QNLLLWT	25.5
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	251-257	NLLLWTL	12.1
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	253-259	LLWTLLL	11.8
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	255-261	WTLIIA	16.2
AEA93559.1 sortase [<i>Enterococcus faecalis</i> OG1RF]	267-273	SGFIIWY	19.3

EbpC: 10871

>AEA93558.1 cell wall surface anchor family protein [*Enterococcus faecalis* OG1RF]

MKQLKKVWYTISTLLLILPLFTSVLGTTTAF AEENGES AQLVIHKKKMTDLPDPLIQNSGKEMSEFDKYQ
GLADVTFISIYNTSEFYEQRAAGASVDAAKQAVQSLTPGKPVAQGTTDANGNVTVQLPKKQNGKDAVYTI
KEEPKEGVVAATNMVVAFPVYEMIKQADGSYKYGTEELAVVHIYPKNVVANDGSLHVKKVGTAENEGNG
AEFVISKSEGSPTVKYIQGVKDGLYTWTTDKAQAKRFITGKSYEIGENDFTEAENGTEGELTVKNLEVG
YILEEVKAPNNAELIENQTKTPFTIEANNQTPVEKTVKNDTSKVDKTTPSLDGKDVAIGEKIKYQISVNI
PLGIADKEGDANKYVKFNLVDKHDAALTFDNVTSGEYAYALYDGDTVIAPENYQVTEQANGFTVAVNPAY
IPTLTPGGTLKFVYFMHLNEKADPTKGFKNEANVDNGHTDDQTPPTVEVVTGGKRFIKVDGDTVATQALA
GASFVVRDQNSDTANYLKIDETTKAATWVKTKAEATFTTTADGLVDITGLKYGTYYLEETVAPDDYVLL
TNRIEFVVNEQSYGTTENLVSPKVPNKHGKTL PSTGGKGIYVYLGSGAVLLLLIAGVYFARRRRKENA

Entry name	Position	Sequence	Score
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	8-14	WYTISTL	17.2
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	13-19	TLLLILP	17.5
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	14-20	LLLILPL	15.9
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	15-21	LLILPLF	15.5
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	124-130	TVQLPKK	11.1
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	362-368	NKYVKFN	14.5
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	430-436	LKFVYFM	15.4
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	545-551	TYYLEET	12.7
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	556-562	DYVLLTN	15.3
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	557-563	YVLLTNR	15.5
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	600-606	GIYVYLG	14.4
AEA93558.1 cell wall surface anchor family	602-608	YVYLGSG	11.9

Entry name	Position	Sequence	Score
protein [<i>Enterococcus faecalis</i> OG1RF]			
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	609-615	AVLLLIA	13.3
AEA93558.1 cell wall surface anchor family protein [<i>Enterococcus faecalis</i> OG1RF]	610-616	VLLLIAG	13.9

Sortase A OG1RF_12327

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>AEA95014.1 sortase SrtA [Enterococcus faecalis OG1RF]
MRPKEKKRGKNWLNINSLVLLFIIGLALIFNNQIRSWVQQNSRSYAVSKLKPADVKKNMARETTDFDSD
VESLSTEAVMKAQFENKNLPVIGAIAPISVEINLPPIFKGLSNVALLTGAGTMKEDQVMGKNNYALASHRT
EDGVSLFSPLETRTKKDELIYITDLSTVYTYKITSVEKIEPTRVELIDDPVPGQNMITLITCGDLQATTRIA
VQGTLAATTPIKDANDDMLKAFQLEQKTLADWVA
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Entry name	Position	Sequence	Score
AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	10-16	KNWLINS	11.1
AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	16-22	SLLVLLF	16.2
AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	17-23	LLVLLFI	15.8
AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	18-24	LVLLFII	15.1
AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	25-31	GLALIFN	12.0

Entry name	Position	Sequence	Score
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AEA95014.1 sortase SrtA [<i>Enterococcus faecalis</i> OG1RF]	166-172	TVYTYKI	11.8
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SecA OG1RF_11476

>AEA94163.1 preprotein translocase subunit SecA [*Enterococcus faecalis* OG1RF]

MKGTQIPMANFLKKMIENDKKELRRLEKIADKIDAHASAMEQLSDEQLREKTDEFKARYQKGETLDELPL
EAFVAVVREAAKRVLGFLFPYRVQLMGGIVLHDGNIPEMRTGEGKTLTATMPVYLNALSGEGVHVTVVNEYL
ATRDSNEMGELYNFLGLSVGLNINSKSSDEKREAYNCDITYSTNNELGFDYLRDNMVVYRSQMVQRPLNY
AIVDEVDSILIDEARTPLIISGQAEKSTALYTRADNFVKRLKEDEYKIDIQSKTIGLTEAGIEKAEQTF
GLDNLYDIENTALTHHLDQALRANYIMLLDIDYVVQDNKVLIVDQFTGRIMDGRRYSDGLHQAI EAKEGV
EIEDETKTMTATITFQNYFRMYKKLAGMTGTAKTEEEEFREIYNIQVIQIPTNRPIIRDDRPDLLYPTLES
KFNAVVEDIKERYHKGQPVLVGTAVETSELLSDKLNAAKIPHEVLNAKNHFKEAEIIMNAGQKGAVTIA
TNMAGRGTDIKGLGVLELGGLAVIGTERHESRRIDNQLRGRAGRQGDPGVSQFYLSLEDDLMKRFGSR
IKTFLERMNVQEEDAVIQSKMFTRQVESAQKRVEGNNDTRKNVLQYDDVMREQREVIYAQRQEVIMEEN
DLSVDLMGMVKRTIGRVVDSHTQLEKEEWNLDGIVDFAASTLVHEDTISKKDLENKSAEEIKDYLVARAQ
EVFEEKSQQLNGQEQLLEFEKVVLIRVVDTKWDHIDAMDQLRQSVGLRAYGQNNPLVEYQTEGYSMYNN
MVGSI EYEVTRLFMKSEIRQNVQREQVAQGQAEHPETEQDAAARSNTSAKRQPVVRVDKKVGRNDLCPCGS
GKKFKNCHGRNA

Entry name	Position	Sequence	Score
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AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	20-26	KKELRRL	11.1
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AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	81-87	KRVLGLF	14.8
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AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	152-158	YNFLGLS	13.2
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AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	303-309	ANYIMLL	12.8
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Entry name	Position	Sequence	Score
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	304-310	NYIMLLD	12.9
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	365-371	QNYFRMY	11.4
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	366-372	NYFRMYK	14.4
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	367-373	YFRMYKK	16.1
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	396-402	VIQIPTN	11.7
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	543-549	QFYLSLE	15.8
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	692-698	KDYLVAR	15.0
AEA94163.1 preprotein translocase subunit SecA [<i>Enterococcus faecalis</i> OG1RF]	720-726	EKVVILR	11.7

11. Conference presentation and publications

- Gordon Research Conference on Bacterial Cell Surfaces (GRC) held on June 26 - July 1, 2016 in Mount Snow, USA. (Poster presentation).
- Gordon Research Seminar on Bacterial Cell Surfaces (GRS) held on June 25-26, 2016 in Mount Snow, USA. (Oral presentation).

Mitra, S. D., **I. Afonina** and K. A. Kline (2016). "Right Place, Right Time: Focalization of Membrane Proteins in Gram-Positive Bacteria." Trends Microbiol 24(8): 611-621.