The role of sequence elements and protein interactions in focal subcellular localization of Sortase A in *Enterococcus faecalis*

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**The role of sequence elements and protein interactions in focal subcellular
localization of Sortase A in *Enterococcus faecalis***

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ABSTRACT

Sortase A (SrtA) is a transmembrane protein responsible for covalently anchoring several virulence factors and adhesins to the cell wall of Gram-positive organisms, including Enterococci. In *E. faecalis*, SrtA localizes to single foci at the septum during early division phases and reorients to multiple foci at sites of nascent cell division during later stages of the cell cycle. Structurally, SrtA consists of an N-terminal positively charged cytoplasmic tail, a single transmembrane helix (TMH), and a C-terminal catalytic domain. In this thesis, we identify factors that govern the localization of SrtA in *E. faecalis*. We carried out alanine scan mutagenesis to identify important residues on the cytoplasmic tail and TMH region that are important for focal localization (fused to GST) and identified seven individual mutations that resulted in mislocalization of GST. While these amino acids, individually, did not perturb localization in full-length SrtA, the Asn residue at position 31 on the TMH showed a defect in SrtA substrate attachment to the cell wall. We also identified novel interacting partners of SrtA through crosslinking and co-immunoprecipitation. Mass spectrometry analysis revealed putative interacting proteins including chaperone proteins DnaK, GroEL, and HtrA; cell division protein DivIVA; signal recognition particle receptor FtsY; and cell wall machinery protein FtsI. We validated the interactions *in vivo* and identified DnaK and FtsY to be the strongest interacting partners of SrtA. We further demonstrate that in a DnaK mutant background, SrtA is mislocalized to one half of the cell in the mid-division growth phase suggesting that DnaK governs SrtA in a cell cycle dependent manner. Together these findings suggest that SrtA localization to distinct foci in *E. faecalis* may be governed, independently or in conjunction, by sequence elements and multiple protein-protein interactions at the septum of the cell.

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ABBREVIATIONS

°C – degree Celsius

A. oris – *Actinomyces oris*

AS – Aggregation substance

Asc10 – aggregation substance

B. subtilis – *Bacillus subtilis*

BCV – Big colony variant

BHI – Brain Heart Infusion

BSA - bovine serum albumin

CWSS – cell wall sorting signal

DSP – Dithiobis(succinimidylpropionate)

E. coli – *Escherichia coli*

E. faecalis – *Enterococcus faecalis*

Ebp – Endocarditis and biofilm associated pili

Esp – Enterococcal surface protein

F-plasmid – Fertility plasmid

GST – Glutathione-S-transferase

HA – Hemagglutinin

HMWL – high-molecular-weight ladder

HRP – Horseradish peroxidase

HSP – Heat shock protein

IFM – Immunofluorescence microscopy

kDa – Kilodalton

µg – microgram

µm – micrometre

M. smegmatis – *Mycobacterium smegmatis*

mins – Minutes

MW – molecular weight

O.D. – Optical density

O/N - overnight

OD – optical density
Pbp – Penicillin binding protein
PBS – Phosphate buffered saline
PFA – Paraformaldehyde
RT – room temperature
S. agalactiae – *Streptomyces agalactiae*
S. aureus – *Staphylococcus aureus*
S. mutans – *Streptococcus mutans*
S. oralis – *Streptococcus oralis*
S. pyogenes – *Streptococcus pyogenes*
SCV – Small colony variant
SrtA – Sortase A
SrtC – Sortase C
SrtE – Sortase E
TCA – Trichloroacetic acid
TMH – Transmembrane helix
Tn – Transposon
TSBG – Tryptic soya broth with glucose
w/v – weight per volume
WT – wild type
v/v – volume per volume

Chapter 1

Introduction

1.1 Localization of proteins in bacteria

Biological processes in all organisms, including bacteria, often require complex protein networks for their efficient execution. This makes protein organization in the bacterial milieu not a stochastic event, but rather a coordinated effort involving protein-protein interactions, protein gradients across the cell cytoplasm, geometric cues such as membrane curvature, protein structure, and sequence elements (Shapiro and Losick 2000, Shapiro, McAdams et al. 2009) (**Fig. 1.1**). Understanding the mechanisms by which proteins localize themselves can provide insight into the correlation between structure and function of proteins, uncover moonlighting proteins, determine the role of non-protein components of the cell such as lipid domains, develop better tools to study single molecules in a cell, and ultimately give us a complete view of how cellular processes are interrelated.

In this thesis, we primarily focus on the localization of the protein secretion and sorting (Sec-Srt) system of Gram-positive bacteria, specifically in *Enterococcus faecalis*, as a model system to define the molecular underpinnings of protein localization-function relationship. The Sec-Srt machinery is responsible for secretion and attachment of several proteins, many of which are virulence factors, making it a gateway between the external and internal environments of the cell.

Four factors contribute to focal localization of Gram-positive proteins on the bacterial membrane.

I. Sequence determinants of protein localization		
Ia. Extracellular domain: mutations (red dots) disrupt PG interactions and result in mislocalization of the protein (dotted arrows). <i>S. pneumoniae:</i> StkP, MapZ <i>M. tuberculosis:</i> PknB	Ib. Intracellular domain: mutations (red dots) cause mislocalization of the protein (dotted arrows). <i>S. pneumoniae:</i> StkP, Wze <i>B. subtilis:</i> YycG	Ic. TMH: mutation result in mislocalization of the protein (dotted arrows). <i>C. perfringens:</i> TcpA, TcpC <i>B. subtilis:</i> DivIVA
II. Protein-protein interactions that mediate focal localization	III. Lipid domains involved in localization	IV. Geometric cues involved in localization
A. Direct interaction at the membrane. B. Initial interactions in the cytoplasm followed by colocalized interaction at the membrane. C. Interaction via an intermediate partner (yellow) to deliver cargo (purple) to the membrane, followed by dissociation of intermediate partner (red arrow). <i>S. pneumoniae:</i> Wze - Wzd; StkP - GpsB; PtkA - MinD <i>E. faecalis:</i> SrtA - AS SrtC - EbpA, EbpC <i>B. subtilis:</i> DivIVA - SecA PtkA - YjoA, YnfE, YvyG, Ugd, SsbA	Depletion of anionic lipids (red) results in mislocalization of the protein (dotted arrows). <i>B. subtilis:</i> SecA DivIVA	Proteins recognize membrane curvature and associate accordingly to the positive curvature (yellow) or to the negative curvature (green). <i>B. subtilis:</i> DivIVA (binds concave) SpoVM (binds convex)

Fig. 1.1: General overview of mechanisms governing localization of membrane proteins in Gram-positive bacteria. Four main factors contribute to the localization of proteins in Gram-positive bacteria: (I) Sequence determinants, (II) Protein-protein interactions, (III) Lipid domains, and (IV) Geometric cues of the cell. Adapted from (Mitra, Afonina et al. 2016)

1.1.1 Protein localization governed by sequence elements

Proteins often rely on signals encoded in their primary amino acid sequence to direct their localization within the cell. Signal peptide sequences in cocci and ovococci

bacteria play a fundamental role in directing cell-wall destined proteins to different parts of the cell (Carlsson, Stalhammar-Carlemalm et al. 2006, DeDent, Bae et al. 2008). A study in *Streptococcus pyogenes* showed that M protein was secreted at the division septum while protein F is preferentially secreted at the old cell poles (Carlsson, Stalhammar-Carlemalm et al. 2006). YSIRK/GS motif peptides direct proteins towards the cross wall of staphylococci while conventional signal peptides direct proteins to peripheral cell poles (DeDent, Bae et al. 2008). This is an added layer of complexity to the existing role of signal sequences in bacterial protein export.

Sortase C2 (SrtC2) in *Actinomyces oris*, responsible for fimbriae polymerization, possesses two transmembrane helices. Mutational studies revealed that the C-terminal transmembrane helix and its 110 amino acid transmembrane-proximal cytoplasmic domain are required for the localization of the protein to distinct domains on the membrane and without which SrtC2 does not localize to the membrane (Wu, Mishra et al. 2012). Mislocalization of SrtC2 in *A. oris* disrupts its ability to co-aggregate with *Streptococcus oralis* and its ability to form biofilms (Wu, Mishra et al. 2012). The cytoplasmic domain may be needed to retain the mature SrtC2 protein within the membrane or may interact with cytosolic factors that coordinate pilus assembly. SrtC in *Enterococcus faecalis* (also called Bps, for biofilm and pilus-associated sortase), which is responsible for covalent assembly of Ebp (endocarditis and biofilm associated pilus) (Nallapareddy, Singh et al. 2006, Kemp, Singh et al. 2007), depends on its positively charged C-terminal cytoplasmic tail for proper localization and efficient pilus assembly (Kline, Kau et al. 2009). SrtE1 in *Streptomyces coelicolor* requires the negatively charged region of its 139 amino acid cytoplasmic extension for aerial hyphae formation (Kattke, Chan et al. 2016) although its role in localizing the protein is unclear. Similarly, in SrtC1 in Group B *Streptococcus*, the two transmembrane helices are

important for function (polymerization of pili) but only the N-terminal helix is needed for localization of SrtC1 to the membrane (Cozzi, Malito et al. 2011). The pilus polymerizing SrtA in *Corynebacterium diphtheria* possesses a single transmembrane helix. In the absence of this transmembrane region, the enzyme is still catalytically active and is able to bind to pilus monomers. However, SrtA is unable to polymerize pili highlighting the need for its transmembrane region and proper localization in the membrane for its function (Guttilla, Gaspar et al. 2009).

1.1.2 Protein localization governed by external cues

Proteins within the cytoplasm diffuse in three-dimensional space, while those on the membrane move in two-dimensional space where they encounter other proteins and potential interacting partners. Two mechanisms seem to govern protein localization: diffuse-and-capture and self-assembly. The diffuse-and-capture phenomenon is wide-spread in bacteria and is a predominant mechanism of protein localization (Shapiro, McAdams et al. 2009).

The localization of SpoIVFB in sporulating *Bacillus subtilis* is a well-characterized example of the diffuse-and-capture mechanism of protein localization. The SpoIVFB is a membrane-embedded metalloprotease that localizes to the outer forespore membrane when the mother cell engulfs the forespore (Rudner, Fawcett et al. 1999).

Fig. 1.2: Diffusion and capture of the metalloprotease SpoIVFB in *B. subtilis*.

SpoIVFB (purple spheres) is recruited by diffusion along the mother cell membrane and captured by SpoIVA (yellow spheres) at the interface between the mother cell and outer forespore membrane. Adapted from (Thanbichler and Shapiro 2008)

SpoIVFB is inserted into the mother cell cytoplasmic membrane and diffuses until captured at the outer forespore membrane by SpoIVA where it catalyzes the cleavage of sigma factors in the forespore (**Fig. 1.2**). Diffuse and capture was also observed in the localization of PleC histidine kinase in *Caulobacter*, where PleC moves through the membrane without any directional bias until captured by its target at the cell pole (Deich, Judd et al. 2004). Thus, some proteins act as anchors to localize process-related proteins in the cell which begs the question: what localizes these anchor proteins in the bacterial cell?

Self-assembly of a protein complex or oligomers can occur by sensing membrane curvature, nucleoid occlusion, or affinity for certain features of the cell envelope (Laloux and Jacobs-Wagner 2014). DivIVA is primarily a cell division protein that self-assembles at sites of negative membrane curvature in *B. subtilis* (Lenarcic, Halbedel et al. 2009) and *S. coelicolor* (Hempel, Wang et al. 2008) by forming oligomers that are able to sense the concave membrane. The concave curvature of the membrane possibly stabilizes the DivIVA oligomer as it reduces the surface area

of the oligomers exposed to the cytosol thereby decreasing chances of detachment and diffusion (Muchova, Kutejova et al. 2002). Proteins also use the lack of nucleoid as a cue for localization since protein oligomers can form more easily in regions that are devoid of bulky polymers such as the nucleoid as was demonstrated in the asymmetric inheritance of protein aggregates in *E. coli*. During cell division under physiological stress conditions in *E. coli*, protein aggregates are found associated with the old-pole cells and not new-pole cells to generate a population free of damaged proteins. Aggregation of damaged proteins to the old-pole cells is driven by nucleoid occlusion where protein aggregates are found in regions lacking chromosomal DNA (Wayne, Sham et al. 2010).

Membrane properties such as the presence of specific phospholipids and lipid rafts can also affect the localization of membrane proteins. Functional membrane microdomains in bacteria, or lipid rafts, play an important role in localization of signal transduction proteins and were first identified in governing localization of KinC, a membrane-associated sensor kinase, in *B. subtilis* (LeDeaux, Yu et al. 1995, Lopez and Kolter 2010). In addition to KinC, many proteins involved in signal transduction and protein secretion were identified in these microdomains (Lopez and Kolter 2010, Lopez 2015). The subcellular helical pattern localization of the secretion machinery ATPase SecA in *B. subtilis* depends on the overall phospholipid content of the bacterial membrane. Depletion of the phospholipid phosphatidylglycerol (PG) results in delocalization of SecA (Campo, Tjalsma et al. 2004).

Taken together, proteins rely not only on their inherent sequence or structure, but also on external cues from within the cell for their localization to specific domains. Moreover, domain-specific localization is often linked to the cellular function of the localized proteins.

1.2 Sortase A: model for protein localization-function relationships

Sortase A (SrtA) belongs to the superfamily of sortases (Ilangovan, Ton-That et al. 2001) and is present in all Gram-positive bacteria. SrtA is a membrane bound cysteine transpeptidase responsible for anchoring a variety of proteins to the cell wall (Mazmanian, Liu et al. 1999). Many sortase substrates are virulence factors, making SrtA itself a viable drug target because inhibition of SrtA may limit the surface display of a variety of virulence-associated proteins (Maresso and Schneewind 2008). SrtA was first characterized in *Staphylococcus aureus* and is homologous to SrtA present in other Gram-positive pathogens such as *Enterococcus faecalis*, *Staphylococcus mutans*, *S. pyogenes*, *Streptococcus pneumoniae*, and *B. subtilis* (Mazmanian, Liu et al. 1999). SrtA has a catalytic domain, a single transmembrane helix, and a short cytoplasmic positively charged tail (Mazmanian, Ton-That et al. 2001). SrtA cleaves its substrates which possess a cell wall sorting signal (CWSS) consisting of an LPxTG motif, a C-terminal hydrophobic region, and a charged tail (Schneewind, Mihaylova-Petkov et al. 1993, Ton-That, Liu et al. 1999). The catalytic domain of SrtA recognizes and cleaves substrate proteins at their LPxTG motif, followed by two nucleophilic reactions that anchor the substrate to the cell wall (Ton-That, Liu et al. 1999, Perry, Ton-That et al. 2002). Specifically, the cysteine residue in the SrtA catalytic domain attacks the carbonyl backbone of the threonine residue within the LPxTG in the substrate to form an acyl-enzyme ester intermediate. The catalytic domain also recognizes the cell wall precursor, lipid II (undecaprenyl-pyrophosphate-MurNac(-l-Ala-d-iGln-l-Lys(NH₂-Gly₅)-d-Ala-d-Ala)-β1-4-GlcNac) as a second substrate and attaches the protein to the amine group of the free lipid II peptide cross bridge (Ruzin, Severin et al. 2002). Lipid II is then incorporated into the cell wall by transglycosylation and transpeptidation

reactions carried out by the cell wall synthesis machinery comprising of penicillin-binding proteins and autolysins.

The process of substrate attachment to lipid II, and not mature peptidoglycan, is significant since crosslinked peptidoglycan does not have free peptide cross-bridges for the nucleophilic reactions necessary to resolve the sortase-protein bond (Petit, Stroming, et al. 1968). This requirement leads to the hypothesis that SrtA localizes at sites of nascent cell wall synthesis. The pattern of SrtA localization therefore varies across different species of Gram-positive bacteria and is often a function of cell morphology, which in turn depends on the location of peptidoglycan synthesis machinery.

1.2.1 SrtA localization in rod-shaped bacteria

Rod-shaped bacteria have two cell wall synthesis machineries: lateral/peripheral and septal. The lateral machinery is responsible for cell elongation prior to cell division and septal machinery for formation of division septa (Zapun, Vernet et al. 2008). Thus, the localization of SrtA and its substrates are probably different in rod-shaped bacteria as compared to cocci that have only possess the septal machinery. To date there is no experimental evidence describing SrtA localization patterns in rod shaped bacteria. However, proteins such as SecA and SecY in *B. subtilis* and the sortase substrates InlA, InlH, InlJ in *Listeria monocytogenes* display a helical distribution along the cell membrane and cell wall, respectively (Campo, Tjalsma et al. 2004, Bruck, Personnic et al. 2011, Dajkovic, Hinde et al. 2016). This pattern of localization is coincident with the helical pattern of cell wall synthesis in *B. subtilis* (Daniel and Errington 2003) and

suggests that SrtA would follow a similar helical localization pattern however, experimental evidence is required.

1.2.2 SrtA localization in cocci and ovococci

Staphylococci and *Neisseria* (except *Neisseria elongata*) are considered true cocci while streptococci, enterococci, and lactococci are considered ellipsoid or ovococci (Zapun, Vernet et al. 2008). Localization of SrtA in *S. aureus* has not been extensively characterized, rather the localization of some of its substrates have been described including protein A (DeDent, McAdow et al. 2007) and heme iron transport proteins IsdA and IsdB (Pishchany, Dickey et al. 2009), all of which localize to distinct foci coincident with site of cell wall synthesis. These findings suggest that *S. aureus* SrtA may also localize to these same domains, but experimental evidence is required.

Ellipsoid bacteria are unique because their cell wall synthesis is a co-ordination between the septal and peripheral cell wall machinery (**Fig. 1.3**) (Pinho, Kjos et al. 2013). The peripheral cell wall machinery is located at the “equatorial plane” which tracks with the site of future division septa. SrtA is predominantly associated with the septum but also localizes at the equatorial plane in ellipsoid bacteria, and often co-localizes with the secretion ATPase SecA, as demonstrated in *S. pyogenes*, *S. mutans*, and *E. faecalis* (Hu, Bian et al. 2008, Raz and Fischetti 2008, Kline, Kau et al. 2009). SrtA localizes to the septum following septation (mid-division phase) in *S. pyogenes* and is observed at the equatorial plane especially during later stages of cell division. SrtA also localizes to the cell poles in a small fraction of cells (Raz and Fischetti 2008). This localization pattern of SrtA during the different stages of cell division is coincident with the localization of the septal and peripheral cell wall synthesis machineries described recently in *S. pyogenes* (Sugimoto, Maeda et al. 2017). *E. faecalis* SrtA shows

a similar pattern of localization as observed in *S. pyogenes*, localizing at single foci during the mid-division phase and multiple foci coincident with the equatorial plane in late divisional cells (Kline, Kau et al. 2009, Kandaswamy, Liew et al. 2013).

Fig 1.3: Septal and peripheral cell wall synthesis machineries in ovococci. In ovococci (like *E. faecalis*), both cell wall machineries are at play during cell division. Septal machinery consists of Pbp1a and Pbp2x. The peripheral machinery consists of Pbp2b and Pbp3 (and possibly Pbp1b and Pbp2a). Autolysin is a cell wall modifying enzyme that is required to split the dividing cell into two equal daughter cells. Adapted from (Pinho, Kjos et al. 2013)

1.3 SrtA substrates – Is focal localization of SrtA important for its function in substrate attachment?

SrtA localization in Gram-positive bacteria is likely a function of the localization of cell wall synthesis and cell division machinery as these two processes are tightly linked. As described above, SrtA in ovococci localizes primarily at the septum during the mid-division phase and at the equatorial plane and to a certain extent the cell pole during the late division phase (Hu, Bian et al. 2008, Raz and Fischetti 2008, Kline, Kau et al. 2009). This pattern of localization suggests that SrtA could attach its substrates at the septum, equatorial plane, and to a certain extent at the cell poles.

In *E. faecalis*, SrtA is responsible for the covalent attachment of over 20 substrates based on the presence of a CWSS, including Enterococcal surface protein (Esp), Ebp, aggregation substance (Asc10), and adhesion to collagen (Ace) (Sillanpaa, Xu et al. 2004). Deletion of *srtA* in *E. faecalis* leads to the accumulation of at least two of its substrates, Ebp and Asc10, at single foci on the cell membrane (Kline, Kau et al. 2009). However, the functional effects of mislocalizing SrtA have yet to be characterized. We hypothesize that for SrtA to efficiently attach its substrates to the cell wall, focal localization to proper domains on the cell membrane is necessary. The effect of mislocalization on the function of a protein has been demonstrated in *E. faecalis* SrtC, the pilin-polymerizing sortase. SrtC co-localizes with SrtA and the general secretion machinery at distinct foci on the cell membrane and mislocalization of SrtC results in an overall decrease in piliated *E. faecalis* cells (Kline, Kau et al. 2009). This

decrease in piliated cells suggest that proper localization of SrtC is necessary for pilus biogenesis.

Inefficient or no attachment of substrates to the cell wall due to enzyme mislocalization may lead to an attenuation in virulence as seen in *srtA* null strains in *Streptococcus gordonii*, *L. monocytogenes*, *S. pneumoniae*, *B. anthracis*, *E. faecalis*, and *S. aureus* (Bolken, Franke et al. 2001, Bierne, Mazmanian et al. 2002, Kharat and Tomasz 2003, Zink and Burns 2005, Kemp, Singh et al. 2007, Maresso and Schneewind 2008).

1.3.1 Endocarditis and biofilm associated pili (Ebp)

One of the most well characterized SrtA substrates in *E. faecalis* are the endocarditis and biofilm associate pili. Pili in *E. faecalis* are made up of three subunits EbpA, EbpB, and EbpC and are co-transcribed along with SrtC in a polycistronic mRNA (**Fig. 1.4**) (Nallapareddy, Singh et al. 2006). SrtC polymerizes the three subunits to form large molecular weight structures that are greater than 200kDa in mass and over 10µm in length (Sillanpaa, Xu et al. 2004, Nielsen, Flores-Mireles et al. 2013). On polymerization, the assembled pili are attached to the cell wall via SrtA (Kline, Kau et al. 2009). Ebp are important for the formation of biofilm and mediate attachment to host fibrinogen and collagen and plays an important role in urinary tract infections and endocarditis (Nallapareddy, Singh et al. 2006, Nallapareddy, Sillanpaa et al. 2011, Flores-Mireles, Pinkner et al. 2014). Ebp expression is phase variable which could be advantageous to the bacterial cell during infection since non-pili expressing cells would not be cleared by pili-specific immune response of the host (Nallapareddy, Singh et al.

2006, Danne, Dubrac et al. 2014). In the absence of SrtA, polymerized pili accumulate in the cell membrane (Kline, Kau et al. 2009, Nielsen, Flores-Mireles et al. 2013).

Fig 1.4: Polymerization and attachment of endocarditis and biofilm associated pili in *E. faecalis*. (A) The pilus operon consisting of *ebpA*, *ebpB*, *ebpC*, and *srtC*. *srtA* is expressed downstream and not part of the operon. (B)-(E) The process of pilin polymerization by SrtC starting from the cap, EbpA, followed by addition of multiple subunits of EbpC, and finally EbpB. The polymerized pili is then attached to the cell wall by SrtA. Adapted from (Nielsen, Flores-Mireles et al. 2013)

1.3.2 Aggregation substances (Asc10)

Another well characterized SrtA substrate in *E. faecalis* is the aggregation substance. Asc10 is encoded by *prgB* on the conjugation plasmid pCF10 in *E. faecalis* that encodes for a type IV secretion system (Li, Alvarez-Martinez et al. 2012). Expression of Asc10 in a plasmid donor cell is triggered by the sensing of pheromones made by a recipient cell (Dunny, Brown et al. 1978, Dunny, Craig et al. 1979). Asc10 is involved in aggregation, biofilm formation, plasmid transfer, increased virulence by binding to human neutrophils, and survival within host cells (Rakita, Vanek et al. 1999, Sussmuth, Muscholl-Silberhorn et al. 2000, Chuang-Smith, Wells et al. 2010, Bhatt, Cruz et al. 2015). The 67.6kb pCF10 plasmid within plasmid donor cells encodes for structural genes necessary for conjugation (Kao, Olmsted et al. 1991) and is responsive to a chromosomally encoded peptide cCF10 (LVTLVFV) produced by recipient cells (Mori, Sakagami et al. 1988, Hirt, Manias et al. 2005). The recipient cell secretes cCF10 as a pre-pheromone that is processed by Eep protease to form a functional pheromone (An, Sulavik et al. 1999). cCF10 is sensed by the donor cell by the lipid-anchored protein PrgZ which then recruits the OppBCDF complex (Leonard, Podbielski et al. 1996). cCF10 enters the donor cell via the OppBCDF channel and binds to the pCF10-encoded repressor PrgX which serves to activate transcription of conjugation machinery genes *prgB*, *pcfC*, and *pcfG* (Hedberg, Leonard et al. 1996, Leonard, Podbielski et al. 1996) (**Fig. 1.5**).

Fig. 1.5: Control of conjugation by two signalling molecules (pheromones) cCF10 and iCF10 in *E. faecalis*. cCF10 and iCF10 (inhibitor) are imported into the donor cell via membrane-anchored PrgZ. The pheromones compete to bind to the repressor PrgX. Derepression, by binding of PrgX/cCF10, allows transcription of prgQ operon to facilitate conjugation. Adapted from (Chatterjee, Cook et al. 2013).

1.4 Scope of the study

E. faecalis is a Gram-positive bacterium and the most common enterococcus species associated with human infection followed by *E. faecium* (Manson, Rauch et al. 2008, Gilmore, Lebreton et al. 2013) causing nosocomial infections such as urinary tract infection, endocarditis, and bacteremia (Nallapareddy, Singh et al. 2006). Nosocomial enterococcal infections are increasingly difficult to treat due to the emergence of multidrug resistant strains (Johnston and Jaykus 2004). *E. faecalis*

employs SrtA to attach its arsenal of virulence factors to the cell wall enabling it to cause infections in its host. Thus, SrtA is considered as a virulence factor and a target for drug design. Many bacterial proteins localize to discrete subcellular sites and there is often a relationship between their localization and function within the cell. Disrupting virulence factor localization could therefore also be a viable anti-virulence strategy.

SrtA of *E. faecalis* localizes to discrete membrane foci in a cell-cycle dependent manner which could be mediated by interactions with cell wall, cell division, and/or secretory machinery components. The two main mechanisms by which proteins localize are through their structural or sequence components and by interactions with domains or proteins. This thesis aims to understand the mechanisms by which, and functional consequences of, SrtA subcellular localization in *E. faecalis*. We therefore sought to identify important residues within SrtA as well as its interacting partners. This study employed both genetic and biochemical approaches to uncover factors involved in localizing SrtA to distinct foci on the membrane of *E. faecalis* and characterize the effect of mislocalized SrtA on its efficiency in substrate attachment to the cell wall. We identified residues within the tail and transmembrane region that could be necessary for localization and/or function of SrtA. We determined the protein expression and quantified attachment of Ebp and Asc10 to the cell wall, as representative examples for the functional analysis of mislocalized SrtA in *E. faecalis*. We also identified two interacting partners of SrtA, FtsY and DnaK. Together these discoveries represent the first information regarding the factors that dictate SrtA subcellular localization, and the functions associated with that localization, in a virulence factor that is conserved across all Gram-positive pathogens.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in **Table 1** and **Table 2**. The experiments and strain construction were carried out in *Enterococcus faecalis* OG1RF and *Escherichia coli* DH5 α strains. We cultured *E. faecalis* in brain-heart infusion medium (BHI) (BD Difco) with incubation at 37°C under static conditions. We cultured *E. coli* in Luria-Bertini medium (LB) (BD Difco) with incubation at 37°C under shaking conditions. We added Bacto-Agar to BHI or LB or Minimal Media to a final concentration of 2% for solid media. Plasmids were maintained during cell growth with 500 μ g/ml kanamycin for *E. faecalis* and 50 μ g/ml kanamycin or 100 μ g/ml ampicillin for *E. coli*.

Table 1: List of strains used in this study

Strain	Characteristics	Antibiotic Resistance	Reference
<i>E. faecalis</i> OG1RF	Human dental isolate	Rifampicin and Fusidic acid 25 μ g/ml	(Dunny, Brown et al. 1978)
<i>E. faecalis</i> OG1RF Δ srtA	In-frame deletion of Sortase A gene	Rifampicin and Fusidic acid 25 μ g/ml	(Kline, Kau et al. 2009)
<i>E. faecalis</i> OG1SS	Derivative of OG1	Streptomycin 500 μ g/ml	(Franke and Clewell 1981)
<i>E. faecalis</i> <i>dnak::Tn</i>	DnaK transposon mutant	Rifampicin and Fusidic acid 25 μ g/ml,	(Kristich, Nguyen et al. 2008)

		Chloramphenicol 10 µg/ml	
<i>E. faecalis</i> <i>groEL::Tn</i>	GroEL transposon mutant	Rifampicin and Fusidic acid 25 µg/ml	(Kristich, Nguyen et al. 2008)
<i>E. faecalis</i> OG1RFΔ <i>htrA</i>	In-frame deletion of HtrA	Rifampicin and Fusidic acid 25 µg/ml	Adeline Yong, unpublished
<i>E. faecalis</i> OG1RFΔ <i>htrAΔsrtA</i>	Contains in-frame deletion of HtrA and SrtA	Rifampicin and Fusidic acid 25 µg/ml	Adeline Yong, unpublished
<i>E. coli</i> DH5α	Derived from <i>E.</i> <i>coli</i> DH5		(Grant, Jessee et al. 1990)
<i>E. coli</i> BTH101	Reporter strain for BACTH assay, F-, <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galK16</i> , <i>rpsL1</i> (<i>Str^r</i>), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i> .		(Karimova, Pidoux et al. 1998)

Table 2: List of plasmids used in this study

Plasmid	Characteristics	Antibiotic resistance	Reference
pCF10	cCF10 pheromone responsive plasmid encoding aggregation	Tetracycline 15 µg/ml	(Mori, Sakagami et al. 1988)

	substance and conjugation apparatus in <i>E. faecalis</i>		
pAK1	Empty vector with multiple cloning site and native SrtA promoter	Kanamycin	(Kline, Kau et al. 2009)
pAK1-SrtA _{tail} -TMH-GST	Encodes for SrtA tail and transmembrane helix fused to GST under the control of native SrtA promoter	Kanamycin	Charles Wang, unpublished
pAK1-SrtA _{tail} -GST	Encodes for SrtA tail fused to GST under the control of native SrtA promoter	Kanamycin	Charles Wang, unpublished
pAK1-GST	Encodes for GST under the control of native SrtA promoter	Kanamycin	This study
pAK1-SrtA _{tail} -TMH-GST ^{R2A}	Arg2 mutated to Ala	Kanamycin	This study
pAK1-SrtA _{tail} -TMH-GST ^{P3A}	Pro3 mutated to Ala	Kanamycin	This study
pAK1-SrtA _{tail} -TMH-GST ^{K4A}	Lys4 mutated to Ala	Kanamycin	This study

pAK1- SrtA _{tail} -TMH- GST ^{E5A}	Glu5 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{K6A}	Lys6 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{K7A}	Lys7 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{R8A}	Arg8 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{K10A}	Lys10 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{N11A}	Asn11 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{W12A}	Trp12 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{L13A}	Leu13 mutated to Ala	Kanamycin	This study

pAK1- SrtA _{tail} -TMH- GST ^{L17A}	Leu17 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{L18A}	Leu18 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{L20A}	Leu20 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{L28A}	Leu28 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{N31A}	Asn31 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{N32A}	Asn32 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{Q33A}	Gln33 mutated to Ala	Kanamycin	This study
pAK1- SrtA _{tail} -TMH- GST ^{I34A}	Ile34 mutated to Ala	Kanamycin	This study

pAK1-SrtA-2HA	Encodes for SrtA with hemagglutinin tag under the control of native SrtA promoter	Kanamycin	(Kline, Kau et al. 2009)
pAK1-SrtA-2HA ^{K6A}	Lys6 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{K10A}	Lys10 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{N11A}	Asn11 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{W12A}	Trp12 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{L17A}	Leu17 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{L18A}	Leu18 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{L28A}	Leu28 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{N31A}	Asn31 mutated to Ala	Kanamycin	This study
pAK1-SrtA-2HA ^{K10AN11A}	Tail combo	Kanamycin	This study
pAK1-SrtA-2HA ^{W12AL17A L18A L28AN31A}	TMH combo	Kanamycin	This study

pKNT25	Encodes T25 fragment of the CyaA protein	Kanamycin	(Karimova , Ullmann et al. 2000)
pUT18	Encodes T18 fragment of the CyaA protein	Ampicillin	(Karimova , Ullmann et al. 2000)
pKT25-zip	T25 fused to the leucine zipper GCN protein	Kanamycin	(Karimova , Ullmann et al. 2000)
pUT18C-zip	T18 fused to the leucine zipper GCN protein	Ampicillin	(Karimova , Ullmann et al. 2000)
pKNT25-SrtA _{tail} -TMH	T25 fused to the SrtA tail and transmembrane helix	Kanamycin	This study
pUT18-(prey protein)	T18 fused to either DivIVA, DnaK, FtsY, general stress protein (GSP), GroEL, HtrA, hypothetical serine protease (HSP), FtsI, SecA, or WxL surface domain protein (WxL).	Ampicillin	This study

2.2 Chemical crosslinking with Dithiobis(succinimidyl propionate)

E. faecalis strains were inoculated in liquid BHI media with appropriate antibiotics, and grown overnight. The next day, we inoculated fresh BHI media with

overnight culture at 1:100 dilution. The cultures were grown statically to mid-logarithmic (mid-log) growth phase (~OD 0.6). The cells were pelleted by centrifugation at 5000 x g for 15 mins at 4°C. The pellet was resuspended in PBS with 0.5% volume per volume (v/v) Triton X-100 to obtain a homogenous suspension. We treated the pellet with 10 mg/ml lysozyme (Sigma-Aldrich) for one hour at 37°C. We sonicated the lysed suspension at 40% amplitude for 2 mins (10 secs ON, 5 secs OFF) followed by centrifugation. The supernatant was transferred to a fresh tube and the crosslinker DSP (Thermo Scientific) was added at varying concentrations and time according to **Fig. 2.1** at room temperature (RT). The crosslinking reaction was stopped by adding Tris-HCl pH 7.5 to a final concentration of 50 mM and incubating for 15 mins. Alternatively, the mid-log phase cells were first treated with DSP at a concentration of 0.5 mM for 15 mins followed by lysis and sonication as described previously.

		Optimization parameter 2 DSP concentration (mM)					
		0.5	1	1.5	2.0	2.5	3.0
Optimization parameter 1 Reaction time (min)	15	✓	✓				
	30	✓					
	60		✓				

Fig 2.1: Optimization of the reaction time and concentration for DSP.

Tick marks indicate presence of crosslinked bands greater than >27 kDa.

2.3 GST Pull-down assay

We equilibrated the glutathione agarose resin (Sigma-Aldrich) with equilibration buffer at pH 8.0 (50 mM Tris + 150 mM NaCl). If the cross-linked suspension was turbid, it was filtered through 0.22 μ m membrane filter to avoid blocking of the column. The crosslinked cell suspension was passed through the gravity flow column twice at a rate of 0.5 ml/minute. The column was washed with 15 ml of equilibration buffer and fractions of 1.5 ml were collected. Elution was carried out using 100 mM reduced glutathione in equilibration buffer. 5 fractions of 1 ml each were collected. The flow-through, the wash fractions and the elution fractions were analysed by SDS-PAGE.

2.4 Co-immunoprecipitation

30ml of overnight or mid-log cultures of *E. faecalis* were pelleted by centrifugation at 3000 x g for 10 mins at 4°C. The pellet was washed with 10 ml of 1X PBS pH 7.4. The pellet was resuspended in 3ml of ice-cold 1X PBS with 1% v/v NP-40 and protease inhibitors. The cells were lysed with 5 mg/ml of lysozyme for 1 hour at 37°C. The suspension was sonicated at an amplitude of 40% for 2 mins (15 secs ON, 5 secs OFF). The sonicated suspension was centrifuged at 12,000 x g for 10 mins at 4°C. The supernatant was transferred to a fresh tube and crosslinked with 0.5 mM DSP for 15-20 mins at RT followed by addition of Tris-HCl pH 7.5 to a final concentration of 50 mM to quench the reaction for 15 mins at RT. Alternatively, the cells were first treated with 0.5 mM DSP followed by Tris-HCl pH 7.5 treatment, lysis, and sonication. Simultaneously, 25 μ l of magnetic protein A/G beads were washed twice, for 10 mins each, with 1 ml of ice-cold 1X PBS. The protein A/G beads were incubated with 50 μ l of 1:20 diluted antibody (anti-SrtA/anti-GST/pre-immune rabbit serum) for 1 hour at

RT on a rotating wheel. Antibody dilutions were prepared in 1X PBS with 1% weight per volume (w/v) bovine serum albumin (BSA). The beads were then washed twice with ice-cold 1X PBS and incubated with 500 μ l cross-linked cell lysate overnight at 4°C on a rotating wheel. The beads were then washed with 1 ml of ice-cold washing buffer (50 mM Tris, 300 mM NaCl, 1 mM EDTA, 0.5% v/v NP-40, 10% v/v glycerol, pH 7.4) four times for 15 mins each at 4°C. Bound proteins from the beads were eluted by either, boiling it at 100°C for 10 mins or incubating at 37°C in case of rabbit antibodies, in 50 μ l of 2X Laemmli buffer.

2.5 Mass spectrometry compatible silver staining

Silver staining was carried out as per the Rockefeller University protocol (2016). Protein samples were run on NuPAGE® Bis-Tris mini gels (ThermoFisher Scientific, USA). For each gel 150 ml of each solution was used to ensure that the gel is completely submerged. First, the gel was fixed in 50% v/v methanol + 5% v/v acetic acid solution for 20 mins, washed with 50% v/v methanol, and then water for 10 mins each. The gel was then sensitized with 0.02% w/v thiosulfate for 1 min, followed by two 1 min washes with water. Silver reaction was carried out for 20 mins with 0.1% w/v silver nitrate in 0.08% v/v formalin. The gel was washed twice for 1 min with water and developed using fresh 2% w/v sodium carbonate with 0.04% v/v formalin solution. Reaction was stopped by incubation with 5% v/v acetic acid for 10 mins with a final 10 mins wash with water. The gel can be preserved by incubating in 8.8% v/v glycerol for 20 mins. Silver-stained bands of interest were sent for mass spectrometric analysis to Taplin Mass Spectrometry Facility, Harvard Medical School (Boston, MA).

2.6 Molecular techniques

2.6.1 Electrocompetent cell preparation

E. coli DH5α electrocompetent cells were prepared as follows. A single colony from an LB plate was inoculated in 5 ml of SB media and incubated overnight at 37°C. The culture was then diluted 1:250 in fresh SB media and incubated at 37°C till O.D.600 of 0.8-0.9 was reached. The culture was then cooled on ice for 15 mins followed by centrifugation at 5000 x g at 4°C for 15 mins. The pelleted cells were re-suspended in 250 µl of ice cold 10% v/v glycerol and centrifuged as before. The above process was repeated twice but with 100 µl and 25 µl of 10% v/v ice cold glycerol respectively. Finally, the cell pellet was re-suspended in 1 µl of 10% v/v glycerol and the cells were aliquoted into 50 µl aliquots. The aliquots were flash frozen in liquid nitrogen and stored at -80°C. Each aliquot was sufficient for one transformation. Transformation was carried out in chilled 0.2 cm cuvettes (BioRad). 2.5 µl of plasmid DNA was added to 50 µl *E. coli* aliquots and electroporated (2.5 kV, 25 µF and 200 Ω). Transformants were recovered in 1 ml of SOC media, incubated at 37°C for 1 hour and plated onto LB media supplemented with appropriate antibiotic.

E. faecalis OG1RF electrocompetent cells were prepared as follows. A single colony from a BHI plate was inoculated in BGYT media and incubated overnight at 37°C. The overnight culture is initially tested in BGYT with 3% w/v, 4% w/v and 5% w/v glycine. Glycine weakens the cell wall of Gram positive bacteria that facilitates the entry of DNA upon applying electrical pulse (Shepard and Gilmore 1995). All tubes showed growth but 3% w/v glycine gave the highest O.D. Overnight culture was inoculated in BGYT media with 3% w/v glycine and incubated at 37°C till O.D.600 reached 0.6-0.8. The culture was cooled on ice for 15 mins followed by centrifugation at 5000 x g for 15 mins at 4°C. The cells were re-suspended consecutively in 50 µl, 25

ml and 10 ml of ice cold 10% v/v glycerol, with centrifugation between each wash to pellet the cells. The cells were finally re-suspended in 1 ml of ice cold 10% v/v glycerol and 50 µl aliquots were made, flash frozen and stored at -80°C. Transformation was carried out in chilled 0.1 cm cuvettes (BioRad). 2.5 µl of plasmid DNA was added to 50 µl *E. faecalis* aliquots and electroporated (1.7 kV, 25 µF and 200 Ω) in the GenePulser (BioRad). Transformants were recovered in recovery media, incubated at 37°C for 2 hours and plated on BHI supplemented with 0.25 M sucrose and appropriate antibiotics.

2.6.2 Construction of Sortase A tail and TMH mutants

Plasmids pAK1 SrtA_{tail-TMH}-GST and pAK1 SrtA-2HA, extracted using the PureLink Quick Plasmid Miniprep Kit (Invitrogen), were used as a template to construct alanine mutations by site directed mutagenesis. The designed primers are listed in **Table 3**. The amino acids were sequentially replaced with alanine. The PCR products were treated with Dpn1 enzyme (Thermo Scientific) for 1 hour at 37°C to remove the parent plasmid. In case of ligation, 50 ng of Dpn1 treated PCR product was reacted with T4 DNA Ligase (Thermo Scientific) for 1 hour at 22°C followed by inactivation at 70°C for 5 mins. This mixture was then used for transformation. Clones were verified with pAK1 primers (**Table 3**) to check the sequence.

Table 3: List of primers used in this study

Primer	Forward sequence (5'- 3')	Reverse sequence (5'- 3')	Description
GST	ATGTCCCCTATA CTAGGTTATTGG	CATATTTTCCCTC CTTTTAATGTATG	Delete the SrtA _{tail-TMH} region to construct pAK1-GST

R2A_GST	AGGAGGGAAAA T ATGGCTCCAAAA GAGAAAAAAG	CTTTTTTCTCTTT TGGAGCCATATTT TCCCTCCT	Mutate Arg2 to Ala on pAK1-SrtA _{tail} -TMH-GST
P3A_GST	AAAATATGCGCG CTAAAGAGAAAA AA	TTTTTCTCTTTAG CGCGCATATTTT	Mutate Pro3 to Ala on pAK1-SrtA _{tail} -TMH-GST
K4A_GST	ATTAAAAGGAG GGAAAATATGCG CCCAGCTGAGAA AAAAAG	TTTTTTTCTCAGC TGGGCGCATATTT TCCCT	Mutate Lys4 to Ala on pAK1-SrtA _{tail} -TMH-GST
E5A_GST	TATGCGCCCAAA AGCTAAAAAAA GAGGAAAAAAA	TTTTTTCCTCTTTT TTAGCTTTTGGG CGCATA	Mutate Glu5 to Ala on pAK1-SrtA _{tail} -TMH-GST
K6A_GST	GCGCCCAAAAG AGGCTAAAAGA GGAAAAA	TTTTTCCTCTTTTA GCCTCTTTTGGGC GC	Mutate Lys6 to Ala on pAK1-SrtA _{tail} -TMH-GST
K7A_GST	GCGCCCAAAAG AGAAAGCTAGA GGAAAAA	TTTTTCCTCTAGC TTTCTCTTTTGGG CGC	Mutate Lys7 to Ala on pAK1-SrtA _{tail} -TMH-GST
R8A_GST	AAAAGAGAAAA AAGCTGGAAAA AATTGGTTAATC	GATTAACCAATTT TTCCAGCTTTTT TCTCTTT T	Mutate Arg8 to Ala on pAK1-SrtA _{tail} -TMH-GST

K10A _GST	AAAAAAAGAGG AGCTAATTGGTT AATCAACAGT	ACTGTTGATTAAC CAATTAGCTCCTC TTTTTTT	Mutate Lys10 to Ala on pAK1-SrtA _{tail} -TMH-GST
N11A _GST	AAAGAGGAAAA GCTTGGTTAATC AACAGTTTATTA G	ATTAACCAAGCTT TTCCTCTTTTTTC TCTTTTGGG	Mutate Asn11 to Ala on pAK1-SrtA _{tail} -TMH-GST
W12A _GST	GAGGAAAAAAT GCTTTAATCAAC AGTTTATT	AATAAACTGTTG ATTAAAGCATTTT TTCCTC	Mutate Trp12 to Ala on pAK1-SrtA _{tail} -TMH-GST
L13A _GST	GAAAAAATTGG GCTATCAACAGT TTATTAGTT	AACTAATAAACT GTTGATAGCCCA ATTTTTTC	Mutate Leu13 to Ala on pAK1-SrtA _{tail} -TMH-GST
L17A _GST	CAACAGTGCTTT AGTTTTACTATT TATCATTGGC	GTAAAACTAAAG CACTGTTGATTAA CCAATTTTT TCC	Mutate Leu17 to Ala on pAK1-SrtA _{tail} -TMH-GST
L18A _GST	TGGTTAATCAAC AGTTTAGCTGTT TACTATTTATC ATTG	CAATGATAAATA GTAAACAGCTA AACTGTTGATTAA CCA	Mutate Leu18 to Ala on pAK1-SrtA _{tail} -TMH-GST
L20A _GST	GTTTATTAGTTG CTCTATTTATCA TTGGCTTAGCC	GATAAATAGAGC AACTAATAAACT GTTGATTA ACC	Mutate Leu20 to Ala on pAK1-SrtA _{tail} -TMH-GST

L28A _GST	GGCTTAGCCGCT ATTTTAAACAAT CAG	GTAAAAAATAGC GGCTAAGCCAAT GATAAATAG	Mutate Leu28 to Ala on pAK1-SrtA _{tail} -TMH-GST
N31A _GST	GGCTTAGCCTTA ATTTTGGCTAAT CAGATATCCCC	GGGGATATCTGA TTAGCAAAAATT AAGGCTAA GCC	Mutate Asn31 to Ala on pAK1-SrtA _{tail} -TMH-GST
N32A _GST	CTTAGCCTTAAT TTTAAACGCTCA GATATCCCC	GGGGATATCTGA GCGTTAAAAATT AAGGCTAAG	Mutate Asn32 to Ala on pAK1-SrtA _{tail} -TMH-GST
Q33A _GST	GCCTTAATTTTT AACAATGCTATA TCCCCTATACT AGG	CCTAGTATAGGG GATATAGCATTGT TAAAAATTAAGG C	Mutate Gln33 to Ala on pAK1-SrtA _{tail} -TMH-GST
I34A _GST	CTTAATTTTTTAA CAATCAGGCTTC CCCTATACTAGG	CCTAGTATAGGG GAAGCCTGATTGT TAAAAATTAAG	Mutate Ile34 to Ala on pAK1-SrtA _{tail} -TMH-GST
K6A _HA	same as GST		Mutate Lys6 to Ala on pAK1-SrtA-2HA
K10A _HA	same as GST		Mutate Lys10 to Ala on pAK1-SrtA-2HA
N11A _HA	same as GST		Mutate Asn11 to Ala on pAK1-SrtA-2HA
W12A _HA	same as GST		Mutate Trp12 to Ala on pAK1-SrtA-2HA

L17A _HA	same as GST		Mutate Leu17 to Ala on pAK1-SrtA-2HA
L18A _HA	same as GST		Mutate Leu18 to Ala on pAK1-SrtA-2HA
L28A _HA	same as GST		Mutate Leu28 to Ala on pAK1-SrtA-2HA
N31A _HA	GCCTTAATTTTT GCTAATCAGATA CGCAGTTGGG	GCGTATCTGATTG CTAAAAATTAAG GCCAAGCCAATG	Mutate Asn31 to Ala on pAK1-SrtA-2HA
TailCo_ GST/HA	GAAAAAAGAG GAGCTGCTTGGT TAATCAACAGTT TATTAGT	GATTAACCAAGC AGCTCCTCTTTTT TTCTCTTTTG GGC	Mutate Lys10 and Asn11 to Ala on pAK1-SrtA _{tail-TMH} -GST and pAK1-SrtA-2HA
TMHCo1_ GST/HA	GGTTAATCAACA GTGCTGCTGTTT TACTATTTATCA TTGGCTTAGCCT	GCCAATGATAAA TAGTAAAACAGC AGCACTGTTGATT AACCAATTTT	Mutate Leu17 and Leu18 to Ala on pAK1-SrtA _{tail-TMH} -GST and pAK1-SrtA-2HA
TMHCo2_ GST	GGCTTAGCCGCT ATTTTGTCTAAT CAGATATCCCCT ATACT	GGATATCTGATTA GCAAAAATAGCG GCTAAGCCAATG ATAAAT	Mutate Leu28 and Asn31 to Ala on pAK1-SrtA _{tail-TMH} -GST ^{L17AL18A}
TMHCo2_ HA	CATTGGCTTGGC CGCTATTTTTCG TAATCAGATACG CAGTTGGGT	CTGCGTATCTGAT TAGCAAAAATAG CGGCCAAGCCAA TGATAAATAGT	Mutate Leu28 and Asn31 to Ala on pAK1-SrtA-2HA ^{L17AL18A}

TMHCo3_ GST/HA	GAGGAAAAAAT <u>GCTTTAATCAAC</u> AGTGCTGCT	ACTGTTGATTAAA GCATTTTTTCCTC TTTTTTTCTC	Mutate Trp12 to Ala on pAK1-SrtA _{tail} -TMH- GST ^{L17AL18AL28AN31A} and pAK1-SrtA- 2HA ^{L17AL18AL28AN31A}
SrtA _{tail} - TMH	ACTCTAGAGGAT CCCATGCGCCCA AAAGAGAAAAA	ATTCGAGCTCGGT ACTTCTGATTGTT AAAAATTAAGGC TAAGCC	Amplifies the SrtA _{tail} - TMH region with 15bp flanks from pAK1- SrtA _{tail} -TMH-GST
DivIVA	ACTCTAGAGGAT CCCATGGCATT ACTCCATTAGAT ATTCA	ATTCGAGCTCGGT ACTTTTGATTCTT CTTCAATTGTTTC TTCAG	Amplifies the DivIVA region with 15bp flanks
DnaK	ACTCTAGAGGAT CCCATGAGTAAA ATTATTGGTATT GACTTAGGA	ATTCGAGCTCGGT ACTTTGTCATCAC CATTTACTTCTTC AAAA	Amplifies the DnaK region with 15bp flanks
FtsY	ACTCTAGAGGAT CCCATGGGATTT TTTGATAAAATT AAAAAAGCT	ATTCGAGCTCGGT ACAACATCTTTTA ATAAGCCTTTAAA TAAGCC	Amplifies the FtsY region with 15bp flanks
GSP	ACTCTAGAGGAT CCCTTGGAGGAG ATTTTTATGGCT AAAAA	ATTCGAGCTCGGT ACCCCTTTAAATT CTTTTTTTACGTC TTT	Amplifies the GSP region with 15bp flanks

GroEL	ACTCTAGAGGAT CCCATGGCAAAA GAGATTAAATTT GCAG	ATTCGAGCTCGGT ACCATCATACCGC CCATGC	Amplifies the GroEL region with 15bp flanks
HtrA	ACTCTAGAGGAT CCCATGCACTTA TTGGGAGGTTAT TTTA	ATTCGAGCTCGGT ACTTGATTGCTGC GATTATTTTGTTG	Amplifies the HtrA region with 15bp flanks
HSP	ACTCTAGAGGAT CCCATGAGTGTA GTATTAGGACTT TTGG	ATTCGAGCTCGGT ACCTGTTTGGGTG CTCGG	Amplifies the HSP region with 15bp flanks
FtsI	ACTCTAGAGGAT CCCTTGACTTTTT TCAACAACAAAA TAATTAAATATT TC	ATTCGAGCTCGGT ACTTTTTTGTACA TTCCATATACGC TTCTAAAA	Amplifies the FtsI region with 15bp flanks
WxL	ACTCTAGAGGAT CCCATGCGAAAC AAACAAAGCC	ATTCGAGCTCGGT ACATTCCTGGTA CGTCTTCTAAC	Amplifies the WxL region with 15bp flanks
SecA	ACTCTAGAGGAT CCCTTGAAAGGA ACACAGATACCA ATG	ATTCGAGCTCGGT ACAGCGTTTCTTC CATGACAAT	Amplifies the SecA region with 15bp flanks

pAK1	TATCCGTGTCGT TCTGT CCA	GCCTAAAGACAA GCCACCTG	Amplifies multiple cloning site on pAK1 derived plasmids
M13	GTAAAACGACG GCCAGTG	CAGGAAACAGCT ATGAC	Amplifies multiple cloning site on empty pAK1
PrgB	GCCAACAGAAGT TGCAC CAG	CGCATGGCCACCT TTAT TCG	Amplifies prgB gene on pCF10 plasmid
T25	ATGACCATGATT ACGCCAAG	GTTATATCGATGG TGCAGCC	Amplifies the multiple cloning site on pKT25/ pKNT25
T18	ATGACCATGATT ACGCCAAG	TTATATCGATTGG CGTTCCACT	Amplifies the multiple cloning site on pUT18/ pUT18C

2.7 SDS-PAGE and Western Blot assay

Protein samples were mixed with equal amount of 2X NuPAGE LDS (Invitrogen) or 2X Laemmli (BioRad) sample buffer and preheated to 95°C. The samples were cooled and loaded onto NuPAGE® Bis-Tris or Tris-Acetate mini gels (Invitrogen) and run in 1x MOPS SDS or Tris Acetate running buffer in XCellSureLock®Mini-Cell for ≥ 50 mins at 200 V. We transferred proteins from the gel to a nitrocellulose membrane using the iBlot™ Dry Blotting system according to the protocol provided by Life Technologies (Invitrogen). The cathode, anode and filter paper are commercially available (Invitrogen). On completion of transfer, we placed the membrane in blocking buffer containing 3% w/v BSA in phosphate buffer saline

with 0.05% v/v Tween-20 (PBS-T) for 1 hour shaking at RT. We then washed the membrane 3 times with PBS-T for 10 mins each with shaking followed by incubation with the primary antibodies (SABio, Singapore; T25 and T18 from Santa Cruz Biotechnology) for 2 hours at RT. The antibodies concentrations are described in **Table 4**. All antibodies were diluted in PBS-T + 3% w/v BSA. The membrane was washed with PBS-T 3 times for 10 mins each with shaking and incubated with appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies (Thermo Scientific) at RT, for 1 hour with shaking. Following incubation, the membrane was washed with PBS-T 3 times for 10 mins each with shaking. We detected the presence of HRP using the SuperSignal Chemiluminescent substrates (Thermo Fischer Scientific).

Table 4: List of antibodies and their concentrations used in this study

*F – Femto, P – Pico

Target protein	Primary antibody concentration, host	Secondary antibody concentration	Substrate:Buffer Water
Anti-GST	1:3000, Rabbit	1:6000	Femto 1:1:8
Anti-HA	1:3000, Rabbit	1:6000	Femto 1:1:8
Anti-SrtA	1:250, Mouse	1:1250	Femto 1:1:8
Anti-SrtC	1:200, Mouse	1:1250	Femto 1:1
Anti-EbpC	1:3000, Guinea pig	1:6000	Femto 1:1:8
Anti-EbpA	1:3000, Rabbit	1:6000	Femto 1:1:8
Anti-SecA	1:3000, Rabbit	1:6000	Femto 1:1:8
Anti-AS	1:1000, Rabbit	1:5000	Femto 1:1
Anti-T18	1:1500, Mouse	1:1250	Femto 1:1:3
Anti-T25	1:1000, Rabbit	1:5000	Femto 1:1:3
Anti-HtrA	1:3000, Guinea Pig	1:6000	Pico 1:1

2.8 Immunofluorescence microscopy

We subcultured overnight cultures of our strain of interest in a 1:10 dilution in BHI with appropriate antibiotics, and grew them statically at 37°C for approximately 105 mins. Cells were harvested at mid-log phase, washed once by resuspension in 1 ml of 0.01M phosphate buffer (PB). We fixed the cells in 1:4 ratios with PB and 4% v/v paraformaldehyde (PFA) for 20 mins at 4°C. Cells were then washed in 1 ml of PB, diluted to 1:4 ratios in PB, gently smeared onto poly-L-lysine pre-coated slides, air-dried at RT for 10 mins, and dried in hybridization oven for 20 mins. Cells were subsequently permeabilized with 20 µl of 10 mg/ml of lysozyme in lysis buffer at 37°C for 1 hour. After incubation, the cells were washed with PB 3 times, dried in the oven, blocked with 2% w/v BSA in PB (P-BSA) for 20 mins, incubated with 20 µl of primary antibody 4°C overnight. Slides then were washed extensively with PB, incubated with Alexa Fluor 488 secondary antibodies (Invitrogen) for visualizing GST diluted to 1:1000 in P-BSA, incubated in dark at RT for 1 hour, and washed with PB extensively. Slides were mounted with 10 µl of Vectashield mounting media (Vecalotor Laboratories, Inc), covered with glass coverslips and sealed. Imaging was done 30 mins after mounting.

We used the inverted epi-fluorescence microscope (Zeiss Axio observer Z1; Carl Zeiss GmbH, Germany) fitted with a 100X oil immersion objective (numerical aperture 1.4, optovar magnification changer 1.6X). We obtained stained images with an AF488/FITC filter cube (460-490 nm band pass excitation filter, 515-550 nm band pass barrier filter) and an AF568/Cy3 filter cube (530-550 nm band pass excitation filter, 590 nm long pass barrier filter). We fixed the exposure times on the wide-field microscope for each experiment for unbiased image analysis.

2.9 Bacterial two-hybrid assay

The bacterial two-hybrid assay was carried out as per the bacterial adenylate cyclase two-hybrid system protocol (Euromedex).

2.9.1 Construction of bait and prey plasmids

SrtA_{tail-TMH} and the prey proteins were constructed by amplifying the regions using the primers listed in **Table 3**. PCR products along with pKNT25 and pUT18 parent plasmids were digested with SmaI (New England Biolabs) at 37°C for 30 mins followed by gel purification (Invitrogen). The purified products were ligated with a 1:3 vector-insert molar ratio and transformed into competent *E. coli* cells. Clones were verified with T25 and T18 primers (**Table 3**) to check sequence.

2.9.2 Analysis of protein-protein interactions by blue-white screening

pKNT25-SrtA_{tail-TMH} was co-transformed with the pUT18 prey protein plasmids individually into competent *E. coli* BTH101. The transformants were spotted onto minimal media supplemented with 0.004% w/v X-gal (Sigma Aldrich), kanamycin, and ampicillin. pKT25-zip and pUT18C-zip were co-transformed and used as positive controls for blue colour development. pUT18 plasmids were co-transformed with pKT25-zip as negative controls for blue colour development. The plates were incubated at 30°C for up to 4 days.

2.10 Mating assay

We performed the mating assay between *E. faecalis* OG1RF recipient strains and donor *E. faecalis* OG1SS/pCF10 strain. The strains were grown overnight in BHI with appropriate antibiotics and diluted 1:10 in fresh media. The cCF10 peptide, LVTLVFV (Axil Scientific, Singapore), was added to the diluted OG1SS/pCF10

culture to a final concentration of 0.12ng/ml to induce AS expression (Antiporta and Dunny 2002) and incubated for 1 hour at 37°C under shaking conditions. To 4.5 ml diluted recipient strains, 0.5 ml of induced OG1SS/pCF10 was added and the two strains were conjugated for 30 mins at 37°C under shaking conditions. 50 µl of this culture was plated on BHI supplemented with appropriate antibiotics. The colonies were screened using *prgB* primers.

2.11 Cell fractionation

Overnight cultures of *E. faecalis* were diluted in fresh media with antibiotics. The cells were treated with 10 mg/ml of lysozyme, followed by centrifugation at 20,000 g for 2 mins. The supernatant was collected and stored as cell wall fraction. The protoplast was either used for protein analysis or fractionated into cytoplasm and cell membrane. The protoplast was sonicated for 2 mins followed by two rounds of centrifugation at 15,700 g for 30 mins to remove unlysed cells in cell fractionation buffer (20mM pH 8 Tris-HCl, 150mM NaCl, 1mM EDTA, 0.1% v/v TritonX) (Nielsen, Flores-Mireles et al. 2013). The supernatant was then subjected to ultracentrifugation for 2.5 hours at 165,000 x g. The pellet contained the membrane and the supernatant was the cytoplasmic fraction. The cytoplasmic fraction was then precipitated using trichloroacetic acid (TCA).

2.12 Biofilm assay

We carried out the crystal violet biofilm assay in a 96 well plate (Thernofisher, USA) as described previously (Bhatty, Cruz et al. 2015) with the following modifications. We normalized overnight cultures to O.D 600 of 0.7. We washed the pellet and resuspended it in 1 ml PBS. We added 8 µl of normalized culture to 200 µl

of Tryptic soya broth (TSB) containing 0.25% w/v of glucose in each well and incubated the plate at 37°C for 24 hours. We carried out the assay in triplicates. We utilized 200 µl of the remaining culture to perform CFU by serial dilution. Post incubation we washed the wells 2 times with PBS, stained with 0.1% w/v crystal violet solution (prepared from 1% w/v aqueous crystal violet solution, Sigma-Aldrich, Germany), and incubated for 30 minutes at 4°C. We washed the wells three times and blot dried. We added 200 µl of ethanol:acetone (80:20) solution to each well to solubilize CV and incubated for 30 mins at R.T. After incubation, O.D. 600 readings were taken using a spectrophotometer (UVmini-1240, Shimadzu, Japan).

2.13 Whole genome sequencing

We isolated genomic DNA from normalized overnight cultures of *E. faecalis* transposon mutants using the Wizard Genomic DNA Purification Kit (Promega, USA). Prior to sequencing, we assessed the extracted DNA by Qubit for purity and agarose gel electrophoresis to detect shearing. The samples were sent to the sequencing facility at SCELSE for whole genome sequencing and DNA library preparation by Illumina MiSeq V3. We detected the transposon insertion using CLC Genomics Workbench 9 software (Qiagen Bioinformatics, Germany).

Chapter 3

Sortase A depends on sequence elements

for focal localization to the septum

Membrane proteins make up 20-30% of the total protein content in a cell and are necessary for communication between the internal and external environment of the bacteria (Wallin and von Heijne 1998). Membrane proteins are anchored to the cell membrane via membrane targeting domains (non-cleavable preprotein), transmembrane helices (TMH), hydrophobic regions, and charged cytoplasmic tails (Dalbey, Wang et al. 2011). These structural and sequence elements are necessary for interaction between membrane proteins and for proper insertion of the proteins into the membrane. SrtA possesses a single transmembrane helix and a positively charged cytoplasmic tail which we hypothesize to play a role in the focal localization of the protein to the septum. Indeed, the positively-charged C-terminal tail of SrtC, the pilin polymerizing sortase in *E. faecalis*, is necessary for its localization and function (Kline, Kau et al. 2009). While SrtA localization in different Gram-positive bacteria has been reported for a number of species, the mechanisms underlying its subcellular localization patterns has yet to be delineated. In this chapter, we explore the contribution of individual amino acids within the cytoplasmic tail and TMH of SrtA in localizing the protein to the septum by alanine scan mutagenesis and immunofluorescence microscopy.

3.1 SrtA localization depends on tail and transmembrane helix (TMH)

In *E. faecalis*, the full length SrtA protein is made up of 233 amino acids and has a molecular weight of 27 kDa. SrtA consists of three domains: an N-terminal

positively-charged cytoplasmic tail, a single transmembrane helix, and a C-terminal extracellular catalytic domain (**Fig. 3.1**). The extracellular catalytic domain makes up the bulk of the protein and is approximately 25 kDa in size while the N-terminal tail and transmembrane helix (TMH) function as a signal peptide and stop transfer signal for membrane anchoring (Mazmanian, Liu et al. 2000). To first determine whether the tail and TMH region were sufficient to focally enrich the protein at the septum, similar to what had been previously reported for *E. faecalis* SrtC we replaced the catalytic domain of SrtA with the 26 kDa glutathione-S-transferase (GST). SrtA_{tail-TMH}-GST fusion construct was expressed from a plasmid under the control of the native SrtA promoter. We simultaneously constructed the SrtA_{tail}-GST and only GST containing plasmids. We transformed each plasmid into *E. faecalis* $\Delta srtA$

Fig. 3.1: Sequence of the tail and transmembrane helix of Sortase A in *E. faecalis*.

Residues 1 to 11 are part of the N-terminal positively charged tail. Residues 12 to 34 form the single transmembrane α -helix.

The three constructs were expressed in an *E. faecalis* $\Delta srtA$ background, as determined by anti-GST immunoblot (**Fig. 3.2a**). The SrtA_{tail-TMH}-GST construct was expressed at a lower level than the other two constructs (**Fig 3.2b**), likely due to presence of the membrane domain (Bernaudat, Frelet-Barrand et al. 2011). Membrane proteins can have low expression levels due to a number of factors such as limited availability of biogenesis factors including signal recognition particle (SRP) and/or

lipid space (Drew, Froderberg et al. 2003) and increased susceptibility to degradation by membrane protease FtsH (Ito and Akiyama 2005). In order to study the localization pattern of the GST fusion proteins, we performed immunofluorescent staining on each strain with antibodies against GST. Here and throughout this thesis, immunofluorescence microscopy images were analyzed using Projected System of Internal Coordinates from Interpolated Contours (PSICIC) (Guberman, Fay et al. 2008) in order to quantify localization patterns.

Fig. 3.2: Synthesis of SrtA_{tail-TMH}-GST in *E. faecalis* Δ*srtA*. (a) Three constructs on the pAK1 plasmid; SrtA_{tail-TMH}-GST, SrtA_{tail}-GST, and GST under the control of native SrtA promoter. (b) Anti-GST immunoblot of the three constructs with *E. faecalis* and

E. faecalis $\Delta srtA$ as negative controls. Anti-SecA immunoblot as loading control for all the samples (no difference observed).

Briefly, PSICIC uses interpolated contours to define the cell borders and measure the fluorescence intensity around the cell perimeter. Using this approach, we observed that SrtA_{tail-TMH}-GST localized to discrete domains at the septum of *E. faecalis* (**Fig. 3.3a**) similar to that previously observed for native SrtA (Kandaswamy, Liew et al. 2013). However, neither the SrtA_{tail}-GST or the GST control displayed focal localization, as expected because they are devoid of membrane domains (**Fig. 3.3b and 3.3c**). From these experiments, we conclude that the tail and transmembrane helix are sufficient to direct the heterologous GST protein to the septum.

Fig. 3.3: Localization of GST constructs in *E. faecalis* $\Delta srtA$ by IFM using anti-GST antibody. (a) SrtA_{tail-TMH}-GST localizes to the septal region as indicated by peaks at 25 and 75 AU. (b) and (c) SrtA_{tail}-GST and GST localize throughout the periphery of the cell membrane without any distinct focal localization. IFM carried out using anti-GST antibody. *E. faecalis* was used as negative control and no fluorescence was detected. Analysis by PSICIC was performed on >100 cells per strain. Scale bar is 2 μ m.

To further confirm that these constructs localize to different compartments of the cell, we performed fractionation in order to separate the cell wall, cell membrane and cytoplasm. In control samples, HtrA was found in the membrane fraction as expected. We observed SrtA_{tail}-TMH-GST and SrtA_{tail}-GST in the membrane fraction while GST was found in the membrane and cytoplasm (**Fig. 3.4**)

Fig. 3.4: Localization of GST constructs in *E. faecalis* Δ srtA by cell fractionation using anti-GST antibody. SrtA_{tail}-TMH-GST and SrtA_{tail}-GST are found associated with the membrane fraction. GST was found associated with both the membrane and cytoplasmic fraction. HtrA was used as loading control and observed only in the membrane fraction as expected.

3.2 Mutating residues within the tail and TMH affect expression of the heterologous GST protein

Once we established that the tail and transmembrane helix were sufficient for GST focal localization, we sought to identify the contribution of individual residues in

the expression and localization of the heterologous protein. We carried out site directed mutagenesis on the plasmid encoding the SrtA_{tail-TMH}-GST protein under the control of the native SrtA promoter using primers listed in **Table 3**. We successfully mutated 9 out of the 10 residues within the tail region and 10 out of the 23 amino acids with the TMH region. The mutated plasmids were transformed into *E. coli* for propagation and verified by capillary sequencing. The plasmids carrying the mutation at the correct region were transformed into *E. faecalis* Δ srtA and assessed protein production by western blot using an anti-GST antibody (**Fig. 3.5**). We observed that the mutations R2A, E5A, K6A, K7A, and R8A individually resulted in decreased expression of the SrtA_{tail-TMH}-GST construct (**Fig. 3.5a**), whereas mutations P3A, K4A, K10A, and N11A resulted in increased expression when compared to the SrtA_{tail-TMH}-GST construct (**Fig. 3.5b**). We hypothesize that the change in protein level may be due to the change in charge of the N-terminal cytoplasmic tail which overlaps the signal peptide which affects stability or rate of degradation. To mutate the remaining residues on the tail and transmembrane region, we altered the PCR extension temperature/time, gradient temperature for annealing, DpnI digestion time, and redesigned the primers, but were unable to mutate the remaining residues. We either did not get any transformants or obtained the wild type parent plasmid post sequencing. Work is ongoing to generate the full panel of alanine mutants across the entire cytoplasmic tail and TMH and assess their contribution to GST synthesis and focal localization.

Fig. 3.5: Immunodetection of SrtA_{tail-TMH}-GST alanine scan mutants using anti-GST antibody. (a) Alanine scan mutants of the tail region of SrtA_{tail-TMH}-GST (b) Alanine scan mutants of the TMH region of SrtA_{tail-TMH}-GST in *E. faecalis* Δ srtA. SecA was used as loading control.

3.3 Residues within the tail and TMH mislocalize SrtA_{tail-TMH}-GST fusion protein

The amino acid point mutations in the SrtA_{tail-TMH}-GST fusion protein allowed us to analyze the effect of each residue on the localization of the heterologous protein by immunofluorescence microscopy. Within the cytoplasmic tail, we observed that mutant proteins that were produced at a lower level (such as K6A, K7A, and R8A), localized to the septum similar to the parental wild type SrtA, indicating that there was no correlation between protein level and localization (**Fig. 3.6**). To further support the observation that protein levels do not dictate subcellular localization patterns, some of the overexpressed constructs, K10A and N11A, did not localize to the septum and were instead found distributed around the periphery of the cell membrane (**Fig 3.6a**). Within the transmembrane region, the mutants W12A, L17A, L18A, L28A, and N31A did not show septal localization (**Fig. 3.6b**), whereas K6A displayed a localization pattern similar to the parental wild type SrtA (**Fig. 3.6a**). Rather than being enriched at the septum, the W12A and N31A mutants localized to discrete foci around the cell

membrane and the other mutants (K10A, N11A, L17A, L18A, L28A) showed a more random distribution. We hypothesize that K10, N11, W12, and N31 could be involved in interactions with the hydrophilic groups of the lipid bilayer while L17, L18, and L28 could be involved in stabilizing intermembrane interactions or interactions with the hydrophobic tails of the lipid bilayer. We predicted the structure of the transmembrane and tail region using I-TASSER (**Fig. 3.7**) (Zhang 2008) and observed that the leucine residues; L17, L18, and L28, correspond to the exposed faces α -helical turns of the TMH.

Fig. 3.6: Localization profile of SrtA_{tail-TMH-GST} alanine scan mutants in *E. faecalis* Δ srtA by IFM using anti-GST antibody.

(a) K6A as a control showing focal localization at the septum. (b)-(h) K10A, N11A, W12A, L17A, L18A, L28A, N31A. Scale bar is

2 μ m.

Fig. 3.7: I-Tasser prediction model of the SrtA tail and transmembrane helix. Residues that result in mislocalization in SrtA_{tail-TMH}-GST are labelled (yellow). The transmembrane helix is depicted in red and the tail region is depicted in green. Labelling done in PyMol.

Leucine, along with alanine, are involved in stabilizing α -helix structures (Lyu, Sherman et al. 1991), substituting leucine with alanine is not predicted to affect the helical structure. In addition, the leucine side chain is neither reactive and nor likely to be involved in protein interactions; however, leucine has been reported to interact with lipids (Barnes 2007). Thus, the L17A, L18A, and L28A mutants could give rise to mislocalized GST due to perturbed protein-lipid interactions within the hydrophobic region of the lipid bilayer.

Amongst the amino acid point mutants constructed to date, only the K10A mutant showed a significantly slower growth phenotype ($p < 0.05$) (**Fig. 3.8 and data not shown**). Based on these growth curves, we calculated the doubling time for *E. faecalis* expressing the K10A mutant to be 122 mins as compared to 72 mins for *E. faecalis* encoding SrtA_{tail-TMH}-GST. We confirmed that this phenotype is specific to *E. faecalis* as the K10A showed normal growth phenotype in the *E. coli* strain.

Interestingly, we observed two colony morphologies in the K10A mutant: a big colony variant – BCV and a small colony variant – SCV (**Fig. 3.9a**). Both of these variants grew on kanamycin, indicating the plasmid backbone on which the constructs were cloned was still present, but they grew at different rates. The BCV showed a growth rate similar to SrtA_{tail}-TMH-GST. We hypothesized that the mutation at K10 was unstable and/or specifically toxic to *E. faecalis* and that the BCV was not expressing the mutated version of the fusion protein, or was giving rise to suppressor mutants. To assess whether suppressor mutations were arising in strains expressing the K10 mutation, we sequenced both the whole genome and the plasmid DNA. We found that the SrtA_{tail}-TMH-GST region was deleted on the plasmids isolated from the BCV strain. We further confirmed this by checking the synthesis of the GST protein in both strains and found that the heterologous GST fusion protein was not produced in the BCV (**Fig. 3.9b**). Since the BCV did not express GST, we conclude that the mislocalization profile of the K10A GST fusion arise solely from the mutant SCV.

Fig. 3.8: K10A shows a slower growth phenotype only in *E. faecalis*. (a) and (c) Growth curves of GST constructs in *E. coli*.

No significant difference in growth rates were observed between the mutant strain and SrtA_{tail-TMH}-GST (b) and (d) Growth curves of GST constructs in *E. faecalis* Δ srtA. Significant growth defect observed ($p < 0.05$) between K10A and SrtA_{tail-TMH}-GST. Statistical analysis carried out by unpaired t-test (GraphPad)

Fig. 3.9: Characterization of BCV and SCV of K10A mutants in *E. faecalis* $\Delta srtA$.

(a) Colony morphologies of *E. faecalis* $\Delta srtA$ /pAK1-SrtA_{tail-TMH}-GST and K10A. K10A showed two distinct colony morphologies: BCV and SCV. (b) PCR amplification of the 1.2 kb region of the pAK1 plasmid containing the SrtA_{tail-TMH}-GST insert. The BCV variant of K10A lacks the 1.2 kb insert (c) Anti-GST immunoblot to verify expression of GST fusion proteins in the two colony variants. SecA was used as loading control (no difference in loading observed)

3.4 Single amino acid mutations in SrtA-2HA do not affect localization

Seven amino acids on the tail and transmembrane region resulted in the mislocalization of SrtA_{tail-TMH}-GST. In order to analyze the effect of these seven mutations on expression and localization in a full-length SrtA, we mutated the amino acids in the full-length native SrtA fused to 2xHA at the C-terminal end of the protein that was expressed from a plasmid and transformed into *E. faecalis* $\Delta srtA$. We mutated

K6A in the full-length SrtA-2HA as a control because it showed a decreased GST fusion protein level but showed focal enrichment in the septum similar to wild-type SrtA. We predicted that the protein synthesis pattern would be similar to the corresponding SrtA_{tail-TMH}-GST mutants. Like mutations within the heterologous GST fusion, K6A was produced at lower levels, K10A was synthesized at higher levels (**Fig. 3.10**) and L17A and L28A (data not shown) were produced at same levels as compared to SrtA-2HA. However, protein levels of the N11A, L18A, and N31A mutants differed from the corresponding GST mutant strains. While N11A in the GST strain resulted in increased protein level, in the SrtA-2HA construct mutation N11 resulted in a decrease. Similarly, for L18A and N31A we observed a decrease in the GST fusion protein but in the SrtA-2HA the protein was synthesized at higher levels compared to the wild type protein. We hypothesize that the differential protein profiles of point mutants on the heterologous GST fusion and full length SrtA proteins are due the difference in the stability or rate of degradation of the GST and full-length constructs (**see Appendix-1 Table 1 for summary**).

Fig. 3.10: Immunodetection of SrtA-2HA alanine scan mutants using anti-HA antibody. SecA was used as loading control (no difference in loading was observed).

While mutating some of the residues the full length SrtA-2HA resulted in altered protein levels in comparison to the wild-type parental protein, we did not observe a change in localization patterns for any of the amino acid point mutants (**Fig. 3.11**). All of the mutants showed enriched foci at the septum, similar to native SrtA. We hypothesize that in the full-length native SrtA protein, a single residue change is not enough to mislocalize the protein and that a combinatorial mutant may have to be constructed to affect focal localization of SrtA. We derived this hypothesis based on a similar finding reported in a study on the dimer-monomer equilibrium of *S. aureus* SrtA in which in a truncated version of the protein containing only the catalytic domain and containing mutations in any of the three catalytic residues (N132A, K137A, Y143A) individually was sufficient to break the SrtA dimer *in vitro* (Lu, Zhu et al. 2007). However, in a follow up study *in vivo*, amino acid point mutations on the full-length version of SrtA did not disrupt dimerization. The full length SrtA dimer was only disrupted in a mutant harboring all three mutations (Zhu, Xiang et al. 2016). We are currently generating strains with combinatorial tail and TMH mutations in the full-length SrtA-2HA.

3.5 Functional effect of single amino acid mutations on SrtA-2HA

Although the single amino acid mutations we created in SrtA-2HA did not affect its subcellular localization, some of them did alter SrtA protein level, which could affect the efficiency of SrtA to attach substrates to the cell wall. Therefore, we tested the functionality of SrtA *in vivo* by measuring its ability to attach two of its substrates, Ebp and Asc10, by wild type SrtA-2HA and the differentially expressed *srtA* point mutant strains. Ebp attachment to the cell wall by SrtA was analyzed directly by immunoblot using anti-EbpA (**Fig. 3.12** and **Fig. 3.13**). We observed that the N31A mutant

accumulated more polymerized pili in the protoplast as compared to the cell wall, indicating the N31 mutant is not functional.

Fig. 3.11: Localization profile of SrtA-2HA alanine scan mutants by IFM using anti-HA antibody. (a) SrtA-2HA as a control showing focal localization at the septum. (b)-(g) K10A, N11A, W12A, L17A, L18A, and N31A.

Fig. 3.12: Immunodetection of EbpA in SrtA-2HA alanine scan mutants in *E. faecalis* Δ srtA on cell wall and protoplast using anti-HA antibody. The cells were fractionated into cell wall and protoplast fractions. Each blot had SrtA-2HA loaded as internal control to compare expression levels between SrtA-2HA and the mutant strains. SecA was used as loading control for the protoplast fraction. No SecA was observed in the cell wall fraction. No sample loaded between the cell wall and protoplast fractions. n=2

Fig. 3.13: N31A SrtA-2HA shows accumulation of EbpA in protoplast. The density values were calculated from the immunoblot in Fig. 3.12 using ImageJ. The relative density values were obtained by comparing absolute density values with SrtA-2HA (SrtA-2HA was loaded on all blots as an internal control). The graph shows the difference between cell wall and protoplast relative density values (GraphPad). Negative values indicate more protein in the protoplast compared to the cell wall. n=2

We next wanted to assess Asc10 attachment efficiency by wild type and mutant SrtA proteins. However, since the strains we were using did not harbour the plasmid encoding Asc10, we first carried out mating experiments between the SrtA-2HA strains and a pCF10-containing *E. faecalis* strain to obtain transconjugants containing both, the pAK1-SrtA-2HA and pCF10 plasmids, confirmed by colony PCR using primers listed in **Table 3**. We harvested cells 2 hours post induction with cCF10 and fractionated the cells into cell wall and protoplast, performed immunoblot using anti-AS antibody, and calculated the difference between Asc10 levels in cell wall and protoplast (**Fig. 3.14** and **Fig. 3.15**). Similar to the Ebp immunoblot, we observed a

decrease in Asc10 attachment to the cell wall for N31A as compared to SrtA-2HA again indicating that N31A is functional defective.

Fig. 3.14: Immunodetection of Asc10 in SrtA-2HA alanine scan mutants on cell wall using anti-AS antibody. The cells were fractionated into cell wall and protoplast fractions. Each blot had SrtA-2HA loaded as internal control to compare protein levels between SrtA-2HA and the mutant strains. SecA was used as loading control for the protoplast fraction. No SecA was observed in the cell wall fraction. n=2

Fig. 3.15: N31A SrtA-2HA mutant shows accumulation of aggregation substance in protoplast fraction. The density values were calculated using ImageJ. Relative density values obtained by comparing absolute density values to SrtA-2HA. The graph shows the difference between cell wall and protoplast relative density values (GraphPad). Negative values indicate more protein in the protoplast compared to the cell wall. n=2

Since both Ebp and Asc10 both contribute to biofilm formation in *E. faecalis* (Chuang-Smith, Wells et al. 2010, Sillanpaa, Chang et al. 2013), we also indirectly assessed the efficiency of SrtA by measuring biofilm formation in the SrtA mutant strains using the crystal violet assay. From the Ebp and Asc10 immunoblots, we compared the cell wall and protoplast fractions of all the strains and found that N31A was least effective in attaching the substrates to the cell wall (**Fig. 3.13** and **Fig. 3.15**). The crystal violet assay measured the biofilm contributed by Ebp since in the $\Delta ebpABC$ strain we observed a significant decrease in biofilm. We did not measure the Asc10-associated biofilm because Asc10 is not expressed from these cells. We observed that none of the mutants was significantly attenuated in their biofilm forming capability. Taken together, these data suggest that the catalytic domain of SrtA may contribute to the focal enrichment of the enzyme at the septum in *E. faecalis* since individual amino acid changes in the cytoplasmic tail or TMH which altered heterologous GST fusion protein localization did not perturb the SrtA localization pattern. However, some of the residues do affect the enzyme's function, as observed in the N31A mutants' inefficiency in attaching substrates to the cell wall, and indicate functionality of SrtA may not solely depend on proper localization.

Fig 3.16: Alanine point mutants in SrtA-2HA do not show altered biofilm phenotype. (a) Optical density values of the biofilm stained by crystal violet. (b) CFU for each strain prior to setting up the biofilm assay. No significant difference was observed in the CFU values. Error bars represent standard deviation from 3 independent experiments. Statistical analysis done using unpaired t-test (GraphPad). * $p < 0.05$, n.s. > 0.05

3.6 Discussion

In this chapter, we addressed the effect of mutating individual amino acid residues in the tail and TMH region of SrtA on protein synthesis, localization, and function. We first established by immunofluorescence microscopy that the tail and TMH region of the enzyme were sufficient for membrane focal localization of the GST fusion protein to the septum in *E. faecalis*. We confirmed that the SrtA_{tail-TMH}-GST was associated solely with the membrane fraction. While SrtA_{tail}-GST and the GST constructs were associated with the membrane, only the SrtA_{tail-TMH}-GST focally localized to the septum, further establishing the need of the tail and transmembrane helix to direct SrtA localization to the septum in *E. faecalis*. However, additional internal controls are still in progress for the other subcellular compartments (i.e. the cell wall and cytoplasm) to confirm clean fractionation. We employed alanine scanning mutagenesis to assess the contribution of each residue to synthesis and localization of the GST fusion protein. Alanine scanning is a widely used approach since alanine is a non-bulky amino acid that does not alter main chain conformation of the protein or introduce steric or electrostatic hindrance (Wells 1991). Using this mutagenesis approach, we identified seven amino acid residues that affected localization of the GST fusion protein; however, mutation of these residues in the full-length protein did not perturb focal localization. To explain this discrepancy, we hypothesized that in the full-length SrtA, the catalytic domain could contribute to stabilizing focal localization at the septum, possibly through its dimerization capability (Zhu, Xiang et al. 2016), which is why we observed mislocalization in the GST fusion protein and not the full-length SrtA. To address this, we are constructing combinatorial mutant strains, including residues involved in dimerization, to see if mutating a combination of the tail and TMH residues would affect localization and probably function of the enzyme.

In addition, we observed protein toxicity in *E. faecalis* in the K10A (GST) mutant, and toxicity is often associated with high expression of heterologous proteins. In the case of the K10A mutant, replacing lysine with alanine may have led to a change in protein folding, especially since lysine is at the interface between the tail and transmembrane region, leading to exposure of hydrophobic surfaces and protein aggregation. The lysine mutation is not a dominant mutation since, in the presence of chromosomal SrtA, we observed septal localization of the GST fusion protein (data not shown), indicating that the GST fusion protein and native SrtA interact (possibly form a dimer) to localize to the septum. Mutating lysine may also interfere with the insertion of the protein into the membrane (Stewart, Bailey et al. 1998), which is why the protein is specifically toxic to *E. faecalis* and not *E. coli*

We also observed a change in protein levels of the alanine scan mutants on both the SrtA_{tail}-TMH-GST and SrtA-2HA constructs. The tail region is also the signal sequence for SrtA and mutating this region could lead to change in protein stability, folding or degradation (Beena, Udgaonkar et al. 2004). The TMH region is rich in hydrophobic residues and substitutions in this region could lead to a change in protein solubility state prior to insertion into the cell membrane. Changes in protein solubility, stability, and/or folding could lead to rapid degradation of the fusion protein and therefore we detect an overall decrease in protein quantity.

Apart from studying the effects of mutations on expression and localization, we also functionally characterized the full-length SrtA tail and TMH mutants. We measured the substrate (Ebp and Asc10) attachment difference between cell wall and protoplast in SrtA-2HA and the mutant strains. N31A showed a significant difference in its ability to attach Ebp and Asc10 to the cell wall, yet did not show a reduced Ebp-associated biofilm phenotype when compared with SrtA-2HA in the crystal violet

biofilm assay. Since immunoblots are semi-quantitative, we aim to validate the difference in substrate attachment in the mutants by immunomicroscopy techniques. We will also measure biofilms that are dependent on *both* Asc10 and Ebp for the point mutant strains to determine if the defect in substrate attachment results in a weaker biofilm. Since the *in vitro* biofilm assay was carried out on polystyrene surfaces, we could assess the efficiency of SrtA mutants to attach substrates to the cell wall that mediate attachment, uptake, and/or survival in host cells such as macrophages. In the next chapter, we address the protein interactions that govern localization of SrtA in *E. faecalis*.

Chapter 4

Sortase A interacts with cytoplasmic and membrane associated proteins

Subcellular organization of proteins, lipids, ribosome, DNA, RNA within a cell is necessary for the survival of bacteria and its adaptation to the external environment. The subcellular localization of these biomolecules are mediated by intra and intermolecular interactions (Laloux and Jacobs-Wagner 2014). Studies over the past two decades have focused on unearthing the mechanisms and themes that govern localization of macromolecules within the cell. Membrane proteins have been the focus of much of this research due to the role they play in linking the inner and outer environment of the cell. Sortase A localization, as described in Chapter 1, is closely linked to cell wall and potentially cell cycle machineries since its localization is cell cycle dependent and tracks with the septum of the cell. SrtA localization could be dependent on the cell machineries at these domains and is mediated by interactions with proteins and lipids at these domains (Raz and Fischetti 2008, Kline, Kau et al. 2009, Kandaswamy, Liew et al. 2013). We hypothesized that interactions mediating localization of SrtA would occur with the cytoplasmic tail and to a certain extent with the transmembrane. In this chapter, we describe studies aimed to elucidate proteins that interact with SrtA and determine if they affect localization of the protein in *E. faecalis*. We carried out protein-protein crosslinking, pull-down, and co-immunoprecipitation followed by mass spectrometric analysis. Proteins identified as putative interacting partners were further analyzed *in vivo* by a bacterial two hybrid assay. Finally, we examined the expression and localization pattern of SrtA in the transposon and deletion mutants of the newly identified protein interacting partners.

4.1 DSP crosslinks proteins in *E. faecalis* both *in vitro* and *in vivo*

DSP (or Lomant's reagent) is a homobifunctional crosslinking agent with a 12 Å^o spacer arm. DSP reacts with amines on the sidechain of lysine residues or primary amines at the N-terminus of proteins. DSP is also more specific than formaldehyde because it does not crosslink DNA (Kurdistani and Grunstein 2003). Moreover, DSP is lipophilic and able to cross the cell membrane making it ideal for crosslinking the lysine rich tail of SrtA with putative interacting partners.

We first set out to optimize the concentrations and reaction time of DSP treatment in order to obtain efficient crosslinking and decrease false positive. For crosslinking optimization and other studies, we used the *E. faecalis* Δ srtA/pAK1-SrtA_{tail-TMH}-GST strain (overexpressing SrtA tail and transmembrane region fused to GST). We took two general approaches for crosslinking studies: 1) we first lysed cells and then performed crosslinking, or 2) we treated intact cells with crosslinking agent prior to cell lysis. In the first approach, we first obtained whole cell lysates by treating 2 ml cultures with 10 mg/ml of lysozyme in phosphate buffered saline (PBS) for 1 hour at 37°C followed by sonication. The buffers were devoid of any primary amine such as Tris since DSP reacts with primary amines rendering it ineffective. The whole cell lysates were then treated with DSP concentrations varying from 0.5 to 3 mM, reaction times of 15, 30, 60 mins at room temperature (**Fig. 2.1**, Chapter 2). We then performed western blot using anti-GST antibodies to determine optimal crosslinking conditions. Effective crosslinking was determined by the presence of molecular weight bands detected above 27 kDa, which is the molecular weight of SrtA_{tail-TMH}-GST, that were immunoreactive with the anti-GST antibody indicative of complexes crosslinked with SrtA_{tail-TMH}-GST. At concentrations of 0.5 mM and 1 mM and reaction times of 15 and 30 mins, we obtained multiple bands above 27 kDa (**Fig. 4.1**). These bands were not

detected in control samples where we added only the solvent dimethyl sulfoxide (DMSO) (data not shown).

Fig. 4.1: Anti-GST immunoblot for optimization of DSP crosslinking post lysis *in vitro* at 0.5 mM and 1 mM using SrtA_{tail}-TMH-GST. Lysed whole cell suspensions of *E. faecalis* Δ srtA/pAK1-SrtA_{tail}-TMH-GST were treated with 0.5 mM and 1 mM of DSP for 15, 30, and 60 mins. Band observed at 27 kDa is the SrtA_{tail}-TMH-GST. Higher molecular weight bands represent complexes crosslinked with SrtA_{tail}-TMH-GST and hence reactive with anti-GST antibody.

Since *E. faecalis* is a Gram-positive bacterium, DSP must penetrate both cell wall and cell membrane. Therefore, in a second approach, we tested the efficacy of DSP to

penetrate the cell wall of *E. faecalis* prior to lysis. We added DSP at a concentration of 0.5 mM for 30 mins to the resuspended pellet of *E. faecalis* Δ srtA/pAK1 SrtA_{tail}-TMH-GST, before and after lysozyme treatment (to remove the cell wall and promote lysis), followed by sonication. We again analyzed the efficacy of crosslinking by western blot and compared the banding pattern between the two samples (**Fig. 4.2**). We observed multiple bands above the 27 kDa SrtA_{tail}-TMH-GST band in the test lanes indicating that that DSP can effectively crosslink proteins pre- and post-lysis. Multiple bands were not observed in the control sample (**Fig. 4.2** Lanes depicting 0 mM) indicating that the bands observed in the test lane were specific to DSP crosslinking. For GST pull down and co-immunoprecipitation experiments, we crosslinked the samples pre-lysis with 0.5 mM DSP for 30 mins.

Fig. 4.2: Efficacy of DSP pre- and post-lysis using *E. faecalis* Δ srtA/pAK1-SrtA_{tail}-TMH-GST. The 27 kDa band corresponds to SrtA_{tail}-TMH-GST. Higher molecular weight

bands correspond to complexes crosslinked to SrtA_{tail-TMH}-GST and hence detected by anti-GST antibody.

4.2 Multiple protein interactions detected by GST pull-down and co-immunoprecipitation

We employed two affinity-based techniques to detect interacting partners of SrtA using the standardized conditions of 0.5 mM of DSP for 30 mins. First, the GST pull-down method was exploited using the test strains *E. faecalis* Δ srtA/pAK1-SrtA_{tail-TMH}-GST and negative control strain *E. faecalis* Δ srtA/pAK1-GST. The eluted fractions were run on an SDS-PAGE gel and probed with anti-GST antibody. We observed higher molecular weight bands in the samples that were crosslinked and subsequently lysed. We did not observe any higher molecular weight bands in the GST control samples indicating that this was not a non-specific interaction with the GST tag.

Unlike GST pull down that utilizes the affinity between GST and glutathione beads, co-immunoprecipitation makes use of antibodies against the protein of interest (SrtA in this case) bound to protein A/G beads. We used magnetic beads that can be separated easily from the solution using a magnetic rack, simplifying washing and subsequent elution steps. The whole cell lysates of *E. faecalis* and *E. faecalis* Δ srtA/pAK1-SrtA (*srtA* overexpressed from the plasmid) were prepared as described earlier and the co-immunoprecipitation eluents were analysed by western blot using anti-SrtA antibody. We used two controls for the co-immunoprecipitation; a non-specific IgG control and *E. faecalis* Δ srtA control to detect non-specific interactions with the protein A/G beads and the antibody itself. We observed higher molecular weight bands at ~40 kDa and between 120-190 kDa in both crosslinking-lysis test

conditions (**Fig. 4.3**). We also observed an intense band at 120 kDa in all samples. This band is probably the IgG antibody as we observed a similar band in the IgG and *ΔsrtA* controls and is expected as we did not add reducing agents to the eluted fractions. We also tested the efficacy of dithiothreitol (DTT) to break the DSP crosslinks by treating the eluted samples with 50mM DTT and incubating them for 30 mins at 37°C. The DTT effectively cleaved the DSP crosslink as none of the bands observed in the non-reduced samples were seen in the reduced samples (**Fig. 4.3**). DTT also affected the antibody band observed at 120 kDa.

Fig. 4.3: Anti-SrtA immunoblot for co-immunoprecipitation using crosslinked whole cell lysates of *E. faecalis* *ΔsrtA*/pAK1-SrtA. Band at 27 kDa corresponds to SrtA. The test lane corresponds to crosslinked pSrtA immunoprecipitated with SrtA

antibody, the IgG lane corresponds to crosslinked pSrtA immunoprecipitated with non-specific IgG antibody, and dSrtA corresponds to *E. faecalis* Δ srtA cell lysate immunoprecipitated with SrtA antibody. The last three lanes are treated with DTT to cleave the DSP crosslink. Arrows indicate bands seen only in the test lane and not in the control lanes.

We stained the gels with mass spectrometry compatible silver stain (Shevchenko, Wilm et al. 1996) and cut out single bands from the test sample lane and corresponding band in the control lane. The samples were subjected to trypsin digestion followed by microcapillary LC/MS/MS analysis using the Orbitrap mass spectrometer (Thermo Scientific) at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, USA). The identification of the peptides and proteins were done at the facility by comparing the readouts to the OG1RF database using the Sequest algorithm (Eng, McCormack et al. 1994). The background proteins in the co-immunoprecipitation were significantly less than those observed in the GST pull down assay, indicating that in our setup the co-immunoprecipitation was more specific. The proteins listed in **Table 5** were obtained after comparing the proteins in the test samples (both, GST pull-down and co-immunoprecipitation) to the control samples, eliminating proteins with a peptide hit of < 3 , molecular weight of < 20 kDa, and a false discovery rate of zero. We also eliminated ribosomal proteins, as they are common contaminants of pull down and co-immunoprecipitation assays, and LPXTG-motif proteins as they would be SrtA substrates. We ensured that SrtA was detected in the test samples as absence of SrtA would indicate a non-specific interaction.

Except for the WxL surface domain protein, all the putative interacting partners were found to be cytoplasmic or membrane proteins. Although this putative partner doesn't contain an LPXTG motif and so is not a predicted sortase substrate, it is part of an operon containing a transmembrane protein and an LPXTG-containing protein indicating a possible novel assembly of this surface protein via SrtA (Galloway-Pena, Liang et al. 2015).

Table 5: List of putative interacting partners with SrtA

Locus	Molecular weight (kDa)	Localization	Function	Peptide hits	No. of experiments
OG1RF_12006	57.11	Cytoplasm	GroEL, chaperone	8-12	3
OG1RF_10735 [#]	26.65	Cytoplasm	DivIVA, cell division	2	2
OG1RF_11455 [#]	21.2	Cytoplasm	General stress protein	5	2
OG1RF_10488	76.8	Cell wall	WxL surface domain protein	3	2
OG1RF_12158	82.6	Cytoplasm	FtsI, cell wall synthesis machinery	5	2
OG1RF_11078	65.6	Cytoplasm	DnaK, chaperone	7	2
OG1RF_12441	42.4	Cytoplasm*	Hypothetical serine protease	7	2
OG1RF_12305	44.7	Membrane	HtrA, Serine protease	4	2
OG1RF_12362	48.6	Membrane associated	Signal recognition particle receptor	3	1

*predicted

[#] detected in GST pull down

Total number of co-immunoprecipitation experiments = 3

Total number of GST pull down experiments = 2

4.3 SrtA tail and TMH show strong interaction with FtsY and DnaK *in vivo*

We validated the *in vitro* interactions between SrtA_{tail-TMH} and the prey proteins by the adenylate cyclase bacterial two-hybrid assay. SrtA_{tail-TMH} was cloned into the pKTN25 plasmid while the nine prey proteins were cloned individually into the pUT18 plasmid. The two plasmids were co-transformed into *E. coli* BTH101 and spotted onto minimal media containing X-gal and IPTG with appropriate antibiotics. The plates were incubated at 30°C for up to four days. Within 24 hours, we observed blue colonies for the FtsY and DnaK test strains indicating a strong interaction between these proteins and SrtA_{tail-TMH}, individually (**Fig. 4.4**). We observed faint blue colour development for GSP, HSP, WxL, HtrA, DivIVA, and GroEL except for FtsI which only gave rise to white colonies (**Fig. 4.4**). As a negative control, we tested the interaction of the prey proteins with an *E. coli* leucine zipper protein (encoded by pKT25-*zip*). We did not observe any colour development in the transformants after 4 days of incubation (**Fig. 4.5**).

Fig. 4.4: Spot assay to test interaction between prey proteins and SrtA_{tail-TMH} by blue-white colony screening. Prey proteins cloned on the pUT18 were transformed with bait protein cloned on pKNT25. The co-transformants were spotted on minimal media containing X-gal and IPTG. Blue color indicates positive interaction. n=3

Fig. 4.5: Negative control for spot assay for by blue-white colony screening. Prey proteins cloned on the pUT18 were transformed with negative control bait leucine zipper protein cloned on pKNT25. The co-transformants were spotted on minimal media containing X-gal and IPTG. Blue color indicates positive interaction. n=3

Fig. 4.6: Immunodetection of plasmid and chromosomal *srtA* in transposon and deletion mutants. (a) Plasmid-encoded *srtA* detected using anti-HA (b) Chromosome-encoded *srtA* detected using anti-SrtA. Both (a) and (b) are from deletion and transposon *E. faecalis* strains transformed with pAK1-SrtA-2HA. (c) Chromosome-encoded *srtA* detected using anti-SrtA in untransformed strains. SecA was used as a loading control.

4.4 SrtA mislocalizes in DnaK mutant background

We identified two potential interactors of SrtA: FtsY and DnaK. We next aimed to identify if SrtA mislocalizes in the absence of these proteins. Since FtsY is essential in *E. faecalis*, we are in the process of making a conditional mutant for this gene. To study the localization of SrtA in a *dnaK* mutant background, we transformed the pAK1-SrtA-2HA plasmid into *dnaK::Tn* and examined SrtA localization using the anti-HA antibody. We simultaneously transformed the pAK1-SrtA-2HA plasmid into *groEL::Tn*, *wxl::Tn*, *ftsI::Tn*, and $\Delta htrA$. Since all these strains contained the chromosomal SrtA, we also transformed the pAK1-SrtA-2HA plasmid into $\Delta htrA\Delta srtA$ as a control for the $\Delta htrA$ strain. We first checked the expression of *srtA* in each of the mutant strains harbouring the SrtA-2HA plasmid (**Fig. 4. 5**). Interestingly, we found that in the *wxl::Tn*/pAK1-SrtA-2HA and *ftsI::Tn*/pAK1-SrtA-2HA, *srtA* was not expressed from the plasmid or chromosome (**Fig. 4.6 a and b**). However, in the *wxl::Tn* and *ftsI::Tn* strains chromosomal *srtA* was expressed at wild-type levels (**Fig. 4.5c**). We transformed these two strains with an empty vector and checked the expression of chromosomal *srtA* and found that it was expressed at wild-type levels (**Appendix-1 Fig 2**). Thus the reduced expression of chromosomal and plasmid *srtA* is not a plasmid effect but most likely due to presence of two copies of *srtA* in the cell. We then sequenced the genomic DNA of the *ftsI::Tn*, *wxl::Tn*, and *groEL::Tn* to verify correct insertion of the transposon. In the respective strains, the transposon was located at the promoter of *ftsI* and inserted at the 3' end near the stop codon of *groEL*. In the *wxl::Tn* mutant, the transposon was inserted within the OG1RF_10104 gene encoding a PpfI family protease (some of the transposon wells contain more than one transposon mutant within the *E. faecalis* transposon library). We are in the process of sequencing another *wxl::Tn* mutant which we hope will be a correct transposon insertion so that we can

study SrtA localization in a true *wxl* mutant. The lack of chromosomal *srtA* expression in the PfpI family protease transposon mutant would also be worth exploring as a possible regulatory mechanism of SrtA in the cell. All the other strains expressed both the plasmid and chromosomal SrtA (**Fig. 4.6 a and b**).

We observed that SrtA-2HA localized to the septum in all the strains except in the *dnaK::Tn* (**Fig. 4.7**). In the DnaK transposon mutant approximately 33% of the cells show an aberrant growth rate and cell morphology where cells appeared elongated and chained (Irina Afonina, unpublished) which we observed in the immunofluorescence images of the *dnaK::Tn* transformed with pAK1-SrtA-2HA. SrtA-2HA in *dnaK::Tn* localized to one half of the cell and we hypothesize that it localizes to either the old pole or new pole cells and not both. Taken together, these data suggest that DnaK plays an integral role in localizing SrtA to the septum in *E. faecalis*.

Fig. 4.7: SrtA-2HA mislocalized in *dnaK::Tn*. (a) PSICIC analysis of immunofluorescence images (anti-HA) showing fluorescence peaking from 25 AU to 50 AU, indicating localization of SrtA-2HA to one half of the cell. (b) and (c) Fluorescence image and corresponding phase contrast image. Not all cells fluoresce which is observed in all strains transformed with the pAK1-SrtA-2HA. Number of cells analysed >100. n=3. Scale bar is 2 μ m.

4.5 Discussion

In this chapter, we identified interacting partners of SrtA using *in vitro* and *in vivo* approaches. We stabilized interactions by using a cleavable crosslinker, DSP. We optimized the crosslinking conditions in whole cell lysates and extended the analysis to pre-lysed cells. We concluded that a DSP concentration of 0.5 mM and a reaction time of 30 mins was optimal for our setup. However, we did not test other concentrations and reaction times for pre-lysed cells. Using this setup, we pulled down nine putative interacting partners for the SrtA tail and transmembrane helix and validated them by a bacterial two hybrid assay. We confirmed two strong interacting partners of SrtA tail and TMH: FtsY and DnaK.

FtsY is an essential membrane-associated protein and acts as a signal-recognition particle (SRP) receptor for the SRP-ribosome nascent chain (RNC) complex. Bacterial FtsY homologs do not contain any transmembrane domains but interact with the SecYEG translocon (Bibi, Herskovits et al. 2001, Angelini, Deitermann et al. 2005). Interaction between FtsY and SecY has been shown to play a role in membrane targeting of FtsY possibly via a conformational change in FtsY (Herskovits, Seluanov et al. 2001). SecYEG co-localizes with SrtA at the septum in *E. faecalis* and interaction between FtsY and SrtA_{tail-TMH} could be necessary for efficient sorting of the substrates at the septum. We are currently investigating if FtsY is necessary for localization of SrtA by creating a conditional mutant of FtsY.

DnaK, or heat-shock protein (Hsp70), is part of the chaperone complex DnaK-DnaJ-GrpE that is elevated in bacteria in response to high temperatures (Schulz, Tzschaschel et al. 1995). In *E. faecalis*, the expression of *dnaK* along with *groEL* are elevated in response to exposure to heat, bile salts, and detergents (Chiappori, Fumian et al. 2015). SrtA-2HA mislocalized in the *dnaK::Tn* mutant but localized to the septum

in the other chaperone transposon mutant, *groEL::Tn*. However, since GroEL is essential in most bacteria (Fayet, Ziegelhoffer et al. 1989, Burnett, Horwich et al. 1994), and since the transposon was inserted at the stop codon of *groEL*, it is possible that the functionality of GroEL is still retained. DnaK is involved in several cellular functions such as folding of nascent polypeptides, refolding of thermally damaged proteins, and membrane integrity in *Mycobacterium smegmatis* (Fay and Glickman 2014), and very recently in stability of DivIVA *S. aureus* suggesting a role in cell division (Bottomley, Liew et al. 2017). Therefore, mislocalization of SrtA-2HA in *dnaK::Tn* may not be related to the general chaperone activity of DnaK, but could be due to the interaction of SrtA with DnaK, directly or in a complex. The interaction between DnaK and SrtA_{tail-TMH} could be necessary for SrtA to recognize the septal region in the parent cell and future division septa in the daughter cell. We identified DivIVA in our *in vitro* pull-down assays, but we did not observe a strong interaction between DivIVA and SrtA in the *in vivo* assay. It is possible that SrtA could be indirectly interacting with DivIVA via DnaK. This would explain the recruitment of SrtA from the septum to sites of nascent division septa in the daughter cell during cell division. Interestingly, we have preliminary data indicating that the mislocalization occurs during mid-division phases and is recovered during later division stages suggesting that the mislocalization is a function of cell cycle. Taken together these data strongly suggest that SrtA localization to the septum is governed by protein-protein interactions with the cell division machinery, which corroborates with earlier knowledge that SrtA localization is a function of the cell cycle.

Chapter 5

Conclusion and Future Studies

Localization of proteins to distinct sites is often linked to the function of the protein within a cell. Proteins in bacteria rely on several factors to localize and interact with other proteins including protein sequence determinants, protein-protein interactions, lipid composition of the membrane, and geometric cues. Understanding how proteins localize can give us a clue to their function and the interrelated, or functionally linked, cellular processes. In this thesis, we addressed two factors that govern SrtA localization in *E. faecalis*: sequence elements and protein interacting partners. We have shown that sequence elements within SrtA are necessary for directing protein localization to distinct foci on the membrane. These sequence elements could be required for proper insertion into the membrane, stabilizing protein-lipid interactions, and/or interaction with other proteins in the membrane or cytoplasm. Of the residues that affected SrtA_{tail}-TMH-GST localization, we speculate that K10, N11, W12, and N31 might interact with the hydrophilic groups of the lipid bilayer while L17, L18, and L28 may be involved in intermembrane interactions. The helix projection (**Appendix-1 Fig. 1**) of the transmembrane region of SrtA showed all the mutation sites except W12 clustered at one face of the α -helix supporting the hypothesis that L17, L18, and L28 could be involved in intermembrane interactions and possibly facilitate insertion of the protein into the membrane. Some residues, such as N31 on the transmembrane helix, may be necessary for efficient sorting of substrates by SrtA. N31, in addition to residues within the catalytic domain, may also be involved in stabilizing the SrtA dimer (Zhu, Xiang et al. 2016). Dimerization dependent localization has been shown in the pole-to-pole oscillation of the Min system in *E. coli*. MinD-ATP bound

dimers are exclusively associated with the membrane starting from the poles, followed by dissociation of the dimers by MinE. Monomers of MinD-ADP are found in the cytosol and dimerization leads to reassociation with the membrane (Lackner, Raskin et al. 2003, Park, Wu et al. 2011). However, this association is also dependent on the ATP to ADP conversion (Lackner, Raskin et al. 2003), which reinforces the idea that a network of factors govern protein localization in a cell.

We aligned *E. faecalis* SrtA with homologs from 41 different bacterial species with >50% total sequence identity (**Appendix-1 Fig. 3a**) and from 12 different *Enterococcus* species to identify the conserved residues within the tail and transmembrane region (**Appendix-1 Fig. 3b**). L28, F30 and I34 were highly conserved amongst the 41 sequences analyzed, while G25 and L26 were weakly conserved (**Appendix-1 Fig. 3a**). Within the 12 *Enterococcus* species (**Appendix-1 Fig. 3b**), the tail and transmembrane region showed significant homology and conservation of residues with N15, L18, G25, L28, F30, Q33, and I34 being fully conserved. L17, L21, F22, I23, I24, and L26 were highly conserved while N11, L13, V19, and N31 were weakly conserved within the enterococcal SrtA sequences. We observed mislocalization in the heterologous SrtA_{tail-TMH}-GST fusion protein upon mutating K10, N11, W12, L17, L18, L28, and N31. Amongst these residues, L28 is the most highly conserved residue within enterococcal SrtA and across other bacterial species. Similar to L28, F30 and I34 are conserved but we were unable to obtain transformants for F30A and did not observe mislocalization on mutating I34. L17 and L18 are highly conserved amongst the enterococcal SrtA supporting our hypothesis that within the transmembrane helix these two residues, along with L28, may be responsible for intermembrane interactions necessary for septal enrichment of SrtA.

We have also identified novel interacting partners of SrtA by using *in vitro* and *in vivo* techniques. DnaK and FtsY strongly interact with SrtA in the bacterial two-hybrid assay. We demonstrate for the first time that in the absence of DnaK, SrtA localizes to one-half of the cell periphery indicating that SrtA localization to distinct foci at the mid-division phase is mediated by DnaK. We have preliminary data demonstrating that this mislocalization of SrtA in a DnaK mutant is cell cycle dependent which supports prior knowledge that SrtA focally localizes to distinct domains at different stages of cell division. In future work, it will be of interest to assess the attachment of SrtA substrates in a DnaK mutant and investigate the virulence of this strain in biofilm assays. Data from the lab (unpublished) shows that DnaK is involved in Ebp biogenesis and it is worth exploring the involvement of DnaK in expression of other SrtA substrates in *E. faecalis* through transcriptomics and immunofluorescence assays. We also hypothesize that DivIVA, through DnaK, may be indirectly involved in SrtA localization and are in the process of making a conditional mutant of DivIVA to assess its role in localizing SrtA to the septum.

Besides sequence elements and protein interactions, lipid domains and geometric cues are involved in protein localization. DivIVA in *B. subtilis* localizes to sites of nascent septum and recognizes these negatively curved sites, which is also rich in cardiolipin, via its N-terminus. Thus, DivIVA exploits both, concave surfaces and cardiolipin rich regions for localization during septation in *B. subtilis* (Barak and Wilkinson 2007, Lenarcic, Halbedel et al. 2009). Lipid domains and geometric cues could also be involved in SrtA localization and have not been explored in this thesis. Cell shape mutants that perturb the septum formation, such as in a RodA mutant, would be ideal to test the localization. The septum in *E. faecalis* is rich in anionic lipid domains (Kandaswamy, Liew et al. 2013), which is a feature of other ovococci such as *S.*

pyogenes (Rosch, Hsu et al. 2007), and it will also be of interest to assess if these lipid domains are necessary for SrtA localization, initially by performing crude lipid pull downs (*in vitro*) to determine enrichment of SrtA with anionic lipids or creating strains depleted in anionic lipids (*in vivo*) and assessing SrtA localization by immunofluorescence imaging.

The two approaches in this thesis to elucidate protein sequence determinants and interacting partners are complementary and allow us to assess if these two factors are inter- dependent for SrtA localization. In the future, it will be important to determine if mutation of the residues, as point and/or combinatorial mutants, that govern localization will abolish interaction with DnaK and FtsY in a reciprocal pull down and bacterial two hybrid assays.

Taken together, this study advances our knowledge of the mechanisms by which SrtA focalizes to the septum in *E. faecalis*. SrtA is conserved in all Gram-positive bacteria and is a virulence factor. Understanding the mechanisms by which this enzyme localizes and efficiently functions is an avenue for identifying potential antivirulence strategies. For example, molecular chaperones, such as DnaK, have recently been in the limelight as potential drug targets (Neckers and Tatu 2008, Chiappori, Fumian et al. 2015). DnaK could therefore serve as a drug target since it mediates SrtA localization and possibly hinders efficient attachment of substrates to the cell wall. Further research into characterizing SrtA interacting partners and identifying hotspot residues within the protein will aid in combating not only enterococcal infections but other Gram-positive pathogens.

APPENDIX-1

Appendix-1 Fig. 1: Helical wheel projection of the transmembrane helix of SrtA.

The transmembrane helix residues (W12 to I34) are represented by the α -helical projection using Helical Wheel Projections by RZ Lab. Hydrophilic residues are shown as circles and hydrophobic residues as diamonds. The most hydrophobic residues is green and the amount of green decreases proportionally to hydrophobicity. The least hydrophobic residue is coded yellow. The most hydrophilic (uncharged) residue is coded in red and the amount of red decreases proportionally to hydrophilicity. Mutations on the transmembrane region affecting localization are circled in blue.

Appendix-1 Table 1:

Mutated residue	SrtA_{tail}-TMH-GST fusion protein level and localization	SrtA-2HA fusion protein level and localization
R2A	-/septal	na
P3A	+/septal	na
K4A	+/septal	na
E5A	-/septal	na
K6A	-/septal	-/septal
K7A	-/septal	na
R8A	-/septal	na
K10A	+/distributed	+/septal
N11A	+/distributed	-/septal
W12A	0/ focally distributed	0/septal
L13A	0/septal	na
L17A	0/distributed	0/septal (data not shown)
L18A	-/distributed	+/septal
L20A	0/septal	na
L28A	0/ distributed	0 / septal (data not shown)
N31A	-/focally distributed	+/septal
N32A	0/septal	na
Q33A	-/septal	na
I34A	+/septal	na

* '+' Higher protein level than SrtA_{tail}-TMH-GST or SrtA-2HA, '-' Lower than SrtA_{tail}-

TMH-GST or SrtA-2HA, '0' Same as SrtA_{tail}-TMH-GST or SrtA-2HA

Appendix-1 Fig. 2: Immunoblot to detect chromosomal SrtA in transposon mutants transformed with empty vector. Chromosomal SrtA in the transposon mutants transformed with pAK1 empty vector (lane 5 and 7) was expressed at wild-type levels (lane 1 and 3). SecA was used as loading control.

(a)

E. faecalis WP_002393168.1
E. faecalis EOL21535.1
Bacillus WP_002354994.1
L. lactis AGY44190.1
L. graminis WP_057907619.1
L. mucosae WP_006499403.1
E. rivorum WP_069699916.1
L. murinus WP_076148970.1
L. concavus WP_057822920.1
V. teuberi WP_084657140.1
V. fluvialis WP_086951281.1
L. saniviri KRO15118.1
L. collinoides WP_056997353.1
L. salivarius WP_080979053.1
E. sulfureus WP_016186156.1
E. faecalis EGG52276.1
M. plutonius WP_013772990.1
L. lactis EQC95333.1
L. lactis AFW91663.1
L. paracasei WP_016367614.1
S. urinalis WP_006738745.1
L. saerimneri WP_027826637.1
L. paracasei WP_071253026.1
L. coleohominis KRM82477.1
M. plutonius KMT25711.1
S. agalactiae WP_095456289.1
S. agalactiae CCW42096.1
G. sanguinis WP_065377896.1
E. quebecensis WP_069635697.1
Streptococcus WP_102777144.1
Streptococcus WP_071793172.1
S. anginosus WP_003024433.1
C. perfringens WP_003457562.1
E. aquimarinus WP_071874571.1
S. constellatus WP_006269590.1
S. devriesei WP_027976025.1
L. aviaries WP_057827005.1
S. entericus WP_018368967.1
L. mutans AKH05478.1
L. kefiri WP_056982698.1
S. agalactiae WP_017646311.1
E. canintestini WP_071865201.1

TAIL TRANSMEMBRANE HELIX
1 10 20 30
-----MRPKEKKRGKNWLNLSLLVLLFFIIGLALIFNNQI
-----MRPKEKKRGKNWLNLSLLVLLFFIIGLALIFNNQI
-----MRPKEKKRGKNWLNLSLLVLLFFIIGLALIFNNQI
----MAERKKTKRGKYNWLLNTFIVLLFIVGLVLFIFNKPI
-----MQKKTKNRLINGLLAILLIMGLVLFIFINPI
-----MHRKLNWLRWTVAVILFVVGGLALIFNQI
MASRKNRHTKNKSNKKNWLVNILLFVLLIGLALIFNTQI
-----MKKVLKYCLIVVLLVIGLALIFNNQI
----MKKTGRKKTTHKVLSTIGVLLFVGLALVFNNQI
-----MMVILFLLGVALIFNNQI
-----MVLLLVGFALIFNNQI
----MKPVKKEHRARTWFVRTLMLVGLIGLALVFNEQI
--MDQEPKKQPKKRGRRLYRTLFAVLIVISLGLIFNEQI
-----MSKIWKVLQWVIVVLMIVGLALIFNEQI
-----MKKKKGVLNWVTNLFVLLVLLIGLALVFNNQI
-----MKLKKILTNTLLVLLFAVGLVLFVNNQI
----MMTKNKKRKLKNWITNILLFFLLSIGLILVFNNQI
--MEERTKNKNKKRKNWLLNTFIVLLFIVGLVLFIFNKPI
--MEERTKNKNKKRKNWLLNTFIVLLFIVGLVLFIFNKPI
-----MQNRKNKYRWLRWTLIMLLIVSLAMIFNEQL
---MKKRQVTKSKVWNFLRWLLVILLTIGLALIFNKSI
---MA---AKKSHK---SWLYRVALLLVIGLALVFNEQI
-----MQYRKNKYRWLRWPLIMLLIVSLAMIFNEQL
-----MKKKEWLRTAIVVLFVSLALVFNNQI
-----MTKNKKRKLKNWITNILLFFLLSIGLILVFNNQI
-----MVLLIIVGLALVFNKPI
-----MVLLIIVGLALVFNKPI
-----MNKKLKNWLNILIVVLLATSVVLFIFKNQI
MASK-KLRPKKKNRKNWLNIFLFLLLIVGLALVFNTQI
----MTTKRNKKKGSSRLRNLLAVVLLIIGLGLMFNKTI
----MTTKRIKKKGSSRLRNLLAVVLLIIGLGLMFNKTI
----MLSTRTKHNKRKNILINIATLLIIVALALIFNSSI
-----MRIKESKTRRYLINILLVTMTIIGIILILNDQI
-----MKRKKRLKNSLINFLFLVLLVIGFALIFNRQI
----MSSRRKKHNKRKNILINIVATLLIIVALALIFNSSI
----MNNMRKPKKKRHFLRSFFILLVIGLALIFNKPI
--MAESRSKKHGKVRKTLVSILVILLVLCGLALIFNEQI
--MTKTRKTKHSKRLGRFLYNLFVLLVIVGLALIFNKSI
----MKKERQSKKKRGFLRIFLPILLVIGLALIFNTPI
-----MRKGFLKLLINIVFVLLIIVALGLIFNRQI
----MRNKKKSHGLENFVRWLLVLLIIVGLALVFNKPI
-----MKKKSREKNWLNIMLLLLLVGLGLTFNNQI
.. : : ::

(b)

Appendix-1 Fig. 3: Multiple sequence alignment of SrtA across (a) different bacterial species and (b) 12 *Enterococcus spp.* The N-terminal tail and TMH region of SrtA of *E. faecalis* OGIRF (the first sequence in a and b) was compared with homologous sortase sequences using Clustal Omega (Goujon, McWilliam et al. 2010, Sievers, Wilm et al. 2011) and are presented in order of total sequence similarity.

‘.’ indicates conservation between groups of amino acids with weakly similar properties, ‘:’ indicates conservation between groups of amino acids with strongly similar properties, ‘*’ indicates complete conservation. The residues highlighted are those that resulted in mislocalization of SrtA_{tail-TMH}-GST.

APPENDIX-2

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

Membrane proteins represent a significant proportion of total bacterial proteins and perform vital cellular functions ranging from exchanging metabolites and genetic material, secretion and sorting, sensing signal molecules, and cell division. Many of these functions are carried out at distinct foci on the bacterial membrane, and this subcellular localization can be coordinated by a number of factors, including lipid microdomains, protein–protein interactions, and membrane curvature. Elucidating the mechanisms behind focal protein localization in bacteria informs not only protein structure–function correlation, but also how to disrupt the protein function to limit virulence. Here we review recent advances describing a functional role for subcellular localization of membrane proteins involved in genetic transfer, secretion and sorting, cell division and growth, and signaling.

Conference abstract: Microbial Adhesions and Signal Transduction Gordon Research Seminar and Conference, July 2017.

Sortase A (SrtA) is a membrane protein responsible for covalently anchoring a number of virulence factors and adhesins to the cell wall of Gram-positive organisms, including Enterococci. In *E. faecalis*, SrtA localizes to single foci at the septum during early division phases and reorients to multiple foci at sites of nascent cell division during later stages of the cell cycle. We aimed to identify the factors that govern the

localization of SrtA in *E. faecalis*. Structurally, SrtA consists of a C-terminal catalytic domain, a single transmembrane helix (TMH), and an N-terminal positively charged cytoplasmic tail. We carried out alanine scan mutagenesis to identify residues important for focal localization (fused to GST). While almost all mutations decreased the expression of the GST-fusion protein, eight individual mutations within the tail and TMH region also resulted in its mislocalization. We also carried out cross-linking with dithiobis(succinimidyl propionate) and co-immunoprecipitation to identify interacting partners of SrtA. Mass spectrometry analysis revealed putative interacting proteins including chaperone proteins DnaK, GroEL, and HtrA; cell division protein DivIVA; signal recognition particle receptor FtsY; and cell wall machinery protein Pbp2B. Together these findings suggest that SrtA localization to distinct foci in *E. faecalis* may be governed, independently or in conjunction, by sequence elements and multiple protein-protein interactions at the septum of the cell.

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