Spatial and temporal localization of cell wall associated pili in *Enterococcus Faecalis*

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**SPATIAL AND TEMPORAL LOCALIZATION OF CELL
WALL ASSOCIATED PILI IN *ENTEROCOCCUS*
*FAECALIS***

CHOO PEI YI

SCHOOL OF BIOLOGICAL SCIENCES

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Master of Science

2020

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I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Abbreviations

Ace	Collagen binding protein
AFM	Atomic force microscopy
AS	Aggregation substance
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BADA	BODIPY-FL 3-amino-D-alanine
BHI	Brain heart infusion
BSA	Bovine serum albumin
CWS	C-terminal cell wall sorting
CWSS	C-terminal cell wall sorting signal
Ebp	Endocarditis and biofilm associated pilus
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>E. faecium</i>	<i>Enterococcus faecium</i>
Esp	Enterococcal surface protein
FDAA	Fluorescent D-amino acid
GeIE	Gelatinase
GI	Gastrointestinal
HADA	7-hydroxycoumarin-3-carboxylic acid 3-amino-D-alanine
HMW	High molecular weight
L-Ala	L-alanine
OD	Optical density
PBPs	Penicillin binding proteins
PB	Phosphate buffer
PBS	Phosphate buffer saline
PG	Peptidoglycan
RT	Room temperature

<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
SIM	Structured illumination microscopy
SprE	Serine protease
SrtA	Sortase A
SrtC	Sortase C
TADA	TAMRA 3-amino-D-alanine
UTI	Urinary tract infection

Abstract

Enterococcus faecalis relies upon a number of cell wall-associated proteins for virulence. One sortase-assembled virulence factor is the endocarditis and biofilm associated pilus (Ebp), an important factor for biofilm formation. The current paradigm for virulence factor assembly in Gram-positive bacteria is that Sortase A recognizes and cleaves at the LPXTG motif within its substrates and covalently attaches them to the growing cell wall at sites of new cell wall synthesis. While the cell wall anchoring mechanism and polymerization of Ebp is well characterized, less is known about the spatial and temporal deposition of this protein on the cell surface. We followed the distribution of Ebp and peptidoglycan (PG) at different growth stages of *E. faecalis* via immunofluorescence, along with fluorescent D-amino acids (FDAA) staining. Surprisingly, cell surface Ebp did not co-localize with newly synthesized PG. Instead, surface-anchored Ebp was localized to the cell hemisphere but never at the septum where new cell wall is deposited. In addition, the older hemisphere of mid-division cells was completely saturated with Ebp, while at the newer hemisphere, Ebp appeared as two foci directly adjacent to the newly synthesized PG. When cell wall synthesis was inhibited by ramoplanin, an antibiotic that inhibits lipid II, new Ebp was still deposited at the cell surface. Based on these data, we propose an alternative paradigm for sortase substrate deposition in *E. faecalis*, in which Ebp can be anchored directly onto uncross-linked cell wall, independent of cell wall synthesis.

1. Introduction

1.1 Enterococci

Enterococci are Gram-positive, ovococcal, opportunistic lactic acid bacteria that are commonly found in the gastrointestinal tracts (GI) of humans and other animals (Kommineni *et al.*, 2015). Apart from human reservoirs, Enterococci can be ubiquitously found in plants, soil and water. Enterococci are able to withstand extreme environmental conditions such as temperatures ranging from 10°C to 45°C, high salt concentrations up to 6.5% NaCl, pH ranging from 4.5 to 10.0, and even in nutrient scarcity (Fisher and Phillips, 2009). Within the *Enterococcus* genus, *Enterococcus faecalis* and *Enterococcus faecium* are the two most common human-associated pathogen where *E. faecalis* is the primary isolate from clinical specimens (Gilmore and Clewell, 2002). Both *E. faecalis* and *E. faecium* are opportunistic pathogens capable of causing life-threatening infections in humans such as infective endocarditis, bacteremia, and urinary tract infection (UTI) (Hidron *et al.*, 2008; Murdoch *et al.*, 2009; Patterson *et al.*, 1995; Weiner-Lastinger *et al.*, 2019). *E. faecalis* in particular, is a rising cause of concern due to its resistance to multiple antimicrobial drugs, thus limiting therapeutic options (Arias and Murray, 2012).

1.2 Virulence factors

E. faecalis can colonize and infect different host niches by possessing multiple virulence factors which are responsible for adhesion and biofilm formation. *E. faecalis* virulence factors can be categorized into two main groups: secreted and surface attached factors. Secreted virulence factors expressed by *E. faecalis* include cytolysin, gelatinase (GelE), and serine protease (SprE) (Kawalec *et al.*, 2005; Waters *et al.*, 2003). Surface attached factors, which are frequently adhesins, make up the majority

of *E. faecalis* virulence factors. These adhesins include aggregation substance (AS), collagen binding protein (Ace), enterococcal surface protein (Esp), and endocarditis and biofilm-associated pilus (Ebp) (Hendrickx *et al.*, 2009), which are the most studied virulence factors to date. Unlike Gram-negative bacteria that possess an outer membrane, the Gram-positive bacterium *E. faecalis* contains a thick peptidoglycan cell wall, which acts as a scaffold for the processing and attachment of these virulence factors. A feature common to each of these cell-wall associated virulence factors is the presence of a C-terminal cell wall sorting (CWS) signal which consists of an LPxTG (leucine, proline, x- any amino acid, threonine and glycine) motif, a hydrophobic domain, and a positively charged cytoplasmic tail (Schneewind *et al.*, 1992). The enzyme responsible for the recognition and anchoring of these surface proteins to the peptidoglycan is the housekeeping enzyme, sortase A (SrtA) (Mazmanian *et al.*, 1999; Ton-That *et al.*, 1999). Sorting of *E. faecalis* virulence factors to the cell wall is crucial to its pathogenicity and long-term persistence during colonization and infection.

1.3 Ebp

Ebp is one of the most well-characterized cell wall anchored surface exposed proteins in *E. faecalis*. Ebp is important for biofilm formation, and is implicated in endocarditis and UTI (Nallapareddy *et al.*, 2011a; Sillanpaa *et al.*, 2013). Ebp plays a role in helping *E. faecalis* bind to platelets, fibrinogen, and collagen for surface attachment and immune evasion (Nallapareddy *et al.*, 2011a; Nallapareddy *et al.*, 2011b). Ebp expression is phase variable where only a subset of cells expresses Ebp in any cell population (Nallapareddy *et al.*, 2006). Ebp is made up of three pilin subunits (EbpA, EbpB, and EbpC), where EbpA and EbpB are minor subunits that form the pilus tip and base, respectively, while EbpC is the major subunit that makes up the pilus

backbone (Nallapareddy *et al.*, 2006; Nielsen *et al.*, 2013). The Ebp encoding genes are in the same operon together with sortase C (*srtC*), where SrtC is responsible for the polymerization of Ebp subunits (Nallapareddy *et al.*, 2011a). Pilus assembly begins at the cell membrane where EbpA is first recognized by SrtC, forming an EbpA-SrtC intermediate. A series of EbpC polymerization reactions then occur, resulting in the formation of the pilus backbone. Lastly, through an unknown mechanism of polymerization termination, EbpB is incorporated onto the growing pilus fibre, forming the base of the pilus. The fully assembled pilus is then anchored onto the cell wall by SrtA. Through this sorting mechanism, the fully polymerized and anchored Ebp is able to bind to multiple external surfaces, aiding *E. faecalis* in attachment to abiotic and biotic surfaces.

1.4 Sortase A

SrtA is a transpeptidase conserved in nearly all Gram-positive bacteria (Marraffini *et al.*, 2006). SrtA contains a transmembrane domain and is focally enriched at the septum of *E. faecalis* cells (Kline *et al.*, 2009). SrtA recognizes the C-terminal cell wall sorting signal (CWSS) of its target substrate and covalently attaches it onto the cell wall. The sorting pathway begins on the cell membrane after sortase substrates are transported from the cytoplasm via the Sec pathway. SrtA recognizes the LPxTG motif on the CWSS of sortase substrates and cleaves them between threonine and glycine. Cleavage is followed by the covalent linkage of the cysteine residue of SrtA to the threonine within the substrate to form a sortase-substrate intermediate. The substrate is then covalently linked to the lipid II cell wall precursor, which is eventually incorporated into the growing peptidoglycan layer via transglycosylation and transpeptidation (Perry *et al.*, 2002; Ruzin *et al.*, 2002). Most SrtA mechanistic studies were conducted in *S. aureus* as a model organism and ubiquitously applied to

most Gram-positive bacteria in which SrtA is conserved. Although there is strong evidence that the crossbridge on lipid II (**Fig. I**) is the anchoring site for sortase substrates, the possibility of anchoring substrates via alternate pathways onto the cross-bridge of mature uncross-linked peptidoglycan has not been eliminated.

1.5 Cell wall

The Gram-positive cell wall is composed of 3 main constituents: peptidoglycan (PG), anionic polymers such as teichoic acids, and wall-associated proteins (Bhavsar and Brown, 2006). Of the three constituents, peptidoglycan is the major component of the enterococcal cell wall. The peptidoglycan layers sit directly above the phospholipid bilayer, forming a lattice structure that protects the cell from osmotic stress and turgor pressure. A single peptidoglycan unit is made up of a disaccharide N-acetylmuramic acid-N-acetylglucosamine (NAM-NAG), a stem and a crossbridge (Vollmer *et al.*, 2008). In *E. faecalis*, the pentapeptide stem consists of L-alanine-D-iso-glutamine-L-lysine-D-alanine-D-alanine and the crossbridge is made up of L-alanine-L-alanine. The crossbridge is attached to the sidechain of L-lysine of the stem (**Fig.I**) (Schleifer and Kandler, 1972). Peptidoglycan subunits are polymerized into glycan chains ranging from 5-30 subunits by the transglycosylase activity of penicillin binding proteins (PBPs) via a process known as transglycosylation. Adjoining glycan chains are then interconnected by a process known as cross-linking, where transpeptidase activity of PBPs forms a peptide bond between the L-alanine crossbridge to the 4th D-alanine of the pentapeptide stem. During cross-linking, the 5th D-alanine of the pentapeptide stem is cleaved. Although the general peptidoglycan subunit is similar for most Gram-positive bacteria, what is unique to different species is the amino acid sequence that forms the crossbridge. The crossbridge of *E. faecalis* is made up of two

L-alanine while the crossbridge of *E. faecium*, *Staphylococcus aureus* and *Streptococcus pneumoniae*(35%) are made up of a single D-Asp, five glycines and L-alanine-L-alanine, and L-serine-L-alanine, respectively (Heijenoort, 2001; Schleifer and Kandler, 1972; Schleifer and Kilpper-Bälz, 1987). The differences in crossbridge length in turn affects the extent of cell wall cross-linking where a longer crossbridge gives rise to more cross-linked cell wall and vice versa (Kim *et al.*, 2014). For example, the percentage of cross-linking of *S. aureus* is in the high values of 74 – 92% (Vollmer and Seligman, 2010), while the percentage cross-linking of *E. faecalis* and *S. pneumoniae* is only approximately 48% and 35% respectively (Bui *et al.*, 2012; Yang *et al.*, 2017). Given that the crossbridge of lipid II is the proposed target for SrtA to anchor its substrates, this dissertation aims to characterize the spatial and temporal relationship between cell wall synthesis with the anchoring of sortase-anchored pili.

1.6 Motivation and hypothesis

While the process of pilus assembly, sorting machinery, and cell wall synthesis are interlinked and well-studied individual processes in *E. faecalis*, little is known about the interplay between these mechanisms. Despite evidence showing that the overall cell wall structure in *E. faecalis* is different, the main paradigm of surface-exposed pathways is based on studies carried out in *S. aureus*. Here, we sought to bridge the gap between cell wall protein anchoring and cell wall synthesis by investigating the spatial and temporal distribution of surface exposed pilus in *E. faecalis*. It has been proposed that sortase processing of cell wall associated proteins are linked to the cell wall synthesis process at discrete locations near the site of cell division (Kline *et al.*, 2009). Since sortases are focally enriched at the cell septum, which coincides with the site of new cell wall synthesis, we hypothesize that Ebp will be also be focally localized at the division septum (**Fig. I**). To test our hypothesis, we used

immunofluorescence, wide field and super resolution imaging methods over different time periods to track surface exposed Ebp and cell wall synthesis. The temporal and spatial information of Ebp localization gained here provide new insights on the emergence of virulence factors during *E. faecalis* infections.

Fig. 1 Model of cell wall synthesis and pilus sorting at the division septum, and schematic of *E. faecalis* cells at mid and late division phases

2 Materials and Methods

2.1 Bacterial culture and strains

The *E. faecalis* OG1RF strain was streaked out onto what BHI agar from 25% glycerol stocks stored at -80°C; single colonies were inoculated into brain heart infusion broth (BHI broth; BD Difco, USA) and grown overnight statically for 16 to 18 hrs at 37 °C. To obtain cells at mid log phase, the overnight culture was subcultured at 1:10 dilution into fresh BHI media and grown to OD₆₀₀ 0.5 ± 0.05. Cells were then normalized to OD₆₀₀ 0.5 in 1 mL 0.1 M phosphate buffer (PB).

2.2 Immunofluorescence microscopy

Cells at mid log phase were normalized in 1 mL PB, were blocked in 2% bovine serum albumin (BSA) in PB for 20 mins at room temperature (RT). For Ebp staining, cells were incubated with guinea pig anti-EbpC serum or rabbit anti-EbpA serum (SABio, Singapore) at 1:500 dilution in PB-2% BSA for 1 hr at RT. Next, cells were washed once in PB and incubated with fluorescence-conjugated secondary antibody at 1:500 dilution in PB-2% BSA (Alexa Fluor 405/488/568–goat anti-guinea pig antibody for EbpC and goat anti-rabbit antibody for EbpA [Invitrogen, Inc., USA]). Cells were then washed once in PB and resuspended in 1 mL PB. Before mounting, microscope glass slides were washed once in filtered 70% ethanol followed by filtered ultrapure water (18.2 ohm) and dried. Hydrophobic wells measuring 1.25 cm in diameter were drawn on the glass slide using a PAP pen (Sigma Aldrich, Singapore). 20 µL of cells were spotted onto each well and allowed to dry in a 60 °C oven. Slides were mounted using 5 µL of mounting media (Vectashield®, USA) and sealed with coverslip. Widefield microscopy was performed using an inverted epi-fluorescence microscope (Zeiss Axio observer Z1, Germany) fitted with a 100x/1.3 oil Ph3

objective lens. Structured illumination imaging (SIM) was performed using a 100x/1.46 oil DICIII objective lens fitted onto Elyra PS.1 microscope (Zeiss).

2.3 Fluorescent D-amino acid (FDAA) labelling

Cells were subcultured 1:10 in 5 mL BHI and grown to early log phase. Cells were normalized to OD₆₀₀ 0.25 in pre-warmed BHI and FDAA, 7-hydroxycoumarin-3-carboxylic acid 3-amino-D-alanine (HADA)(Kuru *et al.*, 2015), was added to a final concentration of 250 μ M. Cells were then grown for either 5, 20, 40 or 120 mins at 37 °C with agitation. To halt the labelling, cells were placed on ice and washed thrice in ice cold PB. Cells were then mounted onto clean glass slides before imaging by SIM. For FDAA sequential labelling, cells were labelled first with the green derivative of BODIPY-FL 3-amino-D-alanine (BADA) for 5 mins or 40 mins at 37 °C in BHI, washed once in ice cold PBS and labelled with the red FDAA derivative, TAMRA 3-amino-D-alanine (TADA) for 5 mins or 40 mins at 37 °C in BHI. Cells were then washed once in ice cold PBS and incubated in BHI with HADA for 10 mins or 20 mins at 37 °C, washed once in PBS and mounted onto glass slides and imaged via Structured illumination microscopy (SIM) using a 100x/1.46 oil DICIII objective lens fitted onto Elyra PS.1 microscope (Zeiss).

2.4 Time Lapse imaging

Cells were grown to mid log phase and stained for Ebp as described above. 1% BHI agarose gel pads were prepared by sandwiching molten BHI agarose between two glass slides. Once solidified, 1 cm by 1 cm agarose gel squares were cut out. 5 μ L of cells were spotted and evenly spread across the agarose gel pad and sealed with a glass cover slip using parafilm wax. Images were then taken every 15 mins at RT for 2.5 hrs using an inverted epi-fluorescence microscope (Zeiss Axio observer Z1, Germany) fitted with a 100x/1.3 oil Ph3 objective lens. SIM was performed using a 100x/1.46 oil

DICIII objective lens fitted onto Elyra PS.1 microscope (Zeiss).

2.5 Triple Ebp chase labelling

E. faecalis OG1RF cells were grown in BHI to mid log phase and stained for EbpC via immunofluorescence first with a green fluorescent secondary antibody, Alexa Fluor 488 (Invitrogen, Singapore). After the first Ebp labelling, cells were washed and further grown in BHI for 1 hr at 37 °C and stained for EbpC with a blue fluorescent secondary antibody, Alexa Fluor 405. Next, the cells were again allowed to grow in BHI for 1 hr at 37 °C and similarly labelled for EbpC but with a red fluorescent secondary antibody, Alexa Fluor 568. After the final staining, cells were mounted and imaged via SIM.

2.6 Determination of sub-bacteriostatic concentration of antibiotics

E. faecalis cells were grown to OD 0.4 at 37 °C and subsequently transferred into a 96-well plate containing varying concentrations of ramoplanin (16 – 32 µL) in BHI. Cells with different concentrations of ramoplanin were incubated at 37 °C incubation for 4 hrs and absorbance (600 nm) reading was taken every 15 mins using Tecan M200 microtiter plate reader. The sub-bacteriostatic concentration of ramoplanin is determined at the lowest dose of antibiotics that showed a decrease in absorbance reading within the first hour of incubation.

2.7 Ramoplanin Ebp-HADA chase experiment

E. faecalis cells were grown to mid log phase, harvested and labelled for EbpC via immunofluorescence first using a green fluorescent secondary antibody, Alexa Fluor 488. After washing once in PB, cells were grown in the presence or absence of ramoplanin with 250 µM HADA in BHI at 37 °C for 1 hr. Cells were washed and, again labelled for EbpC via immunofluorescence, instead with a red fluorescent secondary antibody. SIM imaging was performed.

2.8 Western blot

Cells that were treated or untreated with 26 µg/mL ramoplanin for 1 hr were normalized to OD₆₀₀ 0.5, washed once in PBS and incubated with 10 mg/mL lysozyme in a 37°C water bath for 1 hr. Cell wall and protoplast fractions were separated by centrifugation at 14 000 rpm for 5 mins. NuPAGE™ LDS sample buffer (Thermo Fisher Scientific, USA) were added to the fractions before boiling for 20 mins. Ebp blots were run in NuPAGE 3 to 8% Tris- acetate pre-cast gels in Tris-acetate buffer while SecA blots were run in NuPAGE 10% Bis-Tris pre-cast gels for 50 mins at 120 V. After SDS-PAGE was completed, proteins were transferred onto a PVDF membrane using an iblot™ gel transfer machine and iblot™ transfer stacks (Thermo Fisher Scientific, USA). The membranes were then blocked in 0.1% v/v Tween- 5% bovine serum albumin-PBS (Sigma Aldrich, Singapore) overnight at 4°C shaking. The next day, blocking buffer was removed and replaced with primary antibody (rabbit anti-EbpA, rabbit anti-EbpB, guinea pig anti-EbpC and rabbit anti-SecA) at a dilution of 1:3000 for 1 hr at RT and washed trice in 0.01% Tween-PBS. After washing, the membranes were incubated in secondary antibody (IgG guinea pig or rabbit conjugated horseradish peroxidase) (ThermoFisher Scientific, Singapore) at a dilution of 1:6000 for 1 hr and washed trice in 0.01% Tween-PBS. Protein bands were detected by chemiluminescence using SuperSignal™ west femto maximum sensitivity substrate kit (ThermoFisher Scientific, USA).

3. Results

3.1 Cell surface-exposed Ebp localization is septum exclusive and is temporally deposited towards the pole

Proper sorting and cell surface-exposure of the *Enterococcus* pilus can facilitate its virulence during infection. To determine the spatial organization of pili on the cell surface, we performed an immunofluorescence assay on *E. faecalis* OG1RF cells labelled with antibody raised against the major pilus subunit, EbpC. We visualized *E. faecalis* pili distribution at three different growth phases, define as 1) early division elongated monococci which have not yet undergone septation (1 μm -1.5 μm), 2) mid division diplococci which are undergoing elongation and septum constriction (1.5 μm -2 μm), and 3) late division cells consisting of two daughter cells just before separation (> 2 μm). During each growth phase, we observed EbpC labelling only at the cell hemispheres and at sites adjacent to the equatorial ring, but not at the septum, which we refer to as “septum exclusive” (**Fig. 2A**). Furthermore, we observed that one hemisphere of the cell is always more saturated with Ebp than the other. The hemispherical saturation and septum exclusive localization patterns were seen in early and mid division phase cells. However, in late division cells, we observed EbpC fully covering both hemispheres with two additional foci adjacent to the nascent septum, mirroring each other (**Fig. 2A**). This transition from asymmetric distribution of Ebp in the mid division phase to symmetric Ebp distribution between the two hemispheres hinted that the two foci adjacent to the septum may seed the eventual coverage of the entire hemisphere. EbpC will eventually be decorated throughout the entire *E. faecalis* cell wall as reflected in late division cells. To gain a more detailed characterization of Ebp distribution, we quantified the localization pattern of EbpC by plotting the Ebp fluorescence intensity against the cell perimeter in mid division phase cells and observed that the fluorescence intensity is lowest at positions that corresponded to the

septum (Fig. 2B).

Fig. 2 Distribution of surface exposed Ebp in *E. faecalis*. (A) Representative immunofluorescence (IF) labelling of surface exposed Ebp in *E. faecalis* at early, mid and late division phase. Scale bar, 1 μ m. (B) Quantification of Ebp fluorescence intensity along the cell periphery (0-100) of mid division cells starting from the cell pole where 25 and 75 corresponds to the cell septum (n=92). The shaded areas on the graph correspond to one hemisphere and two foci adjacent to the equatorial ring with peaked fluorescence intensity. (C) Time-lapse of pre-labelled live *E. faecalis* cells mounted on BHI agarose pad and imaged every 15 mins. (D) Chase labelling of Ebp via immunofluorescence at 1 hour growth intervals using green, blue and red fluorescent conjugated secondary antibodies as shown in the

schematic. Images on the second row are merged images of either green and blue or blue and red. Representative mid and late division phase cells are shown. Scale bar: 1 μm

The low fluorescence intensity at the septum further is consistent with our visual observation that cell wall associated Ebp localization is septum exclusive. Moreover, the highest fluorescence intensity plateaus from positions 38 to 63, which corresponds to the beginning and end of one hemisphere of the cell, again consistent with our visual observation that one side of the cell is more saturated with Ebp than the other. Two smaller peaks were observed at positions 13 and 87, which correspond to the two foci located adjacent to the septum on the other hemisphere of the cell (**Fig. 2B**). Because the maximum fluorescence intensity of these two foci is lower than the other hemisphere, we hypothesized that these two foci are likely newly emerged Ebp and hence there is a lower number of surface exposed Ebp compared to the other hemisphere. We speculate that additional Ebp will continue to emerge to eventually complete the hemispherical coverage. Overall, our findings indicate that pili localization is septum exclusive and that Ebp decoration begins at one hemisphere of the cell.

Because we observed that Ebp becomes surface exposed (and therefore available for binding by extracellular antibodies for immunofluorescence) and saturates one cell hemisphere before the other, we hypothesized two possible scenarios to describe how one hemisphere becomes fully piliated. In the first scenario, the two foci adjacent to the septum remain immobile and newer pili emerge next to them, towards the pole where they eventually cover the hemisphere. In the second scenario, the two foci migrate towards the pole while new Ebp continue to emerge behind the migrating foci from the site adjacent to the septum. To determine which of these two scenarios could explain temporal pilus deposition on the cell wall, we performed time-lapse

fluorescence microscopy on live EbpC-labelled cells. We traced the original fluorescent Ebp foci on the cell and observed that as the cell elongates and divides, the single focus does not move along the cell periphery. Instead, the stained EbpC focus remains where it was originally labelled, while new cell material is synthesized at the septum as the cell divides (**Fig. 2C**). This lack of foci movement suggested that once deposited on the cell wall, Ebp are immobile and that newer Ebp are likely deposited towards the cell pole to achieve their hemispherical localization pattern. To trace for newly emerged Ebp that potentially emerge towards the cell pole, we performed an Ebp pulse chase labelling experiment where Ebp were labelled three consecutive times via immunofluorescence using green, blue and red fluorescently labelled secondary antibodies sequentially with 1 hour of growth between each labelling. We imaged the cells using structured illumination microscopy (SIM) and observed that Ebp were labelled in a sequential manner towards the pole (**Fig. 2D**). In the left hemisphere where the two Ebp foci were initially labelled in green, we observed blue-labelled Ebp overlapping with the older green-labelled Ebp, in addition to new blue-label localizing to segments adjacent to the green foci. On the same hemisphere, red-labelled Ebp, which are the newest Ebp deposited on the cell surface, overlapped with both green and blue and filled up the entire cell hemisphere. These data were consistent with our observation that Ebp deposited on the cell wall are fixed and that newer Ebp are deposited in a temporal manner towards the cell pole where over time, newer Ebp are exposed closer to the pole while older ones remain closer to the equatorial rings and eventually, the whole hemisphere is saturated with Ebp. These data also hinted that Ebp deposition and cell wall synthesis are coordinated where new Ebp may appear together with newly synthesized cell wall. Thus, we followed up by investigating the process of cell wall synthesis in *E. faecalis*.

3.2 New cell wall is synthesized at the septum and is driven towards the cell hemisphere as the cell elongates

The current paradigm in Gram-positive bacteria, largely defined in *S. aureus*, is that SrtA substrates are anchored to the cell wall via lipid II, a cell wall precursor (Ton-That and Schneewind, 1999; Ton-That *et al.*, 1997). Similarly, we postulated that sortase-anchored Ebp is attached to lipid II and becomes surface exposed as this lipid II-substrate intermediate is incorporated into the growing peptidoglycan cell wall. To test this, we first characterized *E. faecalis* cell wall synthesis dynamics using fluorescent D-amino acid (FDAA) probes. FDAAs are fluorescent conjugated D-amino acids that are actively incorporated into the cell wall by penicillin-binding proteins (PBPs) via transpeptidation (Hsu *et al.*, 2017; Kuru *et al.*, 2015). We incubated exponential growing cells with the blue derivative of FDAA, HADA for 5, 20, 40 or 120 minutes and visualized the staining patterns using super-resolution microscopy (**Fig. 3A**). After 5 minutes of HADA exposure, we saw cell wall labelling exclusively at the septum, indicating that new cell wall is synthesized at mid cell, as supported by other groups (Boersma *et al.*, 2015; Hsu *et al.*, 2019). After labelling for 20 minutes, we saw a distinctive “cross-like” localization pattern where cell wall was stained from the equatorial ring to the septum and unlabeled at both hemispheres (**Fig. 3A**). This “cross-like” localization pattern supports that new cell wall is synthesized at mid cell. By contrast, after 40 minutes of labelling, most cells were stained at the septum and one side of the hemisphere while the other hemisphere remained unlabeled (**Fig. 3A**). This contrasting distribution of PG staining is consistent with one side of each diplococci being more mature than the other. After 120 minutes of HADA exposure, most cells were uniformly labelled, indicating that they have gone through at least 2 replication cycles (**Fig. 3A**). These cell wall labelling patterns that were

observed after HADA labelling for different durations led us to postulate that PG incorporation in *E. faecalis* is coordinated asymmetrically. We proposed that new PG is always synthesized at midcell and older PG is consistently, constantly, and evenly pushed out by newer cell wall with little or no intercalation of new and old cell wall material.

To spatially distinguish between old and new cell wall, and to determine the temporal distribution of PG, we next performed a similar sequential labelling experiment, but instead carried out labelling of new PG using three different coloured FDAAs. A series of short pulses was carried out, in which we first incubated the cells for 5 mins with a green FDA, BODIPY-FL 3-amino-d-alanine (BADA) followed by 10 minutes with a red FDA, TAMRA 3-amino-D-alanine (TADA) and lastly, 10 mins with HADA (**Fig. 3B**). The three stains appeared as distinct fluorescent bands parallel to each other on the cell with minimal overlap, suggesting immobility of the cell wall PG after it is incorporated. BADA and TADA fluorescent bands appeared narrower than that of HADA's, suggesting splitting of these fluorescent bands at midcell as newer PG is synthesized and incorporated at the septum. Furthermore, BADA labelled PG formed the outermost bands, flanking both TADA and HADA labelled PG with TADA sandwiched between BADA and HADA (**Fig 3B**). This chronological labelling pattern supports our hypothesis that PG at midcell is pushed away from the septum as the cell elongates and divides. We further demonstrated the ability to visualize the older hemisphere of the cell, we extended the serial FDA labelling of BADA, TADA and HADA to 40 minutes, 40 minutes, and 20 minutes, respectively (**Fig. 3C**). We chose 40 minutes for the first two pulses because we observed single hemispherical PG staining for this staining duration and if our hypothesis is true, we predict that the

older hemisphere of the cell will be labelled green, the other hemisphere labelled red, and the middle of the cell labelled blue. Consistent with this prediction, we observed distinct labelling of the cell wall where BADA labelling was seen predominantly at one hemisphere of the cell while TADA labelled the other cell hemisphere, and HADA staining was observed in the middle of the cell (**Fig. 3C**). These data supported that the cell wall of one hemisphere is always older than the other. Our findings demonstrate that cell wall synthesis in *E. faecalis* occurs strictly at the septum and there is minimal overlap between the old and new cell wall.

Fig. 3 Fluorescent D-amino acid (FDAA) labeling of *E. faecalis* cell wall throughout the cell cycle. (A) Representative images of HCC-amino-D-alanine (HADA) labeled *E. faecalis* cell wall, pseudocolored cyan, at various time points. Cells were grown to exponential phase and labeled with HADA for 5, 20, 40 and 120 minutes. Cells were imaged by structured illumination microscopy (SIM). HADA has been pseudo-coloured cyan for ease of visualization. Scale bar: 1 μ m. (B & C) Representative images after sequential labelling of *E. faecalis* cell wall at short (B) and long (C) pulses and imaged by SIM. In (B), cells were labelled with BADA (5 minutes), TADA (5 minutes) and HADA (10 minutes). In (C), cells were labelling with BADA (40 minutes), TADA (40 minutes) and HADA (20 minutes). Scale bar: 0.5 μ m.

3.3 Ebp does not co-localize with newly synthesized cell wall

Ebp is a surface-exposed virulence factor that is covalently attached onto the cell wall by SrtA. Based largely on studies in *S. aureus* (Perry *et al.*, 2002; Ton-That and Schneewind, 1999; Ton-That *et al.*, 1997), SrtA is thought to anchor its substrates onto the growing cell wall via linkage to a lipid II precursor. Therefore, we postulated that Ebp is exposed to the cell surface together with newly synthesized cell wall at the septum, via a similar interaction with lipid II precursors. To test if Ebp exposure coincides with the new cell wall, we performed co-localization studies by co-staining both the cell wall and pili (**Fig. 4AB**). We performed both short and long incubations with HADA to determine if there were any differences in co-localization patterns. Surprisingly, Ebp did not co-localize with the newly synthesized labelled cell wall regardless of the length of HADA exposure. Instead, we saw Ebp staining where HADA labelling was absent. Ebp labelling also appeared more saturated at the older hemisphere. Our results illustrate that a mature cell wall may be required before *Enterococcus* pilus can be exposed on the surface and accessible to antibody labeling.

To test if PG synthesis precedes Ebp deposition, we performed a HADA-Ebp chase experiment. The experimental design was similar to the above assay, but included additional steps where cells were allowed to grow in BHI for an hour (in the absence of any stain) and then stained again for Ebp with a green secondary antibody. When cells were stained were pulsed in HADA for 1 hour, costained with Ebp and allowed to grow and stained a second time for Ebp, we saw that newly labelled Ebp overlapped with the pre-labelled cell wall (**Fig. 4C**). This confirmed our hypothesis that the cell wall must be synthesized first before Ebp is anchored onto it. Taken together, our results showed that pilus deposition on the cell wall is dependent on the age of

Enterococcus cell wall and septal lipid II may not be necessary for anchoring of pili.

Fig. 4 Co-staining of peptidoglycan and Ebp. (A & B) Exponential growing cells were labelled with HADA for short pulse (5 minutes) or long (120 minutes), stained for Ebp via immunofluorescence and imaged via SIM. Scale bar = 2 μ m. (B), Representative image of cells at early, mid and late division phases are shown. Scale bar = 1 μ m. (C) HADA staining followed by chase labelling of Ebp. Exponentially growing cells were harvested, stained with HADA for 1 hr followed by labelling of EbpC via immunofluorescence first with a green and then a red secondary antibody after 1 hr growth in BHI. Images were taken via SIM and two representative images are shown. Scale bar = 1 μ m.

3.4 New pilus deposition at the cell hemisphere is independent of cell wall synthesis

The synthesis and assembly of the *E. faecalis* cell wall components are crucial in promoting its virulence, however the coordination between these synthesis between these different components remain un-elucidated. Polymerized pili are anchored onto the cell wall by SrtA. The sorting machinery was characterized in a *S. aureus* model whereby SrtA catalyzes the formation of a substate-lipid II intermediate which is eventually incorporated into the cell wall via transglycosylation and transpeptidation reaction (Perry *et al.*, 2002). Our observations that new cell surface exposure of Ebp and formation of the Enterococcal cell wall do not occur simultaneously led us to postulate that Ebp may bypass precursor lipid II and instead be directly anchored onto the uncrosslinked cell wall by SrtA. The *E. faecalis* cell wall is about 50% cross-linked compared to 85% in *S. aureus* (Kim *et al.*, 2015; Yang *et al.*, 2017). This lack of complete cell wall cross-linking suggests that there is an abundance of free L-Ala-L-Ala crossbridges in *E. faecalis* for anchoring of cell surface proteins. When *E. faecalis* was stained with BODIPY™ FL vancomycin (Vanc-FL), a fluorescently labelled antibiotic that targets the D-Ala-D-Ala residues in the cell wall, we saw enrichment of fluorescence at the septum and staining at the cell periphery (**Fig 5A**). The intense fluorescence at the cell septum is indicative of the D-Ala-D-Ala on lipid II cell wall precursor, while the staining at the cell periphery indicates the D-Ala-D-Ala on uncrosslinked cell wall. To test if Ebp deposition is dependent on cell wall synthesis, and hence the availability of lipid II precursors, we treated cells with ramoplanin to inhibit cell wall synthesis. Ramoplanin primarily targets lipid II precursors, rendering them unavailable for transglycosylation by PBP1b, and hence inhibiting new cell wall synthesis (Fang *et al.*, 2006). We performed growth kinetics in increasing concentrations of ramoplanin and determined the sub-bacteriostatic

concentration to be 26 $\mu\text{g/mL}$ in *E. faecalis* (**Fig. 5B**). To verify that cell wall synthesis was inhibited at this concentration we grew ramoplanin-treated cells together with HADA. After 1 hour of incubation, no newly synthesised cell wall labelling was observed in ramoplanin-treated cells, confirming that cell wall synthesis was inhibited (**Fig 5C**). To determine if new Ebp was deposited onto the cell surface when cell wall synthesis was inhibited, we performed a HADA-Ebp chase experiment in the presence and absence of ramoplanin. Interestingly, at sub-bacteriostatic concentrations of ramoplanin, we saw new Ebp deposition at the cell hemisphere despite cell wall synthesis inhibition (**Fig. 5D**). To eliminate the possibility that new Ebp in ramoplanin treated cells were membrane bound, we performed western blot to examine presence of the three subunits of Ebp in both the cell wall and protoplast fractions of both ramoplanin treated and untreated cells. However, the high molecular weight (HMW) ladder, representing polymerized pili, visible on the Ebp immunoblots were present only in the cell wall fraction in ramoplanin treated cells (**Fig. 7**), suggesting that the new Ebp are being incorporated into the cell wall via uncross-linked PG away from the septum. Accordingly, when we stained ramoplanin treated cells with Vanc-FL, we observed homogeneous cell wall staining throughout the cell and lack of fluorescence enrichment at the septum, indicative of abundance of uncross-linked PG in the cell and inhibition of new cell wall synthesis respectively (**Fig. 5A**). In contrast to the current paradigm for sortase substrate incorporation into the cell wall, our data suggest that in *E. faecalis*, Ebp can be anchored onto uncross-linked cell wall, which facilitates exposure to the cell exterior. We propose a new paradigm for *E. faecalis* Ebp deposition where SrtA mediated Ebp attachment of pili bypasses the need for lipid II-substrate intermediates and directly anchors pili onto uncross-linked PG.

Fig. 5 Cell wall synthesis inhibition by ramoplanin and its effect on pilus deposition. (A) Cells were stained with BODIPY® FL vancomycin and imaged. Scale bar: 1 μm **(B)** Exponentially growing bacteria were treated with increasing concentrations of ramoplanin for 4 hrs at 37°C. Growth was measured at an absorbance of 600nm. Scale bar: 1 μm **(C)** HADA labelling of bacteria treated in the presence or absence of 26 $\mu\text{g/mL}$ ramoplanin. **(D)** Exponentially growing cells were harvested and first labeled for Ebp in green via immunofluorescence. Cells were then allowed to grow in the presence or absence of ramoplanin with HADA for 1hr. A second Ebp labelling was performed via immunofluorescence.

4 Discussion

Spatial localization of cell wall-anchored surface proteins has been studied in various species of Gram-positive cocci. In *S. aureus* and *Streptococcus pyogenes*, cell-wall associated proteins are localized at the septal region or at the poles depending in the presence or absence of a conserved YSIRK motif within the substrates signal sequence, respectively (Carlsson *et al.*, 2006; Raz *et al.*, 2012). In *S. pneumoniae*, sortase-assembled pili are distributed in a non-homogenous manner, focally localized at the cell surface at discrete sites (Fälker *et al.*, 2008). In *E. faecium*, two distinct types of pili are expressed, namely PilA and PilB. PilA are evenly distributed around the cell, while polymerized PilB are observed at the older pole at exponential phase (Hendrickx *et al.*, 2008; Hendrickx *et al.*, 2010). Hence, pili localization in Gram-positive cocci is determined in both a spatial and temporal manner.

Here we show that surface exposed *E. faecalis* Ebp are distributed in an asymmetrical manner such that the older hemisphere is saturated with Ebp while new Ebp becomes surface exposed at the newer hemisphere. This heterogeneous localization pattern has been reported for other *E. faecalis* surface exposed virulence factors such as aggregation substance (AS) (Olmsted *et al.*, 1993; Wanner *et al.*, 1989). Although localization of surface proteins has been explored in *E. faecalis*, the spatiotemporal distribution of these proteins has not been characterized. Using time-lapse microscopy, we traced the movement of labelled pili along the cell hemisphere and showed that they remain at a fixed position on the cell wall. These pili are subsequently pushed further towards the equatorial rings by newly synthesized cell wall from the septum. The Ebp turnover appears to be low as labelled pili remain intact throughout cell division. We addressed Ebp turnover by performing a double Ebp

immunofluorescence chase experiment using different fluorescently labelled antibodies, and observed that new pili are deposited and/or become surface-exposed temporally towards the poles and eventually, the deposited pili will fill up the whole cell hemisphere as the pole ages.

The sequential deposition and surface exposure of cell wall anchored pili is analogous to that of cell wall synthesis where both exhibits hemispherical localization. The similarities in cell wall and Ebp labelling, together with the predicted mechanism of protein attachment to the cell wall, led us to speculate that Ebp is emerging together with new cell wall. When different fluorescently labelled FDAA labels were successively pulsed in short (5 mins) or long waves (40 mins) in *E. faecalis*, there was little to no overlap between each colour that was pulsed, suggesting there is minimal cell wall turnover, similar to *S. pneumoniae* (Boersma *et al.*, 2015). This finding is in contrast to *Bacillus subtilis*, where rapid cell wall turnover is observed when similar FDAA labelling experiments were performed (Boersma *et al.*, 2015; Kuru *et al.*, 2015). The minimal cell wall turnover trait in *E. faecalis* supports the observation that pre-labelled pili remain locked in position as the cell continues to grow and divide. Long pulse chase of FDAA peptidoglycan labelling in *E. faecalis* showed distinct hemispherical labelling of the older (green) and newer (red) cell wall, indicating that one hemisphere of the cell is always more mature than the other, as would be expected. This hemispherical labelling pattern is reflective of Ebp localization where labelling is saturated at one hemisphere of the cell. Together, these similarities led us to speculate that new pili could be intercalated with newly synthesized cell wall. Unexpectedly, our Ebp and cell wall co-staining results showed that previous studies do not fit with the present model. The labelling of new peptidoglycan and surface

exposed pili was almost mutually exclusive, where there was little overlap between the two. When a second Ebp staining was performed after growth in fresh media, new pili appeared to be deposited onto the mature pre-labelled cell wall. This result was unexpected as the current paradigm is that SrtA anchors its substrates onto the cell wall precursor, lipid II, which is subsequently incorporated onto the cell wall via transglycosylation and transpeptidation (Ton-That and Schneewind, 1999; Ton-That *et al.*, 1997). The lack of correlation between newly synthesised peptidoglycan and surface exposed pili indicated that pilus deposition could be independent of lipid II precursors, and that pili might be deposited via an alternative mechanism.

In other Gram-positive bacteria, a cell wall precursor is needed for surface protein attachment. The results of our experiments with *E. faecalis* showed that with and without inhibition of cell wall synthesis by ramoplanin, an antibiotic targeting lipid II, pilus deposition was not affected. We observed new pili appearing at the cell hemispheres and not at septum where new cell wall synthesis takes place. Based on these observations, it is tempting to speculate that SrtA in *E. faecalis* can anchor cell wall proteins onto uncross-linked, mature cell wall. Compared to *S. aureus*, which has approximately 85% of cross-linked peptidoglycan, the percentage of cross-linked cell wall in *E. faecalis* is lower at approximately 50% (Kim *et al.*, 2014; Yang *et al.*, 2017). The lower percentage of PG cross-linking in *E. faecalis* is attributed to the shorter peptidoglycan crossbridge of only 2 L-Ala-L-Ala compared to the pentaglycine crossbridge in *S. aureus* (Yang *et al.*, 2017). The abundance of uncross-linked peptidoglycan in *E. faecalis* may serve as anchor points for SrtA to anchor polymerized pili. Taken together, we propose a model where the older hemisphere of *E. faecalis* will always be more saturated with polymerized pili than the younger hemisphere, where newer pili are just beginning to be anchored and exposed to the

surface. As the cell grows and elongates during division, new pili are anchored onto uncross-linked cell wall in chronological order towards the pole and eventually saturate the whole hemisphere (**Fig. 6**). This study provides an in-depth characterization of the spatiotemporal dynamics of peptidoglycan and surface exposed virulence factors in *E. faecalis* and highlights the possibility of an alternative route to cell wall protein anchoring by SrtA.

Fig 6. Spatial-temporal models of *E. faecalis* pili cell wall anchoring and pili deposition. Polymerized pili can either be anchored onto the cell wall via lipid II at the cell septum or via uncross-linked cell wall at the cell periphery. Surface exposed pili are localized asymmetrically where the older hemisphere is more saturated with pili. Over time, the cell elongates and newer pili are deposited in a chronological manner towards the pole, eventually populating the whole hemisphere.

5 Future work

Proper sorting and exposure of virulence factors is essential for *E. faecalis* attachment and pathogenesis. Our study proposes an alternative route where cell surface proteins are exposed through attachment to uncross-linked cell wall. This section aims address the open questions surrounding our alternate model.

To provide further evidence in our model that uncross-linked L-Ala in the cell wall can be used as a substrate for cell wall anchoring, we can knockout the enzymes involved in the addition of the first and second L-Ala of the cross-bridge. The enzymes responsible for the addition of the first and second L-Ala in *E. faecalis* are FemA and FemB respectively. In the absence of a peptidoglycan crossbridge, we would likely see fewer to no Ebp anchored on the cell wall. Knocking out FemB in *E. faecalis* would result in shortened PG crossbridges which likely affects the cross-linking in *E. faecalis*. A consequence of reduced PG cross-linking in the absence of FemB would be increased Ebp anchoring onto the cell wall. If no increased Ebp exposure is observed, this suggests that Ebp anchoring may reach saturation whereby increasing the number of anchor points does not increase the number of Ebp anchored onto the cell wall due to limiting factors such as surface area of the cell wall. After successful deletion of *femA* or *femB* in *E. faecalis*, the extent of PG crosslinking can be determined by PG extraction and semi-quantified by high performance liquid chromatography (HPLC). To further quantify Ebp on the cell surface, Ebp detection via immunofluorescence can be compared between the mutants and WT to measure surface exposed Ebp. An increase in fluorescence intensity would indicate that there is an increase in labelled surface exposed Ebp. Furthermore, cell fractionation followed by western blot analysis can accompany these analyses to further verify if there is indeed a change in cell wall anchored Ebp. These assays will give more evidence in

observing changes in cell wall anchored substrates in the presence of a decreased cross-linked cell wall.

Another outstanding question is identifying the location of Ebp polymerization and anchor points on the cell are. Since SrtC and SrtA are the enzymes responsible for polymerization and anchoring of Ebp onto the cell wall respectively, we speculate that these Ebp anchor points coincide with sortase localization. Both SrtA and SrtC are shown to be focally enriched at the cell septum (Kline *et al.*, 2009). Yet in this study, we show that surface exposed Ebp is cell septum exclusive and new pili are appearing at the cell hemispheres. It is possible that SrtA can also be found along the cell hemispheres to catalyze Ebp cell wall anchoring. One way to detect SrtA under the cell wall is to remove the cell wall by lysozyme treatment and label membrane bound SrtA via immunofluorescence. Another possible explanation to why Ebp is only seen at the cell hemispheres could be due to the penetration limitation of primary and secondary antibodies. The Ebp labelling technique used in this study only stains for surface exposed Ebp. To address the possibility of incomplete detection of Ebp and to stain for unexposed Ebp, we can subject cells to lysozyme treatment to remove the cell wall and to allow the antibodies to access Ebp which are buried under the cell wall. After removing the cell wall, we can co-stain the cell for SrtA and Ebp and observe their localization. Areas where SrtA and Ebp staining co-localize would strongly suggest sites of cell wall anchoring. Alternatively, we could look at where Ebp localizes in a SrtA mutant background. In the absence of SrtA, Ebp are retained on the membrane after polymerization. By staining Ebp in a SrtA mutant, we can locate membrane anchored Ebp and predict where Ebp are localized before cell wall attachment.

Despite the questions above quantifying sortase-assembled substrates on the cell surface, the mechanisms on how polymerized pili are exposed to the cell surface remains unsolved. Our study show that Ebp is displayed in a non-random manner at the cell hemispheres and is more saturated at the older hemisphere of the cell. Since pili are long fibrous structures and the cell wall is dense and tightly packed, there are likely channels on the cell wall that allow surface proteins to protrude out of the cell wall. A recent study showed via atomic force microscopy (AFM) that the Gram-positive cell wall is denser at the division septa where new cell wall is synthesized, while the mature cell wall constitutes large pores of up to 60 nm in diameter (Pasquina-Lemonche *et al.*, 2020). The presence of large pores at the mature cell wall which are absent at the new cell wall is indicative of cell wall modification by cell wall hydrolases as the cell wall matures. Perhaps why we are only seeing surface exposed Ebp at the cell hemisphere is because the cell wall at the hemisphere is older and hence have undergone modifications by cell wall hydrolases to allow Ebp to protrude out. To explore the possibility of cell wall modification is playing a role in pili exposure, we could knock out known *E. faecalis* cell wall hydrolases and observe if there is less surface exposed pili. By addressing these questions, these findings can give us a clearer picture of what is happening beneath the cell wall and bring us a step closer to understanding the process of sortase substrate anchoring in *E. faecalis*.

7 Conclusion

Altogether, this study has unraveled the dynamics of surface deposition of cell wall anchored protein in *E. faecalis* and raised the paradigm-shifting possibility of an alternative sorting mechanism where SrtA can directly anchor its substrates onto uncross-linked cell wall. The novelty of this study lies in the combined approach of investigating both cell wall synthesis and cell wall anchored proteins surface exposure at the same time and not as mutually exclusive subjects. This study implicates that virulence factors sorting mechanisms may not be universally conserved in Gram-positive bacteria and that alternate sorting mechanisms have to be taken into consideration during targeted treatment development.

7. Appendix

Fig 7. New pili on ramoplanin treated cells are cell wall bound. Ebp immunoblots of ramoplanin treated and untreated cells in three fractions – whole cell lysate (WCL), cell wall fraction (CWF) and protoplast fraction. All three Ebp subunits were blotted with SecA as a loading control.

8. References

- Arias, C.A., Murray, B.E., 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nature reviews. Microbiology* 10, 266-278.
- Bhavsar, A.P., Brown, E.D., 2006. Cell wall assembly in *Bacillus subtilis*: how spirals and spaces challenge paradigms. *Molecular Microbiology* 60, 1077-1090.
- Boersma, M.J., Kuru, E., Rittichier, J.T., VanNieuwenhze, M.S., Brun, Y.V., Winkler, M.E., 2015. Minimal Peptidoglycan (PG) Turnover in Wild-Type and PG Hydrolase and Cell Division Mutants of *Streptococcus pneumoniae* D39 Growing Planktonically and in Host-Relevant Biofilms. *J Bacteriol* 197, 3472-3485.
- Bui, N.K., Eberhardt, A., Vollmer, D., Kern, T., Bougault, C., Tomasz, A., Simorre, J.P., Vollmer, W., 2012. Isolation and analysis of cell wall components from *Streptococcus pneumoniae*. *Anal Biochem* 421, 657-666.
- Carlsson, F., Stålhammar-Carlemalm, M., Flärdh, K., Sandin, C., Carlemalm, E., Lindahl, G., 2006. Signal sequence directs localized secretion of bacterial surface proteins. *Nature* 442, 943-946.
- Fälker, S., Nelson, A.L., Morfeldt, E., Jonas, K., Hultenby, K., Ries, J., Melefors, Ö., Normark, S., Henriques-Normark, B., 2008. Sortase-mediated assembly and surface topology of adhesive pneumococcal pili. 70, 595-607.
- Fang, X., Tiyanont, K., Zhang, Y., Wanner, J., Boger, D., Walker, S., 2006. The mechanism of action of ramoplanin and enduracidin. *Mol. BioSyst.* 2, 69-76.
- Fisher, K., Phillips, C., 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* 155, 1749-1757.
- Gilmore, M.S., Clewell, D.B., 2002. The Enterococci: pathogenesis, molecular biology, and antibiotic resistance.
- Heijenoort, J.V., 2001. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. *Glycobiology* 11, 25R-36R.
- Hendrickx, A.P., Willems, R.J., Bonten, M.J., van Schaik, W., 2009. LPxTG surface proteins of enterococci. *Trends Microbiol* 17, 423-430.

- Hendrickx, A.P.A., Bonten, M.J.M., Van Luit-Asbroek, M., Schapendonk, C.M.E., Kragten, A.H.M., Willems, R.J.L., 2008. Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate. *Microbiology* 154, 3212-3223.
- Hendrickx, A.P.A., Schapendonk, C.M.E., Van Luit-Asbroek, M., Bonten, M.J.M., Van Schaik, W., Willems, R.J.L., 2010. Differential PilA pilus assembly by a hospital-acquired and a community-derived *Enterococcus faecium* isolate. 156, 2649-2659.
- Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A., Fridkin, S.K., 2008. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control & Hospital Epidemiology* 29, 996-1011.
- Hsu, Y.P., Booher, G., Egan, A., Vollmer, W., VanNieuwenhze, M.S., 2019. d-Amino Acid Derivatives as in Situ Probes for Visualizing Bacterial Peptidoglycan Biosynthesis. *Acc Chem Res* 52, 2713-2722.
- Hsu, Y.P., Rittichier, J., Kuru, E., Yablonowski, J., Pasciak, E., Tekkam, S., Hall, E., Murphy, B., Lee, T.K., Garner, E.C., Huang, K.C., Brun, Y.V., VanNieuwenhze, M.S., 2017. Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem Sci* 8, 6313-6321.
- Kawalec, M., Potempa, J., Moon, J.L., Travis, J., Murray, B.E., 2005. Molecular diversity of a putative virulence factor: purification and characterization of isoforms of an extracellular serine glutamyl endopeptidase of *Enterococcus faecalis* with different enzymatic activities. *J Bacteriol* 187, 266-275.
- Kim, S.J., Chang, J., Singh, M., 2015. Peptidoglycan architecture of Gram-positive bacteria by solid-state NMR. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1848, 350-362.
- Kim, S.J., Singh, M., Sharif, S., Schaefer, J., 2014. Cross-link formation and peptidoglycan lattice assembly in the FemA mutant of *Staphylococcus aureus*. *Biochemistry* 53, 1420-1427.
- Kline, K.A., Kau, A.L., Chen, S.L., Lim, A., Pinkner, J.S., Rosch, J., Nallapareddy, S.R., Murray, B.E., Henriques-Normark, B., Beatty, W., Caparon, M.G., Hultgren, S.J., 2009. Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in *Enterococcus faecalis*. *J Bacteriol* 191, 3237-3247.
- Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C.J., Salzman, N.H., 2015. Bacteriocin production

augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526, 719-722.

Kuru, E., Tekkam, S., Hall, E., Brun, Y.V., Van Nieuwenhze, M.S., 2015. Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth in situ. *Nat Protoc* 10, 33-52.

Marraffini, L.A., Dedent, A.C., Schneewind, O., 2006. Sortases and the Art of Anchoring Proteins to the Envelopes of Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews* 70, 192-221.

Mazmanian, S.K., Liu, G., Ton-That, H., Schneewind, O., 1999. Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science (New York, N.Y.)* 285, 760-763.

Murdoch, D.R., Corey, G.R., Hoen, B., Miro, J.M., Fowler, V.G., Jr., Bayer, A.S., Karchmer, A.W., Olaison, L., Pappas, P.A., Moreillon, P., Chambers, S.T., Chu, V.H., Falco, V., Holland, D.J., Jones, P., Klein, J.L., Raymond, N.J., Read, K.M., Tripodi, M.F., Utili, R., Wang, A., Woods, C.W., Cabell, C.H., International Collaboration on Endocarditis-Prospective Cohort Study, I., 2009. Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis-Prospective Cohort Study. *Arch Intern Med* 169, 463-473.

Nallapareddy, S.R., Singh, K.V., Sillanpaa, J., Zhao, M., Murray, B.E., 2011a. Relative contributions of Ebp Pili and the collagen adhesin ace to host extracellular matrix protein adherence and experimental urinary tract infection by *Enterococcus faecalis* OG1RF. *Infect Immun* 79, 2901-2910.

Nallapareddy, S.R., Singh, K.V., Sillanpaa, J., Garsin, D.A., Hook, M., Erlandsen, S.L., Murray, B.E., 2006. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *Journal of Clinical Investigation* 116, 2799-2807.

Nallapareddy, S.R., Sillanpaa, J., Mitchell, J., Singh, K.V., Chowdhury, S.A., Weinstock, G.M., Sullam, P.M., Murray, B.E., 2011b. Conservation of Ebp-type pilus genes among Enterococci and demonstration of their role in adherence of *Enterococcus faecalis* to human platelets. *Infect Immun* 79, 2911-2920.

Nielsen, H.V., Flores-Mireles, A.L., Kau, A.L., Kline, K.A., Pinkner, J.S., Neiers, F., Normark, S., Henriques-Normark, B., Caparon, M.G., Hultgren, S.J., 2013. Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *J Bacteriol* 195, 4484-4495.

Olmsted, S.B., Erlandsen, S.L., Dunny, G.M., Wells, C.L., 1993. High-resolution visualization by field emission scanning electron microscopy of *Enterococcus faecalis* surface proteins encoded by the pheromone-inducible conjugative plasmid pCF10. *J Bacteriol* 175, 6229-6237.

Pasquina-Lemonche, L., Burns, J., Turner, R.D., Kumar, S., Tank, R., Mullin, N., Wilson, J.S., Chakrabarti, B., Bullough, P.A., Foster, S.J., Hobbs, J.K., 2020. The architecture of the Gram-positive bacterial cell wall. *Nature* 582, 294-297.

Patterson, J.E., Sweeney, A.H., Simms, M., Carley, N., Mangi, R., Sabetta, J., Lyons, R.W., 1995. An analysis of 110 serious enterococcal infections. Epidemiology, antibiotic susceptibility, and outcome. *Medicine (Baltimore)* 74, 191-200.

Perry, A.M., Ton-That, H., Mazmanian, S.K., Schneewind, O., 2002. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* 277, 16241-16248.

Raz, A., Talay, S.R., Fischetti, V.A., 2012. Cellular aspects of the distinct M protein and SfbI anchoring pathways in *Streptococcus pyogenes*. *Molecular Microbiology* 84, 631-647.

Ruzin, A., Severin, A., Ritacco, F., Tabei, K., Singh, G., Bradford, P.A., Siegel, M.M., Projan, S.J., Shlaes, D.M., 2002. Further evidence that a cell wall precursor [C(55)-MurNAc-(peptide)-GlcNAc] serves as an acceptor in a sorting reaction. *J Bacteriol* 184, 2141-2147.

Schleifer, K.H., Kandler, O., 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* 36, 407-477.

Schleifer, K.H., Kilpper-Bälz, R., 1987. Molecular and Chemotaxonomic Approaches to the Classification of Streptococci, Enterococci and Lactococci: A Review. *Systematic and Applied Microbiology* 10, 1-19.

Schneewind, O., Model, P., Fischetti, V.A., 1992. Sorting of protein A to the staphylococcal cell wall. *Cell* 70, 267-281.

Sillanpaa, J., Chang, C., Singh, K.V., Montealegre, M.C., Nallapareddy, S.R., Harvey, B.R., Ton-That, H., Murray, B.E., 2013. Contribution of individual Ebp Pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. *PloS one* 8, e68813.

- Ton-That, H., Schneewind, O., 1999. Anchor Structure of Staphylococcal Surface Proteins. *Journal of Biological Chemistry* 274, 24316-24320.
- Ton-That, H., Faull, K.F., Schneewind, O., 1997. Anchor Structure of Staphylococcal Surface Proteins: A BRANCHED PEPTIDE THAT LINKS THE CARBOXYL TERMINUS OF PROTEINS TO THE CELL WALL. 272, 22285-22292.
- Ton-That, H., Liu, G., Mazmanian, S.K., Faull, K.F., Schneewind, O., 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proceedings of the National Academy of Sciences of the United States of America* 96, 12424-12429.
- Vollmer, W., Seligman, S.J., 2010. Architecture of peptidoglycan: more data and more models. *Trends Microbiol* 18, 59-66.
- Vollmer, W., Blanot, D., de Pedro, M.A., 2008. Peptidoglycan structure and architecture. *FEMS Microbiol Rev* 32, 149-167.
- Wanner, G., Formanek, H., Galli, D., Wirth, R., 1989. Localization of aggregation substances of *Enterococcus faecalis* after induction by sex pheromones. *Archives of Microbiology* 151, 491-497.
- Waters, C.M., Antiporta, M.H., Murray, B.E., Dunny, G.M., 2003. Role of the *Enterococcus faecalis* GeIE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol* 185, 3613-3623.
- Weiner-Lastinger, L.M., Abner, S., Edwards, J.R., Kallen, A.J., Karlsson, M., Magill, S.S., Pollock, D., See, I., Soe, M.M., Walters, M.S., Dudeck, M.A., 2019. Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network, 2015-2017. *Infect Control Hosp Epidemiol*, 1-18.
- Yang, H., Singh, M., Kim, S.J., Schaefer, J., 2017. Characterization of the tertiary structure of the peptidoglycan of *Enterococcus faecalis*. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1859, 2171-2180.