The role of anionic lipids in enterococcus faecalis physiology

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**NANYANG
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SINGAPORE

**THE ROLE OF ANIONIC LIPIDS IN *ENTEROCOCCUS*
FAECALIS PHYSIOLOGY**

CHEN QINGYAN

SCHOOL OF BIOLOGICAL SCIENCES

2019

The role of anionic lipids in *Enterococcus faecalis* physiology

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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 Doctor of Philosophy

2019

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Abbreviations

%	percent
°C	degree Celsius
μg	microgram
μl	microliter
Ala-PG	alanyl-phosphatidylglycerol
Arg-PG	arginyl-phosphatidylglycerol
AS	aggregation substance
ATc	anhydrotetracycline
AU	arbitrary unit
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BADA	BODIPY-FL 3-amino-d-alanine
BHI	Brain Heart Infusion
bp	base pair
BSA	bovine serum albumin
CAMP	cationic antimicrobial peptide
CAUTI	catheter-associated urinary tract infection
CDP-DAG	CDP-diacylglycerol
CFU	colony-forming unit
CI	competitive index
CL	cardiolipin
Cm	chloramphenicol
CMP	cytidine monophosphate
CW	cell wall
DAP	daptomycin
DMPE	dimethyl phosphatidylethanolamine

DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>E. faecium</i>	<i>Enterococcus faecium</i>
<i>E. casseliflavus</i>	<i>Enterococcus casseliflavus</i>
<i>E. mundtii</i>	<i>Enterococcus mundtii</i>
<i>E. sulfureus</i>	<i>Enterococcus sulfureus</i>
Ebp	endocarditis- and biofilm-associated pili
EDTA	ethylenediaminetetraacetic acid
Epa	enterococcal polysaccharide antigen
Erm	erythromycin
Esp	enterococcal surface protein
FBS	fetal bovine serum
FC	fold change
FDAA	fluorescent d-amino acid
FDR	false discovery rate
FISH	fluorescent <i>in situ</i> hybridization
Fus	fusidic acid
G3P	glycerol 3-phosphate
GelE	gelatinase
GFP	green fluorescent protein
GI	gastrointestinal
HA	haemagglutinin
hBD2	human β -defensin 2
hBD3	human β -defensin 3
HCl	hydrochloric acid

HMWL	high molecular weight ladder
HNP	human neutrophil peptide
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HtrA	high temperature requirement A
IFM	immunofluorescence microscopy
IM	inner membrane
IPTG	isopropyl- β -D-thiogalactoside
Kan	kanamycin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	lactic acid bacteria
LB	Luria-Bertani
LC-MS-MS	liquid chromatography with tandem mass spectrometry
lys-PG	lysyl-phosphatidylglycerol
MBC	Minimum bactericidal concentration
mins	minutes
ml	milliliters
mM	millimolar
MOI	multiplicity of infection
MprF	multiple peptide resistance factor
MRM	multiple reaction monitoring
NaCl	sodium chloride
NAO	10- <i>N</i> -nonyl-acridine orange
ng	nanogram
OD	optical density
OM	outer membrane
PA	phosphatidic acid

PAP _{2s}	Type 2 phosphatidic acid phosphatase
PB	phosphate buffer
PBP	penicillin-binding protein
PBS	phosphate buffer saline
PBST	PBS with 0.1% Tween 20
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PG	phosphatidylglycerol
PGN	peptidoglycan
PGP	phosphatidylglycerol-phosphate
PgsA	CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase
PI	phosphatidylinositol
PIP	phosphatidylinositol-phosphate
PL	phospholipid
PLD	phospholipase D
PMN	polymorphonuclear leukocyte
Proto	protoplast
PS	phosphatidylserine
PSICIC	Projected System of Internal Coordinates from Interpolated Contours
p-value	probability value
qPCR	quantitative PCR
Rif	rifampicin
RNA	ribonucleic acid
RT	reverse transcription
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. coelicolor</i>	<i>Streptomyces coelicolor</i>

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SrtA	sortase A
SrtC	sortase C
SUR	surotomycin
TADA	TAMRA 3-amino-d-alanine
Tc	tetracycline
TCS	two component system
TEM	transmission electron microscopy
TetR	tet-repressor
Tn	transposon
UTI	urinary tract infection
WCL	whole cell lysate
WT	wild type

Abstract

Enterococcus faecalis is a Gram-positive, facultative anaerobic, diplococcal, opportunistic pathogen, causing many nosocomial infections. Virulence factor secretion and assembly occurs at spatially restricted foci at the septum of the cell in *E. faecalis*. The foci coordinating these processes occur at the cell division plane, which is coincident with anionic lipid domains. The anionic lipids phosphatidylglycerol (PG) and cardiolipin (CL) are the major phospholipids (PLs) in Gram-positive bacterial membranes. Here, to determine the role of anionic PLs in *E. faecalis* physiology, we constructed a *pgsA* inducible mutant and *cls* deletion mutants to investigate the roles of PG and CL in the localization of secretion and virulence factor assembly machinery, as well as to antimicrobial resistance. We observed that *pgsA* is essential for cell viability in *E. faecalis*, but *cls1* and *cls2* are not. As expected for an essential gene, depletion of *pgsA* and its gene product, which is required for PG biosynthesis, resulted in severe cell morphology defects and cell death.

Protein expression of the sortases involved in focal virulence factor assembly were all decreased in the *pgsA*_{ind} at low anhydrotetracycline (ATc) concentrations (when PG is presumed to be depleted) and *cls2*-deficient mutants. However, delocalization of the sortases was only observed in the PG-depletion mutant, but not in *cls*-deficient mutants, suggesting independent roles for these two anionic PLs in virulence factor assembly. Furthermore, *cls2*-deficiency resulted in increased EbpC production on a single cell level, whereas neither the piliation on a population level nor the localization of EbpC was altered. In addition, in *pgsA*_{ind} at low ATc concentrations, the cell wall was less cross-linked and cell surface deposition was altered, which may serve as a compensatory reaction to PG depletion.

Finally, we demonstrated that *pgsA*_{ind} at low ATc concentrations became more sensitive to cell membrane targeting antimicrobials but more resistant to cell wall targeting antimicrobials, and *Cls2* contributed to daptomycin (DAP) sensitivity, suggesting that PG and CL differently contribute to antimicrobial resistance in *E. faecalis*. In summary, both PG and CL contribute to virulence factor assembly and antimicrobials resistance, but in different ways. These findings pave the way for future studies on the roles of phospholipids in *E. faecalis* biology and virulence, and may shed light on the development of new antimicrobials and treatment strategies.

Chapter 1 Introduction and literature review

1.1 Enterococci

Enterococci are Gram-positive, facultative anaerobic, diplococcal, opportunistic and non-spore-forming pathogens. Previously, enterococci were considered part of the genus *Streptococcus* but were later classified into their own genus and named *Enterococcus* (Schleifer and Kilpper-Bälz 1984, Murray 1990). Enterococci are found in plants, soil, and as commensals of the gastrointestinal (GI) tract of humans and animals (Kommineni et al. 2015). Enterococci can survive in a wide range of environments, including extreme temperature from 10°C to 45 °C, pH from 4.5 to 10.0, and high sodium chloride concentrations up to 6.5% NaCl (Fisher and Phillips 2009). As a member of lactic acid bacteria (LAB) group, enterococci have been widely used as probiotics or as starter cultures in the food industry for many years (Foulquie Moreno et al. 2006). However, enterococci have been reported as the third most common cause of nosocomial infections, including urinary tract infection (UTI), bacteremia, endodontic infection, and wound infections (Arias and Murray 2012). The *Enterococcus* genus can be divided into five groups based on genetic similarity: *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. mundtii* and *E. sulfureus*, in which *E. faecalis* and *E. faecium* are the most commonly studied (Staley et al. 2014). *E. faecalis* is responsible for a large majority (80-90%) of human enterococcal infections and the remaining enterococcal infections are mostly caused by *E. faecium* (Bennett and Dunny 2010, Lempiäinen et al. 2005, Tendolkar et al. 2003). *E. faecalis* is also one of the most commonly isolated species of enterococci associated with nosocomial infections and is one of the most common causative agents of catheter-associated urinary tract infections (CAUTI) (Abat et al. 2016, Byappanahalli et al. 2012). Many *Enterococcus*-associated nosocomial infections are increasingly resistant to multiple antimicrobials, limiting therapeutic options. Thus, understanding the mechanism of their antibiotic resistance is extremely urgent in order to develop new treatment strategies to overcome resistance (Handwerger et al. 1993, Protonotariou et al. 2010, Miller et al. 2014).

During enterococcal infection, biofilm formation is an important factor for enterococcal pathogenesis. Enterococcal biofilm formation contributes to their virulence and tolerance to various antimicrobials, rendering their eradication extremely difficult (Mohamed et al. 2004, Ch'ng et al. 2019). The ability of enterococci to adhere and form biofilm on abiotic surfaces is partly due to their multi-subunit surface appendages such as pili, which are directly involved

in adhesion to and association with the host (Mohamed and Huang 2007, Garsin and Willems 2010, Fallah et al. 2017).

1.2 Bacterial cell membrane lipids

Bacterial membranes are complex and dynamic assemblies of proteins and lipids, in which lipids form lamellar membrane bilayers and incorporate membrane proteins to contribute to many cellular processes (Strahl and Errington 2017). Many essential signaling processes are present at cell membrane, as part of two component systems (TCS). The sensor kinase of TCS systems, coupled with their cognate response regulator proteins in the cytoplasm, enable the bacterial cell to sense and respond to the extracellular environment (Wagner et al. 2017). In principle, the bacterial membrane also contributes to bacterial shape and cell division. The integrity and organization of the bacterial membrane is crucial for cell survival and understanding the role of membrane organization during different environmental stresses has been an important topic in past decades (Lindner and Naim 2009, Escriba et al. 2015).

Bacterial membrane lipid homeostasis contributes to bacterial survival in different environments. Bacterial membrane lipids are formed by amphiphilic lipids which in most cases and conditions studied are phospholipids (PLs). In these studies, bacterial membranes consist of approximately 60% proteins and 40% phospholipids (PLs), although the proportion varies depending on the specific bacterial species. In addition, the PL diversity and distribution also varies in different bacterial species (Dowhan 1997). PLs are the determinants of membrane protein topology, and the composition of PLs can be regulated in response to different cell growth stressors. The PL component in bacterial membranes can play multiple functional roles, such as acting as basic building blocks of the cell membrane bilayers, forming membrane vesicles, functioning as signal receptors, acting as primary or secondary messengers, and forming lipid enriched domains for protein functions (Epand et al. 2010, Kaneti et al. 2013, Zaknoon et al. 2012, Epand et al. 2016).

PLs consist of a hydrophilic phosphate head group and two hydrophobic fatty acyl chains. The special amphipathic characteristic of PLs contributes to creation of a physical barrier in an aqueous environment. In a given species, PL varies in length, saturation, and branching of fatty acyl chains as well as the structure, polarity and charge of the head groups (Lin and Weibel 2016). Based on the identification of the head groups in PLs, there are three major PLs in

bacterial membranes: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (Ernst et al. 2016). Minor quantities of anionic phosphatidic acid (PA), and some PL derivatives, such as lysyl phosphatidylglycerol (lys-PG), alanyl-phosphatidylglycerol (Ala-PG) and dimethyl PE (DMPE) also exist in bacterial membranes (Yao and Rock 2013, Slavetinsky et al. 2012, Niedermann et al. 1991). In addition, there are also some other types of PLs in bacterial membranes, such as phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS), which are less characterized in bacteria (Mazzella et al. 2004, Fang et al. 2000). The fatty acyl chains of PLs determine the viscosity of bacterial membranes, affecting many membrane-associated functions including the passive permeability of hydrophobic molecules, membrane sensing, and remodeling (Parsons and Rock 2011, de Carvalho and Caramujo 2018).

The biosynthesis of phospholipids starts from the synthesis of phosphatidic acid (PA), which is produced through the acylation of glycerol 3-phosphate (G3P) with fatty acids (Yao and Rock 2013, Bond 2017). Using PA as a precursor, the biosynthesis pathways and enzymes involved in PL synthesis are summarized (**Figure 1.1**). PA is catalyzed by the CDP-diacylglycerol (CDP-DAG) synthase CdsA to form CDP-DAG, which is the key intermediate in glycerophospholipid synthesis in bacteria (Kishida et al. 1993). The *cdsA* gene is essential in *E. coli*, and decreased activity of CdsA results in accumulation of PA and membrane dysfunction (Ganong et al. 1980, Ganong and Raetz 1982). The glycerophospholipid synthesis pathway from CDP-DAG and the functions of different glycerophospholipids are described in the following sections.

Figure 1.1 Overall biosynthesis pathway of bacterial phospholipid. The unique chemical structure of head group of different phospholipids (blue) with are shown, and the synthesis enzymes are indicated in red. Enzymes with known structures are underlined. The reversible reactions are marked with “*”. *PA*, phosphatidic acid; *CDP-DAG*, diphosphate-diacylglycerol; *PGP*, phosphatidylglycerol-phosphate; *PG*, phosphatidylglycerol; *CL*, cardiolipin; *PIP*, phosphatidylinositol-phosphate; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PE*, phosphatidylethanolamine; *PC*, Phosphatidylcholine (Tang et al. 2018).

Based on differences in cell envelope structures, most bacteria are separated into one of two families: Gram-positive and Gram-negative according to their characteristic Gram-staining phenotype (Gram 1884). The Gram-negative bacteria contain three principle layers in the envelope: the outer membrane (OM), the inner membrane (IM) and a thin cell wall layer between the two membrane layers. By contrast, the Gram-positive bacteria contain two layers: one cytoplasmic membrane and a thick cell wall exterior to it (Silhavy et al. 2010). PLs are distributed heterogeneously in the bacterial membrane and dynamically communicate with the extracellular environment (Matsumoto et al. 2006, Barak and Muchova 2013). In the studies of human pathogens and model bacteria, the PL composition in bacterial membrane differs in different species (**Table 1.1**). The major PLs in the membrane of Gram-negative bacteria are

PE, PG, and CL whereas the major PLs in Gram-positive bacteria are PG, and CL (Lin and Weibel 2016). In bacterial membranes, PE is zwitterionic whereas PG and CL are anionic. In addition, PG or CL can be modified with the amino acid residues lysine, alanine, or arginine to convert the anionic lipid to cationic or zwitterionic form (Peschel et al. 2001, Vences-Guzman et al. 2012).

Table 1.1 Major PL composition in cell membrane of different bacteria

Bacteria strain	Percentage of PLs in membranes (%) ^a			References
	PE	PG	CL	
Gram-negative				
<i>Escherichia coli</i>	79	18	4	(Romantsov et al. 2007)
<i>Pseudomonas aeruginosa</i>	60	22	16	(Ghorbal et al. 2013)
<i>Proteus mirabilis</i>	76	13	6	(Gmeinfr and Martin 1976)
Gram-positive				
<i>Bacillus subtilis</i>	12	70	4	(Bittman et al. 1985)
<i>Streptococcus pneumonia</i>	ND ^b	57	38	(Trombe et al. 1979)
<i>Staphylococcus aureus</i>	ND ^b	57	5	(Fischer 1994)
<i>Enterococcus faecalis</i>	ND ^b	30	32	(Mishra et al. 2012)
^a PL composition was calculated in cells at exponential phase				
^b Not detected				

1.2.1 Phosphatidylglycerol (PG)

Phosphatidylglycerol (PG) is a major anionic phospholipid in many bacterial membranes (Dowhan 1997, Dugail et al. 2017). Structurally, PG molecules consist of two fatty acyl chains and a glycerol 3-phosphate backbone (**Figure 1.2A**) and can be separated into a polar head group and a nonpolar tail group (**Figure 1.2B**). PG species differ from each other in the identity of the two fatty acyl chains, including the number of carbon atoms per fatty acyl chain as well

as the number and position of carbon-to-carbon double bonds in each unsaturated fatty acyl chain (**Figure 1.2C**).

Figure 1.2 Structure of phosphatidylglycerol (PG). (A) General chemical structure of PG with two fatty acyl chains (R₁ and R₂, red). (B) Schematic structure of PG with a polar head and nonpolar tails. (C) Molecular structure of one species of PG, PG (16:0/18:1(7Z)), which means PG contains 16 carbon atoms and 0 carbon-to-carbon double bond for the acyl group at position *sn*-1, 18 carbon atoms and 1 carbon-to-carbon double bond (at position 7Z) for the acyl group at position *sn*-2. The corresponding molecules in the polar head (black) and nonpolar tails (red) are highlighted.

In the process of PG formation, PG is synthesized from CDP-DAG in two steps (Sohlenkamp and Geiger 2016). Firstly, the phosphatidyl group of CDP-DAG is transferred to glycerol-3-phosphate (G3P) to form phosphatidylglycerol-phosphate (PGP). This reaction is catalyzed by the enzyme CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase (PgsA), which is encoded by *pgsA*. In addition, PgsA can also catalyze the reverse reaction (**Tang et al. 2018**). Secondly, PGP is rapidly dephosphorylated by phosphatidylglycerol-phosphate phosphatase (Pgp) to produce PG (**Figure 1.1**). In *E. coli*, an alternative PG synthesis pathway

is demonstrated both *in vivo* and *in vitro*, in which PE is catalyzed by a CL synthase, ClsB, to react with glycerol to form PG (Li et al. 2016). Three different phosphatidylglycerol-phosphate phosphatases, PgpA, PgpB and PgpC are characterized in *E. coli*, and deletion of the three *pgp* genes resulted in cell death (Lu et al. 2011). In *B. subtilis*, the previous Type 2 phosphatidic acid phosphatase (PAP_{2s}) was found to be a principle Pgp enzyme, subsequently renamed as bsPgpB (Ghachi et al. 2017). However, the Pgp in enterococci has not defined yet. Green fluorescent protein (GFP) fusions to the PgsA enzyme are septally localized in *B. subtilis* (Nishibori et al. 2005), whereas the florescent GFP-PgsA in *E. coli* appeared as dots distributed around the cells (Matsumoto et al. 2006, Millman et al. 2001).

The PL compositions are varied in different growth phases and in different species. For example, in *B. subtilis*, the PL composition remains unchanged during the entire exponential phase, but in stationary phase PG is decreased, PE is increased, but lys-PG is still unchanged (Gidden et al. 2009). In *E. faecalis*, the overall PG levels gradually decrease whereas lys-PG levels increase when cells are grown from early exponential phase to late exponential phase (Rashid et al. 2017).

PG plays a crucial role in the stability of bacterial membranes (Zhao et al. 2008). PG is essential for cell viability because mutation of *pgsA* is lethal in many bacteria including *S. aureus* (Martin et al. 1999) and *B. subtilis* (Kobayashi et al. 2003). An *E. coli* *pgsA* null mutant, in which PG is undetectable, is still viable when also harboring *lpp* and *rcsF* mutations, in which the *lpp* mutation prevents accumulation of a nascent major outer membrane lipoprotein Lpp which requires PG for its modification, whereas the *rcsF* mutation prevents the lysis of Δ *pgsA* cells at the temperature of 37–42 °C (Kikuchi et al. 2000, Suzuki et al. 2002). In *B. subtilis*, an induced *pgsA* mutant was constructed to control *pgsA* transcription under a *Pspac* promoter (dose-dependent inducible by isopropyl- β -D-thiogalactoside, IPTG), in which removal or reduced levels of IPTG, and consequently, reduced production and accumulation of PgsA, led to decreased viability. (Lopez et al. 2006). Whether PG is essential in enterococcal species is unknown.

PG plays important roles in protein functions. In *E. coli*, PG is required for SecA function and high-affinity binding to plasma membrane (de Vrije et al. 1988, Hendrick and Wickner 1991). In addition, using liposome models, FtsY, the membrane-associated signal recognition particle receptor, was found to interact with PE and PG with high affinity (De Leeuw et al. 2000,

Millman et al. 2001). The spiral-like anionic lipid distribution in *B. subtilis* is disrupted by PG depletion, and the spiral-like localization of lipid is coincident with the localization of the cell division protein MinD (Barák et al. 2008). The membrane association of MinD also relies on the anionic PG in *E. coli* (Mileykovskaya et al. 2003). SecA focal localization in *B. subtilis* disappears upon depletion of PG, suggesting that PG may determine the subcellular sites in the secretion pathway (Campo et al. 2004).

PG is also involved in antimicrobial resistance. The head group of PG can be modified by different aminoacyl residues, such as lysine, alanine, or arginine in response to certain stress conditions (Geiger et al. 2010). The multiple peptide resistance factor (MprF) mediates the reaction of anionic PG with positively charged lysyl-tRNA to produce lys-PG (Oku et al. 2004, Bao et al. 2012, Geiger et al. 2010). This modification of PG can convert the anionic charge of PG to positively charged lys-PG, resulting in resistance to many cationic antimicrobial peptides (CAMPs) including different human defensins, daptomycin, and vancomycin (Kristian et al. 2003, Ernst and Peschel 2011, Ernst et al. 2009, Nishi et al. 2004). In addition, studies using giant unilamellar vesicles demonstrate that daptomycin (DAP) prefers to bind with PG in the presence of Ca^{2+} (Kreutzberger et al. 2017). Both in DAP-resistant *E. faecalis* and DAP-resistant *E. faecium*, the PG compositions are decreased whereas CL compositions remain unchanged, and the distribution of anionic lipids is also altered (Mishra et al. 2012, Tran et al. 2013).

1.2.2 Cardiolipin (CL)

Cardiolipin, which was first isolated in the year 1942 (Pangborn 1942), consists of two phosphatidic acid moieties linked through a glycerol bridge (**Figure 1.3A**). Based on its structure, CL can be spatially separated into a polar head group and a nonpolar tail group (**Figure 1.3B**). Structurally, CL molecular species differ from each other in the identity of their four fatty acyl moieties, including the number of carbon atoms per fatty acyl chain as well as the number and position of carbon-to-carbon double bonds in each unsaturated fatty acyl chain (Schlame et al. 2005, Minkler and Hoppel 2010). Cardiolipin has gained interest in recent years because of its special dimeric structure and related functions in the cell membrane.

Figure 1.3 Structure of cardiolipin (CL). (A) General chemical structure of CL with four fatty acyl chains (R₁-R₄, red). (B) Schematic structure of CL with a polar head and nonpolar tails. (C) Molecular structure of one species of CL, CL (1'-[16:0/18:1(7Z)], 3'-[16:0/18:1(7Z)]), which means CL contains 16 carbon atoms and 0 carbon-to-carbon double bonds for the acyl group at position *sn*-1, 18 carbon atoms and 1 carbon-to-carbon double bond (at position 7Z) for the acyl group at position *sn*-2, 16 carbon atoms and 0 carbon-to-carbon double bond for the acyl group at position *sn*-3, 18 carbon atoms and 1 carbon-to-carbon double bond (at position 7Z) for the acyl group at position *sn*-4. The corresponding molecules in the polar head (black) and nonpolar tails (red) are highlighted.

Using phosphatidylglycerol (PG) as precursor, CL biosynthesis mainly relies on two pathways (**Figure 1.4**): (1) the bacteria-like CL synthase (Cls-I) pathway in which one reversible phosphatidyl moiety is transferred from one PG molecule to the 3'-hydroxyl group of another PG to form CL and glycerol (Lopez-Lara and Geiger 2017); (2) the eukaryote-like CL synthase (Cls-II) pathway in which the phosphatidyl group is provided by one cytidine diphosphate-diacylglycerol (CDP-DAG) molecule and reacts with one PG molecule to yield CL and cytidine monophosphate (CMP) (Horvath and Daum 2013). In most bacteria and some eukaryotic organisms like *Trypanosoma brucei* and all other trypanosomiasis (Serricchio and Bütikofer 2012), CL is synthesized through the Cls-I pathway in which the cardiolipin synthase (Cls-I)

belongs to the phospholipase D (PLD) superfamily. In eukaryotes and some bacteria, like *Streptomyces coelicolor* and most actinobacteria (Sandoval-Calderon et al. 2009), CL is catalyzed through the Cls-II pathway in which the cardiolipin synthase (Cls-II) belongs to the CDP-alcohol phosphotransferase superfamily. However, an unusual CL synthase (ClsC) in *E. coli* has been described in which the phosphatidyl group is provided by phosphatidylethanolamine (PE), a zwitterionic membrane phospholipid, and reacts with one PG molecule to produce CL and ethanolamine (Nishijima et al. , Tan et al. 2012). CL synthase is not essential in mitochondria and most bacteria, whereas the CL synthase is essential in *S. coelicolor*, and adequate levels of the CL enzyme are required for proper morphogenesis in *Streptomyces* (Jyothikumar et al. 2012).

Figure 1.4 CL biosynthesis pathways from PG. Using a PG molecule as precursor, most bacteria synthesize CL through the “bacteria-like” (Cls-I) pathway, in which one PG molecule reacts with another PG to form CL and glycerol. In eukaryotes and some bacteria, CL is synthesized through the “eukaryote-like” (Cls-II) pathway, in which PG reacts with CDP-DAG to form CL and CMP. An unusual pathway for CL synthesis is found in *E. coli*, in which PG is reacted with PE to form CL and ethanolamine by an unusual cardiolipin synthase encoded by the *clsC* gene. *PG*, phosphatidylglycerol; *CDP-DAG*, CDP-diacylglycerol; *CMP*, cytidine monophosphate; *PE*, phosphatidylethanolamine; *CL*, cardiolipin.

The hydrophobic fluorescent dye 10-*N*-nonyl-acridine orange (NAO) has been used as a CL specific staining dye to visualize and quantify CL distribution in mitochondrial membranes of eukaryotic cells (Septinus et al. 1985, Jacobson et al. 2002, Petit et al. 1992) and in the cell membrane of bacteria (Mileykovskaya and Dowhan 2000, Kawai et al. 2004). It has been reported that NAO emits green fluorescence when it binds to anionic phospholipids, whereas NAO emits red fluorescence only when it binds to CL, in which the nonyl group of NAO inserts in the bilayer generated by the four acyl chains of CL to form a special dimer that emits in the red channel (Garcia Fernandez et al. 2004, Eugenia Mileykovskaya 2001). Based on the observation of NAO enrichment at cell poles and septa in some bacteria including *E. coli*, it has been assumed that CL is enriched at bacterial poles and at the septal region of dividing cells (Kawai et al. 2004, Mileykovskaya and Dowhan 2009). However, subsequent research found that red fluorescence of NAO can also be observed in a CL-deficient *E. coli* strain (Oliver et al. 2014, Wood 2018), suggesting that NAO is also able to bind to other molecules and NAO is not a CL-specific probe. Nevertheless, it has been reported that a GFP-ClfA fusion protein in *B. subtilis* is localized at the poles and septa (Nishibori et al. 2005, Kusaka et al. 2016), suggesting that CL synthesis is localized at polar and septal membranes.

In order to synthesize CL, many bacteria possess multiple CL synthase genes (Dowhan 1997), and these CL synthases contribute differently to CL synthesis in different growth conditions. For example, in gram-negative *E. coli*, three CL synthase genes have been identified: *clsA* (Nishijima et al. 1988), *clsB* (Guo and Tropp 2000) and *clsC* (Tan et al. 2012), in which each CL synthase contributes to CL accumulation differently in different growth phases. ClfA, encoded by *clsA*, is the major enzyme responsible for CL synthesis from two PG molecules (Clf-I pathway) during exponential and stationary phase. ClfB, encoded by *clsB*, is also able to produce CL from two PG molecules (Clf-I pathway) during stationary phase *in vivo*, whereas its activity *in vitro* has not demonstrated so far. It has also reported that ClfB is involved in PG synthesis both *in vivo* and *in vitro* (Li et al. 2016). Unlike ClfA and ClfB, ClfC synthase, encoded by *clsC*, produces CL in a different manner using one PE molecule as the phosphatidyl donor. CL is undetectable in exponential phase in a $\Delta clsAB$ double mutant and is not detectable in any growth phase or growth condition in a $\Delta clsABC$ mutant (Tan et al. 2012, Jeucken et al. 2018, Dagang Guo 2000). In the study of the $\Delta clsABC$ mutant during stationary phase, expressing either *clsA* or *clsB* on a plasmid *in trans* in the $\Delta clsABC$ mutant can restore the CL lipid levels to wild type levels. However, co-expression of *clsC* and its neighboring gene, *ymdB*

which encodes a protein containing a domain with a predicted adenosine diphosphate (ADP) ribose-binding potential (Karras et al. 2005), from the native promoter and in a single operon is required to restore CL lipid levels in the $\Delta clsABC$ mutant during the stationary phase (Tan et al. 2012). Another gram-negative bacteria, *Agrobacterium tumefaciens*, contains two distinct CL synthase genes, named *cls1* and *cls2*, in which *cls2* contributes to CL production in early exponential phase, whereas *cls1* and *cls2* are both responsible for CL synthesis during stationary phase (Czolkoss et al. 2016).

Gram-positive bacteria *B. subtilis* also contains three CL synthase paralogous genes, previously named as *ywiE*, *ywnE* and *ywjE*, in which mutation of *ywnE* results in absolute absence of CL, whereas the *ywiE* and *ywjE* single mutants contain a similar lipid profile compared with wild type, suggesting that *ywnE* gene, also named as *clsA* now, is the main gene responsible for CL synthesis in *B. subtilis* (Kawai et al. 2004, Lopez et al. 2006). Another gram-positive, *S. aureus*, harbors two distinct CL synthase genes, named *cls1* and *cls2*, which both contribute to CL accumulation in *S. aureus* (Kuhn et al. 2015, Ohniwa et al. 2013). Expressing either *cls1* or *cls2* of *S. aureus* in *E. coli* is sufficient to induce CL accumulation in a CL synthesis-deficient *E. coli* mutant. Although knocking out of either *cls1* or *cls2* in *S. aureus* results in reduced CL levels, the CL level in $\Delta cls1$ single mutant is higher than it in $\Delta cls2$ mutant, and the $\Delta cls1\Delta cls2$ double mutant is not able to accumulate CL at stationary phase (Koprivnjak et al. 2011). These data suggest that *cls1* and *cls2* in *S. aureus* contribute unequally to CL yield.

E. faecalis contains two putative *cls* genes, *cls1* and *cls2*, but the regulation and contribution of each CL synthase to CL accumulation in different growth phases and growth conditions has not been previously addressed. Previously, *in vivo* (in human) evolved daptomycin (DAP) resistant *E. faecalis* mutations mapped to *liaF* (encoding a member of a three-component regulatory system LiaFSR) and genes related to cell membrane phospholipid metabolism, such as *gdpD* (encodes glycerophosphoryl diester phosphodiesterase) and *cls1* (encodes cardiolipin synthase, mutation was Lys61del) (Arias et al. 2011). During *in vitro* evolution of DAP-resistant *E. faecalis* strains, (Palmer et al. 2011), two different mutations in *cls1* (N77_Q79del and R218Q) were identified, and expressing either of the two alleles of *cls1* under control of the *cls1* native promoter in a plasmid was able to confer trans-dominant resistance to DAP in an otherwise DAP susceptible strain. The R218Q substitution in *cls1* was also found in a clinical isolate of DAP-resistant *E. faecium*, however the delivery of this *cls1* allele (R218Q) to the chromosome of a susceptible strain was not able to confer DAP resistance (Arias et al.

2011, Tran et al. 2013). Enzyme activity analysis of the Cls1 protein purified from the DAP-resistant *E. faecium* suggested that increased Cls1 activity is likely to facilitate DAP resistance (Davlieva et al. 2013). The mutations occurring in *cls1* were also found in the *in vitro* evolution of surotomycin-resistant (SUR-resistant) *E. faecium* (*cls1* mutation: R211L) and SUR-resistant *Clostridium difficile* (*cls* mutation: D79N) (Adams et al. 2015). What's more, a point mutation in *cls2* was associated with the development of DAP resistance in *S. aureus* (Peleg et al. 2012). These data imply a potential role of CL synthase in antibiotic resistance.

1.3 Virulence factors

Virulence factors are pathogen-produced molecules that promote the establishment and maintenance of disease so that the pathogenic bacteria can sense and respond to the change of the local environments. Many bacteria can produce virulence factors to aid their pathogenesis.

Most virulence factors are restricted to a single or several closely related species, but the virulence factors use similar strategies to cause disease in the host. One of the earliest bacterial virulence factors that contribute to infection are probably the adhesins, which contribute to effective colonization by the bacteria. Governed by the adhesins, bacteria can specifically or non-specifically recognize host receptors and can mediate attachment to the host (Berne et al. 2015). After primary attachment to the host, adhesins also facilitate persistence in the face of the host immune response (Patel et al. 2017). Bacteria use different types of adhesins including, but not limited to, fimbriae and pili (Proft and Baker 2009). Bacteria also form biofilms, which are surface-attached microbial communities and the cells within a biofilm are encased in an extracellular matrix. Biofilms promote the adhesion to surfaces and decrease the susceptibility to many antimicrobial agents (Hall and Mah 2017, O'Toole et al. 2000).

Enterococci also use a variety of virulence factors to cause infection and persist within their hosts (Sava et al. 2010, Jett et al. 1994). For example, aggregation substance (AS) is a surface protein which can facilitate *E. faecalis* adherence and internalization into epithelial cells (Wells et al. 2000, Saringen et al. 2000). The enterococcal surface protein (Esp) contributes to biofilm formation and pathogenesis of UTI (Toledo-Arana et al. 2001, Tendolkar et al. 2004, Shankar et al. 2001). The gelatinase (GelE) also promotes biofilm formation and pathogenesis of *E. faecalis* (Qin et al. 2000, Mylonakis et al. 2002). GelE expression in *E. faecalis* is under the control of the Fsr two-component system (TCS), which consists of four genes (*fsrA*, *fsrB*,

fsrD, and *fsrC*) (Nakayama et al. 2006). The *E. faecalis* adhesin, Ace, mediates attachment of *E. faecalis* to collagen and promotes its pathogenesis (Kowalski et al. 2006, Singh et al. 2010, Nallapareddy et al. 2000, Mandlik et al. 2007).

Another important virulence factor in *E. faecalis* are the endocarditis- and biofilm-associated pili (Ebp) (Nallapareddy et al. 2006, Abbot et al. 2007). *E. faecalis* relies on a conserved secretory and sorting pathway for the translocation, biogenesis, and subsequent attachment of cell wall associated virulence factors, including Ebp, to the cell surface (Kline et al. 2009). The Ebp are high molecular weight polymers, which consist of three subunits, EbpA, EbpB and EbpC. Ebp expressing cells are usually observed on only a subpopulation of *E. faecalis* (Nallapareddy et al. 2006). The three subunits are transcribed from the *ebp* operon, and controlled by an EbpR positive regulator (Bourgogne et al. 2007). EbpC is the main subunit of the pilus, EbpA is the tip adhesion, and EbpB serves as the base of the pilus (**Figure 1.5**). The sortase SrtC, which is encoded downstream of the *ebp* operon but independently transcribed from its own promoter, is responsible for the polymerization of Ebp resulting in polymerized pili with different molecular weights. Upon assembly, the pilus is attached to the cell wall by another sortase-SrtA, and deletion of *srtA* results in increased levels of Ebp in whole cell lysates (Nielsen et al. 2013). The sortase enzymes, SrtA and SrtC, colocalize with SecA, a subunit in the general secretion pathway, at the division plane, which is also enriched with anionic phospholipids (Kandaswamy et al. 2013, Kline et al. 2009).

Figure 1.5 Mechanism of Ebp assembly. (A) The *ebp* operon consists of 3 pilus subunits (*ebpA*, *ebpB* and *ebpC*), independently transcribed *srtC*, and another independently transcribed *srtA*. (B) EbpA incorporation with SrtC. (C) Polymerization of EbpC requires SrtC transpeptidation. (D) SrtA participates the polymerized EbpC. (E) Ebp is anchored to cell wall by SrtA (Nielsen et al. 2013).

1.4 Significance of this study

The increasing resistance of *E. faecalis* to multiple antimicrobials limits therapeutic options for their infections.

In this study, we investigated the role of anionic phospholipids including PG and CL in *E. faecalis* physiology. Using genetic approaches, we constructed a *pgsA* inducible mutant and *cls* deletion mutants and performed imaging, transcriptomics, and infection studies to investigate the role of anionic phospholipids in *E. faecalis*. We examined the roles of PG and CL in both virulence factor assembly and antimicrobial sensitivity. Understanding the

mechanisms underlying cell wall associated virulence factor assembly may contribute to discovery of novel antimicrobials to treat bacterial infections. In addition, understanding of the role of anionic phospholipids in antimicrobial resistance in *E. faecalis* may also provide meaningful insights for advancing treatment strategies for enterococcal infections.

1.5 References

- [1]. Abat, C., M. Huart, V. Garcia, G. Dubourg & D. Raoult (2016) *Enterococcus faecalis* urinary-tract infections: Do they have a zoonotic origin? *J Infect*, 73, 305-13.
- [2]. Abbot, E. L., W. D. Smith, G. P. Siou, C. Chiriboga, R. J. Smith, J. A. Wilson, B. H. Hirst & M. A. Kehoe (2007) Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. *Cellular microbiology*, 9, 1822-1833.
- [3]. Adams, H. M., X. Li, C. Mascio, L. Chesnel & K. L. Palmer (2015) Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and Enterococcus species. *Antimicrobial agents and chemotherapy*, 59, 4139-4147.
- [4]. Arias, C. A. & B. E. Murray (2012) The rise of the Enterococcus: beyond vancomycin resistance. *Nat Rev Microbiol*, 10, 266-78.
- [5]. Arias, C. A., D. Panesso, D. M. McGrath, X. Qin, M. F. Mojica, C. Miller, L. Diaz, T. T. Tran, S. Rincon, E. M. Barbu, J. Reyes, J. H. Roh, E. Lobos, E. Sodergren, R. Pasqualini, W. Arap, J. P. Quinn, Y. Shamoo, B. E. Murray & G. M. Weinstock (2011) Genetic basis for in vivo daptomycin resistance in enterococci. *N Engl J Med*, 365, 892-900.
- [6]. Bao, Y., T. Sakinc, D. Laverde, D. Wobser, A. Benachour, C. Theilacker, A. Hartke & J. Huebner (2012) Role of mprF1 and mprF2 in the pathogenicity of *Enterococcus faecalis*. *PLoS One*, 7, e38458.
- [7]. Barak, I. & K. Muchova (2013) The role of lipid domains in bacterial cell processes. *Int J Mol Sci*, 14, 4050-65.
- [8]. Barák, I., K. Muchová, A. J. Wilkinson, P. J. O'Toole & N. Pavlendová (2008) Lipid spirals in *Bacillus subtilis* and their role in cell division. *Molecular microbiology*, 68, 1315-1327.
- [9]. Bennett, R. J. & G. M. Dunny (2010) Analogous telesensing pathways regulate mating and virulence in two opportunistic human pathogens. *Mbio*, 1, e00181-10.
- [10]. Berne, C., A. Ducret, G. G. Hardy & Y. V. Brun (2015) Adhesins Involved in Attachment to Abiotic Surfaces by Gram-Negative Bacteria. *Microbiol Spectr*, 3.
- [11]. Bittman, R., S. Clejan, B. P. Robinson & N. M. Witzke (1985) Kinetics of cholesterol and phospholipid exchange from membranes containing crosslinked proteins or crosslinked phosphatidylethanolamines. *Biochemistry*, 24, 1403-1409.
- [12]. Bond, P. (2017) Phosphatidic acid: biosynthesis, pharmacokinetics, mechanisms of action and effect on strength and body composition in resistance-trained individuals. *Nutrition & metabolism*, 14, 12-12.
- [13]. Bourgonne, A., K. V. Singh, K. A. Fox, K. J. Pflughoeft, B. E. Murray & D. A. Garsin (2007) EbpR Is Important for Biofilm Formation by Activating Expression of the

Endocarditis and Biofilm-Associated Pilus Operon (ebpABC) of *Enterococcus faecalis* OG1RF. *Journal of Bacteriology*, 189, 6490-6493.

- [14]. Byappanahalli, M. N., M. B. Nevers, A. Korajkic, Z. R. Staley & V. J. Harwood (2012) Enterococci in the environment. *Microbiology and molecular biology reviews* : *MMBR*, 76, 685-706.
- [15]. Campo, N., H. Tjalsma, G. Buist, D. Stepniak, M. Meijer, M. Veenhuis, M. Westermann, J. P. Muller, S. Bron, J. Kok, O. P. Kuipers & J. D. Jongbloed (2004) Subcellular sites for bacterial protein export. *Mol Microbiol*, 53, 1583-99.
- [16]. Ch'ng, J. H., K. K. L. Chong, L. N. Lam, J. J. Wong & K. A. Kline (2019) Biofilm-associated infection by enterococci. *Nat Rev Microbiol*, 17, 82-94.
- [17]. Czolkoss, S., C. Fritz, G. Holzl & M. Aktas (2016) Two Distinct Cardiolipin Synthases Operate in *Agrobacterium tumefaciens*. *PLoS One*, 11, e0160373.
- [18]. Dagang Guo, B. E. T. (2000) A second *Escherichia coli* protein with CL synthase activity. *Biochimica et Biophysica Acta*, 1483, 263-274.
- [19]. Davlieva, M., W. Zhang, C. A. Arias & Y. Shamoo (2013) Biochemical characterization of cardiolipin synthase mutations associated with daptomycin resistance in enterococci. *Antimicrobial agents and chemotherapy*, 57, 289-296.
- [20]. de Carvalho, C. C. C. R. & M. J. Caramujo (2018) The Various Roles of Fatty Acids. *Molecules (Basel, Switzerland)*, 23, 2583.
- [21]. De Leeuw, E., K. Te Kaat, C. Moser, G. Menestrina, R. Demel, B. De Kruijff, B. Oudega, J. Luirink & I. Sinning (2000) Anionic phospholipids are involved in membrane association of FtsY and stimulate its GTPase activity. *The EMBO journal*, 19, 531-541.
- [22]. de Vrije, T., R. L. de Swart, W. Dowhan, J. Tommassen & B. de Kruijff (1988) Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. *Nature*, 334, 173-5.
- [23]. Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity why are there so many lipids?
- [24]. Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu Rev Biochem*, 66, 199-232.
- [25]. Dugail, I., B. D. Kayser & M. Lhomme (2017) Specific roles of phosphatidylglycerols in hosts and microbes. *Biochimie*, 141, 47-53.
- [26]. Epand, R. F., J. E. Pollard, J. O. Wright, P. B. Savage & R. M. Epand (2010) Depolarization, bacterial membrane composition, and the antimicrobial action of ceragenins. *Antimicrobial agents and Chemotherapy*, 54, 3708-3713.

- [27]. Epand, R. M., C. Walker, R. F. Epand & N. A. Magarvey (2016) Molecular mechanisms of membrane targeting antibiotics. *Biochim Biophys Acta*, 1858, 980-7.
- [28]. Ernst, C. M. & A. Peschel (2011) Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. *Mol Microbiol*, 80, 290-9.
- [29]. Ernst, C. M., P. Staubitz, N. N. Mishra, S. J. Yang, G. Hornig, H. Kalbacher, A. S. Bayer, D. Kraus & A. Peschel (2009) The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog*, 5, e1000660.
- [30]. Ernst, R., C. S. Ejsing & B. Antonny (2016) Homeoviscous Adaptation and the Regulation of Membrane Lipids. *J Mol Biol*, 428, 4776-4791.
- [31]. Escriba, P. V., X. Busquets, J. Inokuchi, G. Balogh, Z. Torok, I. Horvath, J. L. Harwood & L. Vigh (2015) Membrane lipid therapy: Modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment. *Prog Lipid Res*, 59, 38-53.
- [32]. Eugenia Mileykovskaya, W. D. (2001) Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. *FEBS Letters*, 507, 187-190.
- [33]. Fallah, F., M. Yousefi, M. R. Pourmand, A. Hashemi, A. Nazari Alam & D. Afshar (2017) Phenotypic and genotypic study of biofilm formation in Enterococci isolated from urinary tract infections. *Microb Pathog*, 108, 85-90.
- [34]. Fang, J., M. J. Barcelona & J. D. Semrau (2000) Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. *FEMS microbiology letters*, 189, 67-72.
- [35]. Fischer, W. (1994) Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. *Medical microbiology and immunology*, 183, 61-76.
- [36]. Fisher, K. & C. Phillips (2009) The ecology, epidemiology and virulence of Enterococcus. *Microbiology*, 155, 1749-57.
- [37]. Foulquie Moreno, M. R., P. Sarantinopoulos, E. Tsakalidou & L. De Vuyst (2006) The role and application of enterococci in food and health. *Int J Food Microbiol*, 106, 1-24.
- [38]. Ganong, B. & C. Raetz (1982) Massive accumulation of phosphatidic acid in conditionally lethal CDP-diglyceride synthetase mutants and cytidine auxotrophs of *Escherichia coli*. *Journal of Biological Chemistry*, 257, 389-394.
- [39]. Ganong, B. R., J. M. Leonard & C. Raetz (1980) Phosphatidic acid accumulation in the membranes of *Escherichia coli* mutants defective in CDP-diglyceride synthetase. *Journal of Biological Chemistry*, 255, 1623-1629.

- [40]. Garcia Fernandez, M. I., D. Ceccarelli & U. Muscatello (2004) Use of the fluorescent dye 10-N-nonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models. *Anal Biochem*, 328, 174-80.
- [41]. Garsin, D. A. & R. J. Willems (2010) Insights into the biofilm lifestyle of enterococci. *Virulence*, 1, 219-21.
- [42]. Geiger, O., N. Gonzalez-Silva, I. M. Lopez-Lara & C. Sohlenkamp (2010) Amino acid-containing membrane lipids in bacteria. *Prog Lipid Res*, 49, 46-60.
- [43]. Ghachi, M. E., N. Howe, R. Auger, A. Lambion, A. Guiseppi, F. Delbrassine, G. Manat, S. Roure, S. Peslier, E. Sauvage, L. Vogeley, J.-C. Rengifo-Gonzalez, P. Charlier, D. Mengin-Lecreulx, M. Foglino, T. Touzé, M. Caffrey & F. Kerff (2017) Crystal structure and biochemical characterization of the transmembrane PAP2 type phosphatidylglycerol phosphate phosphatase from *Bacillus subtilis*. *Cellular and Molecular Life Sciences*, 74, 2319-2332.
- [44]. Ghorbal, S. K. B., A. Chatti, M. M. Sethom, L. Maalej, M. Mihoub, S. Kefacha, M. Feki, A. Landoulsi & A. Hassen (2013) Changes in Membrane Fatty Acid Composition of *Pseudomonas aeruginosa* in Response to UV-C Radiations. *Current Microbiology*, 67, 112-117.
- [45]. Gidden, J., J. Denson, R. Liyanage, D. M. Ivey & J. O. Lay (2009) Lipid Compositions in *Escherichia coli* and *Bacillus subtilis* During Growth as Determined by MALDI-TOF and TOF/TOF Mass Spectrometry. *Int J Mass Spectrom*, 283, 178-184.
- [46]. Gmeinfr, J. & H. H. Martin (1976) Phospholipid and Lipopolysaccharide in *Proteus mirabilis* and Its Stable Protoplast I - Form: Difference in Content and Fatty Acid Composition. *European journal of biochemistry*, 67, 487-494.
- [47]. Gram, C. (1884) Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. *Fortschritte der Medicin*, 2, 185-189.
- [48]. Guo, D. & B. E. Tropp (2000) A second *Escherichia coli* protein with CL synthase activity. *Biochim Biophys Acta*, 1483, 263-74.
- [49]. Hall, C. W. & T. F. Mah (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol Rev*, 41, 276-301.
- [50]. Handwerger, S., B. Raucher, D. Altarac, J. Monka, S. Marchione, K. V. Singh, B. E. Murray, J. Wolff & B. Walters (1993) Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin, and gentamicin. *Clinical Infectious Diseases*, 16, 750-755.
- [51]. Hendrick, J. & W. Wickner (1991) SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. *Journal of Biological Chemistry*, 266, 24596-24600.
- [52]. Horvath, S. E. & G. Daum (2013) Lipids of mitochondria. *Prog Lipid Res*, 52, 590-614.

- [53]. Jacobson, J., M. R. Duchon & S. J. Heales (2002) Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. *J Neurochem*, 82, 224-33.
- [54]. Jett, B. D., M. M. Huycke & M. S. Gilmore (1994) Virulence of enterococci. *Clin Microbiol Rev*, 7, 462-78.
- [55]. Jeucken, A., J. B. Helms & J. F. Brouwers (2018) Cardiolipin synthases of *Escherichia coli* have phospholipid class specific phospholipase D activity dependent on endogenous and foreign phospholipids. *Biochim Biophys Acta Mol Cell Biol Lipids*, 1863, 1345-1353.
- [56]. Jyothikumar, V., K. Klanbut, J. Tiong, J. S. Roxburgh, I. S. Hunter, T. K. Smith & P. R. Herron (2012) Cardiolipin synthase is required for *Streptomyces coelicolor* morphogenesis. *Mol Microbiol*, 84, 181-97.
- [57]. Kandaswamy, K., T. H. Liew, C. Y. Wang, E. Huston-Warren, U. Meyer-Hoffert, K. Hultenby, J. M. Schröder, M. G. Caparon, S. Normark, B. Henriques-Normark, S. J. Hultgren & K. A. Kline (2013) Focal targeting by human β -defensin 2 disrupts localized virulence factor assembly sites in *Enterococcus faecalis*. *Proc Natl Acad Sci U S A*, 110, 20230-5.
- [58]. Kaneti, G., H. Sarig, I. Marjeh, Z. Fadia & A. Mor (2013) Simultaneous breakdown of multiple antibiotic resistance mechanisms in *S. aureus*. *The FASEB Journal*, 27, 4834-4843.
- [59]. Karras, G. I., G. Kustatscher, H. R. Buhecha, M. D. Allen, C. Pugieux, F. Sait, M. Bycroft & A. G. Ladurner (2005) The macro domain is an ADP-ribose binding module. *Embo j*, 24, 1911-20.
- [60]. Kawai, F., M. Shoda, R. Harashima, Y. Sadaie, H. Hara & K. Matsumoto (2004) Cardiolipin domains in *Bacillus subtilis* marburg membranes. *J Bacteriol*, 186, 1475-83.
- [61]. Kikuchi, S., I. Shibuya & K. Matsumoto (2000) Viability of an *Escherichia coli* *pgsA* null mutant lacking detectable phosphatidylglycerol and cardiolipin. *J Bacteriol*, 182, 371-6.
- [62]. Kishida, S., H. Shirataki, T. Sasaki, M. Kato, K. Kaibuchi & Y. Takai (1993) Rab3A GTPase-activating protein-inhibiting activity of Rabphilin-3A, a putative Rab3A target protein. *Journal of Biological Chemistry*, 268, 22259-22261.
- [63]. Kline, K. A., A. L. Kau, S. L. Chen, A. Lim, J. S. Pinkner, J. Rosch, S. R. Nallapareddy, B. E. Murray, B. Henriques-Normark, W. Beatty, M. G. Caparon & S. J. Hultgren (2009) Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in *Enterococcus faecalis*. *J Bacteriol*, 191, 3237-47.
- [64]. Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron & K.

- Bunai (2003) Essential *Bacillus subtilis* genes. *Proceedings of the National Academy of Sciences*, 100, 4678-4683.
- [65]. Kommineni, S., D. J. Bretl, V. Lam, R. Chakraborty, M. Hayward, P. Simpson, Y. Cao, P. Bousounis, C. J. Kristich & N. H. Salzman (2015) Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature*, 526, 719-22.
- [66]. Koprivnjak, T., D. Zhang, C. M. Ernst, A. Peschel, W. M. Nauseef & J. P. Weiss (2011) Characterization of *Staphylococcus aureus* cardiolipin synthases 1 and 2 and their contribution to accumulation of cardiolipin in stationary phase and within phagocytes. *J Bacteriol*, 193, 4134-42.
- [67]. Kowalski, W. J., E. L. Kasper, J. F. Hatton, B. E. Murray, S. R. Nallapareddy & M. J. Gillespie (2006) *Enterococcus faecalis* adhesin, Ace, mediates attachment to particulate dentin. *J Endod*, 32, 634-7.
- [68]. Kreutzberger, M. A., A. Pokorny & P. F. Almeida (2017) Daptomycin-Phosphatidylglycerol Domains in Lipid Membranes. *Langmuir*, 33, 13669-13679.
- [69]. Kristian, S. A., M. Durr, J. A. Van Strijp, B. Neumeister & A. Peschel (2003) MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. *Infect Immun*, 71, 546-9.
- [70]. Kuhn, S., C. J. Slavetinsky & A. Peschel (2015) Synthesis and function of phospholipids in *Staphylococcus aureus*. *Int J Med Microbiol*, 305, 196-202.
- [71]. Kusaka, J., S. Shuto, Y. Imai, K. Ishikawa, T. Saito, K. Natori, S. Matsuoka, H. Hara & K. Matsumoto (2016) Septal localization by membrane targeting sequences and a conserved sequence essential for activity at the COOH-terminus of *Bacillus subtilis* cardiolipin synthase. *Research in Microbiology*, 167, 202-214.
- [72]. Lempiäinen, H., K. Kinnunen, A. Mertanen & A. Von Wright (2005) Occurrence of virulence factors among human intestinal enterococcal isolates. *Letters in applied microbiology*, 41, 341-344.
- [73]. Li, C., B. K. Tan, J. Zhao & Z. Guan (2016) In Vivo and in Vitro Synthesis of Phosphatidylglycerol by an *Escherichia coli* Cardiolipin Synthase. *J Biol Chem*, 291, 25144-25153.
- [74]. Lin, T. Y. & D. B. Weibel (2016) Organization and function of anionic phospholipids in bacteria. *Appl Microbiol Biotechnol*, 100, 4255-67.
- [75]. Lindner, R. & H. Y. Naim (2009) Domains in biological membranes. *Exp Cell Res*, 315, 2871-8.
- [76]. Lopez-Lara, I. M. & O. Geiger (2017) Bacterial lipid diversity. *Biochim Biophys Acta Mol Cell Biol Lipids*, 1862, 1287-1299.

- [77]. Lopez, C. S., A. F. Alice, H. Heras, E. A. Rivas & C. Sanchez-Rivas (2006) Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity. *Microbiology*, 152, 605-16.
- [78]. Lu, Y.-H., Z. Guan, J. Zhao & C. R. Raetz (2011) Three phosphatidylglycerol-phosphate phosphatases in the inner membrane of *Escherichia coli*. *Journal of Biological Chemistry*, 286, 5506-5518.
- [79]. Mandlik, A., A. Swierczynski, A. Das & H. Ton - That (2007) *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. *Molecular microbiology*, 64, 111-124.
- [80]. Martin, P. K., T. Li, D. Sun, D. P. Biek & M. B. Schmid (1999) Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol*, 181, 3666-73.
- [81]. Matsumoto, K., J. Kusaka, A. Nishibori & H. Hara (2006) Lipid domains in bacterial membranes. *Mol Microbiol*, 61, 1110-7.
- [82]. Mazzella, N., J. Molinet, A. D. Syakti, A. Dodi, P. Doumenq, J. Artaud & J.-C. Bertrand (2004) Bacterial phospholipid molecular species analysis by ion-pair reversed-phase HPLC/ESI/MS. *Journal of lipid research*, 45, 1355-1363.
- [83]. Mileykovskaya, E. & W. Dowhan (2000) Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. *J Bacteriol*, 182, 1172-5.
- [84]. Mileykovskaya, E. & W. Dowhan (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. *Biochim Biophys Acta*, 1788, 2084-91.
- [85]. Mileykovskaya, E., I. Fishov, X. Fu, B. D. Corbin, W. Margolin & W. Dowhan (2003) Effects of phospholipid composition on MinD-membrane interactions in vitro and in vivo. *Journal of Biological Chemistry*, 278, 22193-22198.
- [86]. Miller, W. R., J. M. Munita & C. A. Arias (2014) Mechanisms of antibiotic resistance in enterococci. *Expert Rev Anti Infect Ther*, 12, 1221-36.
- [87]. Millman, J. S., H.-Y. Qi, F. Vulcu, H. D. Bernstein & D. W. Andrews (2001) FtsY binds to the *Escherichia coli* inner membrane via interactions with phosphatidylethanolamine and membrane proteins. *Journal of Biological Chemistry*, 276, 25982-25989.
- [88]. Minkler, P. E. & C. L. Hoppel (2010) Separation and characterization of cardiolipin molecular species by reverse-phase ion pair high-performance liquid chromatography-mass spectrometry. *J Lipid Res*, 51, 856-65.
- [89]. Mishra, N. N., A. S. Bayer, T. T. Tran, Y. Shamoo, E. Mileykovskaya, W. Dowhan, Z. Guan & C. A. Arias (2012) Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content. *PLoS One*, 7, e43958.

- [90]. Mohamed, J. A. & D. B. Huang (2007) Biofilm formation by enterococci. *J Med Microbiol*, 56, 1581-8.
- [91]. Mohamed, J. A., W. Huang, S. R. Nallapareddy, F. Teng & B. E. Murray (2004) Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect Immun*, 72, 3658-63.
- [92]. Murray, B. E. (1990) The life and times of the Enterococcus. *Clin Microbiol Rev*, 3, 46-65.
- [93]. Mylonakis, E., M. Engelbert, X. Qin, C. D. Sifri, B. E. Murray, F. M. Ausubel, M. S. Gilmore & S. B. Calderwood (2002) The *Enterococcus faecalis* *fsrB* gene, a key component of the *fsr* quorum-sensing system, is associated with virulence in the rabbit endophthalmitis model. *Infect Immun*, 70, 4678-81.
- [94]. Nakayama, J., S. Chen, N. Oyama, K. Nishiguchi, E. A. Azab, E. Tanaka, R. Kariyama & K. Sonomoto (2006) Revised model for *Enterococcus faecalis* *fsr* quorum-sensing system: the small open reading frame *fsrD* encodes the gelatinase biosynthesis-activating pheromone propeptide corresponding to staphylococcal AgrD. *Journal of bacteriology*, 188, 8321-8326.
- [95]. Nallapareddy, S. R., X. Qin, G. M. Weinstock, M. Hook & B. E. Murray (2000) *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun*, 68, 5218-24.
- [96]. Nallapareddy, S. R., K. V. Singh, J. Sillanpaa, D. A. Garsin, M. Hook, S. L. Erlandsen & B. E. Murray (2006) Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest*, 116, 2799-807.
- [97]. Niedermann, G., V. Weissig, B. Sternberg & J. Lasch (1991) Carboxyacyl derivatives of cardiolipin as four-tailed hydrophobic anchors for the covalent coupling of hydrophilic proteins to liposomes. *Biochim Biophys Acta*, 1070, 401-8.
- [98]. Nielsen, H. V., A. L. Flores-Mireles, A. L. Kau, K. A. Kline, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *Journal of Bacteriology*, 195, 4484-4495.
- [99]. Nishi, H., H. Komatsuzawa, T. Fujiwara, N. McCallum & M. Sugai (2004) Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 48, 4800-4807.
- [100]. Nishibori, A., J. Kusaka, H. Hara, M. Umeda & K. Matsumoto (2005) Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. *Journal of bacteriology*, 187, 2163-2174.

- [101]. Nishijima, S., Y. Asami, N. Uetake, S. Yamagoe, A. Ohta & I. Shibuya (1988) Disruption of the *Escherichia coli* *cls* gene responsible for cardiolipin synthesis. *Journal of Bacteriology*, 170, 775-780.
- [102]. O'Toole, G., H. B. Kaplan & R. Kolter (2000) Biofilm formation as microbial development. *Annu Rev Microbiol*, 54, 49-79.
- [103]. Ohniwa, R. L., K. Kitabayashi & K. Morikawa (2013) Alternative cardiolipin synthase *Cls1* compensates for stalled *Cls2* function in *Staphylococcus aureus* under conditions of acute acid stress. *FEMS Microbiol Lett*, 338, 141-6.
- [104]. Oku, Y., K. Kurokawa, N. Ichihashi & K. Sekimizu (2004) Characterization of the *Staphylococcus aureus* *mprF* gene, involved in lysinylation of phosphatidylglycerol. *Microbiology*, 150, 45-51.
- [105]. Oliver, P. M., J. A. Crooks, M. Leidl, E. J. Yoon, A. Saghatelian & D. B. Weibel (2014) Localization of anionic phospholipids in *Escherichia coli* cells. *J Bacteriol*, 196, 3386-98.
- [106]. Palmer, K. L., A. Daniel, C. Hardy, J. Silverman & M. S. Gilmore (2011) Genetic basis for daptomycin resistance in enterococci. *Antimicrob Agents Chemother*, 55, 3345-56.
- [107]. Pangborn, M. C. (1942) Isolation and purification of a serologically active phospholipid.pdf. *J. Biol. Chem.*, 143, 247-256.
- [108]. Parsons, J. B. & C. O. Rock (2011) Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery? *Current opinion in microbiology*, 14, 544-549.
- [109]. Patel, S., N. Mathivanan & A. Goyal (2017) Bacterial adhesins, the pathogenic weapons to trick host defense arsenal. *Biomed Pharmacother*, 93, 763-771.
- [110]. Peleg, A. Y., S. Miyakis, D. V. Ward, A. M. Earl, A. Rubio, D. R. Cameron, S. Pillai, R. C. Moellering, Jr. & G. M. Eliopoulos (2012) Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus*. *PLoS One*, 7, e28316.
- [111]. Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung & A. Tarkowski (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *Journal of Experimental Medicine*, 193, 1067-1076.
- [112]. Petit, J. M., A. Maftah, M. H. Ratinaud & R. Julien (1992) 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur J Biochem*, 209, 267-73.
- [113]. Proft, T. & E. N. Baker (2009) Pili in Gram-negative and Gram-positive bacteria - structure, assembly and their role in disease. *Cell Mol Life Sci*, 66, 613-35.

- [114]. Protonotariou, E., E. Dimitroulia, S. Pournaras, V. Pitiriga, D. Sofianou & A. Tsakris (2010) Trends in antimicrobial resistance of clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium* in Greece between 2002 and 2007. *J Hosp Infect*, 75, 225-7.
- [115]. Qin, X., K. V. Singh, G. M. Weinstock & B. E. Murray (2000) Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun*, 68, 2579-86.
- [116]. Rashid, R., A. Cazenave-Gassiot, I. H. Gao, Z. J. Nair, J. K. Kumar, L. Gao, K. A. Kline & M. R. Wenk (2017) Comprehensive analysis of phospholipids and glycolipids in the opportunistic pathogen *Enterococcus faecalis*. *PLoS One*, 12, e0175886.
- [117]. Romantsov, T., S. Helbig, D. E. Culham, C. Gill, L. Stalker & J. M. Wood (2007) Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*. *Mol Microbiol*, 64, 1455-65.
- [118]. Sandoval-Calderon, M., O. Geiger, Z. Guan, F. Barona-Gomez & C. Sohlenkamp (2009) A eukaryote-like cardiolipin synthase is present in *Streptomyces coelicolor* and in most actinobacteria. *J Biol Chem*, 284, 17383-90.
- [119]. Sartingen, S., E. Rozdzinski, A. Muscholl-Silberhorn & R. Marre (2000) Aggregation substance increases adherence and internalization, but not translocation, of *Enterococcus faecalis* through different intestinal epithelial cells *in vitro*. *Infect Immun*, 68, 6044-7.
- [120]. Sava, I. G., E. Heikens & J. Huebner (2010) Pathogenesis and immunity in enterococcal infections. *Clin Microbiol Infect*, 16, 533-40.
- [121]. Schlame, M., M. Ren, Y. Xu, M. L. Greenberg & I. Haller (2005) Molecular symmetry in mitochondrial cardiolipins. *Chem Phys Lipids*, 138, 38-49.
- [122]. Schleifer, K. H. & R. Kilpper-Bälz (1984) TTransfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 34, 31-34.
- [123]. Septinus, M., T. Berthold, A. Naujok & H. W. Zimmermann (1985) [Hydrophobic acridine dyes for fluorescent staining of mitochondria in living cells. 3. Specific accumulation of the fluorescent dye NAO on the mitochondrial membranes in HeLa cells by hydrophobic interaction. Depression of respiratory activity, changes in the ultrastructure of mitochondria due to NAO. Increase of fluorescence in vital stained mitochondria in situ by irradiation]. *Histochemistry*, 82, 51-66.
- [124]. Serricchio, M. & P. Bütikofer (2012) An essential bacterial-type cardiolipin synthase mediates cardiolipin formation in a eukaryote. *Proceedings of the National Academy of Sciences of the United States of America*, 109, E954-E961.

- [125]. Shankar, N., C. V. Lockatell, A. S. Baghdayan, C. Drachenberg, M. S. Gilmore & D. E. Johnson (2001) Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infection and Immunity*, 69, 4366-4372.
- [126]. Silhavy, T. J., D. Kahne & S. Walker (2010) The bacterial cell envelope. *Cold Spring Harbor perspectives in biology*, 2, a000414-a000414.
- [127]. Singh, K. V., S. R. Nallapareddy, J. Sillanpää & B. E. Murray (2010) Importance of the collagen adhesin ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. *PLoS pathogens*, 6, e1000716-e1000716.
- [128]. Slavetinsky, C. J., A. Peschel & C. M. Ernst (2012) Alanyl-phosphatidylglycerol and lysyl-phosphatidylglycerol are translocated by the same MprF flippases and have similar capacities to protect against the antibiotic daptomycin in *Staphylococcus aureus*. *Antimicrob Agents Chemother*, 56, 3492-7.
- [129]. Sohlenkamp, C. & O. Geiger (2016) Bacterial membrane lipids: diversity in structures and pathways. *FEMS Microbiol Rev*, 40, 133-59.
- [130]. Staley, C., G. M. Dunny & M. J. Sadowsky (2014) Environmental and animal-associated enterococci. *Adv Appl Microbiol*, 87, 147-86.
- [131]. Strahl, H. & J. Errington (2017) Bacterial Membranes: Structure, Domains, and Function. *Annu Rev Microbiol*, 71, 519-538.
- [132]. Suzuki, M., H. Hara & K. Matsumoto (2002) Envelope disorder of *Escherichia coli* cells lacking phosphatidylglycerol. *J Bacteriol*, 184, 5418-25.
- [133]. Tan, B. K., M. Bogdanov, J. Zhao, W. Dowhan, C. R. Raetz & Z. Guan (2012) Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. *Proc Natl Acad Sci U S A*, 109, 16504-9.
- [134]. Tang, Y., H. Xia & D. Li. 2018. Membrane Phospholipid Biosynthesis in Bacteria. In *Advances in Membrane Proteins: Part I: Mass Processing and Transportation*, ed. Y. Cao, 77-119. Singapore: Springer Singapore.
- [135]. Tendolkar, P., A. Baghdayan & N. Shankar (2003) Pathogenic enterococci: new developments in the 21st century. *Cellular and Molecular Life Sciences CMLS*, 60, 2622-2636.
- [136]. Tendolkar, P. M., A. S. Baghdayan, M. S. Gilmore & N. Shankar (2004) Enterococcal Surface Protein, Esp, Enhances Biofilm Formation by *Enterococcus faecalis*. *Infection and Immunity*, 72, 6032-6039.
- [137]. Toledo-Arana, A., J. Valle, C. Solano, M. a. J. Arrizubieta, C. Cucarella, M. Lamata, B. Amorena, J. Leiva, J. R. Penadés & I. Lasa (2001) The Enterococcal Surface Protein, Esp, Is Involved in *Enterococcus faecalis* Biofilm Formation. *Applied and Environmental Microbiology*, 67, 4538-4545.

- [138]. Tran, T. T., D. Panesso, H. Gao, J. H. Roh, J. M. Munita, J. Reyes, L. Diaz, E. A. Lobos, Y. Shamoo, N. N. Mishra, A. S. Bayer, B. E. Murray, G. M. Weinstock & C. A. Arias (2013) Whole-genome analysis of a daptomycin-susceptible *enterococcus faecium* strain and its daptomycin-resistant variant arising during therapy. *Antimicrobial agents and chemotherapy*, 57, 261-268.
- [139]. Tran, T. T., D. Panesso, N. N. Mishra, E. Mileykovskaya, Z. Guan, J. M. Munita, J. Reyes, L. Diaz, G. M. Weinstock, B. E. Murray, Y. Shamoo, W. Dowhan, A. S. Bayer & C. A. Arias (2013) Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. *MBio*, 4.
- [140]. Trombe, M. C., M. A. Laneelle & G. Laneelle (1979) Lipid composition of aminopterin-resistant and sensitive strains of *Streptococcus pneumoniae*. Effect of aminopterin inhibition. *Biochim Biophys Acta*, 574, 290-300.
- [141]. Vences-Guzman, M. A., O. Geiger & C. Sohlenkamp (2012) Ornithine lipids and their structural modifications: from A to E and beyond. *FEMS microbiology letters*, 335, 1-10.
- [142]. Wagner, R. M., L. Kricks & D. Lopez (2017) Functional Membrane Microdomains Organize Signaling Networks in Bacteria. *The Journal of Membrane Biology*, 250, 367-378.
- [143]. Wells, C. L., E. A. Moore, J. A. Hoag, H. Hirt, G. M. Dunny & S. L. Erlandsen (2000) Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. *Infect Immun*, 68, 7190-4.
- [144]. Wood, J. M. (2018) Perspective: challenges and opportunities for the study of cardiolipin, a key player in bacterial cell structure and function. *Curr Genet*, 64, 795-798.
- [145]. Yao, J. & C. O. Rock (2013) Phosphatidic acid synthesis in bacteria. *Biochim Biophys Acta*, 1831, 495-502.
- [146]. Yao, J. & C. O. Rock (2013) Phosphatidic acid synthesis in bacteria. *Biochimica et biophysica acta*, 1831, 495-502.
- [147]. Zaknoon, F., K. Goldberg, H. Sarig, R. F. Epand, R. M. Epand & A. Mor (2012) Antibacterial properties of an oligo-acyl-lysyl hexamer targeting Gram-negative species. *Antimicrobial agents and chemotherapy*, 56, 4827-4832.
- [148]. Zhao, W., T. Róg, A. A. Gurtovenko, I. Vattulainen & M. Karttunen (2008) Role of phosphatidylglycerols in the stability of bacterial membranes. *Biochimie*, 90, 930-938.

Chapter 2 Construction and characterization of *pgsA* inducible mutant in *Enterococcus faecalis*

2.1 Abstract

Enterococcus faecalis is a Gram-positive, facultative anaerobic, diplococcal, opportunistic pathogen, causing many nosocomial infections. As a major anionic phospholipid component in the cell membrane, phosphatidylglycerol (PG) is essential for cell viability in most bacteria. Whether PG is essential in enterococcal species is unknown. Using a TetR repressible promoter to drive *pgsA* transcription, we have constructed an inducible *pgsA* mutant, *pgsA*_{ind}, to deplete PG levels by removal of the inducer anhydrotetracycline (ATc) in *E. faecalis*. As expected for an essential gene, depletion of *pgsA* and its gene product, which is required for PG biosynthesis, resulted in severe cell morphology defects and cell death. Similar to other bacteria, PgsA is focally localized at the septum of *E. faecalis*. This is the first demonstration of construction a regulatable mutant of an essential gene in *E. faecalis*. Upon decreasing ATc concentrations, we observed two distinct populations that may result from the presence of suppressor mutants. Nevertheless, the subpopulation without suppressor mutations in *pgsA*_{ind} can be used to further characterize the role of PG in *E. faecalis* physiology.

2.2 Introduction

Enterococci are Gram-positive, facultative anaerobic, diplococcal, opportunistic pathogens. Enterococci are found in plants, soil, and as commensals of the gastrointestinal (GI) tract (Kommineni et al. 2015). Enterococci have recently emerged as the third most common cause of nosocomial infections, including urinary tract infection (UTI), bacteremia, and wound infections (Arias and Murray 2012). *Enterococcus faecalis* is one of the two most commonly isolated species of enterococci associated with nosocomial infections and is one the most common causative agent of catheter-associated urinary tract infections (CAUTI) (Abat et al. 2016).

Phospholipids (PLs) are a major component of the bacterial membrane. In bacteria, there are three major classes of phospholipids: phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylethanolamine (PE) (Zhang and Rock 2008), in which PG is the major anionic phospholipid comprising around 25% of lipids in the Gram positive bacterial membrane (Dowhan 1997). In most bacteria, cytidine diphosphate-diacylglycerol (CDP-DAG) is the precursor of PL and is synthesized from phosphatidic acid (PA) by CDP-DAG synthase (CdsA) (Parsons and Rock 2013). In the process of PG formation, PG is synthesized from CDP-DAG in two steps (Sohlenkamp and Geiger 2016). Firstly, the phosphatidyl group of CDP-DAG is transferred to glycerol-3-phosphate (G3P) to form phosphatidylglycerol-phosphate (PGP). This reaction is catalyzed by the enzyme CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase (PgsA), which is encoded by *pgsA*. Secondly, PGP is rapidly dephosphorylated by phosphatidylglycerophosphate phosphatase (Pgp) to produce PG (**Figure 1.1**). It has been reported that PG is essential for cell viability because mutation of *pgsA* is lethal in many bacteria including *Staphylococcus aureus* (Martin et al. 1999) and *Bacillus subtilis* (Kobayashi et al. 2003). However, an *Escherichia coli* *pgsA* null mutant is still viable when also harboring *lpp* and *rscF* mutations and PG is even undetectable (Kikuchi et al. 2000). Whether PG is essential in enterococcal species is unknown.

To deplete PG levels in *E. faecalis*, we constructed an inducible *pgsA* mutant using a “tet-off” system (**Figure 2.1**). The “tet-off” system was first identified in *E. coli* and then developed to regulate target gene expression based on the concentration of the inducer, anhydrotetracycline (ATc) or tetracycline (Tc) (Orth et al. 2000, Berens and Hillen 2004). According to the

mechanism of “tet-off” system, we created a *pgsA* inducible mutant (*pgsA_{ind}*) in this chapter to regulate *pgsA* gene expression, and subsequently deplete PG levels.

Figure 2.1 Schematic representation of tet-off system. (A) TetR repressor protein (blue) binds to the *tetO* operator region (dark blue) which exists in the promoter region of the target gene, resulting in shutting down of gene transcription. (B) In the presence of inducer anhydrotetracycline (ATc, green), ATc binds to TetR dimer with high affinity, resulting in dissociation from *tetO*. Thus, the transcription of the target gene is regulated by ATc in a dose-dependent manner.

2.3 Materials and methods

2.3.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this chapter are provided in **Table 2.1**.

E. coli strains were grown in Luria-Bertani (LB, Difco, France) broth or agar, whereas *E. faecalis* strains were grown in Brain Heart Infusion (BHI, Becton, Dickinson, USA) broth or agar. For all experiments in this chapter, bacterial strains were streaked from a 25% (v/v) glycerol stock stored at -80°C onto 1.5% agar plates and grown overnight at 37°C. The next day, a single colony from an agar plate was inoculated into liquid medium and incubated at 37°C overnight, followed by 1:10 dilution into fresh media and growth to an optical density of 600 nm wavelength ($OD_{600\text{ nm}} = 0.45-0.6$) to reach mid-exponential phase. Respective antibiotics listed in **Table 2.1** were added to liquid or agar media at the concentrations indicated for growth of strains with plasmids.

Table 2.1 List of bacterial strains and plasmids used in this chapter

Strains or plasmids	Characteristic(s)	Resistance, concentrations	References
Strains			
<i>Enterococcus faecalis</i> OG1RF (WT)	ATCC 47077, <i>E. faecalis</i> OG1RF wild type strain, human oral isolate	Rif ^r and Fus ^r , 25 µg/ml	(Dunny et al. 1978)
<i>Enterococcus faecalis</i> OG1RF pGCP023::tetR	<i>E. faecalis</i> OG1RF harboring pGCP023::tetR	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>Enterococcus faecalis</i> OG1RF P _{srtA-tetO} -pgsA pGCP023::tetR, abbreviate to <i>E. faecalis</i> OG1RF pgsA _{ind}	<i>E. faecalis</i> OG1RF pgsA inducible mutant harboring pGCP023::tetR, the promoter of pgsA (OG1RF_12410) gene was modified with srtA (OG1RF_12327) promoter and tet operator	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work

<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S1	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S2	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S3	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S4	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S5	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S6	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S7	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work

<i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} S8	Suppressor mutant isolated from <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind} by serial passaging in the absence of ATc and picking a single colony	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>Escherichia coli</i> DH5α	<i>E. coli</i> competent cells for cloning		
Plasmids			
pGCP213	Shuttle vector temperature-sensitive plasmid for generation of mutants	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	(Nielsen et al. 2012)
pGCP213::P _{srtA} - <i>tetO</i> - <i>pgsA</i>	Derivative of pGCP213, containing 500 bp upstream of the -35 promoter region of <i>pgsA</i> , tet inducible promoter and 500 bp downstream of -10 promoter region of <i>pgsA</i>	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work
pABG5	Shuttle vector	Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml (<i>E. faecalis</i>)	(Granok et al. 2000)
pGCP023:: <i>tetR</i>	Derivative of pABG5, encoding Tet repressor under <i>guaB</i> promoter	Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml (<i>E. faecalis</i>)	(Vega et al. 2013)
pGCP023:: <i>tetR</i> -ΔKan	Derivative of pGCP023:: <i>tetR</i> lacking Kan ^r cassette	Cm ^r , 10 µg/ml (<i>E. faecalis</i>)	This work
pMSP3535	Shuttle vector carrying nisin inducible promoter	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	(Bryan et al. 2000)
pMSP3535:: <i>pgsA</i> -2HA	Derivative of pMSP3535, encoding C-terminal 2HA-tagged <i>pgsA</i> (OG1RF_12410) under nisin inducible promoter	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work

Abbreviations: Rif, rifampicin; Fus, fusidic acid; Kan, kanamycin; Erm, erythromycin; Cm, chloramphenicol
Superscript “r” indicate resistance.

2.3.2 Genetic manipulation and construction of *pgsA* inducible mutant and complementation strain in *Enterococcus faecalis* OG1RF

The CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase (*pgsA*) gene (Genebank gene ID: 12289159) was identified in the complete genome of *Enterococcus faecalis* OG1RF (Genebank accession number: NC_017316) based on sequence homology with previously described *pgsA* genes in other organisms (Dowhan 2013, Dryden and Dowhan 1996). Native -35 and -10 promoter regions of *pgsA* gene were predicted using an online prediction algorithm (<http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) and then replaced with the corresponding promoter region sequences of constitutively expressed *srtA* (Sortase A) and then the region between -35 and -10 region was replaced with the tet-operators site *tetO1* and a second *tetO2* was inserted downstream of the -10 region (**Figure 2.3**). The native promoter sequence of *pgsA* (from -35 region to -10 region) was replaced by corresponding inducible promoter region (**Table 2.2**). The inducible promoter region was constructed by sewing together the 500bp downstream and 500bp upstream flanking sequences of native *pgsA* promoter sequence by performing PCR using primer pairs PsrtA-NotI-F/PsrtA-tetO-R and PsrtA-tetO-F/PsrtA-PstI-R, respectively, followed by overlap PCR using primer pair PsrtA-NotI-F/ PsrtA-PstI-R (**Table 2.3**). The amplified approximately 1000bp PCR products were cloned into a temperature sensitive vector pGCP213 and transformed into *E. coli* DH5 α competent cells. The plasmid pGCP213::PsrtA-*tetO*-*pgsA* was extracted from *E. coli*, sequenced, and correct plasmids were then transformed into *E. faecalis* OG1RF.

Figure 2.2 Diagram of *pgsA* native and inducible promoter construction. The inducible promoter consists of -35, -10 region of *srtA* and two tetO operator regions. The native -35 and -10 region (blue) of *pgsA* was replaced with the -35 and -10 region of *srtA* (pink). The region between the *srtA* -35 and -10 region was replaced with tetO1 (orange) and tetO2 (orange) was inserted downstream of the -10 region.

Table 2.2 Gene promoter and operator sequence used in this work

Gene promoter	Sequence (5'-3') ^a	Reference
Native <i>pgsA</i> promoter	<u>GTGACA</u> CTGCCAGCAAGATA <u>ACGTAAAT</u>	<i>E. faecalis</i> OG1RF
Native <i>srtA</i> promoter	<u>ATAAAT</u> AGGTTTTTCAAATAAATTG <u>TGGTAAACT</u>	<i>E. faecalis</i> OG1RF
Inducible <i>pgsA</i> promoter	<u>ATAAAT</u> ACTCTATCATTGATAGAGT <u>TGGTAAACTT</u> CCCTATCAGTGATAGAGA	This work
Operator		
<i>tetO1</i>	ACTCTATCATTGATAGAGT	
<i>tetO2</i>	TCCCTATCAGTGATAGAGA	
^a -10 region (red) and -35 region (blue) is underlined		

To generate the chromosomal *pgsA* mutant containing the *pgsA* inducible promoter, the strain *E. faecalis* OG1RF pGCP213::PsrtA-tetO-*pgsA* was grown by serial passage at 30°C in the presence of erythromycin (Erm) for two days to maintain the plasmid, followed by serial passage for three days at 42°C in the presence of Erm to select for chromosomal integration of this temperature sensitive plasmid. Subsequently, cells were grown at 30°C in the absence of Erm to select for excision of the integrated plasmid by homologous recombination. Erm

sensitive cells were screened using primer pairs OG1RF_pgsA_mutant_F/OG1RF_pgsA_mutant_R (**Table 2.3**) to get the chromosomal *pgsA* mutant OG1RF $P_{srtA-tetO}$ -*pgsA*. The accuracy of the chromosomal promoter mutation, and absence of secondary mutations, was confirmed by whole genome sequencing.

To construct the accomplished inducible promoter system, a plasmid pGCP023::*tetR* expressing the tet-repressor (TetR), was constructed based on the shuttle vector pAGB5 (Granok et al. 2000, Vega et al. 2013). The vector backbone of pGCP023::*tetR* encoded both kanamycin and chloramphenicol resistance encoding genes. For downstream applications, pGCP023::*tetR* was also modified to knock out the kanamycin resistance cassette. The PCR product amplified from pGCP023::*tetR* using primer pair kanacut-F/kanacut-R (**Table 2.3**) was digested by NotI and self-ligated to form a new plasmid named pGCP023::*tetR*-ΔKan.

The resulting plasmids (pGCP023::*tetR* or pGCP023::*tetR*-ΔKan) were transformed into the mutant containing the *pgsA* inducible promoter (**Table 2.2**) to achieve the regulatable *pgsA* mutant *E. faecalis* OG1RF $P_{srtA-tetO}$ -*pgsA* pGCP023::*tetR* or *E. faecalis* OG1RF $P_{srtA-tetO}$ -*pgsA* pGCP023::*tetR*-ΔKan. The former is abbreviated to *E. faecalis* OG1RF *pgsA*_{ind} used for the characterizations of the strain in **Chapter 2**. *E. faecalis* OG1RF $P_{srtA-tetO}$ -*pgsA* pGCP023::*tetR*-ΔKan is used only when a second plasmid is transformed into the *pgsA* mutant strain.

The complementation plasmid pMSP3535::*pgsA*-2HA was generated with the shuttle vector pMSP3535 (Bryan et al. 2000). To clone the C-terminal 2HA (haemagglutinin) tagged *pgsA* gene (*pgsA*-2HA) as along with the upstream ribosome-binding sites of *pgsA*, the PCR product amplified from *E. faecalis* OG1RF using primer pair *pgsA*-2HA-F1/ *pgsA*-2HA-R (**Table 2.3**) was subsequently amplified using primer pair *pgsA*-2HA-F2/ *pgsA*-2HA-R (**Table 2.3**). The final PCR product was cloned into the linearized plasmid pMSP3535 (digested by BamHI and SphI) by In-Fusion (In-Fusion® HD Cloning Kit, Takara Bio, USA) cloning. The plasmid pMSP3535::*pgsA*-2HA was confirmed by sequencing and transformed into *E. faecalis* OG1RF $P_{srtA-tetO}$ -*pgsA* pGCP023::*tetR*-ΔKan to achieve the complementation strain *E. faecalis* *pgsA*_{ind} pMSP3535-*pgsA*-2HA.

Table 2.3 Primers used for construction of *pgsA* inducible mutant and complementation plasmid

Primer name	Sequence (5'→3') ^a
PsrtA-NotI-F	ATTG <u>GCGGCCGCT</u> TGGCGATGCAAGGTATCTT
PsrtA-PstI-R	ATCCCTGCAGGGAGTGCTGAAAATGGAATG
PsrtA-tetO-R	GGAAGTTTACCAACTCTATCAATGATAGAGTATTTA TGAAAAAAGAAAAGAATCTG
PsrtA-tetO-F	TGATAGAGTTGGTAAACTTCCCTATCAGTGATAGAG AAGAACAGGAGAAGGAAGGG
OG1RF_pgsA_mutant_F	AGCAATCCTGACATGACGCA
OG1RF_pgsA_mutant_R	CCTGTTCTTCTCTATCACTGATAGGG
kanacut-F	TAGCTATC <u>GCGGCCGCT</u> GTTTCCTTGCATTCTAAAAC
kanacut-R	ATACACAAG <u>GCGGCCGCA</u> CTGTCCATACTCTGATGTT
pgsA-2HA-F1	TCGTATGGGTAAGCGTAATCTGGAACATCGTATGGG TACATCGAACCTTTAAAAACAT
pgsA-2HA-F2	CTGTGACGAGCTCGTTAAGCGTAATCTGGAACATC GTATGGGTAAGCGTAATC
pgsA-2HA-R	GACTCTGCATGGATCAGAACAGGAGAAGGAAG
^a Restriction sites underlined	

2.3.3 Whole genome sequencing

Strains were inoculated from frozen stocks into liquid media and grown statically overnight. Overnight cultures were diluted 10-fold into fresh media and incubated to obtain an optical density at 600 nm equal to 0.5. The isolation of DNA was performed using PureLink® Genomic DNA Kit (Thermo Fisher, USA) according to manufacturer's protocols. Quantification of DNA was performed using Qubit™ DNA Assay Kits and Qubit™ RNA Assay Kits (Invitrogen, USA), respectively. Integrity of DNA was analyzed by gel electrophoresis using Agilent DNA ScreenTape (Agilent Technologies, Singapore). Whole genome sequencing was performed using an Illumina MiSeq sequencing (300 bp pair end) by the sequencing facility of Singapore Centre of Life Science Engineering (SCELSE) in

Singapore and data were analyzed by CLC Genomics Workbench software using *E. faecalis* OG1RF as template.

2.3.4 Growth kinetic assays

Bacteria were grown to exponential stage and normalized to OD_{600 nm}=0.5 as described above. Cultures were then diluted 1000-fold into fresh media to achieve colony-forming unit (CFU)= ~ 10⁵/ml. 200 µl of the diluted cells were added to individual wells in a 96-well plate and incubated for 20 hours at 37°C statically. At every 0.5-hour interval, the absorbance was read using an Infinite 200 PRO microtiter plate reader (Tecan Group Ltd., Männedorf, Switzerland). All the experiments were performed with technical triplicates and biological triplicates.

2.3.5 10-N-nonyl-acridine orange (NAO) and Hoechst co-staining

Cells were harvest from exponential phase as described above, and optical density was normalized to OD_{600 nm}=0.5 in 1 ml 0.01M phosphate (PB, PH7.2) buffer (Beisson et al. 2010). Normalized cells were incubated with 10 µl Hoechst 33342 (1:1000, Thermofisher, USA) for 5 mins at 37°C statically. Cells were centrifuged to remove supernatant, washed 3 times with 0.01M PB buffer, and suspended in 1 ml 0.01M PB buffer. Subsequently, cells were incubated with 10 µl 0.1mM 10-N-nonyl-acridine orange (NAO, Thermofisher, USA) for 5 mins at 37°C statically. Cells were spun down, washed 3 times in 0.01M PB buffer, and suspended in 1 ml 0.01M PB buffer. 20 µl of cells were smeared on Poly-L-Lysine pre-coated slides (Polysciences Inc., USA), dried in a hybridization oven at 45°C, and mounted with 5 µl VectaShield mounting medium. The slides were visualized using an inverted widefield epifluorescence microscope (Zeiss Axio Observer.Z1, Germany), using 100x oil immersion objective. Phase contract and fluorescent images were captured using AxioVision software and merged using Image-J software.

2.3.6 Immunoblot and quantification

Cells were harvested from exponential phase as described above, and density was normalized to OD_{600 nm}=0.5 in 1 ml 0.01M PB buffer. Normalized cells were centrifuged to obtain a cell pellet. The cell pellet was incubated with 100 µl of 10 mg/ml of lysozyme (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid- EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour. After lysozyme treatment, the whole cell

lysates were added with 33 μ l of NuPAGE® LDS Sample Buffer (4 $\times$) and 10 μ l of 1M dithiothreitol (DTT). Then samples were boiled at 100°C for 20 mins and cooled in ice. Subsequently, 10 μ l of samples and 3 μ l of PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermos Scientific, USA) were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The samples were loaded onto NuPAGE Novex 4-12% Bis-Tris gels in NuPAGE MOPS SDS running buffer and run in the XCell SureLock Mini-Cell Electrophoresis System (Thermos Scientific, USA) at 200V for 50 mins. Subsequently, proteins from the gels were transferred to membrane using iBlot® Transfer Stacks (Life technologies Corp. USA). The membrane was then blocked with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) with 0.1% Tween 20 (PBST) at 4°C for 1 hour and then incubated with primary antibodies at 4°C for overnight. The antibodies and respective concentrations (diluted in 5% BSA in PBST) used in this chapter were described in **Table 2.4**. The next day, the membrane was washed with PBST for 3 times and incubated with Pierce™ horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature in dark for 1 hour. Then, the membrane was washed with PBST for 3 times and incubated with Super Signal West Femto substrate (Thermos Fisher Scientific, Inc., USA) for 5 mins to develop the membrane. Subsequently, the membrane was exposed to the Green X-ray film (Care stream, USA) with a Kodak processor (Kodak X-OMAT processor 2000) in dark room. The film was captured, and the images were quantified with image J software.

Table 2.4 Antibodies used for immunoblotting assay in this chapter

Protein of interest	Primary antibody concentration	Host	Secondary antibody concentration
SecA	1:3000	Rabbit	1:6000
HA	1:3000	Rabbit	1:6000

2.3.7 Immunofluorescence microscopy (IFM)

Cells were harvested from exponential phase as described above, and optical density was normalized to OD_{600 nm}=1.0 in 1 ml 0.01M PB buffer. After washing once with 0.01M PB buffer once, cells were fixed in 1 ml 4% paraformaldehyde (PFA) for 20 mins at 4°C. After fixation, cells were washed once with 0.01M PB buffer, resuspended in 1 ml 0.01M PB buffer,

and diluted at a 1:4 ratio by adding 250 μ l of fixed cells to 750 μ l of PB buffer. Then, 20 μ l of diluted cells were smeared onto the ethanol and water-washed Poly-L-Lysine pre-coated slides and dried in a hybridization oven at 45°C. Next, cells were permeabilized with 20 μ l of 10 mg/ml of lysozyme (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour, washed 3 times with PB buffer, and dried in the hybridization oven. Cells were permeabilized again with 20 μ l 0.1% Triton in PB at 37°C for 10 mins, washed 3 times with PB buffer, and dried. Cells were incubated with 20 μ l blocking buffer 2% bovine serum albumin in PB (2% BSA-PB) for 20 mins at room temperature, washed 3 times with PB buffer, and dried. Subsequently, cells were smeared with 20 μ l primary antibody anti-HA raised in rabbit (1:500 dilution in 2% BSA-PB) and incubated at 4°C overnight. After primary antibody incubation, cells were washed 3 times with PB buffer and dried. Subsequently, cells were smeared with 20 μ l fluorescence conjugated secondary antibody Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-rabbit IgG (1:1000 dilution in 2% BSA-PB) and incubated in the dark at room temperature for 1 hour. Then, cells were washed 3 times with PB buffer, dried, and smeared with 5 μ l VectaShield mounting medium. The slides were visualized by an inverted widefield epifluorescence microscope (Zeiss Axio Observer.Z1, Germany), using 100x oil immersion objective. Phase contract and florescent and merged images were captured using AxioVision software and analyzed by Matlab software. The Projected System of Internal Coordinates from Interpolated Contours (PSICIC) (Werner et al. 2009) was used to quantify the mean fluorescence intensity at the perimeter of *E. faecalis* cells at the early division stage, in which cell perimeter is between 3.6–6 μ m.

2.3.8 Isolation of *pgsA*_{ind} suppressor mutant

The *pgsA*_{ind} strain was streaked from a 25% (v/v) glycerol stock stored at -80°C onto BHI plates (1.5% agar) supplemented with kanamycin (600 μ g/ml) and ATc (1 μ g/ml) and grown overnight at 37°C. The next day, 30 single colonies from an agar plate were inoculated into 5 ml BHI liquid medium supplemented with kanamycin (600 μ g/ml) *without* ATc and incubated at 37°C. After 2 days' incubation, the cells which were able to grow in the absence of ATc were streaked onto BHI plates (1.5% agar) supplemented with kanamycin (600 μ g/ml) and grown overnight at 37°C. A single colony from each plate was inoculated into 5 ml BHI liquid medium supplemented with kanamycin (600 μ g/ml) and grown overnight at 37°C. Then the overnight culture was streaked onto the same plates in the absence of ATc and grown overnight at 37°C. A single colony from each plate was inoculated in 5 ml liquid medium in the absence

of ATc and grown overnight at 37°C, respectively. The next day, 500 µl of the overnight culture was mixed with 500 µl sterile 50% (v/v) glycerol and stocked at -80°C.

Figure 2.3 Schematic diagram of isolation of suppressor mutants from *pgsA_{ind}* in the absence of ATc. Single colonies from *pgsA_{ind}* are inoculated into BHI media supplemented with kanamycin (in the absence of ATc) and incubated for 2 days. The grown cultures are streaked onto agar plates (in the absence of ATc) and inoculated into same liquid media. The next day, the cultures in which cells can replicate are streaked onto same plates, the isolation is repeated for 3 times to generate 8 stable *pgsA_{ind}* suppressor mutants which can replicate in the absence of ATc.

2.3.9 Real-time quantitative PCR (qPCR)

Cells were grown to exponential phase as described above and harvested. RNA extraction was performed according to the manufacture's protocol using Pure Link™ RNA Mini Kit (Thermo Scientific, USA). RNA samples were treated with TURBO DNA-free™ kit (Invitrogen™, USA) to remove DNA from RNA samples and purified with Agencourt RNA clean XP (Beckman Coulter, USA) magnetic beads. RNA concentration and DNA contamination were measured using Qubit™ RNA HS Assay Kit (Thermo Scientific, USA) and Qubit™ dsDNA HS Assay Kit (Thermo Scientific, USA), respectively. The RNA samples with less than 10% DNA contamination were screened using Tape Station (Agilent Technologies, USA) to measure RIN values. Reverse transcription (RT)-PCR was performed according to manufacturer's instruction using Super Script® III First-Strand Synthesis System for RT-PCR (Invitrogen™, USA). Random hexamer primers provided in the kit were used for cDNA synthesis in this work. The cDNA samples were stored at -20°C for subsequent analysis.

Real-time quantitative PCR (qPCR) was performed according to the manufacturer's instruction using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (Kapa biosystem, USA). Primer pairs (**Table 2.5**) used for targeting genes and the housekeeping gene 16s rRNA (16s) in qPCR were synthesized from Integrated DNA Technologies (IDT). The qPCR reactions were performed in the Step One Plus™ Systems (Life Technologies, USA) and the cycling program was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and finally annealing and extension at 60°C for 30 seconds.

Table 2.5 Primers used for qPCR in this chapter

Primer name	Sequence (5'→3')
16s_F	GGATAACACTTGGAAACAGG
16s_R	TCCTTGTTCTTCTCTAACAA
qPCR-pgsA-F	CGACACAACGTTAGCCGTTA
qPCR-pgsA-R	AAGGCGGTCATCACTAGCAT

2.3.10 Lipid extraction and mass spectrometry (MS) analysis

Cells were harvested from exponential phase as described above, optical density was normalized to OD_{600 nm}=10. Cell pellets were dried, weighted and used for lipid extraction using a modified Bligh & Dyer method in which the extraction solvent contained chloroform/methanol in a ratio of 1:2 (v/v) (Rashid et al. 2017). During the proceed of lipid extraction, an internal standard (PG 14:0) was added for normalization. The extracted total lipid was dissolved in chloroform and methanol (1:1 v/v) to achieve a final lipid concentration of 10 mg/ml and stored at -80°C until mass spectrometry analysis. 2ul of diluted (10 x) sample was injected into an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, USA) using the Advion Nanomate interface. Total PG quantification was performed using the Agilent 6490 QqQ mass spectrometer connected to the 1290 series chromatography system with multiple reaction monitoring (MRM) according to the protocol by Rashid et al (Rashid et al. 2017). The toal PG

levels in each sample were normalized to either the weight of cell pellet or the weight of total lipid extracted.

2.3.11 Statistical analysis

All experiments were performed at least three biological replicates and the mean value was calculated and is presented. All graphs indicate the standard deviation from independent experiments (and not technical replicates). Statistical analysis was performed by Graphpad software using either one-way ANOVA, two-way ANOVA or unpaired t-test unless stated otherwise. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, ns: $p > 0.05$).

2.4 Results

2.4.1 *pgsA* expression is essential for cell survival in *E. faecalis*

It has been reported that *pgsA* is essential in other Gram-positive bacteria, including *S. aureus* *S. aureus* (Martin et al. 1999) and *B. subtilis* (Kobayashi et al. 2003). To investigate if *pgsA* is also essential in *E. faecalis*, we constructed a strain containing a tetracycline inducible *pgsA* gene named *E. faecalis* OG1RF P_{srtA-tetO}-*pgsA* pGCP023::tetR (abbreviated to *pgsA*_{ind}) to enable the depletion of *pgsA* transcription. A constitutively expressed, strong promoter, SrtA promoter, was chosen to maximize *pgsA* gene expression under non-depleting conditions. Based on the mechanism of the tetracycline-inducible promoter system, in which addition of anhydrotetracycline (ATc) will bind the TetR repressor and remove it from the operator to enable transcription, we predicted that *pgsA* gene expression could be regulated in a dose-dependent manner by the ATc inducer. To test the essentiality of *pgsA* in *E. faecalis*, *pgsA*_{ind} mutant was cultured in the presence or absence of ATc and compared to wild type (WT). Both optical density (OD) readings at 600 nm (**Figure 2.4A**) and colony-forming unit (CFU) measurements (**Figure 2.4B**) showed that the *pgsA*_{ind} mutant was able to grow as well as wild type in the presence of ATc but was unable to grow in the absence of ATc, whereas the growth of WT strain was not affected by the absence of ATc. The lack of growth of the *pgsA*_{ind} mutant in the absence of ATc suggests that *pgsA* is essential in *E. faecalis*.

Figure 2.4 *E. faecalis* *pgsA_{ind}* is only able to replicate in the presence of ATc. WT and *pgsA_{ind}* mutant cells (CFU=10⁴/ml) were cultured overnight (~18 hours) in the presence or absence of 1 µg/mL ATc. (A) Optical density of overnight cultures was measured at 600 nm using a UV spectrophotometer. (B) CFU of overnight cultures were enumerated by dilution plating. Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001 and ** indicates p-value <0.01.

Subsequently, to more carefully quantify the effect of ATc on growth, we assessed growth after 18 hours at decreasing ATc concentrations, comparing the *pgsA_{ind}* to WT. We observed that the *pgsA_{ind}* mutant was able to grow well at high concentrations of ATc, from 0.98 ng/ml to 1000 ng/ml but was unable to replicate at concentrations of ATc lower than 0.49 ng/ml (**Figure 2.5B**). By contrast, growth of WT strains was not affected by any concentration of ATc (**Figure 2.5A**). We also performed growth kinetic assays to measure the growth of *pgsA_{ind}* under relatively low concentrations of ATc, compared with WT. Slower growth of *pgsA_{ind}* mutant was observed at from 0.24ng/ml to 0.98 ng/ml ATc (**Figure 2.5D**), while the growth curve of WT was not affected by ATc (**Figure 2.5C**). These data revealed that a certain transcription of *pgsA* was required for cell survival, suggesting that *pgsA* expression is essential in *E. faecalis*.

Figure 2.5 Growth of *E. faecalis* *pgsA_{ind}* is inhibited at low ATc concentrations. Cells were grown in decreasing concentrations of ATc in a 96-well plate. Absorbance readings were measured at every 0.5-hour interval. (A) Absorbance of WT at decreasing concentrations of ATc after 18 hours of growth. (B) Absorbance of *pgsA_{ind}* mutant at decreasing concentrations of ATc after 18 hours of growth. (C) Growth kinetics of WT after incubation in decreasing concentrations of ATc. (D) Growth kinetics of *pgsA_{ind}* mutant in decreasing concentrations of ATc. Data graphed are the mean from three independent biological replicates.

To verify that the growth defect of *pgsA_{ind}* at low concentrations of ATc resulted from low expression levels of *pgsA*, we performed complementation experiments in which *pgsA* was expressed under control of a nisin-inducible promoter on plasmid pMSP3535. In the absence of ATc and Nisin, the complementation strain *pgsA_{ind} ppgsA-2HA* and *pgsA_{ind}* with empty vector pMSP3535 (*pgsA_{ind} pEmpty*) was unable to grow, compared to WT strain (**Figure 2.6**). However, the complemented strain, *pgsA_{ind} ppgsA-2HA* but not *pgsA_{ind} pEmpty*, was able to replicate in the presence of nisin and in the absence of ATc (**Figure 2.6**). Complementation of

pgsA in *pgsA_{ind}* was able to restore cell growth defect in the absence of ATc, suggesting that cell growth defect in *pgsA_{ind}* resulted from lack of *pgsA* gene expression.

Figure 2.6 Growth defect is restored in *E. faecalis* *pgsA_{ind}* complementation strain. Cells were inoculated in media supplemented without ATc and nisin (-ATc /-Nisin), with 200 ng/ml nisin (-ATc /+Nisin) or with 1 µg/mL ATc (+ATc /-Nisin), optical density of overnight cultures (~18 hours) was measured at 600 nm using a UV spectrophotometer. Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001.

Subsequently, to investigate the role of *pgsA* expression in cell growth, we performed kinetic growth assay of *pgsA_{ind}* ppgsA-2HA in decreasing nisin concentrations in the absence of ATc, comparing to WT and to WT ppgsA-2HA. We observed that overnight growth of WT and WT ppgsA-2HA was not affected by different concentrations of nisin (**Figure 2.7A and B**), and growth kinetics were also not affected after 18 hours of incubation (**Figure 2.7D and E**). Overnight growth of *pgsA_{ind}* ppgsA-2HA was not affected at high nisin concentrations, from 200 ng/ml to 20 ng/ml, whereas cell growth was inhibited at concentrations of nisin lower than 10 ng/ml (**Figure 2.7C**). Similarly, the growth curve of *pgsA_{ind}* ppgsA-2HA was also not affected at high nisin concentrations, whereas slower growth was observed at lower concentrations, and cells were not able to replicate at nisin concentrations lower than 2 ng/ml (**Figure 2.7F**).

Figure 2.7 Growth of *pgsA*_{ind} *ppgsA*-2HA is inhibited at low nisin concentrations compared to WT and WT *ppgsA*-2HA. Cells were grown in decreasing concentrations of nisin in a 96-well plate. Absorbance readings were measured at every 0.5-hour interval. Absorbance of (A) WT, (B) WT *ppgsA*-2HA, and (C) *pgsA*_{ind} *ppgsA*-2HA at decreasing concentrations of nisin after 18 hours of growth. Growth kinetics of (D) WT, (E) WT *ppgsA*-2HA, and (F) *pgsA*_{ind} *ppgsA*-2HA after incubation in decreasing concentrations of nisin. Data graphed in are the mean from three independent biological replicates; the

SD is graphed in panels A, B, and C but was not included in D, E, and F for clarity due to the large number of samples.

To examine the relationship between nisin concentration and PgsA protein level in the complementation strain *pgsA_{ind} ppgsA-2HA* and WT *ppgsA-2HA*, we performed western blot as described previously (Nielsen et al. 2013) to quantify the HA tagged PgsA expression from the plasmid at different concentrations of nisin in the absence of ATc (ie no *pgsA* expressed from the chromosome). Cells harvested from mid-exponential phase were used for western blot analysis using antibodies raised either against SecA or HA. Both in WT *ppgsA-2HA* and *pgsA_{ind} ppgsA-2HA* cells, we observed similar PgsA-2HA expression at high nisin concentrations, from 200 ng/ml to 10 ng/ml, and we observed no detectable PgsA-2HA at concentrations of nisin less than 10 ng/ml (**Figure 2.8A**). To quantify PgsA-2HA expression in different nisin concentrations, PgsA-2HA band intensity was normalized to SecA band intensity for the same sample. We observed a dose-dependent decrease in PgsA-2HA expression with decreasing nisin concentrations from 200 ng/ml to 10 ng/ml, and we did not observe any detectable PgsA-2HA at concentrations of nisin lower than 10 ng/ml (**Figure 2.8B**).

Taken together, the growth defect results and low expression level of PgsA in *pgsA_{ind} ppgsA-2HA* at low concentrations of nisin suggested that the cell growth defect of *pgsA_{ind}*, when grown in low concentrations of ATc, resulted from low expression of *pgsA*.

Figure 2.8 PgsA-2HA protein is expressed under a nisin inducible promoter on pMSP3535 in a dose-dependent manner. WT *ppgsA*-2HA and *pgsA*_{ind} *ppgsA*-2HA were grown in decreasing concentrations of nisin in the absence of ATc, and cells were harvested and lysed for western blot analysis using antibodies raised either against SecA or HA. (A) Representative images of western blot data used to quantify PgsA-2HA protein expression levels. (B) Quantification of PgsA-2HA protein in WT *ppgsA*-2HA and *pgsA*_{ind} *ppgsA*-2HA harvested after growth in decreasing nisin concentrations, relative to SecA for the same sample. Data graphed are the mean from three independent biological replicates.

2.4.2 PgsA is focally localized on *E. faecalis* cell envelope

It has been reported that PgsA enzyme is septal localized in *B. subtilis*, (Nishibori et al. 2005), to investigate whether the PgsA in *E. faecalis* is focally localized at the septum as anionic lipid is enriched, we performed immunofluorescence microscopy (IFM). Cells from *pgsA*_{ind} *ppgsA*-2HA and WT *ppgsA*-2HA were harvested from media supplemented with nisin (100 ng/ml) and were examined for PgsA-2HA localization using an anti-HA antibody. We observed a similar PgsA localization pattern in both *pgsA*_{ind} *ppgsA*-2HA and WT *ppgsA*-2HA cell, but varied by the stage of cell division (**Figure 2.9A**). Single foci were observed at the septa of pre-division cells, and multiple distinct foci were observed at the septa of cells at early-division

and late-division stages (**Figure 2.9B**). To quantify PgsA-2HA localization patterns, we used Projected System of Internal Coordinates from Interpolated Contours (PSICIC) (Guberman et al. 2008) to quantify the mean fluorescence intensity at the perimeter of *E. faecalis* cells at the early division stage. At this stage of the cell cycle, position 25 (in arbitrary units, AU) corresponds to one side of the septum, and position 75 AU corresponds to the other side of the septum. The mean fluorescence intensity of WT *ppgsA*-2HA and *pgsA*_{ind} *ppgsA*-2HA peaked at the septa (**Figure 2.9C and D**), which was consistent with visual observations. The fluorescence intensity of WT *ppgsA*-2HA cells was higher than *pgsA*_{ind} *ppgsA*-2HA, suggesting that *pgsA* expression levels might be different between the two strains even at same nisin concentrations.

Figure 2.9 PgsA is focally localized at the septa of *E. faecalis*. PgsA-2HA localization was visualized via IFM using an anti-HA antibody in mid-log phase cultures and analyzed by PSICIC software. (A) Cartoons depict the representative localization pattern of PgsA observed over many cells at each stage of cell division (see Material and Methods for division stage determination). (B) Representative images of PgsA localization in *E. faecalis* cells at different cell division stages, scale bar = 0.5 μ m. The mean fluorescent intensity of (C) WT *ppgsA*-2HA and (D) *pgsA*_{ind} *ppgsA*-2HA harvested from different concentrations of nisin. The images are showing representative localization pattern of PgsA during early division stage at nisin 200 ng/ml, scale bar = 0.5 μ m. The cell perimeter was quantified by PSICIC software from at least 200 early-division cells, in which cells without fluorescence were excluded from

the analyses. Units are displayed in arbitrary units (AU). Cartoons depict predominant site of PgsA localization, and the coordinates of the perimeter correspond with the x-axes.

2.4.3 Low expression of PgsA results in morphology changes in *E. faecalis* *pgsA*_{ind}

Since PG is a major phospholipid of the Gram-positive cell membrane, we hypothesized that PG depletion would affect cell membrane lipid integrity and therefore influence cell morphology. To test the hypothesis, we stained both WT and *pgsA*_{ind} with NAO lipid dye and Hoechst nucleic acid stain at difference concentrations of ATc and performed fluorescence microscopy. We observed that WT cells displayed uniform diplococcal shape and had similar width and length, regardless of ATc concentrations (**Figure 2.10A**), suggesting that ATc does not affect WT cell morphology. The *pgsA*_{ind} mutant under high concentrations of ATc, such as 16 ng/ml and 2 ng/ml, displayed similar morphologies to WT (**Figure 2.10B**). At low concentrations of ATc, for example 0.5 ng/ml and 0.25 ng/ml, *pgsA*_{ind} mutant contained two populations, in which one population displayed WT-like morphology while the other population showed very heterogeneous sizes and shapes, in which cells became longer or wider, compared with WT cells (highlighted by red headarrows in **Figure 2.10B**).

Upon NAO staining, we observed that all WT cells harvested at different ATc concentrations had unique green foci at the septa suggesting that ATc does not influence lipid distribution. Meanwhile, in the *pgsA*_{ind} cells which displayed WT-like morphology, including cells harvested from high ATc concentrations and some cells harvested from low ATc concentrations, unique green foci were observed at the septa. However, in the cells displaying changed morphology harvested from low 0.5 ng/ml and 0.25 ng/ml ATc, green fluorescence was uniformly distributed throughout the cell membrane, indicating that lipid distribution was altered in *pgsA*_{ind} at low ATc concentrations. Similarly, in the WT and *pgsA*_{ind} cells with WT-like morphology, DNA appeared condensed within each coccal unit of a diplococcus, suggesting that nucleoid shape and distribution were similar in these cells. However, in the *pgsA*_{ind} cells with altered morphology, blue fluorescence was altered and appeared extended or distributed across the length of chaining cells, with no break at the septa or between individual cocci. In addition, the blue fluorescence intensity was increased in cells with altered morphology and Hoechst staining patterns, suggesting that DNA may be accumulating in morphology altered cells.

To quantify the morphology changes in WT and *pgsA_{ind}* at different concentrations of ATc, we measured the cell width and length in each growth condition using MicrobeJ (Ducret et al. 2016) as depicted in the cartoon (**Figure 2.10C**). Data plotted on a scatter diagram of cell width and length distribution shows that cell width and length of WT cells fell within in a limited distribution, regardless of ATc concentration (**Figure 2.10D**). In *pgsA_{ind}*, cell width and length distributions were not altered at high concentrations of ATc, whereas cell width and length of a subpopulation of *pgsA_{ind}* cells at low concentrations were increased reflecting that some cells became wider or longer (**Figure 2.10E**). We also calculated the percentage of morphology altered *pgsA_{ind}* cells at each ATc concentration in which the cell width or length value exceeded the largest value for WT cells grown in the absence of ATc (ie no *pgsA* expressed from the chromosome) (**Figure 2.10F**). We observed that approximately 60% of the *pgsA_{ind}* cells displayed altered morphology at ATc 0.5 ng/ml and approximately 50% of the *pgsA_{ind}* cells showed altered morphology ATc 0.25 ng/ml, demonstrating that low concentrations of ATc resulted in morphology changes in *E. faecalis pgsA_{ind}*.

Figure 2.10 Morphology of *E. faecalis* $pgsA_{ind}$ is altered at low ATc concentrations. Cells were stained with NAO and Hoechst. (A, B) Representative images of WT and $pgsA_{ind}$ at decreasing ATc concentrations. Scale bar = 2 μ m. The morphology altered cells were highlighted by red arrowheads. (C) Cartoon depiction of the measurement of *E. faecalis* cell width and length. (D) Scatter plot of cell width and length distribution of WT under different ATc concentrations. (E) Scatter plot of cell width and length distribution of $pgsA_{ind}$ under different ATc concentrations compared with WT without ATc. One hundred cells in each condition were counted and are shown. (F) Data in panel D and E were quantified and the percentage of morphology altered cells in $pgsA_{ind}$ and WT at different ATc concentrations are

shown relative to WT without ATc which is 0% (not shown). Two hundred cells in each condition were analyzed.

Based on the growth defect and low *pgsA* expression results for *pgsA*_{ind} *ppgsA*-2HA at low nisin concentrations, along with our previous observation of aberrant cell morphology in *pgsA*_{ind} at low ATc concentrations, we hypothesized that cell morphology of *pgsA*_{ind} *ppgsA*-2HA would be altered at low concentrations of nisin (ie where there is no chromosomal expression of *pgsA* and low plasmid expression of *pgsA*). To test this, we examined the cell morphology of *pgsA*_{ind} *ppgsA*-2HA and WT *ppgsA*-2HA at different concentrations of nisin in the absence of ATc (ie no *pgsA* expressed from the chromosome), compared to WT. As predicted, all WT and WT *ppgsA*-2HA cells at different nisin concentrations displayed uniform diplococcal morphology, which is similar to WT cells without nisin treatment (**Figure 2.11A and B**), suggesting that nisin alone or high expression of *pgsA* does not affect WT and WT *ppgsA*-2HA cell morphology. In the complementation strain *pgsA*_{ind} *ppgsA*-2HA, at high concentrations of nisin (200-20 ng/ml), cells displayed similar morphologies to WT (**Figure 2.11C**). However, in the *pgsA*_{ind} *ppgsA*-2HA cells at low concentrations of nisin (5-1 ng/ml), cells became longer or wider, which was similar to what we observed in the inducible mutant *pgsA*_{ind} at low ATc concentrations (**Fig 2.10**). Taken together, complementation of *pgsA* in *pgsA*_{ind} restored cellular morphology in *pgsA*_{ind} at low concentrations of ATc to WT morphology, suggesting that the aberrant cell shape in *pgsA*_{ind} at low concentrations of ATc resulted from low expression of *pgsA*.

Figure 2.11 Morphology of *E. faecalis* *pgsA*_{ind} *ppgsA*-2HA is altered at low concentrations of nisin. Representative images of (A) WT, (B) WT *ppgsA*-2HA and (C) *pgsA*_{ind} *ppgsA*-2HA harvested from decreasing nisin concentrations. Scale bar =2 μ m.

2.4.4 Suppressor mutations were found in surviving *pgsA*_{ind} in the absence of ATc

We observed two different populations in *pgsA*_{ind} at low ATc concentrations, in which one population displayed “WT-like” morphology and the other population displayed altered

morphology (**Figure 2.10**). We also verified that *pgsA* was essential in *E. faecalis*, which was supported by the growth defect in *pgsA*_{ind} and restored growth in the complementation strain *pgsA*_{ind} *ppgsA*-2HA (**Figure 2.5-2.8**). In *E. coli*, a *pgsA* null mutant was viable if also harbouring *lpp* and *rcsF* mutations even though PG was undetectable in this *pgsA* null mutant (Kikuchi et al. 2000, Suzuki et al. 2002). Thus, we hypothesized that the population displaying “WT-like” morphology was a population of cells suffering suppressor mutations resulting in cell viability (or health) even though *pgsA* expression was extremely decreased. To obtain pure suppressor mutants, we performed serial passaging of *pgsA*_{ind} in the absence of ATc for several days. After several days’ passaging, we obtained 8 stable *pgsA*_{ind} suppressor mutants, which were able to grow well in the absence of ATc (**Figure 2.12A**). We also visualized the cell morphology of these *pgsA*_{ind} ATc suppressor mutants in the absence of ATc, and the morphology of *pgsA*_{ind} suppressor mutant 1 (*pgsA*_{ind} S1) suggested that the suppressor mutants converted to WT morphology even in the absence of ATc (**Figure 2.12B**). Similar WT morphologies were also observed in other *pgsA*_{ind} suppressor mutants (data was not shown).

Figure 2.12 The *pgsA*_{ind} ATc suppressor mutants were able to replicate in the absence of ATc. WT, *pgsA*_{ind} mutant and *pgsA*_{ind} suppressor mutant cells (CFU=10⁵/ml) were cultured overnight (~18 hours) in the absence of 1 µg/mL ATc. (A) Optical density of overnight cultures was measured at 600 nm using a UV spectrophotometer. (B) Representative images of *pgsA*_{ind} S1 and WT in the absence of ATc are shown. Statistical significance was determined by two-way ANOVA with Sidak’s test for multiple comparisons, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001 and ** indicates p-value <0.01.

Next, we hypothesized that mutations occurred in these *pgsA*_{ind} ATc suppressor mutants that support cell growth. One possibility was that another enzyme was induced or activated to synthesize PG, since it was reported that *clsB*, which encodes a CL synthase in *E. coli*, was also involved in the synthesis of PG (Li et al. 2016). Alternatively, mutations could have occurred in *pgsA* or other membrane related genes that supported cell viability in the absence or low expression of *pgsA*, since an *E. coli pgsA* null is still viable when harboring *lpp* and *rcsF* mutations (Kikuchi et al. 2000). To test the hypothesis, we performed whole genome sequencing to sequence 8 *pgsA*_{ind} suppressor mutants and compared their sequence to *pgsA*_{ind} which was not able to replicate in the absence of ATc. However, surprisingly, we did not find any other chromosomal mutations in these 8 *pgsA*_{ind} suppressor mutants, compared to *pgsA*_{ind}. Therefore, we went on to sequence the plasmid *ptetR* in these suppressor mutants. We found different types of mutations occurred in the plasmids, as compared to original *ptetR* (**Table 2.6**). Most of the mutations occurred in the coding sequencing of *tetR* (*pgsA*_{ind} S1-S5), which may alter the structure of TetR and subsequently affect the ability of TetR to bind the *tetO* operator in the *pgsA*_{ind} inducible system. The plasmid *ptetR* was undetectable in the isolate S7. Some other mutations which were identified in S6 and S8 may affect the function of TetR as the *pgsA* gene transcription in these suppressor mutants was similar to *P_{srtA}-tetO-pgsA* (which is *pgsA*_{ind} without *ptetR*) by qPCR analysis (Figure 2.). Thus, we hypothesized that the two different populations we observed in *pgsA*_{ind} at low concentrations of ATc (**Figure 2.9**) may result from similar suppressor mutations in the plasmid *ptetR*.

Table 2.6 Mutations of the plasmid *ptetR* in the *pgsA*_{ind} suppressor mutants, compared to *pgsA*_{ind}

Strain	Plasmid size (bp)	Type	Mutations in <i>tetR</i>	Other mutations excluding <i>tetR</i>
<i>pgsA</i> _{ind} S1	7123	Substitution	204 Phe (AAG) → Leu (AAT)	-
<i>pgsA</i> _{ind} S2	7105	Deletion	118 His (GTA), 1bp deletion → GA	-
<i>pgsA</i> _{ind} S3	7128	Insertion	TTATT, 5bp insertion of the promoter of <i>tetR</i>	-

<i>pgsA</i> _{ind} S4	7105	Insertion/ deletion	95 Asp (CTA), 1bp insertion→ CTAA;GGTCTTATTTTAAATC AAA, 19bp deletion of the promoter of <i>tetR</i>	-
<i>pgsA</i> _{ind} S5	7105	Insertion/ deletion	31 Ala (CGT), 1bp insertion → (CGGT)/	19bp deletion of the Cam resistance cassette (7032-7053)
<i>pgsA</i> _{ind} S6	7116	Substitutio n/ deletion	-	(GAACAATCTTCAT TCT,1916-1926) to (CATACTTCC),
<i>pgsA</i> _{ind} S7	Nd	-	-	
<i>pgsA</i> _{ind} S8	7105	Substitutio n/ deletion	39 Gly (CCT) → Arg(TCT)	18bp deletion of the downstream of Kan terminator(4165-4183)
Nd: no detectable plasmid

Figure 2.13 *pgsA* expression in the suppressor mutant was similar to *P_{srtA}-tetO-pgsA* and *pgsA_{ind}* at high ATc concentration. qPCR was analyzed in *pgsA_{ind}* under different ATc concentrations, compared to WT without ATc treatment. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates repeated on non-consecutive days. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001.

2.4.5 *pgsA* expression is decreased in *pgsA*_{ind} mutant at all ATc concentrations

Based on the mechanism of the “tet-off” system used to construct the *pgsA*_{ind} inducible mutant in *E. faecalis* and the growth defect observed at low ATc concentrations, we hypothesized that *pgsA* expression was decreased in *pgsA*_{ind} when ATc is limiting whereas *pgsA* expression should not be altered in *pgsA*_{ind} at high ATc concentrations. To verify this, we performed real-time quantitative PCR (qPCR) to quantify relative *pgsA* expression in *pgsA*_{ind} at different ATc concentrations, compared to WT without ATc. Total RNA from cells harvested from exponential phase was extracted and we performed real-time qPCR according to the methods described in **Section 2.3.8**. However, we observed significant transcriptional decrease of *pgsA* in *pgsA*_{ind} at all concentrations of ATc tested, even though the cell growth and cell morphology were not affected at high ATc concentrations, and the relative *pgsA* expression at 0.25 ng/ml ATc (in which cell growth was defective and cell morphology was altered) was higher than *pgsA* transcript levels at higher ATc concentrations (**Figure 2.14**).

Figure 2.14 *pgsA* expression was decreased in *pgsA*_{ind}. qPCR was analyzed in *pgsA*_{ind} under different ATc concentrations, compared to WT without ATc treatment. Statistical significance was determined by one-way ANOVA, N = 6 independent biological replicates repeated on non-consecutive days. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001.

Because this was not the expected result, next, we included WT *ptetR* as control since the *pgsA*_{ind} (*E. faecalis* OG1RF P_{srtA-tetO}-*pgsA ptetR*) to exclude any influence of the plasmid or growth in antibiotic to *pgsA* transcription. The qPCR results revealed that *pgsA* expression was similar for WT and WT *ptetR* (**Figure 2.15A**). Even so, to exclude a possible influence caused by ATc, all strains including WT, WT *ptetR* and *pgsA*_{ind} were inoculated in different concentrations of ATc. Relative *pgsA* expression was normalized to WT *ptetR* at each ATc concentration. However, we still observed significantly decreased *pgsA* expression in *pgsA*_{ind} at all ATc concentrations, even at very high ATc concentration (1000 and 100 ng/ml) (**Figure 2.15B**). What's more, the relative *pgsA* expression in *pgsA*_{ind} at 0.25 ng/ml was also higher than at higher ATc concentrations. Again, the qPCR results were unexpected, because we previously observed growth defect and morphology change in a subpopulation of *pgsA*_{ind} at low ATc concentrations, which suggested *pgsA* depletion in those populations.

Figure 2.15 Relative *pgsA* expression was decreased in *pgsA*_{ind}, compared to WT *ptetR*. (A) Relative transcriptional fold change of *pgsA* in WT *ptetR* without ATc treatment, compared to WT without ATc treatment. (B) Relative transcriptional fold change of *pgsA* in WT and *pgsA*_{ind} at different concentrations of ATc, compared to WT *ptetR*. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates repeated on non-consecutive days. Error bars represent standard error of the mean (SEM), the symbol ns indicates p-value >0.05.

2.4.6 PG levels were not altered in *pgsA*_{ind} at high ATc concentration

Although the *pgsA* transcription results obtained by qPCR were not consistent with the growth defect and morphology phenotype we observed (**Figure 2.5 and Figure 2.10**), we nonetheless tried to measure PG levels in *pgsA*_{ind} at different concentrations using liquid chromatography with tandem mass spectrometry (LC-MS-MS). The total PG levels of each sample was normalized to either the weight of total extracted lipid, or the weight of dried cell pellet.

When PG levels were normalized to the cell weight, total PG levels of *pgsA*_{ind} at 16 ng/ml ATc was not significantly altered, compared to WT at 16 ng/ml, whereas the PG levels of *pgsA*_{ind} at 0.25 ng/ml was significantly increased, compared to WT at 0.25 ng/ml (**Figure 2.16A**). When PG levels were normalized to total lipid weight, total PG levels of all sample were similar (**Figure 2.16B**). The different conclusions from these two normalization methods may have resulted from a variation in lipid extraction, since total lipid extracted from *pgsA*_{ind} at 0.25 ng/ml was significantly higher than other conditions (**Figure 2.16C**). Nevertheless, the lipid analysis of *pgsA*_{ind} at 16 ng/ml suggested that PG levels were not significantly altered, even though qPCR results showed that *pgsA* expression levels were significantly decreased under the same conditions. Hypotheses and future work to reconcile these unexpected and inconsistent results are discussed below.

Figure 2.16 PG levels were not altered in *pgsA_{ind}* at high ATc concentration. Total lipid was extracted from cells harvested after growth with ATc 16 ng/ml and 0.25 ng/ml. Total PG amount in each sample were calculated by mass spectrometry and normalized to (A) total cell weight, and (B) total lipid weight. (C) Total lipid extracted was normalized to cell weight for each sample. Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, N = 5 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol ns indicates p-value >0.05, ** indicates p-value <0.01, *** indicates p-value <0.001

2.5 Discussion and conclusion

In this chapter, we constructed a tetracycline inducible *pgsA*_{ind} mutant, *E. faecalis pgsA*_{ind} to deplete PG and a corresponding complementation strain *E. faecalis pgsA*_{ind} *ppgsA*-2HA using a nisin inducible plasmid. To our knowledge, this is the first demonstration of construction an inducible mutant of an essential gene in *E. faecalis*. Using the *pgsA*_{ind} mutant, we observed a growth defect in *pgsA*_{ind} at low ATc concentrations and *pgsA*_{ind} *ppgsA*-2HA at low nisin concentrations (in the absence of ATc), whereas complementation of PgsA in *pgsA*_{ind} *ppgsA*-2HA at high nisin concentrations (in the absence of ATc, ie no *pgsA* expressed from the chromosome) restored growth to WT levels. These data suggest that *pgsA* is essential in *E. faecalis*, which is consistent with reports in many other species of bacteria (Martin et al. 1999, Kobayashi et al. 2003, van Beilen et al. 2016). Although an *E. coli pgsA* null mutant is viable when also harboring *lpp* and *rscF* mutations, expression of a native Lpp from a plasmid caused cell lysis in this mutant (Kikuchi et al. 2000, Suzuki et al. 2002). In *B. subtilis*, green fluorescent protein (GFP) fusions to PgsA (GFP-PgsA) are septum localized, suggesting that PG may be enriched at the septum (Nishibori et al. 2005). In this chapter, we also observed PgsA focal localization at the septum by visualizing an HA tagged PgsA (PgsA-2HA), suggesting that PG may also be enriched at the septum of *E. faecalis*.

Both in *pgsA*_{ind} at low ATc concentrations and *pgsA*_{ind} *ppgsA*-2HA at low nisin concentrations (in absence of ATc), we observed two populations, in which one population displayed WT-like morphology with uniform diplococcal shape while the other population showed very heterogeneous sizes and shapes, in which cells became longer or wider, compared with WT cells. These data also suggest that *pgsA* is essential in *E. faecalis*. Based on the observation of accumulated DNA in the subpopulation with altered cell morphology, we hypothesized that low expression of *pgsA* in *pgsA*_{ind} at low ATc concentrations affects cell division.

The lipid analysis of *pgsA*_{ind} at 16 ng/ml suggested that PG levels were not significant altered even though qPCR results suggested that *pgsA* expression levels were significantly decreased under all conditions tested. To explain these results, we hypothesize that the *P_{srtA}-tetO* promoter is not strong enough to reach WT-level *pgsA* transcription, but there is a *pgsA* expression threshold, in which low expression of *pgsA* is still able to support synthesis of enough PG levels for cell division to occur. For the analysis of *pgsA*_{ind} at low ATc concentrations, we observed *pgsA*_{ind} was able to grow after long incubation times, even without the inducer ATc, due to

suppressor mutations in TetR. Thus, we hypothesize that the subpopulation with WT-like morphology in *pgsA*_{ind} at low ATc concentrations consists of this kind of suppressor mutant. However, this hypothesis still cannot explain why the *pgsA* expression in *pgsA*_{ind} at low ATc (0.25 ng/ml) is higher than it is at high ATc concentrations as *pgsA* expression in the suppressor mutant is similar to *P_{srtA}-tetO-pgsA* (which is *pgsA*_{ind} without *ptetR*) and *pgsA*_{ind} at high ATc (16 ng/ml). Thus, the apparent greater transcription in the whole population should be resulted from the morphology-altered subpopulation, which may because the cell growth stage of this subpopulation was different from the WT-like subpopulation. Further analysis of *pgsA* expression in individual cells or different subpopulations at different growth stage is required to confirm the hypothesis. For now, the existence of possible suppressor mutants needs to be considered for further analysis at a population level.

In conclusion, in this chapter:

- 1) We constructed an inducible *pgsA*_{ind} mutant, which is the first demonstration of an inducible mutant of an essential gene in *E. faecalis*.
- 2) PgsA is focally localized at the septum of *E. faecalis*.
- 3) Putative low PgsA expression results in cell growth defect and morphology change in *E. faecalis pgsA*_{ind} which can be restored upon complementation with *pgsA* in trans.
- 4) A subpopulation of suppressor mutants, which can grow without the inducer ATc, are found in *pgsA*_{ind} when grown at low ATc concentrations.

2.6 References

- [1]. Abat, C., M. Huart, V. Garcia, G. Dubourg & D. Raoult (2016) *Enterococcus faecalis* urinary-tract infections: Do they have a zoonotic origin? *J Infect*, 73, 305-13.
- [2]. Arias, C. A. & B. E. Murray (2012) The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol*, 10, 266-78.
- [3]. Beisson, J., M. Betermier, M. H. Bre, J. Cohen, S. Duharcourt, L. Duret, C. Kung, S. Malinsky, E. Meyer, J. R. Preer, Jr. & L. Sperling (2010) Immunocytochemistry of *Paramecium* cytoskeletal structures. *Cold Spring Harb Protoc*, 2010, pdb.prot5365.
- [4]. Berens, C. & W. Hillen (2004) Gene regulation by tetracyclines. *Genet. Eng. (N.Y.)*, 26, 255-77.
- [5]. Bryan, E. M., T. Bae, M. Kleerebezem & G. M. Dunny (2000) Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid*, 44, 183-90.
- [6]. Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu Rev Biochem*, 66, 199-232.
- [7]. Dowhan, W. (2013) A retrospective: use of *Escherichia coli* as a vehicle to study phospholipid synthesis and function. *Biochim Biophys Acta*, 1831, 471-94.
- [8]. Dryden, S. C. & W. Dowhan (1996) Isolation and expression of the *Rhodobacter sphaeroides* gene (*pgsA*) encoding phosphatidylglycerophosphate synthase. *J Bacteriol*, 178, 1030-8.
- [9]. Ducret, A., E. M. Quardokus & Y. V. Brun (2016) MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat Microbiol*, 1, 16077.
- [10]. Dunny, G. M., B. L. Brown & D. B. Clewell (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci U S A*, 75, 3479-83.
- [11]. Granok, A. B., D. Parsonage, R. P. Ross & M. G. Caparon (2000) The *RofA* binding site in *Streptococcus pyogenes* is utilized in multiple transcriptional pathways. *J Bacteriol*, 182, 1529-40.
- [12]. Guberman, J. M., A. Fay, J. Dworkin, N. S. Wingreen & Z. Gitai (2008) PSICIC: noise and asymmetry in bacterial division revealed by computational image analysis at sub-pixel resolution. *PLoS Comput Biol*, 4, e1000233.
- [13]. Kikuchi, S., I. Shibuya & K. Matsumoto (2000) Viability of an *Escherichia coli pgsA* null mutant lacking detectable phosphatidylglycerol and cardiolipin. *J Bacteriol*, 182, 371-6.
- [14]. Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron & K.

- Bunai (2003) Essential *Bacillus subtilis* genes. *Proceedings of the National Academy of Sciences*, 100, 4678-4683.
- [15]. Kommineni, S., D. J. Bretl, V. Lam, R. Chakraborty, M. Hayward, P. Simpson, Y. Cao, P. Bousounis, C. J. Kristich & N. H. Salzman (2015) Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature*, 526, 719-22.
- [16]. Li, C., B. K. Tan, J. Zhao & Z. Guan (2016) In Vivo and in Vitro Synthesis of Phosphatidylglycerol by an *Escherichia coli* Cardiolipin Synthase. *J Biol Chem*, 291, 25144-25153.
- [17]. Martin, P. K., T. Li, D. Sun, D. P. Biek & M. B. Schmid (1999) Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol*, 181, 3666-73.
- [18]. Nielsen, H. V., A. L. Flores-Mireles, A. L. Kau, K. A. Kline, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *Journal of Bacteriology*, 195, 4484-4495.
- [19]. Nielsen, H. V., P. S. Guiton, K. A. Kline, G. C. Port, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2012) The metal ion-dependent adhesion site motif of the *Enterococcus faecalis* EbpA pilin mediates pilus function in catheter-associated urinary tract infection. *MBio*, 3, e00177-12.
- [20]. Nishibori, A., J. Kusaka, H. Hara, M. Umeda & K. Matsumoto (2005) Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. *Journal of bacteriology*, 187, 2163-2174.
- [21]. Orth, P., D. Schnappinger, W. Hillen, W. Saenger & W. Hinrichs (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor–operator system. *Nature Structural Biology*, 7, 215-219.
- [22]. Parsons, J. B. & C. O. Rock (2013) Bacterial lipids: metabolism and membrane homeostasis. *Prog Lipid Res*, 52, 249-76.
- [23]. Rashid, R., A. Cazenave-Gassiot, I. H. Gao, Z. J. Nair, J. K. Kumar, L. Gao, K. A. Kline & M. R. Wenk (2017) Comprehensive analysis of phospholipids and glycolipids in the opportunistic pathogen *Enterococcus faecalis*. *PLoS One*, 12, e0175886.
- [24]. Sohlenkamp, C. & O. Geiger (2016) Bacterial membrane lipids: diversity in structures and pathways. *FEMS Microbiol Rev*, 40, 133-59.
- [25]. Suzuki, M., H. Hara & K. Matsumoto (2002) Envelope disorder of *Escherichia coli* cells lacking phosphatidylglycerol. *J Bacteriol*, 184, 5418-25.
- [26]. van Beilen, J., C. J. Blohmke, H. Folkerts, R. de Boer, A. Zakrzewska, W. Kulik, F. M. Vaz, S. Brul & A. Ter Beek (2016) RodZ and PgsA Play Intertwined Roles in

Membrane Homeostasis of *Bacillus subtilis* and Resistance to Weak Organic Acid Stress. *Frontiers in microbiology*, 7, 1633-1633.

- [27]. Vega, L. A., G. C. Port & M. G. Caparon (2013) An association between peptidoglycan synthesis and organization of the *Streptococcus pyogenes* ExPortal. *mBio*, 4, e00485.
- [28]. Werner, J. N., E. Y. Chen, J. M. Guberman, A. R. Zippilli, J. J. Irgon & Z. Gitai (2009) Quantitative genome-scale analysis of protein localization in an asymmetric bacterium. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 7858-7863.
- [29]. Zhang, Y. M. & C. O. Rock (2008) Membrane lipid homeostasis in bacteria. *Nat Rev Microbiol*, 6, 222-33.

Chapter 3 Role of phosphatidylglycerol in *Enterococcus faecalis* physiology

3.1 Abstract

Virulence factor secretion and assembly occurs at spatially restricted foci at the septum of the cell in *Enterococcus faecalis*. Given the essentiality of the general secretion pathway in bacteria and the contribution of virulence factors to disease progression, the foci coordinating these processes are attractive antimicrobial targets. The anionic lipid phosphatidylglycerol (PG) is a major lipid in bacterial membranes and reducing the PG composition in the cell membrane of *Bacillus subtilis* results in the delocalization of SecA, suggesting that membrane lipid composition also affects focal virulence factor secretion and assembly. Hence, in this project, we investigated the contribution of PG to the localization of secretion and virulence factor assembly machinery in *E. faecalis*, as well as to antimicrobial resistance. Using a regulatable *pgsA* mutant, *pgsA*_{ind}, we observed delocalization of enzymes involved in focal virulence factor assembly, from discrete foci enriched at the septum to diffuse staining around the cell membrane. Furthermore, *pgsA*_{ind} at low ATc concentrations became more sensitive to cell membrane targeting antimicrobials but more resistant to cell wall targeting antimicrobials, consistent with cell membrane disruption and cell wall enhancement induced upon PG depletion. In addition, the cell wall of *pgsA*_{ind} at low ATc concentrations was less cross-linked and cell wall deposition was altered, which may serve as a compensatory reaction to PG depletion. In conclusion, these studies suggest that focal virulence factor assembly and antimicrobial resistance in *E. faecalis* is dependent on cellular PG levels.

3.2 Introduction

E. faecalis related infections are difficult to treat because of their intrinsic tolerance to many antibiotics and frequently acquired drug resistance (Guzman Prieto et al. 2016). In addition to antibiotic resistance, enterococci use a variety of virulence factors to cause infect and persist within their hosts (Sava et al. 2010).

Virulence factors are pathogen-produced molecules that promote the establishment and maintenance of disease. In the research of enterococcal infections, *E. faecalis* relies on a conserved secretory and sorting pathway for the translocation, biogenesis, and subsequent attachment of cell wall associated virulence factors to cell surface. The sortase enzymes, sortase A (SrtA) and sortase C (SrtC), colocalize with SecA, a highly conserved cell membrane-associated subunit in general secretion pathway (Kline et al. 2009). This colocalization occurs at single foci at the division plane and is proposed to be coincident with anionic lipid domains (Rashid et al. 2016), since SecA colocalizes with anionic lipid microdomains in Group A Streptococci (Rosch et al. 2007). However, in *E. faecalis*, further research about the lipids and other factors that governs the formation and organization of these foci, where virulence factors involved in secretion and sorting co-localize, is required.

In bacteria, anionic PG can be modified by adding cationic amino acids, such as lysine, alanine or arginine to the head group of PG to form Lys-PG, Ala-PG, or Arg-PG, respectively (Roy et al. 2009, Slavetinsky et al. 2017). The result of this modification is an overall increase in the surface charge of the membrane and subsequently an increased resistance to cationic antimicrobial peptides (CAMPs) via electrostatic repulsion (Ernst et al. 2009). These modifications are mediated by membrane embedded aminoacyl-phosphatidylglycerol synthases, the prototype of which is multiple peptide resistance factor (MprF), first described to add lysine to the anionic head group of PG in *S. aureus* (Peschel et al. 2001), and later in *B. anthracis* (Samant et al. 2009), *Listeria monocytogenes* (Thedieck et al. 2006), and *E. faecalis* (Kumariya et al. 2015). In *E. faecalis*, modification of PG by MprF limits focal targeting by the antimicrobial peptide human β -defensin 2 (hBD2) to discrete (presumably anionic lipid foci), resulting in resistance to hBD2 (Kandaswamy et al. 2013). What's more, the localization of SrtA and SecA can be disrupted by hBD2 exposure, hypothesized to be due to hBD2 disruption of anionic lipid domains (Kandaswamy et al. 2013). In the Gram-positive pathogen *Streptococcus pyogenes*, the "ExPortal" membrane microdomain is the site of secretion and

secreted protein maturation, and the Exportal is enriched in anionic lipids such as PG and CL as evidenced by NAO staining (Rosch and Caparon 2005, Rosch et al. 2007). Furthermore, in *B. subtilis*, reducing the PG composition in the cell membrane results in the delocalization of SecA (Campo et al. 2004). Taken together, these results suggest a conserved phenomenon in Gram positive bacteria in which protein secretion and virulence factor maturation or coordinated and discrete focal membrane microdomains which are enriched for anionic lipids.

In this chapter, we test the hypothesis that virulence factor secretion and sorting processes are spatially restricted to microdomains enriched in anionic phospholipids in *E. faecalis*. To test this, we created a conditional *pgsA* depletion strain and show that PG depletion alters virulence factor expression and focal localization at the lipid microdomains.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this chapter are provided in **Table 3.1**.

E. coli strains were grown in Luria-Bertani (LB, Difco, France) broth or agar, whereas *E. faecalis* strains were grown in Brain Heart Infusion (BHI, Becton, Dickinson, USA) broth or agar. For all experiments in this chapter, bacterial strains were streaked from a 25% (v/v) glycerol stock stored at -80°C onto 1.5% agar plates and grown overnight at 37°C. The next day, a single colony from an agar plate was inoculated into liquid medium and incubated at 37°C overnight, followed by 1:10 dilution into fresh media and growth to an optical density of 600 nm wavelength ($OD_{600\text{ nm}} = 0.45-0.6$) to reach mid-exponential phase. Respective antibiotics listed in **Table 3.1** were added to liquid or agar media at the concentrations indicated for growth of strains with plasmids.

Table 3.1 List of bacterial strains and plasmids used in this chapter

Strains or plasmids	Characteristic(s)	Resistance, concentrations	References
Strains			
<i>Enterococcus faecalis</i> OG1RF (WT)	ATCC 47077, <i>E. faecalis</i> OG1RF wild type strain, human oral isolate	Rif ^r and Fus ^r , 25 µg/ml	(Dunny et al. 1978)
<i>Enterococcus faecalis</i> OG1RF pGCP023:: <i>tetR</i>	<i>E. faecalis</i> OG1RF harboring pGCP023:: <i>tetR</i>	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work
<i>Enterococcus faecalis</i> OG1RF P _{srtA} - <i>tetO</i> - <i>pgsA</i> pGCP023:: <i>tetR</i> , abbreviate to <i>E. faecalis</i> OG1RF <i>pgsA</i> _{ind}	<i>E. faecalis</i> OG1RF <i>pgsA</i> inducible mutant harboring pGCP023:: <i>tetR</i> , the promoter of <i>pgsA</i> (OG1RF_12410) gene was modified with <i>srtA</i>	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml	This work

	(OG1RF_12327) promoter and tet operator		
<i>Enterococcus faecalis</i> OG1RF P _{srtA} -tetO-pgsA pGCP023::tetR-ΔKan	<i>E. faecalis</i> OG1RF pgsA inducible mutant harboring pGCP023::tetR-ΔKan, used to transform a second plasmid	Rif ^r , Fus ^r , 25 μg/ml; Cm ^r , 10 μg/ml	This work
<i>Enterococcus faecalis</i> OG1RF_11710::Tn pgsA _{ind}	<i>E. faecalis</i> OG1RF pgsA inducible mutant containing OG1RF_11710 transposon	Rif ^r , Fus ^r , 25 μg/ml; Cm ^r , 10 μg/ml and Kan ^r , 600 μg/ml	This work
<i>Enterococcus faecalis</i> OG1RF epaI::Tn pgsA _{ind}	<i>E. faecalis</i> OG1RF pgsA inducible mutant containing epaI (OG1RF_11730) transposon	Rif ^r , Fus ^r , 25 μg/ml; Cm ^r , 10 μg/ml and Kan ^r , 600 μg/ml	This work
<i>Enterococcus faecalis</i> OG1RF ΔcroRSΔcisRS pgsA _{ind}	<i>E. faecalis</i> OG1RF pgsA inducible mutant containing in-frame-deletion of croR, croS, cisR and cisS	Rif ^r , Fus ^r , 25 μg/ml; Cm ^r , 10 μg/ml and Kan ^r , 600 μg/ml	This work
<i>Enterococcus faecalis</i> OG1RF fabG2::Tn pgsA _{ind}	<i>E. faecalis</i> OG1RF pgsA inducible mutant containing fabG2 (OG1RF_11485) transposon	Rif ^r , Fus ^r , 25 μg/ml; Cm ^r , 10 μg/ml and Kan ^r , 600 μg/ml	This work
Plasmids			
pABG5	Shuttle vector	Cm ^r , 10 μg/ml and Kan ^r , 600 μg/ml (<i>E. faecalis</i>)	(Granok et al. 2000)
pGCP213	Shuttle vector temperature-sensitive plasmid for generation of mutants	Erm ^r , 500 μg/ml (<i>E. coli</i>); 25 μg/ml (<i>E. faecalis</i>)	(Nielsen et al. 2012)

pGCP213::P _{srtA-tetO} - <i>pgsA</i>	Derivative of pGCP213, containing 500 bp upstream of the -35 promoter region of <i>pgsA</i> , tet inducible promoter and 500 bp downstream of -10 promoter region of <i>pgsA</i>	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work
pGCP023::tetR	Derivative of pABG5, encoding tet repressor under <i>guaB</i> promoter	Cm ^r , 10 µg/ml and Kan ^r , 600 µg/ml (<i>E. faecalis</i>)	(Vega et al. 2013)
pGCP023::tetR-ΔKan	Derivative of pGCP023::tetR lacking Kan ^r cassette	Cm ^r , 10 µg/ml (<i>E. faecalis</i>)	This work
pAK1::srtA-2HA	Encodes C-terminal HA-tagged <i>srtA</i> under native <i>srtA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	(Kline et al. 2009) (Kandaswamy et al. 2013)
pAK1::srtC-2HA	Encodes C-terminal HA-tagged <i>srtC</i> under native <i>srtA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	This work
pGCP123::htrA-HA	Encodes C-terminal HA-tagged <i>htrA</i> under native <i>htrA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	(Yong 2019)
Abbreviations: Rif, rifampicin; Fus, fusidic acid; Kan, kanamycin; Erm, erythromycin; Cm, chloramphenicol Superscript “r” indicate resistance.			

3.3.2 Genetic manipulation and construction of *pgsA* inducible mutant

The *pgsA* inducible mutant *E. faecalis* OG1RF P_{srtA-tetO}-*pgsA* pGCP023::tetR (abbreviated to *E. faecalis* OG1RF *pgsA*_{ind}) constructed in **Chapter 2** was further characterized in this chapter.

E. faecalis OG1RF $P_{srtA-tetO-pgsA}$ pGCP023::*tetR*- Δ Kan is used only when a second plasmid is transformed into the *pgsA* mutant strain. Subsequently, a second plasmid, for example pAK1::*srtA*-2HA, pAK1::*srtC*-2HA or pGCP123::*htrA*-HA was transformed into *E. faecalis* OG1RF $P_{srtA-tetO-pgsA}$ pGCP023::*tetR*- Δ Kan to generate *E. faecalis* *pgsA*_{ind} pAK1::*srtA*-2HA, *E. faecalis* *pgsA*_{ind} pAK1::*srtC*-2HA and *E. faecalis* *pgsA*_{ind} pGCP123::*htrA*-HA, respectively.

The plasmid pGCP213:: $P_{srtA-tetO-pgsA}$ constructed in **Chapter 2** was transformed into the following strains OG1RF_11710::*Tn*, *epal*::*Tn*, Δ *croRS* Δ *cisRS*, OG1RF_10252::*Tn* and *fabG2*::*Tn* mutant, respectively. The *pgsA* inducible promoter was screened according to the method previously described in **Section 2.3.2**. Then the plasmid pGCP023::*tetR* was transformed into the resulted double mutant to construct *pgsA* inducible mutant in different chromosomal background.

The plasmid pAK1::*srtC*-2HA was constructed based on the shuttle vector pAK1::*srtA*-2HA, the *srtC* sequence was amplified based on the genome of *E. faecalis* OG1RF using primer pair pAK1-SrtC-F/ pAK1-SrtC-R (**Table 3.2**). The resulted PCR product was cloned into linearized plasmid via In-fusion (In-Fusion® HD Cloning Kit, Takara Bio, USA) cloning, in which the linearized plasmid was amplified from plasmid pAK1::*srtA*-2HA using primer pair pAK1-linear-F/ pAK1-linear-R (**Table 3.2**). The sequence of resulted plasmid pAK1::*srtC*-2HA was confirmed by PCR sequencing.

Table 3.2 Primers used for genetic manipulation in this chapter.

Primer name	Sequence (5'→3')
pAK1-SrtC-F	AAAAGGAGGGAAAATATGAAGTCAAAAAAGAAACG
pAK1-SrtC-R	AACATCGTATGGGTACTTTGGTTTTCTGGTCG
pAK1-linear-F	TACCCATACGATGTTCCAGATTACGCTTACCCATACGATG
pAK1-linear-R	ATTTTCCCTCCTTTTAATGTATG

3.3.3 Minimum bactericidal concentration (MBC) test

Cells were grown to exponential stage as described above and normalized to OD_{600 nm}=0.5 in 1 ml 0.01M phosphate (PB, PH7.2) buffer (Beisson et al. 2010). Cells were washed once in 0.01M PB buffer and suspended in 1 ml 0.01M PB buffer, followed by 500-fold dilution in 0.01M PB buffer to achieve colony-forming unit (CFU)= $\sim 2 \times 10^5$ /ml, and the antimicrobials stock were also diluted in 0.01M PB buffer. 100 μ l of the diluted cells were incubated with 100 μ l of decreasing concentrations of antimicrobials in 96-well plate at 37°C statically. After 2 hours incubation, the CFU of each well was measured by enumerated by dilution plating, and the MBC result was defined as the lowest concentration of antimicrobial in which all cells were dead. Experiments were performed with biological triplicates and technical triplicates.

3.3.4 Immunoblot and quantification

Cells were harvested from exponential phase as described above, and density was normalized to OD_{600 nm}=0.5 in 1 ml 0.01M PB buffer. Normalized cells were centrifuged to obtain a cell pellet. The cell pellet was incubated with 100 μ l of 10 mg/ml of lysozyme (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour. After lysozyme treatment, the whole cell lysates were added with 33 μ l of NuPAGE® LDS Sample Buffer (4 $\times$) and 10 μ l of 1M dithiothreitol (DTT). Then samples were boiled at 100°C for 20 mins and cooled in ice. Subsequently, 10 μ l of samples and 3 μ l of PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermos Scientific, USA) were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The samples were loaded onto NuPAGE Novex 4-12% Bis-Tris gels in NuPAGE MOPS SDS running buffer and run in the XCell SureLock Mini-Cell Electrophoresis System (Thermos Scientific, USA) at 200V for 50 mins. Subsequently, proteins from the gels were transferred to membrane using iBlot® Transfer Stacks (Life technologies Corp. USA). The membrane was then blocked with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) with 0.1% Tween 20 (PBST) at 4°C for 1 hour and then incubated with primary antibodies at 4°C for overnight. The antibodies and respective concentrations (diluted in 5% BSA in PBST) used in this chapter were described in **Table 3.3**. The next day, the membrane was washed with PBST for 3 times and incubated with Pierce™ horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature in dark for 1 hour. Then, the membrane was washed with PBST for 3 times and incubated with Super Signal West Femto substrate (Thermos Fisher Scientific,

Inc., USA) for 5 mins to develop the membrane. Subsequently, the membrane was exposed to the Green X-ray film (Care stream, USA) with a Kodak processor (Kodak X-OMAT processor 2000) in dark room. The film was captured, and the images were quantified with image J software.

Table 3.3 Antibodies used for immunoblotting assay in this chapter

Protein of interest	Primary antibody concentration	Host	Secondary antibody concentration
SecA	1:3000	Rabbit	1:6000
SrtA	1:200	Mouse	1:1250

3.3.5 Immunofluorescence microscopy (IFM)

Cells were harvested from exponential phase as described above, and optical density was normalized to OD_{600 nm}=1.0 in 1 ml 0.01M PB buffer. After washing once with 0.01M PB buffer once, cells were fixed in 1 ml 4% paraformaldehyde (PFA) for 20 mins at 4°C. After fixation, cells were washed once with 0.01M PB buffer, resuspended in 1 ml 0.01M PB buffer, and diluted at a 1:4 ratio by adding 250 µl of fixed cells to 750 µl of PB buffer. Then, 20 µl of diluted cells were smeared onto the ethanol and water-washed Poly-L-Lysine pre-coated slides and dried in a hybridization oven at 45°C. Next, cells were permeabilized with 20 µl of 10 mg/ml of lysozyme (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour, washed 3 times with PB buffer, and dried in the hybridization oven. Cells were permeabilized again with 20 µl 0.1% Triton in PB at 37°C for 10 mins, washed 3 times with PB buffer, and dried. Cells were incubated with 20ul blocking buffer 2% bovine serum albumin in PB (2% BSA-PB) for 20 mins at room temperature, washed 3 times with PB buffer, and dried. Subsequently, cells were smeared with 20 µl primary antibody anti-HA raised in rabbit (1:500 dilution in 2% BSA-PB) and incubated at 4°C overnight. After primary antibody incubation, cells were washed 3 times with PB buffer and dried. Subsequently, cells were smeared with 20 µl fluorescence conjugated secondary antibody Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-rabbit IgG (1:1000 dilution in 2% BSA-PB) and incubated in the dark at room temperature for 1 hour. Then, cells were

washed 3 times with PB buffer, dried, and smeared with 5 μ l VectaShield mounting medium. The slides were visualized by an inverted widefield epifluorescence microscope (Zeiss Axio Observer.Z1, Germany), using 100x oil immersion objective. Phase contract and florescent and merged images were captured using AxioVision software and analyzed by Matlab software. The Projected System of Internal Coordinates from Interpolated Contours (PSICIC) (Werner et al. 2009) was used to quantify the mean fluorescence intensity at the perimeter of *E. faecalis* cells at the early division stage, in which cell perimeter is between 3.6–6 μ m.

3.3.6 Transmission electron microscopy (TEM)

Cells were harvested from exponential phase as described above, and optical density was normalized to $OD_{600\text{ nm}}=10$ in 1 ml PBS. Cells were fixed in 2.5% glutaraldehyde in PBS (pH7.4) at 4°C overnight. After fixation, cells were washed twice with PBS for 20 mins, and post fixed in 1% osmium tetroxide and 0.01% potassium ferrocyanide at room temperature for 2 hours. Cells were then pelleted in gelatin to solidify and trimmed into 2 mm³ cubes before being dehydrated in an ethanol series (25, 50, 75, 95, and 100%) and acetone. Sample cubes were placed into embedding molds filled with araldite and allowed to polymerize at 60°C for 24 hours. Ultrathin sections of 100 nm thick were sectioned using a Leica Ultracut E ultramicrotome and contrasted with lead citrate before imaging under a Jeol JEM-1010 transmission electron microscope at 100kV.

3.3.7 Cell wall peptidoglycan cross-link analysis

A single colony was inoculated into 5 ml media at 37°C overnight, and a 1ml overnight culture was inoculated in 500 ml fresh media and grown at 37°C until optical density $OD_{600\text{ nm}}=0.7$. Cell pellets were harvested, suspended in 10 ml water, and frozen in liquid nitrogen. The cell suspension was then placed in a boiling water bath prior to addition of 8 ml boiling 8% SDS. After boiling for 30 mins, cells were cooled and washed with water 5 times. Samples were analyzed for cross-linking of cell wall peptidoglycan (PGN) according to the protocol by Smith et al (Smith et al. 2019). Briefly, peptidoglycan from sample was extracted, digested by mutanolysin, and reduced. Disaccharide-peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) and the relative abundance of mucopeptides was quantified using UV absorbance at 202nm.

3.3.8 Fluorescent d-amino acids (FDAA) staining

Cells were harvested from exponential phase as described above, and optical density was normalized to $OD_{600\text{ nm}}=0.5$ in 1 ml fresh media. Cells were incubated with 1ml fresh media supplemented with 10 μ l BODIPY-FL 3-amino-d-alanine (BADA) in a 37°C shaking incubator for 1 hour and then washed 3 times with PBS buffer. Subsequently, cells were incubated with 1 ml fresh media supplemented with 10 μ l TAMRA 3-amino-d-alanine (TADA) in a 37°C shaking incubator for 1 hour and washed 3 times with PBS buffer. Cells were suspended in 1 ml PBS and 20 μ l cells were smeared on Poly-L-Lysine pre-coated slides and observed by Zeiss Elyra PS.1 super resolution microscope (Zeiss, Germany).

3.3.9 Real-time quantitative PCR (qPCR)

Cells were grown to exponential phase as described above and harvested. RNA extraction was performed according to the manufacture's protocol using Pure Link™ RNA Mini Kit (Thermo Scientific, USA). RNA samples were treated with TURBO DNA-free™ kit (Invitrogen™, USA) to remove DNA from RNA samples and purified with Agencourt RNA clean XP (Beckman Coulter, USA) magnetic beads. RNA concentration and DNA contamination were measured using Qubit™ RNA HS Assay Kit (Thermo Scientific, USA) and Qubit™ dsDNA HS Assay Kit (Thermo Scientific, USA), respectively. The RNA samples with less than 10% DNA contamination were screened using Tape Station (Agilent Technologies, USA) to measure RIN values. Reverse transcription (RT)-PCR was performed according to manufacturer's instruction using Super Script® III First-Strand Synthesis System for RT-PCR (Invitrogen™, USA). Random hexamer primers provided in the kit were used for cDNA synthesis in this work. The cDNA samples were stored at -20°C for subsequent analysis.

Real-time quantitative PCR (qPCR) was performed according to the manufacturer's instruction using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (Kapa biosystem, USA). Primer pairs (**Table 3.4**) used for targeting genes and the housekeeping gene 16s rRNA (16s) in qPCR were synthesized from Integrated DNA Technologies (IDT). The qPCR reactions were performed in the Step One Plus™ Systems (Life Technologies, USA) and the cycling program was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and finally annealing and extension at 60°C for 30 seconds.

Table 3.4 Primers used for qPCR in this chapter

Primer name	Sequence (5'→3')
16s_F	GGATAACACTTGGAAACAGG
16s_R	TCCTTGTTCTTCTCTAACAA
qPCR-croR-F	TCCAAATTGCACACAACACC
qPCR-croR-R	TTCACCCGAGCCATTACTTC
qPCR-11710-F	TCGTTGTCTTGTGCGACTTC
qPCR-11710-R	CGGGTGCAATTTTGGTAGT
qPCR-epaI-F	TTCTGGCTCGGGATAATTG
qPCR-epaI-R	TTTTCGATTGGCTCTCGTTT

3.3.10 RNA sequencing

Cells were grown to exponential stage as described above and harvested. RNA extraction was performed according to the manufacture's protocol using Pure Link™ RNA Mini Kit (Thermo Scientific, USA). RNA samples were treated with TURBO DNA-free™ kit (Invitrogen™, USA) to remove DNA from RNA samples and purified with Agencourt RNA clean XP (Beckman Coulter, USA) magnetic beads. RNA concentrations and DNA contaminations were measured using Qubit™ RNA HS Assay Kit (Thermo Scientific, USA) and Qubit™ dsDNA HS Assay Kit (Thermo Scientific, USA), respectively. The RNA samples with less than 10% DNA contamination were screened using Tape Station (Agilent Technologies, USA) to measure RIN value. Subsequently, RNA samples were treated with Ribo-Zero® rRNA Removal Kit (Illumina, USA) to delete ribosomal RNA and purified with Agencourt RNA clean XP (Beckman Coulter, USA) magnetic beads. The rRNA-depleted RNA was used for cDNA synthesis, using NEB Next Ultra II RNA First Strand Synthesis Module (Thermo Scientific, USA) and NEB Next Ultra II Directional RNA Second Strand Synthesis Module (Thermo Scientific, USA). The cDNA was purified using Agencourt AMPURE XP beads XP (Beckman Coulter, USA) and sequenced using an Illumina MiSeq sequencer (MiSeq V2 Run, 150 bp pair end) by the sequencing facility of Singapore Centre of Life Science Engineering

(SCELSE) in Singapore. After checking with Fast QC and adapter trimming using bbdut from the BBMap tools, the trimmed sequencing reads were mapped to the *E. faecalis* OG1RF (NCBI accession: GCF_000172575.2) reference genomes using Burrows-Wheeler Alignment tool (Li and Durbin 2009). Reads mapping to the predicted open reading frames of reference genome were quantified using HTSeq (Anders et al. 2015). Subsequently, the sequencing results were checked with FastQC and adapter trimmed using bbdut from the BBMap tools and data were analyzed in the R environment using Bioconductor package, *edgeR* (Robinson et al. 2010). False discovery rate (FDR) < 0.05 and P value <0.05 were chosen to filter the significant differentially expressed genes. Gene annotation and related pathway analysis were performed using the R package cluster Profiler (Yu et al. 2012) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

3.3.11 Statistical analysis

All experiments were performed at least three biological replicates and the mean value was calculated and is presented. All graphs indicate the standard deviation from independent experiments (and not technical replicates). Statistical analysis was performed by Graphpad software using either one-way ANOVA, two-way ANOVA or unpaired t-test unless stated otherwise. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, ns: $p > 0.05$).

3.4 Results

3.4.1 PG is involved in antimicrobial resistance

Based on the growth defect of *pgsA*_{ind} mutant at low concentrations of ATc, we hypothesized that PG is depleted at low concentrations of ATc, and subsequently would be more sensitive to cell membrane targeting antimicrobials since PG is a principle element of cell membrane. To test this, we assessed the MBC for different cell membrane targeting antimicrobials, human β -defensin 2 (hBD2) and colistin, for the *pgsA*_{ind} mutant at decreasing concentrations of ATc. At the same time, we also determined the MBC for the *pgsA*_{ind} mutant for the cell wall targeting antimicrobials human β -defensin 3 (hBD3) and ramoplanin, with the prediction that PG depletion would not affect the MBC of cell wall targeting antimicrobials but would affect the MBC for membrane targeting antimicrobials. As we predicted, the MBCs of hBD2 and colistin were lower for the mutant at low concentrations of ATc (**Table 3.5**) and remained unchanged at high concentrations of ATc when compared to WT without ATc. Surprisingly, the MBCs for the cell wall targeting antimicrobials hBD3 and ramoplanin were higher for the mutant at low concentrations of ATc compared to WT (**Table 3.5**). The altered sensitivity to different antimicrobials suggests that PG contributes in multiple ways to antimicrobial resistance.

Table 3.5 MBC results of *E. faecalis pgsA*_{ind} against different antimicrobials.

Strains	ATc concentration (ng/ml)	MBC(μ g/ml)			
		Cell membrane targeting		Cell wall targeting	
		hBD2	Colistin	hBD3	Ramoplanin
WT	0	>50	64	1	2
<i>pgsA</i> _{ind}	16	>50	64	1	2
<i>pgsA</i> _{ind}	2	>50	64	1	2
<i>pgsA</i> _{ind}	0.5	>50	16	2	4
<i>pgsA</i> _{ind}	0.25	50	16	4	4

3.4.2 Sortase foci are delocalized in *E. faecalis* *pgsA*_{ind} at low ATc

To explore the relationship between PG levels and virulence factor assembly sites, we studied the localization of membrane proteins sortase A (SrtA) and sortase C (SrtC) in *pgsA*_{ind} because *E. faecalis* primarily relies on these sorting pathways for virulence factor assembly. Based on the growth defect and aberrant morphology in *E. faecalis* *pgsA*_{ind} at low ATc concentrations, we questioned whether SrtA and SrtC localization was similarly affected by PG depletion under the same conditions. To test this, we constructed *E. faecalis* *pgsA*_{ind} pAK1::*srtA*-2HA to visualize HA tagged SrtA localization via immunofluorescence microscopy (IFM). Exponentially growing cells harvested from media supplemented with different ATc concentrations were examined for SrtA localization and visualized using an α -HA antibody. In the control sample, SrtA foci were observed at the septa of WT cells at all concentrations of ATc (**Figure 3.1A**), suggesting that the ATc concentrations used in IFM did not influence SrtA localization in *E. faecalis*. SrtA foci were localized at the septa in *pgsA*_{ind} after growth in ATc 16 ng/ml and 2 ng/ml (**Figure 3.1B**), similar to WT. In the *pgsA*_{ind} cells from ATc 0.5 ng/ml and 0.25 ng/ml, similar SrtA foci were observed in the cells with WT-like morphology, while in the cells which were wider or longer (indicated by green arrowheads), SrtA foci were not observed and instead were delocalized in *pgsA*_{ind} at low ATc concentrations, indicating that PG levels are involved in normal SrtA focal localization.

Figure 3.1 SrtA localization in *E. faecalis* pgsA_{ind} is altered at low ATc concentrations. SrtA-HA localization was visualized via IFM using an anti-HA antibody in log phase cultures. Representative images are shown. (A) *E. faecalis* pAK1::srtA-2HA and (B) *E. faecalis* pgsA_{ind} pAK1::srtA-2HA after growth at different ATc concentrations, scale bar =2 μm. The green arrowheads are indicating altered SrtA localization.

To test whether SrtC localization was similarly affected by PG depletion under the same conditions, SrtC localization was visualized via IFM as described above in *E. faecalis* pgsA_{ind} pAK1::srtC-2HA. In the control group, SrtC foci were observed at the septa of WT cells grown in all concentrations of ATc (**Figure 3.2A**), suggesting that ATc concentrations used in this experiment did not affect SrtC localization in *E. faecalis*. Similarly, SrtC foci were observed at the septa of pgsA_{ind} at ATc 16 ng/ml and 2 ng/ml (**Figure 3.2B**), suggesting that SrtC localization in pgsA_{ind} was not altered at high ATc concentrations, compared to WT. In the pgsA_{ind} cells at ATc 0.5 ng/ml and 0.25 ng/ml, similar SrtC septal localization was observed in the cells with WT-like morphology, whereas multiple foci were observed and randomly localized in the morphology altered pgsA_{ind} cells, suggesting that SrtC localization was altered at low ATc concentrations. Taken together, these data indicate that PG levels are also involved in SrtC focal localization.

Figure 3.2 SrtC localization in *E. faecalis* pgsA_{ind} is altered at low ATc concentration. SrtC-HA localization was visualized via IFA using an anti-HA antibody. Representative images are shown. (A) *E. faecalis* pAK1::srtC-2HA and (B) *E. faecalis* pgsA_{ind} pAK1::srtC-2HA after growth at different ATc concentrations, scale bar = 2 μm.

To examine the relationship between SrtA expression and SrtA localization, we performed western blot (Nielsen et al. 2013) to quantify SrtA protein levels in pgsA_{ind} cells grown in different ATc concentrations. Cells harvested after growth in different ATc concentrations were used for western blot analysis using antibodies raised either against SecA or SrtA. To quantify SrtA expression in different ATc concentrations, SrtA band intensity was normalized to SecA band intensity for the same sample. Normalized SrtA intensity of pgsA_{ind} at 16 ng/ml and 2 ng/ml was not significantly changed ($p > 0.05$) compared to WT (**Figure 3.3**), whereas SrtA intensity was significantly decreased in pgsA_{ind} at ATc 0.5 ng/ml ($p < 0.01$) and 0.25 ng/ml ($p < 0.01$), suggesting that SrtA levels are affected by PG levels in the cells.

Figure 3.3 SrtA expression is decreased in *E. faecalis* *pgsA_{ind}* at low ATc concentrations. Relative western blot quantification of SrtA protein in WT or *pgsA_{ind}* harvested after growth in decreasing ATc concentrations. Cells were harvested and lysed for western blot analysis using antibody raised either against SecA or SrtA. (A) Representative images of western blot data used to quantify SrtA protein expression levels. (B) Quantification of normalized SrtA to SecA levels for the same sample is shown. All data graphed are the mean of three independent biological replicates, error bars represent standard error of the mean (SEM). Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, the symbol ** indicates p-value <0.01.

Since both SrtA and SrtC localization was altered in *pgsA_{ind}* at low ATc concentrations, we questioned whether the localization of other membrane proteins was also affected in *pgsA_{ind}* at low ATc concentrations. To test this, we performed IFM to visualize another membrane protein HtrA (high temperature requirement A) localization in *pgsA_{ind}* pGCP123::HtrA-HA. Similarly, HtrA foci were observed at the septa in WT cells at all ATc concentrations (**Figure 3.4A**). HtrA foci were observed at the septa of *pgsA_{ind}* at ATc 16 ng/ml and 2 ng/ml and in *pgsA_{ind}* cells with WT-like morphology at ATc 0.5 ng/ml and 0.25 ng/ml (**Figure 3.4B**). However, multiple paired foci were observed in *pgsA_{ind}* cells with altered morphology at ATc 0.5 ng/ml

and 0.25 ng/ml. We speculate that the multiple paired HtrA foci are coincident with the septa, as they are for WT cells, but in the morphology-altered cells where there are multiple septa, HtrA foci are now coincident with multiple septa. Therefore, these data suggest that PG levels may not be directly involved in HtrA focal localization in *E. faecalis*.

Figure 3.4 HtrA is localized at septum in *E. faecalis* *pgsA*_{ind} at different ATc concentration. HtrA-HA was visualized via IFM using an anti-HA antibody. Representative images of HA labeled *E. faecalis* *pgsA*_{ind} pAK1::htrA-HA are shown. (A) *E. faecalis* pGCP123::htrA-HA and (B) *E. faecalis* *pgsA*_{ind} pGCP123::htrA-HA after growth at different ATc concentrations, scale bar =2 μm.

3.4.3 Cell wall is thicker in *pgsA*_{ind} at low ATc concentrations

We observed that cell morphology was altered in *pgsA*_{ind} at low ATc concentrations (**Figure 2.10**) by widefield microscopy, we performed transmission electron microscopy (TEM) to further visualize the cell morphology of *pgsA*_{ind} at different ATc concentrations, compared to WT. Cells were harvested at exponential phase after growth in media supplemented with different ATc concentrations. Sectioned cells were imaged, and the percentage of cell wall altered cells in each group was measured from 100 total cells. Similar to WT, *pgsA*_{ind} at ATc

16 ng/ml and 2 ng/ml displayed uniform diplococcal shape with a clear membrane (black line) and cell wall (gray layer) (**Figure 3.5A-C**). However, *pgsA*_{ind} grown at ATc 2 ng/ml and 0.25 ng/ml displayed irregular and incomplete cell division, and multiple cells without obvious cell separation were apparent (**Figure 3.5D-E**), which was consistent with the increased width-length ratio measured in a subpopulation of cells from these growth conditions (**Figure 2.10**). The cell wall of the morphology-altered cells was much thicker than in *pgsA*_{ind} cells displaying a WT-like morphology, and lacked a clear cell wall layer structure, suggesting that the cell wall was altered in *pgsA*_{ind} at low ATc concentrations. The percentage of *pgsA*_{ind} cells with thicker cell wall in each ATc concentration, compared to WT, was measured (**Figure 3.5F**), and the percentage of cells with thicker cell wall was increased at low ATc concentrations.

Figure 3.5 Cell wall is thicker in morphology-altered *E. faecalis* *pgsA_{ind}* cells after growth in low ATc concentrations. Representative images from TEM of (A) WT and *pgsA_{ind}* at ATc concentrations (B) 16 ng/ml, (C) 2 ng/ml, (D) 0.5 ng/ml, (E) 0.25 ng/ml, scale bar = 0.2 μm. (F) The percentage of cells with thicker cell wall in *pgsA_{ind}* at different ATc concentrations, based on blinded visual analysis by two independent viewers, compared with WT. One hundred cells in each condition were quantified.

3.4.4 Cell wall synthesis in *E. faecalis* *pgsA*_{ind} is altered at low ATc concentration

E. faecalis *pgsA*_{ind} at low ATc concentrations had thicker cell wall and became more resistant to cell wall targeting antimicrobials (**Table 3.5**). The cell wall of Gram-positive bacteria is a thick layer consisting several layers of cross-linked peptidoglycan (Smith et al. 2019). To measure whether the cross-linking in *pgsA*_{ind} at low ATc concentrations was increased as the cell wall was thicker, we performed cell wall peptidoglycan (PGN) cross-link analysis of *pgsA*_{ind} at different ATc concentrations, compared to WT. The normalized mucopeptides abundance showed that all strains displayed a very similar mucopeptides profile suggesting no major changes in mucopeptides compositions (**Figure 3.6A**). Surprisingly, we found that the relative abundances of crosslinked mucopeptides fragments (dimers, trimers and tetramers) in *pgsA*_{ind} were decreased at low ATc concentration, but not significantly altered at high ATc concentration, compared to WT (**Figure 3.6B**). The data suggested that cell wall of *pgsA*_{ind} at low ATc concentrations were less crosslinked, compared to WT.

Figure 3.6 Cell wall peptidoglycan of *pgsA_{ind}* at low ATc concentration was less crosslinked. Peptidoglycan (PGN) was extracted, digested with mutanolysin, reduced, and disaccharide-peptides were separated by reverse-phase HPLC. (A) Representative image of mucopeptides profiles from different samples are shown, including WT (black), *pgsA_{ind}* at ATc 16 ng/ml (purple), and *pgsA_{ind}* at ATc 0.25 ng/ml (red), in which the purple trace and red trace are shifted in parallel for visualization. The peaks of uncrosslinked mucopeptides (monomers) and differently crosslinked mucopeptides (dimers, trimers and tetramers) are indicated. (B) Relative abundance of crosslinked mucopeptides in different samples. Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, N = 3 independent biological replicates. Error bars represent SEM, the symbol **** indicates p-value <0.0001.

Meanwhile, to explore whether cell wall synthesis in *pgsA_{ind}* was altered after growth in low ATc concentrations, we performed cell wall staining using PGN targeting fluorescent d-amino acids (FDAA) (Hsu et al. 2017). FDAA dyes stain newly synthesized cell wall during cell growth because the D-Ala-like FDAA molecule covalently incorporates into the bacterial cell wall at sites of active penicillin-binding protein (PBP) transpeptidase activity. Cells harvested from mid-exponential phase were incubated with green-emission probes, BODIPY-FL 3-amino-d-alanine (BADA) for 1 hour, followed by yellow-to-orange emission probe, TAMRA 3-amino-d-alanine (TADA) for 1 hour. Old cell wall, synthesized in the first hour was stained by the green FDAA probe, BADA, and new cell wall synthesized in the second hour was stained by the red FDAA probe, TADA. In the *pgsA_{ind}* cells grown at ATc 16ng/ml and WT cells at all ATc concentrations, we observed that one half of a coccoid was always older than the other (**Figure 3.7A-C**), suggesting that the green half of the coccoid is more matured than the other red half. However, two populations were found in *pgsA_{ind}* at 0.25 ng/ml ATc (**Figure 3.7D**), in which one population contains similar cell wall synthesis pattern compared to WT. In the other population, multiple BADA and TADA foci were observed indicating that cell division is defective in this population, resulting in cell wall synthesis not initiated at mid cell. This is consistent with our observation that there are two distinct populations displaying different cellular morphology when *pgsA_{ind}* is grown at low ATc concentrations.

Figure 3.7 Cell wall deposition pattern is aberrant in *E. faecalis* $pgsA_{ind}$ at low concentrations of ATc. Cells were labeled with BADA (green; old PGN synthesis) for 1h, washed, and then labeled with TADA (red; new PGN synthesis). Representative images of *E. faecalis* WT at ATc concentration (A) 16 ng/ml, (B) 0.25ng/ml, and *E. faecalis* $pgsA_{ind}$ at ATc concentration (C) 16 ng/ml, (D) 0.25ng/ml, scale bar = 2 μ m.

3.4.5 Identification of cell response in *E. faecalis* $pgsA_{ind}$ at low ATc concentrations

Since we observed that cell growth and cell morphology were altered in $pgsA_{ind}$ at low ATc concentrations, we interrogated the cell response of *E. faecalis* $pgsA_{ind}$ at low ATc concentrations, using RNA sequencing to measure gene regulation profile of $pgsA_{ind}$ at ATc 0.25 ng/ml, compared to $pgsA_{ind}$ at ATc 16 ng/ml. Significantly differentially regulated genes

were determined by filtering P value < 0.05 and false discovery rate (FDR) < 0.05. Then the significantly differentially regulated genes (105 genes) were grouped into categories based on their functions and pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (**Appendix Figure 1**). The transcriptional data revealed that several gene categories were upregulated in *pgsA*_{ind} at ATc 0.25 ng/ml (**Figure 3.8**).

Figure 3.8 Transcriptional gene expression profile of *pgsA*_{ind} at ATc 0.25 ng/ml, compared to *pgsA*_{ind} at ATc 16 ng/ml. Significantly differentially regulated genes in corresponding functional categories were determined by the Bioconductor package EdgeR (P < 0.05; FDR < 0.05). Data is plotted as log₂ fold change (log₂FC), and the median value of each functional category is indicated by the short black line. Data graphed here are the mean from three independent biological replicates.

Since we observed thick cell wall in *pgsA*_{ind} at low ATc concentrations, we first looked at the cell envelope related genes or gene clusters. We observed that cell envelope related genes were upregulated, such as glycosyltransferases which are involved in enterococcal polysaccharide antigen (Epa) biosynthesis, D-alanine metabolism (involved in D-alanylation of cell wall teichoic acid) and glycan biosynthesis and metabolism (involved in peptidoglycan synthesis and cross-linking) (**Table 3.6**). Secondly, to identify any two-component systems (TCS) that might respond to the low ATc concentration in *pgsA*_{ind}, and since some TCS genes are differentially regulated upon activation, we also looked at any significantly differentially regulated TCS genes, and we found *croR*, *croS* and *yxdK* were upregulated in *pgsA*_{ind} at ATc 0.25 ng/ml (**Table 3.6**).

Table 3.6 Expression of differentially regulated cell envelope related genes in *pgsA*_{ind} at ATc 0.25 ng/ml^a.

Gene locus	Log ₂ FC	Symbol	Annotation
OG1RF_11710	3.43	- ^b	polymerase
OG1RF_11724	2.33	<i>epaO</i>	glycosyl transferase family 2
OG1RF_11727	2.76	<i>epaL</i>	ABC transporter permease
OG1RF_11728	3.13	<i>epaK</i>	hypothetical protein
OG1RF_11730	1.88	<i>epaI</i>	group 2 glycosyl transferase
OG1RF_11735	3.02	<i>epaD</i>	alpha-L-Rha alpha-1,3-L-rhamnosyltransferase
OG1RF_11736	2.70	<i>epaC</i>	glycosyl transferase family 2
OG1RF_11737	3.12	<i>epaB</i>	glycosyl transferase family 2
OG1RF_11738	2.74	<i>epaA/mraY2</i>	undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase
OG1RF_12109	3.68	<i>dltD</i>	D-alanyl-lipoteichoic acid biosynthesis protein
OG1RF_12110	3.50	<i>dltC</i>	D-alanine--poly(phosphoribitol) ligase subunit 2
OG1RF_12111	3.53	<i>dltB</i>	D-alanyl-lipoteichoic acid biosynthesis protein
OG1RF_12112	3.31	<i>dltA</i>	D-alanine--poly(phosphoribitol) ligase
OG1RF_10281	1.90	-	serine protease
OG1RF_10328	3.52	-	peptidase M23, peptidoglycan binding protein
OG1RF_10417	2.28	<i>pbp2A</i>	penicillin-binding protein 2A
OG1RF_11870	3.08	<i>uppP</i>	undecaprenyl-diphosphatase

OG1RF_12030	1.90	<i>femA</i>	methicillin resistance factor FemA
OG1RF_12158	2.45	<i>penA</i>	penicillin-binding protein 2B
OG1RF_12331	3.16	-	lipase
OG1RF_12535	2.59	<i>croR</i>	DNA-binding response regulator
OG1RF_12536	2.60	<i>croS</i>	two-component sensor histidine kinase
OG1RF_10654	1.63	<i>yxdK</i>	two-component sensor histidine kinase
<p>^a: significantly differentially regulated genes are classified into different categories and labeled with different colors: enterococcal polysaccharide antigen (green); D-alanylation of teichoic acid (blue); peptidoglycan associated (orange); two-component system (pink)</p> <p>^b: “-” indicates the gene symbols are not identified</p>			

Both the thicker cell wall phenotype and the RNA sequencing revealed cell wall compensation upon the cell membrane deficiency in *pgsA*_{ind} at low ATc concentrations. The upregulated TCS serve as stimulus-responses to many cell stresses such as cell morphology and environmental stress (Skerker et al. 2005, Tomaras et al. 2008). Thus, we hypothesized that deficiency of these cell envelope related and TCS genes would make *pgsA*_{ind} more sensitive to the inducer ATc (ie *pgsA*_{ind} which cannot compensate via cell wall alterations or TCS responses would be less viable upon *pgsA* depletion). To address this, we identified transposon mutants in genes involved in Epa biosynthesis: OG1RF_11710::*Tn* and *epaI*::*Tn* in the *E. faecalis* OG1RF mariner transposon library (Kristich et al. 2008, Dale et al. 2018) and transformed pGCP213::*P_{srtA}-tetO-pgsA* into the transposon mutants to construct double *pgsA* inducible mutants. Even though CroR activity can be phosphorylated by CroS, there is a cross-talk between CroRS and another TCS CisRS (Kellogg and Kristich 2016). Thus, we constructed *pgsA* inducible mutant in a Δ *croRS* Δ *cisRS* background. Before the construction of double *pgsA* mutant, we confirmed the increase of gene expression by qPCR (**Figure 3.9**), expression of OG1RF_11710::*Tn*, *epaI* and *croR* was increased in *pgsA*_{ind} at 0.25 ng/ml, compared to 16 ng/ml.

Figure 3.9 Relative expression of OG1RF_11710, *epaI* and *croR* is increased in *pgsA*_{ind} at 0.25 ng/ml, compared to 16 ng/ml. Real time qPCR analysis was performed to quantify relative gene expression. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol **** indicates p-value <0.0001

After construction of double *pgsA* mutant in the background described above, we performed growth kinetic assays to measure the growth of these *pgsA* double mutants, *E. faecalis* OG1RF_11710::*Tn pgsA*_{ind}, *E. faecalis epaI*::*Tn pgsA*_{ind}, and *E. faecalis ΔcroRSΔcisRS pgsA*_{ind} under different concentrations of ATc, compared with *E. faecalis pgsA*_{ind}. The growth curve revealed that neither these three *pgsA* double mutants nor the *pgsA*_{ind} mutant were able to replicate in the absence of ATc (**Figure 3.10A-D**). However, contrary to our hypothesis, the growth of each *pgsA* double mutant was actually better than the *pgsA*_{ind} single mutant which displayed an extended lag phase when grown at ATc 0.25 ng/ml, which was not apparent in the double mutants (**Figure 3.10B-D**), suggesting that the *pgsA* double mutants were more resistant ATc depletion.

Figure 3.10 Growths of *pgsA*_{ind} in other background are inhibited at low ATc concentrations. Cells were grown in decreasing concentrations of ATc in a 96-well plate. Absorbance readings were measured at every 0.5-hour interval. Growth kinetics of different strains (A) *E. faecalis* *pgsA*_{ind}, (B) *E. faecalis* OG1RF_11710::Tn *pgsA*_{ind}, (C) *E. faecalis* *epal*::Tn *pgsA*_{ind}, (D) *E. faecalis* Δ *croRS* Δ *cisRS* *pgsA*_{ind} in decreasing concentrations of ATc. Data graphed are the mean from three independent biological replicates.

Meanwhile, to test whether the morphologies of these *pgsA* double mutants were altered, we visualized these *pgsA* double mutant cells grown at ATc 0.25 ng/ml using widefield microscopy. Similar to *pgsA*_{ind}, we observed that these *pgsA* double mutants displayed WT-like morphology at high ATc concentration of 16 ng/ml (**Figure 3.11**). However, the morphologies of the *pgsA* double mutants at ATc 0.25 ng/ml were not significantly altered compared to *pgsA*_{ind}. Instead, the *pgsA* double mutants at ATc 0.125 ng/ml displayed longer and wider morphology, confirming that the *pgsA* double mutants were more resistant ATc limitation.

Figure 3.11 Morphology alteration of *pgsA* double mutants required lower ATc concentrations, compared to *pgsA_{ind}*. Representative images of (A) *E. faecalis pgsA_{ind}*, (B) *E. faecalis* OG1RF_11710::Tn *pgsA_{ind}*, (C) *E. faecalis epal::Tn pgsA_{ind}*, (D) *E. faecalis* Δ *croRS* Δ *cisRS pgsA_{ind}* at different ATc concentrations are shown, in which the *E. faecalis pgsA_{ind}* at ATc 0.125 ng/ml is not shown because *pgsA_{ind}* was not able to grow at ATc 0.125 ng/ml.

Subsequently, to identify the global gene expression profile of *pgsA_{ind}* at different ATc concentrations compared to expression in the WT chromosomal background at matching ATc

concentrations to control for ATc effects, we performed another RNA sequencing analysis of *pgsA*_{ind} at ATc 16 ng/ml and 0.25 ng/ml, compared to WT *ptetR* at the same ATc concentrations, respectively. Significantly differentially regulated genes were again determined by filtering P value < 0.05 and false discovery rate (FDR) <0.05. We identified 1141 genes which were significantly differentially regulated in *pgsA*_{ind}, compared WT *ptetR* to at ATc 16 ng/ml, and 1468 genes were identified at ATc 0.25 ng/ml, in which 496 genes were unique regulated in *pgsA*_{ind} at 0.25 ng/ml. The significantly regulated genes were grouped into categories based on their functions and pathways using the KEGG database. Then we summarized the top most abundant gene categories at each ATc concentrations. The transcriptomic data revealed that metabolic related categories, quorum sensing, aminoacyl-tRNA biosynthesis, and ABC transporters were significantly differentially regulated in *pgsA*_{ind} at ATc 16 ng/ml (**Figure 3.12A**), whereas the ribosome, fatty acid biosynthesis and fatty acid metabolism related categories were significantly differentially regulated in *pgsA*_{ind} at ATc 0.25 ng/ml(**Figure 3.12B**), compared to WT *ptetR* at same ATc concentrations, respectively.

Figure 3.12 Top KEGG categories significantly differentially regulated in *pgsA*_{ind} at ATc (A) 16 ng/ml and (B) 0.25 ng/ml. The size of the circle represents the number (count) of genes significantly regulated in the respective KEGG category. GeneRatio (x-axis) represents the ratio of the number of genes differentially regulated in each KEGG category over the total number of unique genes in the same KEGG category. The color of the circle represents the adjust p-values.

In summary, at ATc 16 ng/ml, *pgsA*_{ind} showed metabolism related categories were most differentially regulated, whereas *pgsA* transcription was significantly decreased (**Figure 2.14**) and PG levels were not significantly altered (**Figure 2.15**). However, at ATc 0.25 ng/ml, ribosome and fatty acid related genes were significantly differentially regulated, whereas cell growth defects (**Figure 2.5**) and cell division defects (**Figure 2.10** and **Figure 3.7**) were observed. Thus, we hypothesized changes in fatty acid lipid biosynthesis was a compensation upon membrane lipid deficiency in *pgsA*_{ind} at ATc 0.25 ng/ml. To test the hypothesis, we identified fatty acid biosynthesis mutants the *E. faecalis* OG1RF mariner transposon library (Kristich et al. 2008, Dale et al. 2018) and constructed *pgsA* inducible mutant in the *fabG2::Tn* mutant background ($\log_2FC=1.58$ in *pgsA*_{ind} at ATc 0.25 ng/ml). We predicted that the *pgsA* double mutant *fabG2::Tn pgsA*_{ind} would be more sensitive to ATc limitation.

After construction of *fabG2::Tn pgsA*_{ind}, we performed growth kinetic assays of this strain in decreasing concentrations of ATc. Similar to *pgsA*_{ind}, the *fabG2::Tn pgsA*_{ind} was not able to replicate in the absence of ATc (**Figure 3.13**). Surprisingly, and again contrary to our hypothesis, the growth of *fabG2::Tn pgsA*_{ind} at ATc 0.125 ng/ml was not significantly altered whereas the growth of *pgsA*_{ind} at ATc 0.125 ng/ml was significantly affected, suggesting that *fabG2::Tn pgsA*_{ind} was more resistant to ATc, compared to *pgsA*_{ind}.

Figure 3.13 Growths of *fabG2::Tn pgsA*_{ind} was more resistant to ATc concentrations, compared to *pgsA*_{ind}. Cells were grown in decreasing concentrations of ATc in a 96-well plate. Absorbance readings were measured at every 0.5-hour interval. Growth kinetics of different strains (A) *E. faecalis pgsA*_{ind}, (B) *E. faecalis fabG2::Tn pgsA*_{ind} in decreasing concentrations of ATc. Data graphed are the mean from three independent biological replicates.

3.5 Discussion and conclusion

In this chapter, we investigated the role of phosphatidylglycerol (PG) in *E. faecalis* physiology using the *pgsA_{ind}* mutant constructed in **Chapter 2**. Since PG is a major anionic phospholipid in bacterial membrane, and many antimicrobials target anionic lipids in the membrane, we measured MBCs of *pgsA_{ind}* at different ATc concentrations against different antimicrobials. We observed that *pgsA_{ind}* at ATc 0.25 ng/ml was more sensitive to the membrane targeting antimicrobials hBD2 and colistin, whereas it was more resistant to cell wall targeting antimicrobials hBD3 and ramoplanin. Both hBD2 and hBD3 are cationic antimicrobial peptides (CAMPs) mainly produced by epithelia and leukocytes cells during inflammation or infections (Sørensen et al. 2005). In general, high concentrations of hBD2, and low concentrations of hBD3 are required to kill Gram-positive bacteria (Harder et al. 1997, García et al. 2001, Harder et al. 2001). This difference is consistent with our findings in that high hBD2 but low hBD3 concentrations were required to kill *E. faecalis* WT cells. While it has been generally assumed that the positively charged defensins kill bacteria by membrane permeation (Matsuzaki 2001, Morgera et al. 2008, Ganz 2003). However, hBD3 selectively binds the lipid II-rich sites of cell wall biosynthesis of *S. aureus*, inhibiting the functions of some cell wall synthesis related enzymes including MraY, MurG, FemX, and PBP2 (Sass et al. 2010), suggesting that hBD3 instead targets the cell wall. In this chapter, we found *pgsA_{ind}* at low ATc concentrations became more sensitive to hBD2, consistent with the membrane being disrupted in *pgsA_{ind}* at low ATc concentrations. The resistance to hBD3 in *pgsA_{ind}* at low ATc concentrations is consistent with the cell wall compensation that cell wall was thicker. In addition, the cell wall peptidoglycan synthesis related gene *pbp2* which encodes PBP2, and *mraY2* which encodes MraY, were all upregulated in *pgsA_{ind}* at ATc 0.25 ng/ml, compared to either *pgsA_{ind}* or WT *ptetR* at ATc 16 ng/ml, consistent with findings in *S. aureus* (Sass et al. 2010). In addition, *pgsA_{ind}* at 0.25 ng/ml is also more sensitive to another cell membrane targeting antibiotic, colistin, but resistant to the cell wall targeting antibiotic ramoplanin. Taken together, these changes in sensitivity to different antimicrobials suggest that the cell membrane is disrupted whereas cell wall synthesis is induced in *pgsA_{ind}* at low ATc concentrations.

Virulence factor anchoring to cell wall in *E. faecalis* relies on a conserved secretion and sorting pathway which occur together at discrete foci at the division septum (Kline et al. 2009). In an *E. coli* PG defective mutant, PG is involved in translocation of cytoplasmically synthesized proteins across the inner membrane, and PG is required for SecA function and high-affinity

binding to plasma membrane (de Vrije et al. 1988, Hendrick and Wickner 1991). In *B. subtilis*, depletion of PG results in delocalization of SecA from a helical organization to a dispersed distribution (Campo et al. 2004). In this chapter, we observed that focal localization of SrtA and SrtC was altered in *pgsA*_{ind} at low ATc concentrations, suggesting that depletion of PG may affect virulence factor assembly by affecting the focal localization of SecA and sortases (SrtA and SrtC) in the secretion and sorting machinery. In another Gram-positive bacteria, *Streptococcus pyogenes*, an Exportal domain where protein secretion occurs in that species, is enriched with anionic lipids (Rosch and Caparon 2004, Rosch et al. 2007). Cationic antimicrobial peptides (CAMPs) such as human β defensins and human neutrophil peptides (HNPs) are able to bind the anionic bacterial membrane via electrostatic attraction (Rashid et al. 2016). In *S. pyogenes*, both polymyxin B and HNP-1 can alter the distribution of anionic phospholipid (PG or CL) in the Exportal and HNP-1 can result in redistribution of the Exportal secreted protein SpeB and HtrA (Vega and Caparon 2012). In *E. faecalis*, focal localization of SrtA and SecA can be disrupted by hBD2 (Kandaswamy et al. 2013). However, the focal localization of HtrA was not affected by depletion of PG, suggesting that CAMP targeting may not affect PG levels but may instead affect anionic lipid distribution. Taken together, we proposed a model to demonstrate cell induction in *pgsA*_{ind} at low ATc concentrations (**Figure 3.14**).

A) Wild type

B) *pgsA*_{ind} at low ATc

Figure 3.14 Model of focal localization pattern of membrane proteins in *E. faecalis* wild-type and *pgsA*_{ind} at low ATc concentrations. Schematic diagram of SrtA, SrtC and HtrA focal localization. (A) The membrane proteins SrtA, SrtC and HtrA focally localize at the septum of WT cell. (B) In the morphology-altered cells of *pgsA*_{ind} at low ATc concentrations, the cell membrane is disrupted, and the cell wall is thicker, in which SrtA and SrtC are mislocalized, whereas HtrA is still septum localized.

We also performed RNA sequencing to understand the cellular response to PG depletion in *pgsA*_{ind} at low ATc concentrations. We observed upregulation of cell wall synthesis and TCS related genes in *pgsA*_{ind} at ATc 0.25 ng/ml, compared to 16 ng/ml, which suggested that these may be compensatory changes in response to PG depletion. To test, we constructed double *pgsA* inducible mutant with mutants in each of OG1RF_11710, *epaI*, and *croRScisRS*. A second RNA sequencing revealed that OG1RF_11710 and *epaI* were also significantly upregulated in *pgsA*_{ind} at ATc 0.25 ng/ml, but neither *croR* nor *croS* were significantly differentially regulated in *pgsA*_{ind} at ATc 16 ng/ml, compared to WT *ptetR*. The second RNA sequencing also suggested an overall upregulation in fatty acid biosynthesis and metabolism genes in *pgsA*_{ind} at ATc 0.25 ng/ml. Thus, we hypothesized that the induction of these genes was also compensatory and would therefore be protective to ATc limitation. However, none of

these hypotheses were proven true. We speculate that the reason for this relates back to the fact that we have two subpopulations in *pgsA*_{ind} at ATc 0.25 ng/ml, which we previously hypothesized in **Chapter 2** to have different PG levels (the morphology-altered cells were decreased for PG and the morphology-normal suppressor mutants were increased for PG). If ATc suppressor mutants exist in a subpopulation of *pgsA*_{ind} at low ATc concentrations, these may affect the accuracy of the RNA sequencing analysis because we calculated the gene expression profile for the whole population. More research needs to be done to quantify gene expression in the individual cell level.

In summary, using the *pgsA*_{ind} mutant, we demonstrate that:

- 1) *pgsA*_{ind} at low ATc concentrations (PG depletion) results in sensitivity to cell membrane targeting antimicrobials and resistance to cell wall targeting antimicrobials in *E. faecalis*.
- 2) Focal localization of SrtA and SrtC is disrupted in *pgsA*_{ind} at low ATc concentrations (PG depletion), whereas focal localization of HtrA is not PG-dependent in *E. faecalis*.
- 3) In *pgsA*_{ind} at low ATc concentrations, the cell wall is less cross-linked and cell wall synthesis is altered as well, which may serve as a compensatory reaction to PG depletion.
- 4) Cell response in *pgsA*_{ind} at low ATc concentrations is complicated and quantitative gene expression analysis needs to be done in a subpopulation level or individual cell level.

3.6 References

- [1]. Anders, S., P. T. Pyl & W. Huber (2015) HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics*, 31, 166-9.
- [2]. Beisson, J., M. Betermier, M. H. Bre, J. Cohen, S. Duharcourt, L. Duret, C. Kung, S. Malinsky, E. Meyer, J. R. Preer, Jr. & L. Sperling (2010) Immunocytochemistry of *Paramecium* cytoskeletal structures. *Cold Spring Harb Protoc*, 2010, pdb.prot5365.
- [3]. Campo, N., H. Tjalsma, G. Buist, D. Stepniak, M. Meijer, M. Veenhuis, M. Westermann, J. P. Muller, S. Bron, J. Kok, O. P. Kuipers & J. D. Jongbloed (2004) Subcellular sites for bacterial protein export. *Mol Microbiol*, 53, 1583-99.
- [4]. Dale, J. L., K. B. Beckman, J. L. E. Willett, J. L. Nilson, N. P. Palani, J. A. Baller, A. Hauge, D. M. Gohl, R. Erickson, D. A. Manias, M. J. Sadowsky & G. M. Dunny (2018) Comprehensive Functional Analysis of the *Enterococcus faecalis* Core Genome Using an Ordered, Sequence-Defined Collection of Insertional Mutations in Strain OG1RF. *mSystems*, 3, e00062-18.
- [5]. de Vrije, T., R. L. de Swart, W. Dowhan, J. Tommassen & B. de Kruijff (1988) Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. *Nature*, 334, 173-5.
- [6]. Dunny, G. M., B. L. Brown & D. B. Clewell (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci U S A*, 75, 3479-83.
- [7]. Ernst, C. M., P. Staubitz, N. N. Mishra, S. J. Yang, G. Hornig, H. Kalbacher, A. S. Bayer, D. Kraus & A. Peschel (2009) The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog*, 5, e1000660.
- [8]. Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*, 3, 710-20.
- [9]. García, J.-R., F. Jaumann, S. Schulz, A. Krause, J. Rodríguez-Jiménez, U. Forssmann, K. Adermann, E. Klüver, C. Vogelmeier & D. Becker (2001) Identification of a novel, multifunctional β -defensin (human β -defensin 3) with specific antimicrobial activity. *Cell and tissue research*, 306, 257-264.
- [10]. Granok, A. B., D. Parsonage, R. P. Ross & M. G. Caparon (2000) The RofA binding site in *Streptococcus pyogenes* is utilized in multiple transcriptional pathways. *J Bacteriol*, 182, 1529-40.
- [11]. Guzman Prieto, A. M., W. van Schaik, M. R. Rogers, T. M. Coque, F. Baquero, J. Corander & R. J. Willems (2016) Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones? *Front Microbiol*, 7, 788.

- [12]. Harder, J., J. Bartels, E. Christophers & J.-M. Schröder (2001) Isolation and characterization of human β -defensin-3, a novel human inducible peptide antibiotic. *Journal of Biological Chemistry*, 276, 5707-5713.
- [13]. Harder, J., J. Bartels, E. Christophers & J. M. Schroder (1997) A peptide antibiotic from human skin. *Nature*, 387, 861.
- [14]. Hendrick, J. & W. Wickner (1991) SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. *Journal of Biological Chemistry*, 266, 24596-24600.
- [15]. Hsu, Y. P., J. Rittichier, E. Kuru, J. Yablonowski, E. Pasciak, S. Tekkam, E. Hall, B. Murphy, T. K. Lee, E. C. Garner, K. C. Huang, Y. V. Brun & M. S. VanNieuwenhze (2017) Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem Sci*, 8, 6313-6321.
- [16]. Kandaswamy, K., T. H. Liew, C. Y. Wang, E. Huston-Warren, U. Meyer-Hoffert, K. Hultenby, J. M. Schröder, M. G. Caparon, S. Normark, B. Henriques-Normark, S. J. Hultgren & K. A. Kline (2013) Focal targeting by human β -defensin 2 disrupts localized virulence factor assembly sites in *Enterococcus faecalis*. *Proc Natl Acad Sci U S A*, 110, 20230-5.
- [17]. Kellogg, S. L. & C. J. Kristich (2016) Functional Dissection of the CroRS Two-Component System Required for Resistance to Cell Wall Stressors in *Enterococcus faecalis*. *Journal of Bacteriology*, 198, 1326-1336.
- [18]. Kline, K. A., A. L. Kau, S. L. Chen, A. Lim, J. S. Pinkner, J. Rosch, S. R. Nallapareddy, B. E. Murray, B. Henriques-Normark, W. Beatty, M. G. Caparon & S. J. Hultgren (2009) Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in *Enterococcus faecalis*. *J Bacteriol*, 191, 3237-47.
- [19]. Kristich, C. J., V. T. Nguyen, T. Le, A. M. Barnes, S. Grindle & G. M. Dunny (2008) Development and use of an efficient system for random mariner transposon mutagenesis to identify novel genetic determinants of biofilm formation in the core *Enterococcus faecalis* genome. *Appl Environ Microbiol*, 74, 3377-86.
- [20]. Kumariya, R., S. K. Sood, Y. S. Rajput, N. Saini & A. K. Garsa (2015) Increased membrane surface positive charge and altered membrane fluidity leads to cationic antimicrobial peptide resistance in *Enterococcus faecalis*. *Biochim Biophys Acta*, 1848, 1367-75.
- [21]. Li, H. & R. Durbin (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25, 1754-60.
- [22]. Matsuzaki, K. (2001) Molecular mechanisms of membrane perturbation by antimicrobial peptides. *Development of novel antimicrobial agents: emerging strategies*, 167-181.
- [23]. Morgera, F., N. Antcheva, S. Pacor, L. Quaroni, F. Berti, L. Vaccari & A. Tossi (2008) Structuring and interactions of human beta-defensins 2 and 3 with model membranes. *J Pept Sci*, 14, 518-23.

- [24]. Nielsen, H. V., A. L. Flores-Mireles, A. L. Kau, K. A. Kline, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *Journal of Bacteriology*, 195, 4484-4495.
- [25]. Nielsen, H. V., P. S. Guiton, K. A. Kline, G. C. Port, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2012) The metal ion-dependent adhesion site motif of the *Enterococcus faecalis* EbpA pilin mediates pilus function in catheter-associated urinary tract infection. *MBio*, 3, e00177-12.
- [26]. Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung, A. Tarkowski, K. P. M. van Kessel & J. A. G. van Strijp (2001) *Staphylococcus aureus* Resistance to Human Defensins and Evasion of Neutrophil Killing via the Novel Virulence Factor Mprf Is Based on Modification of Membrane Lipids with L-lysine. *The Journal of Experimental Medicine*, 193, 1067-1076.
- [27]. Rashid, R., M. Veleba & K. A. Kline (2016) Focal Targeting of the Bacterial Envelope by Antimicrobial Peptides. *Front Cell Dev Biol*, 4, 55.
- [28]. Robinson, M. D., D. J. McCarthy & G. K. Smyth (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139-40.
- [29]. Rosch, J. & M. Caparon (2004) A microdomain for protein secretion in Gram-positive bacteria. *Science*, 304, 1513-5.
- [30]. Rosch, J. W. & M. G. Caparon (2005) The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in *Streptococcus pyogenes*. *Mol Microbiol*, 58, 959-68.
- [31]. Rosch, J. W., F. F. Hsu & M. G. Caparon (2007) Anionic lipids enriched at the ExPortal of *Streptococcus pyogenes*. *J Bacteriol*, 189, 801-6.
- [32]. Roy, H., K. Dare & M. Ibba (2009) Adaptation of the bacterial membrane to changing environments using aminoacylated phospholipids. *Mol Microbiol*, 71, 547-50.
- [33]. Samant, S., F.-F. Hsu, A. A. Neyfakh & H. Lee (2009) The *Bacillus anthracis* Protein MprF Is Required for Synthesis of Lysylphosphatidylglycerols and for Resistance to Cationic Antimicrobial Peptides. *Journal of Bacteriology*, 191, 1311-1319.
- [34]. Sass, V., T. Schneider, M. Wilmes, C. Korner, A. Tossi, N. Novikova, O. Shamova & H. G. Sahl (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in *Staphylococci*. *Infect Immun*, 78, 2793-800.
- [35]. Sava, I. G., E. Heikens & J. Huebner (2010) Pathogenesis and immunity in enterococcal infections. *Clin Microbiol Infect*, 16, 533-40.
- [36]. Skerker, J. M., M. S. Prasol, B. S. Perchuk, E. G. Biondi & M. T. Laub (2005) Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. *PLoS biology*, 3, e334-e334.

- [37]. Slavetinsky, C., S. Kuhn & A. Peschel (2017) Bacterial aminoacyl phospholipids - Biosynthesis and role in basic cellular processes and pathogenicity. *Biochim Biophys Acta Mol Cell Biol Lipids*, 1862, 1310-1318.
- [38]. Smith, R. E., B. Salamaga, P. Szkuta, N. Hajdamowicz, T. K. Prajsnar, G. S. Bulmer, T. Fontaine, J. Kolodziejczyk, J. M. Herry, A. M. Hounslow, M. P. Williamson, P. Serror & S. Mesnage (2019) Decoration of the enterococcal polysaccharide antigen EPA is essential for virulence, cell surface charge and interaction with effectors of the innate immune system. *PLoS Pathog*, 15, e1007730.
- [39]. Sørensen, O. E., D. R. Thapa, A. Rosenthal, L. Liu, A. A. Roberts & T. Ganz (2005) Differential Regulation of β -Defensin Expression in Human Skin by Microbial Stimuli. *The Journal of Immunology*, 174, 4870-4879.
- [40]. Thedieck, K., T. Hain, W. Mohamed, B. J. Tindall, M. Nimtz, T. Chakraborty, J. Wehland & L. Jansch (2006) The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. *Mol Microbiol*, 62, 1325-39.
- [41]. Tomaras, A. P., M. J. Flagler, C. W. Dorsey, J. A. Gaddy & L. A. Actis (2008) Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology*, 154, 3398-409.
- [42]. Vega, L. A. & M. G. Caparon (2012) Cationic antimicrobial peptides disrupt the *Streptococcus pyogenes* ExPortal. *Molecular microbiology*, 85, 1119-1132.
- [43]. Vega, L. A., G. C. Port & M. G. Caparon (2013) An association between peptidoglycan synthesis and organization of the *Streptococcus pyogenes* ExPortal. *mBio*, 4, e00485.
- [44]. Werner, J. N., E. Y. Chen, J. M. Guberman, A. R. Zippilli, J. J. Irgon & Z. Gitai (2009) Quantitative genome-scale analysis of protein localization in an asymmetric bacterium. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 7858-7863.
- [45]. Yong, M. H. 2019. HtrA and CroRS two-component signal transduction system monitor sortase-assembled pilus biogenesis in *Enterococcus faecalis*. Nanyang Technological University
- [46]. Yu, G., L.-G. Wang, Y. Han & Q.-Y. He (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics : a journal of integrative biology*, 16, 284-287.

Chapter 4 Role of cardiolipin in virulence factor assembly and antimicrobial resistance in *Enterococcus faecalis*

4.1 Abstract

Enterococcus faecalis is a Gram-positive bacterium which can become highly resistant to various antibiotics. Cardiolipin (CL) is a unique anionic phospholipid in bacterial cell membranes. CL levels are changed in response to many cell stresses in other bacteria, however, the role of CL in *E. faecalis* physiology is less well-studied. Here, we investigate the role of CL in virulence factor assembly and antimicrobial resistance in *E. faecalis*. Even though two putative CL synthase genes, *cls1* and *cls2*, are identified in *E. faecalis*, our data suggests that *cls2* contributes more to CL production during exponential phase in *E. faecalis*. The *cls2* is responsible for daptomycin (DAP) sensitivity and *E. faecalis* fitness during wound infection. The *cls2*-deficiency results in increased expression of the virulence factor endocarditis- and biofilm-associated pili (Ebp) and more Ebp deposited on the cell wall of single cells, despite decreased expression of the sortases (SrtA and SrtC) involved in focal virulence factor assembly. However, overall piliation in the population and the localization of the Ebp subunit, EbpC, is not altered, nor is the localization SrtA altered. Our results therefore demonstrate that CL levels can impact pilus biogenesis at a single cell level and contribute to antibiotic resistance in *E. faecalis*. These findings may contribute to further antimicrobial strategies for enterococcal infections.

4.2 Introduction

Cardiolipin (CL), also called diphosphatidylglycerol, is a unique anionic phospholipid, which is mostly found in the inner membrane of mitochondria of eukaryotes and in the energy-transducing membrane of bacteria (Musatov and Sedlak 2017, Mileykovskaya and Dowhan 2009). In bacteria, the CL and other phospholipid compositions of cell membranes are altered in different growth phases. In general, a minor amount of CL is synthesized during exponential phase, and CL levels are increased when cells reach stationary phase due to differential regulation of the CL synthases in different growth phases (Randle et al. 1969, Cronan 1968, Short and White 1971). The phospholipid composition of cell membranes and the transcriptional levels of phospholipid metabolism related genes are also altered in different growth conditions, such as osmotic stress, pH stress, or phagocytosis.

In *E. coli*, when exposed to osmotic stress, the proportion of zwitterionic PE is decreased while the proportion of anionic CL is increased, which is partly due to the upregulation of the *cls* genes (Romantsov et al. 2009, Romantsov et al. 2007). Similarly in *B. subtilis*, the *clsA* and *pgsA* genes, responsible for CL and PG synthesis, respectively, are both osmotically regulated, resulting in CL increase and PG decrease in response to osmotic stress (Lopez et al. 2006, Lopez et al. 2000). In *S. aureus*, although the *cls2* encoded enzyme is the dominant synthase responsible for CL accumulation, *cls1* is active for CL synthesis under high salt conditions in a $\Delta cls2$ mutant (Tsai et al. 2011). In *S. aureus*, *cls1* is also responsible for CL accumulation and survival under acute acid stress (Ohniwa et al. 2013). CL levels are also increased in *S. aureus* after phagocytosis by polymorphonuclear leukocytes (PMN) (Femling et al. 2005, Koprivnjak et al. 2011). As a predominant phospholipid class of the cell membrane, CL is also involved in antibiotic resistance toward membrane targeting antibiotics including daptomycin (Zhang et al. 2014), thioridazine (Wassmann et al. 2018) and other antimicrobial peptides, including LL-37, hemolysins and Aurein 1.2 (Hernandez-Villa et al. 2018, Kornspan et al. 2014, Scheinpflug et al. 2015, Poger et al. 2018).

Enterococcus faecalis contains two putative *cls* genes, *cls1* and *cls2*, but the regulation and contribution of each CL synthase to CL accumulation in different growth phases and growth conditions has not been previously addressed. In previous research, different mutations in *cls1* were found in daptomycin (DAP) resistant *E. faecalis* and *E. faecium* (Arias et al. 2011, Palmer et al. 2011, Humphries et al. 2012, Kelesidis et al. 2013) as well as surotomycin (SUR) resistant

E. faecium and *Clostridium difficile* (Adams et al. 2015). Point mutation in *cls2* was associated with the development of DAP resistance in *S. aureus* (Peleg et al. 2012). These data imply a potential role of CL synthase in antibiotic resistance.

In this chapter, single and double *cls* mutants including *cls1::Tn*, Δ *cls2*, *cls1::Tn* Δ *cls2* were constructed to investigate the role of *cls* genes in CL synthesis under different stress conditions. I also explored the role of *E. faecalis* CL in the localization of virulence factor assembly and DAP resistance.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this chapter are provided in **Table 4.1**.

E. coli strains were grown in Luria Bertani (LB, Difco, France) broth or agar, whereas *E. faecalis* strains were grown in Brain Heart Infusion (BHI, Becton, Dickinson, USA) broth or agar. For all experiments in this chapter, bacterial strains were streaked from a 25% (v/v) glycerol stock stored at -80°C onto 1.5% agar plates and grown overnight at 37°C. The next day, a single colony from an agar plate was inoculated into liquid medium and incubated at 37°C overnight, followed by 1:10 dilution into fresh media and growth to an optical density of 600 nm wavelength ($OD_{600\text{ nm}}$) = 0.45-0.6 to reach mid-exponential phase. When appropriate, antibiotics listed in **Table 4.1** were added to liquid or agar media at the concentrations indicated for growth of strains with plasmids.

Table 4.1 List of bacterial strains and plasmids used in this chapter

Strains or plasmids	Characteristic(s)	Resistance, concentrations	References
Strains			
<i>Enterococcus faecalis</i> OG1RF (WT)	ATCC 47077, <i>E. faecalis</i> OG1RF wild type strain, human oral isolate	Rif ^r and Fus ^r , 25 µg/ml;	(Dunny et al. 1978)
<i>Enterococcus faecalis</i> OG1RF <i>cls1::Tn</i>	<i>E. faecalis</i> OG1RF <i>cls1</i> (OG1RF_10364) transposon mutant	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml	(Kristich et al. 2008, Dale et al. 2018)
<i>Enterococcus faecalis</i> OG1RF Δ <i>cls2</i>	<i>E. faecalis</i> OG1RF mutant containing in-frame-deletion of <i>cls2</i> (OG1RF_11324)	Rif ^r , Fus ^r , 25 µg/ml;	This work
<i>Enterococcus faecalis</i> OG1RF <i>cls1::Tn</i> Δ <i>cls2</i>	<i>E. faecalis</i> OG1RF <i>cls1</i> transposon mutant containing in-	Rif ^r , Fus ^r , 25 µg/ml; Cm ^r , 10 µg/ml	This work

	frame-deletion of <i>cls2</i> (OG1RF_11324)		
Plasmids			
pGCP213	Shuttle vector temperature-sensitive plasmid for generation of mutants	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	(Nielsen et al. 2012)
pGCP213::Δ <i>cls2</i>	Derivative of pGCP213, containing 600 bp upstream of the <i>cls2</i> start codon and 600 bp downstream of <i>cls2</i> stop codon	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work
pMSP3535	Shuttle vector carrying nisin inducible promoter	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	(Bryan et al. 2000)
pMSP3535:: <i>cls1</i>	Derivative of pMSP3535, encoding <i>cls1</i> (OG1RF_10364) under nisin inducible promoter	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work
pMSP3535:: <i>cls2</i>	Derivative of pMSP3535, encoding <i>cls2</i> (OG1RF_11324) under nisin inducible promoter	Erm ^r , 500 µg/ml (<i>E. coli</i>); 25 µg/ml (<i>E. faecalis</i>)	This work
pAK1:: <i>srtA</i> -2HA	Encodes C-terminal HA-tagged <i>srtA</i> under native <i>srtA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	(Kline et al. 2009) (Kandaswamy et al. 2013)
pAK1:: <i>srtC</i> -2HA	Encodes C-terminal HA-tagged <i>srtC</i> under native <i>srtA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	This work
pGCP123:: <i>htrA</i> -HA	Encodes C-terminal HA-tagged <i>htrA</i> under native <i>htrA</i> promoter	Kan ^r , 50 µg/ml (<i>E. coli</i>); 600 µg/ml (<i>E. faecalis</i>)	(Yong 2019)
Abbreviations: Rif, rifampicin; Fus, fusidic acid; Kan, kanamycin; Erm, erythromycin; Cam, chloramphenicol Superscript “r” indicate resistance.			

4.3.2 Genetic manipulation and construction of $\Delta cls2$, $cls1::Tn\Delta cls2$ mutant and complementation strains in *Enterococcus faecalis* OG1RF

The cardiolipin synthase genes, *cls1* (OG1RF_10364) and *cls2* (OG1RF_11324), were identified in the complete genome of *Enterococcus faecalis* OG1RF (Genebank accession number: NC_017316) based on sequence homology with previously described cardiolipin synthase genes in other organisms. The 600bp upstream and 600bp downstream flanking sequence of *cls2* coding sequence was sewn together by performing PCR using primer pairs *cls2*-del-1/*cls2*-del-2 and *cls2*-del-3/*cls2*-del-4, respectively, followed by overlap PCR using primer pair *cls2*-del-2/*cls2*-del-3 (**Table 4.2**). The amplified approximately 1200bp PCR products were digested with BamHI/NotI, then cloned into a temperature sensitive vector pGCP213 and transformed into *E. coli* DH5 α competent cells. The resulting plasmid pGCP213:: $\Delta cls2$ was extracted from *E. coli*, sequenced, and correct plasmids were then transformed into *E. faecalis* OG1RF or *E. faecalis* OG1RF *cls1::Tn*.

To generate in-frame-deletion of *cls2* in *E. faecalis* OG1RF and *E. faecalis* OG1RF *cls1::Tn*, the strains *E. faecalis* OG1RF pGCP213:: $\Delta cls2$ and *E. faecalis* OG1RF *cls1::Tn* pGCP213:: $\Delta cls2$ were grown by serial passage at 30°C in the presence of erythromycin (Erm) for two days to maintain the plasmid, followed by serial passage for three days at 42°C in the presence of Erm to select for chromosomal integration of this temperature sensitive plasmid. Subsequently, cells were grown at 30°C in the absence of Erm to select for excision of the integrated plasmid by homologous recombination. Erm sensitive cells were screened using the primer pair *cls2*-del-F/*cls2*-del-R (**Table 4.2**) to identify the in-frame-deletion of *cls2* in *E. faecalis* OG1RF and *E. faecalis* OG1RF *cls1::Tn*.

Table 4.2 Primers used for construction of in-frame-deletion of *cls2* and complementation strains

Primer name	Sequence (5' → 3') ^a
cls2-del-1	ACTTGCTACT <u>GCGGCCGCG</u> TTTTTTCTTGGGGAAAGAA
cls2-del-2	ATTCAGTCTGATTTATTGACGCTTACCTCCTTCTTACTT
cls2-del-3	AAGTAAGAAGGAGGTAAGCGTCAATAAATCAGCAGTGAAT
cls2-del-4	CGTAGACTTGGGATCCTCGCGGCAACAGTATTTAAA
cls2-del-F	GAAATGACGCCGCTTCTTAG
cls2-del-R	AATTGTTTGCGACCATCACA
cls1-com-F	TCGCATGCGAATTCCTTAAAGAATTGGTGAAAATAATCG
cls1-com-R	ATCCGGTACCACTAGTTGATTCTTAGCGTCTTAACAG
cls2-com-F	TCGCATGCGAATTCCTTACAAGACTGGTGACAACA
cls2-com-R	ATCCGGTACCACTAGATGAAAATTTTTGTTTGGATTTT
^a Restriction sites underlined	

The complementation plasmids pMSP3535::*cls1* and pMSP3535::*cls2* was generated with the shuttle vector pMSP3535 (Bryan et al. 2000). The coding region (CDS) of *cls1* and *cls2* was amplified from *E. faecalis* OG1RF genomic DNA using primer pairs cls1-com-F/cls1-com-R and cls2-com-F/cls2-com-R (**Table 4.2**), respectively. The amplified PCR products were cloned into the linearized plasmid pMSP3535 (digested by SpeI and PstI) by In-Fusion (In-Fusion® HD Cloning Kit, Takara Bio, USA) cloning. The resulting plasmid pMSP3535::*cls1* was confirmed by sequencing and transformed into *E. faecalis* OG1RF *cls1*::*Tn*, *E. faecalis* OG1RF Δ *cls2*, and *E. faecalis* OG1RF *cls1*::*Tn* Δ *cls2*. The resulting plasmid pMSP3535::*cls2* was confirmed by sequencing and transformed into *E. faecalis* OG1RF Δ *cls2* and *E. faecalis* OG1RF *cls1*::*Tn* Δ *cls2*.

4.3.3 Whole genome sequencing

Strains were inoculated from frozen stocks into liquid media and grown statically overnight. Overnight cultures were diluted 10-fold into fresh media and incubated to obtain an optical density at 600 nm equal to 0.5. The isolation of DNA was performed using Pure Link® Genomic DNA Kit (Thermo Fisher, USA) according to manufacturer's protocols. Quantification of DNA was performed using Qubit™ DNA Assay Kits and Qubit™ RNA Assay Kits (Invitrogen, USA), respectively. Integrity of DNA was analyzed by gel electrophoresis using Agilent DNA Screen Tape (Agilent Technologies, Singapore). Whole genome sequencing was performed using an Illumina MiSeq sequencing (300 bp pair end) by the sequencing facility of Singapore Centre of Life Science Engineering (SCELSE) in Singapore and data was analyzed by CLC Genomics Workbench software using *E. faecalis* OG1RF as template.

4.3.4 Growth kinetic assays

Bacteria were grown to exponential stage and normalized to OD_{600 nm}=0.5 as described above. Cultures were then diluted 1000-fold into fresh media to achieve colony-forming unit (CFU)= ~ 10⁵/ml. 200 µl of the diluted cells were added to individual wells in a 96-well plate and incubated for 20 hours at 37°C statically. At every 0.5-hour interval, the absorbance was read using an Infinite 200 PRO microtiter plate reader (Tecan Group Ltd., Männedorf, Switzerland). All the experiments were performed with technical triplicates and biological triplicates.

4.3.5 10-N-nonyl-acridine orange (NAO) and Hoechst co-staining

Cells were harvest from exponential phase as described above, and optical density was normalized to OD_{600 nm}=0.5 in 1 ml 0.01M phosphate (PB, PH7.2) buffer (Beisson et al. 2010). Normalized cells were incubated with 10 µl Hoechst 33342 (1:1000, Thermofisher, USA) for 5 mins at 37°C statically. Cells were centrifuged to remove supernatant, washed 3 times with 0.01M PB buffer, and suspended in 1 ml 0.01M PB buffer. Subsequently, cells were incubated with 10 µl 0.1mM 10-N-nonyl-acridine orange (NAO, Thermofisher, USA) for 5 mins at 37°C statically. Cells were spun down, washed 3 times in 0.01M PB buffer, and suspended in 1 ml 0.01M PB buffer. 20 µl of cells were smeared on Poly-L-Lysine pre-coated slides (Polysciences Inc., USA), dried in a hybridization oven at 45°C, and mounted with 5 µl VectaShield mounting medium. The slides were visualized using an inverted widefield

epifluorescence microscope (Zeiss Axio Observer.Z1, Germany), using 100x oil immersion objective. Phase contract and fluorescent images were captured using AxioVision software and merged using Image-J software.

4.3.6 Immunoblot and quantification

Cells were harvested from exponential phase as described above, and cell density was normalized to $OD_{600\text{ nm}}=0.5$ in 1 ml 0.01M PB buffer. Normalized cells were centrifuged to obtain a cell pellet. The cell pellet was incubated with 100 μ l of 10 mg/ml of lysozyme to remove the cell wall (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour. After lysozyme treatment, the whole cell lysates were centrifuged at 14,000 RPM for 5 mins. The supernatant (containing the cell wall fraction) was collected and the pellet (protoplast fraction) was washed once with lysis buffer and resuspended in 100 μ l lysis buffer.

The whole cell lysates or the bacterial fractions described were combined with 33 μ l of NuPAGE® LDS Sample Buffer (4 $\times$) and 10 μ l of 1M dithiothreitol (DTT). The samples were then boiled at 100°C for 20 mins and cooled on ice. Subsequently, 10 μ l of each sample or 3 μ l of Page Ruler™ Plus Prestained Protein Ladder (10 to 250 kDa, Thermos Scientific, USA) were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For analysis of SecA and PgsA, the samples were loaded onto NuPAGE Novex 4-12% Bis-Tris gels in NuPAGE MOPS SDS running buffer, whereas for pili, the samples were loaded onto NuPAGE Novex 3 to 8% Tris-acetate gels in NuPAGE Tris-acetate SDS running buffer. All gels were run in the XCell SureLock Mini-Cell Electrophoresis System (Thermos Scientific, USA) at 200V for 50 mins. Subsequently, proteins from the gels were transferred to membrane using iBlot® Transfer Stacks (Life technologies Corp. USA). The membrane was then blocked with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) with 0.1% Tween 20 (PBST) at 4°C for 1 hour and then incubated with primary antibodies at 4°C overnight. The antibodies and their respective concentrations (diluted in 5% BSA in PBST) used in this chapter are described in **Table 3.2**. The next day, the membrane was washed with PBST 3 times and incubated with Pierce™ horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature in the dark for 1 hour. Then the membrane was washed with PBST 3 times and incubated with Super Signal West Femto substrate (Thermos Fisher Scientific, Inc., USA) for 5 mins. Subsequently, the membrane was exposed to the Green X-ray film (Care stream,

USA) with a Kodak processor (Kodak X-OMAT processor 2000) in the dark room. The film was captured, and the images were quantified with image J software.

Table 4.3 Antibodies used for immunoblotting

Protein of interest	Primary antibody concentration	Host	Secondary antibody concentration
SecA	1:3000	Rabbit	1:6000
HA	1:3000	Rabbit	1:6000
SrtA	1:200	Mouse	1:1250
EbpC	1:3000	Guinea pig	1:6000

4.3.7 Immunofluorescence microscopy (IFM)

Cells were harvested from exponential phase as described above, and the optical density was normalized to OD_{600 nm}=1.0 in 1 ml 0.01M PB buffer. After washing once with 0.01M PB buffer, cells were fixed in 1 ml 4% paraformaldehyde (PFA) for 20 mins at 4°C. After fixation, cells were washed once with 0.01M PB buffer, resuspended in 1 ml 0.01M PB buffer, and diluted at a 1:4 ratio by adding 250 µl of fixed cells to 750 µl of PB buffer. Then, 20 µl of diluted cells were smeared onto the ethanol and water-washed Poly-L-Lysine pre-coated slides and dried in a hybridization oven at 45°C.

For visualization of cell membrane protein, such as SrtA-2HA and HtrA-HA, cells were permeabilized with 20 µl of 10 mg/ml of lysozyme (Sigma Inc., Cat no: 62971-50G-F) in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 0.75 M sucrose) at 37°C for 1 hour, washed 3 times with PB buffer, and dried in the hybridization oven. Cells were permeabilized again with 20 µl 0.1% Triton in PB at 37°C for 10 mins, washed 3 times with PB buffer, and dried. For visualization of cell wall protein (EbpC), cells were not permeabilized.

Cells were incubated with 20ul blocking buffer 2% bovine serum albumin in PB (2% BSA-PB) for 20 mins at room temperature, washed 3 times with PB buffer, and dried. Subsequently, cells were smeared with 20 µl primary antibody anti-HA raised in rabbit or anti-EbpC raised in

Guinea pig (1:500 dilution in 2% BSA-PB) and incubated at 4°C overnight. After primary antibody incubation, cells were washed 3 times with PB buffer and dried. Subsequently, cells were smeared with 20 µl fluorescence conjugated secondary antibody Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-rabbit IgG (1:1000 dilution in 2% BSA-PB) and incubated in the dark at room temperature for 1 hour. Then, cells were washed 3 times with PB buffer, dried, and smeared with 5 µl VectaShield mounting medium. The slides were visualized by an inverted widefield epifluorescence microscope (Zeiss Axio Observer.Z1, Germany), using 100x oil immersion objective. Phase contract and florescent and merged images were captured using AxioVision software and analyzed by Matlab software. The Projected System of Internal Coordinates from Interpolated Contours (PSICIC) (Werner et al. 2009) was used to quantify the mean fluorescence intensity at the perimeter of *E. faecalis* cells at the early division stage, in which cell perimeter is between 3.6–6 µm.

4.3.8 Minimum bactericidal concentration (MBC) determination for daptomycin (DAP)

Cells were grown to exponential stage as described above and normalized to OD_{600 nm}=0.5 in 1 ml 0.01M phosphate buffer (PB) buffer. Cells were washed once in 0.01M PB buffer and suspended in 1 ml 0.01M PB buffer, followed by 500-fold dilution in H₂O to achieve CFU= ~ 2*10⁵/ml, and daptomycin (DAP) stocks were also diluted in H₂O. 100 µl of the diluted cells were incubated with 100 µl of decreasing concentrations of DAP supplemented with CaCl₂ (final Ca²⁺ concentration equals 50mg/L) in a 96-well plate at 37°C statically. After 2 hours incubation, the CFU of each well was enumerated by dilution plating, and the MBC result was defined as the lowest concentration of DAP in which all cells were dead. Experiments were performed with biological triplicates and technical triplicates in each.

4.3.9 Sodium chloride (NaCl) sensitivity analysis

Cells were grown to exponential stage as described above and normalized to OD_{600 nm}=0.5 in 1 ml PBS buffer. Cells were washed once in PBS buffer and suspended in 1 ml PBS buffer, followed by 500-fold dilution in 2× medium supplemented with 2× antibiotics if required, to achieve CFU= ~ 2*10⁵/ml and NaCl was diluted in H₂O. Despite of the NaCl in BHI medium, 100 µl of the diluted cells were added to 100 µl of decreasing percentage of NaCl in a 96-well plate and incubated for 18 hours at 37°C statically. At every 0.5-hour interval, the absorbance was read using an Infinite 200 PRO microtiter plate reader (Tecan Group Ltd., Männedorf,

Switzerland). All the experiments were performed with technical triplicates and biological triplicates.

4.3.10 Mouse wound excisional model

Mouse wound infections were modified were performed as previously described (Chong et al. 2017), with the following modifications. Male C57BL/6 mice (7-8 weeks old, 22 to 25 g; InVivos, Singapore) were used. After anesthesia with 3% isoflurane and trimming of dorsal hair with a shaver, the dorsal hair then fully removed with Nair cream (Church and Dwight Co) to ensure that the wound dressing could be affixed and remain for more than 3 days without detachment. After disinfection with 70% ethanol, the skin was punched with a 6-mm biopsy punch (Integra Miltex, New York), inoculated with 10 µl of the bacteria culture (1:1 ratio of *E. faecalis* OG1RF and *E. faecalis* OG1X, at 10⁶ CFU per inoculum), and sealed with a transparent dressing (Tegaderm, 3M, St Paul Minnesota) to cover the wound site. After 3 days post-infection, mice were euthanized and a 1 × 1 cm piece of skin surrounding the wound site was excised into sterile PBS buffer. Excised wounds were homogenized, and viable bacterial CFU counts were enumerated by plating onto BHI agar without or with selective antibiotics based on different antibiotic resistances in the infecting strains used (*E. faecalis* OG1RF, Rifampicin, 25 µg/ml; *E. faecalis* OG1X, Streptomycin, 500 µg/ml). Comparison to CFU on plain BHI allowed to also monitor for contamination in the wound site. Mice with contaminants were excluded from the datasets and subsequent analysis. The competitive index (CI) was determined with the following formula:

$$CI = \frac{OG1RF(output)/OG1X(output)}{OG1RF(input)/OG1X(input)}$$

4.3.11 Macrophage survival assay

E. faecalis cells were grown to exponential stage as described above and normalized to OD_{600 nm}=0.5. The normalized cells were diluted in PBS to achieve CFU=3 × 10⁸/ml. Macrophage cell line (RAW-Blue) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco® - Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS). 5 × 10⁵ RAW-Blue cells/well were seeded in 6-well plates and incubated overnight to allow for attachment to the surface. The next day, the 6-well plates were washed once with PBS to remove unattached RAW-Blue cells. For each well, 5 × 10⁶ *E. faecalis* cells were added to the

macrophages (multiplicity of infection, MOI =10) and centrifuged to promote cell-bacteria contact. After incubation at 37°C with 5% CO₂ for 1 hour, the plates were washed with PBS three times. Cell culture medium (DMEM + 10% FBS) with antibiotics (vancomycin 10 µg/ml + gentamicin 150 µg/ml) was then added to the wells to kill remaining extracellular bacteria. The macrophages with intracellular bacteria were then incubated until indicated time points. Macrophages were lysed with 0.1% Triton X-100 at indicated time points and the lysates were collected for CFU enumeration. The percent survival was calculated by comparing the normalized bacterial CFU counts at 0 hour to the CFU recovered at the indicated time points.

4.3.12 Real-time quantitative PCR (qPCR)

Cells were grown to exponential phase as described above and harvested. RNA extraction was performed according to the manufacturer's protocol using Pure Link™ RNA Mini Kit (Thermo Scientific, USA). RNA samples were treated with TURBO DNA-free™ kit (Invitrogen™, USA) to remove DNA from RNA samples and purified with Agencourt RNA clean XP (Beckman Coulter, USA) magnetic beads. RNA concentration and DNA contamination were measured using Qubit™ RNA HS Assay Kit (Thermo Scientific, USA) and Qubit™ dsDNA HS Assay Kit (Thermo Scientific, USA), respectively. The RNA samples with less than 10% DNA contamination were screened using Tape Station (Agilent Technologies, USA) to measure RIN values. Reverse transcription (RT)-PCR was performed according to manufacturer's instruction using Super Script® III First-Strand Synthesis System for RT-PCR (Invitrogen™, USA). Random hexamer primers provided in the kit were used for cDNA synthesis in this work. The cDNA samples were stored at -20°C for subsequent analysis.

Real-time quantitative PCR (qPCR) was performed according to the manufacturer's instruction using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (Kapa biosystem, USA). Primer pairs (**Table 4.3**) used for targeting genes and the housekeeping gene 16s rRNA (16s) in qPCR were synthesized from Integrated DNA Technologies (IDT). The qPCR reactions were performed in the Step One Plus™ Systems (Life Technologies, USA) and the cycling program was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and finally annealing and extension at 60°C for 30 seconds.

Table 4.4 Primer sequence used for real-time qPCR in this chapter

Primer name	Sequence (5'→3')
16s_F	GGATAACACTTGGAAACAGG
16s_R	TCCTTGTTCTTCTCTAACAA
qPCR-htrA-F	GATCCTTTTGGGGGATTGTT
qPCR-htrA-R	CGACGTGGTTATTTGTCACG
qPCR-SrtA-F	GCC TTG GCT AGT CAT CGA AC
qPCR-SrtA-R	TTG CTT GTA AAT CGC CAC AG
qPCR-SrtC-F	ATCCTACAGGTGGTGCGAAT
qPCR-SrtC-R	TCTTGGCCAGACTCAATGTG

4.3.13 Statistical analysis

All experiments were performed with at least three biological replicates and the mean value was calculated and is presented. All graphs indicate the standard deviation from independent experiments (and not technical replicates). Statistical analysis was performed by Graphpad software using either one-way ANOVA, two-way ANOVA or unpaired t-test unless stated otherwise. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$, ns: $p > 0.05$).

4.4 Results

4.4.1 Cardiolipin synthase is not essential in *E. faecalis*

It has been reported that CL synthase is not essential in most bacteria, whereas it is essential in *Streptomyces coelicolor* (Jyothikumar et al. 2012). To investigate whether the putative CL synthases, *cls1* and *cls2*, are essential in *E. faecalis*, we identified multiple transposon insertions in *cls1* within a sequenced and arrayed *E. faecalis* OG1RF mariner transposon library (Kristich et al. 2008, Dale et al. 2018), suggesting that *cls1* is not essential in *E. faecalis*. While there were no *cls2* transposon mutants in the same transposon library, we successfully constructed an in-frame-deletion of *cls2* in *E. faecalis* OG1RF and *E. faecalis* OG1RF *cls1::Tn*. To verify that there were no suppressor mutations arising in the $\Delta cls2$ mutant strains that could mask essentiality, we performed whole genome sequencing on the resulting mutant strains and confirmed that there were no secondary mutations present. Therefore, these data suggest that *cls2* is also not essential in *E. faecalis*. Subsequently, to quantify whether *cls* mutants displayed a growth defect, we performed growth kinetic assays of *cls1::Tn*, $\Delta cls2$ and *cls1::Tn* $\Delta cls2$, compared to WT. We observed that the absorbance readings of $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ were slightly higher than WT, whereas *cls1::Tn* grew similar to WT (**Figure 4.1A**). The CFU measurements for all strains at each time point were similar to WT (**Figure 4.1B**). See below for morphological differences between the strains that may explain this discrepancy between absorbance and CFU. Moreover, we performed growth kinetic experiments for the complementation strains of all *cls* mutants, including single mutants (*cls1::Tn* and $\Delta cls2$) and double mutant (*cls1::Tn* $\Delta cls2$). Similar growth curves were observed in the *cls* mutants complemented with a nisin inducible empty vector pMSP3535 (**Figure 4.1C**) and *cls* mutants complemented with either *cls1* or *cls2* (**Figure 4.1D**), compared to WT. These growth data (CFU) suggest that the deficiency of CL synthase does not influence cell growth in *E. faecalis*, and that these CL synthases are not essential in *E. faecalis*.

Figure 4.1 Growth is similar for *cls1::Tn*, $\Delta cls2$, *cls1::Tn\Delta cls2*, and WT strains. Cells were cultured overnight (~18 hours), subcultured to mid-exponential phase, and normalized to CFU=10⁵/ml, and 200 μ l cells were inoculated into 96-well plates. Absorbance readings of (A) *cls* mutants, (C) *cls* mutants with empty vectors, (D) complemented *cls* mutants were measured at every 0.5-hour interval. Data graphed are the mean from three independent biological replicates. (B) CFU of *cls* mutants at each time point were enumerated by dilution plating. N=3 independent biological replicates. Error bars represent standard error of the mean (SEM).

Whereas we observed no differences in CFU in growth curve analysis we did observe a slight difference in absorbance readings in $\Delta cls2$ and *cls1::Tn\Delta cls2*, compared to WT. We hypothesized that the difference in absorbance readings could result from morphology variation in $\Delta cls2$ and *cls1::Tn\Delta cls2*. It was also reported that *cls* deletion resulted in decreased cell size in *Pseudomonas putida* (Bernal et al. 2007). Thus, we tried to visualize the *cls* mutants under microscopy to quantify whether the morphology was altered in *cls* mutants. Historically, the hydrophobic fluorescent dye NAO has been used as a CL specific staining dye to visualize and quantify CL distribution in cell membranes because NAO emits green fluorescence when it binds to anionic phospholipids, whereas NAO emits red fluorescence only when it binds to CL. However, subsequent research found that red fluorescence of NAO can also be observed in a CL-deficient *E. coli* strain (Oliver et al. 2014, Wood 2018), suggesting that NAO is also able

to bind to other molecules and is not a CL-specific probe. To test whether red fluorescence could be observed in our *cls*-deficient *E. faecalis* strains, we also performed NAO and Hoechst co-staining to visualize the morphology and NAO fluorescence of different *cls* mutants.

We observed that all *cls* mutants displayed uniform diplococcal shape, compared to WT (**Figure 4.2A**). Using MicrobeJ software (Ducret et al. 2016), we quantified the cell width and length of each strain as depicted in the cartoon (**Figure 4.2B**). Data plotted on a scatter diagram of cell width and length distributions shows that the cell length of all *cls* mutants fell within in a similar limited distribution, while the width distribution of $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ was slightly higher than *cls1::Tn* and WT (**Figure 4.2C**). This observation likely explained why the absorbance readings of $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ were slightly higher than WT, while the CFU results of $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ at different time points were similar to WT (**Figure 4.1**). Upon NAO staining, we observed unique foci at the septa of all *cls* mutants in both green and red channels (**Figure 4.2A**), suggesting that anionic phospholipid distribution is not affected in *cls*-deficient mutant.

Figure 4.2 Morphology of *cls* mutants is not altered and NAO is not a CL-specific staining dye. Cells were stained with NAO and Hoechst. (A) Representative images of WT all *cls* mutants including phase contrast, NAO staining in the green channel, NAO staining in the red channel, Hoechst staining and merged images. No color was observed in NAO-unstained negative controls (data not shown). Scale bar = 2 μ m. (B) Cartoon depiction of the measurement of *E. faecalis* cell width and length. (C) Scatter plot of cell width and length distribution of all *cls* mutants. 100 cells of each strain were counted and are shown.

4.4.2 Cls2 protein expression is restored to WT levels upon complementation with plasmid pMSP3535::*cls2*

To address whether Cls1 and Cls2 protein expression is deficient in *cls* mutants and can be restored in the respective complementation strains, we tried to purify the Cls1 and Cls2 proteins of *E. faecalis* OG1RF to generate corresponding primary antibodies at the PPP (Protein

Purification Platform) facility in NTU. We successfully purified Cls2 and generated the α -Cls2 primary antibody. Since *cls2* in the complementation was controlled by a nisin inducible promoter in plasmid pMSP3535, we performed western blot to measure Cls2 expression in the different *cls* mutants in the absence or presence of nisin (200ng/ml). Cells harvested from mid-exponential phase were used for western blot analysis and probed using antibodies raised either against Cls2 and SecA as a protein loading control. In the absence of nisin, Cls2 expression was not observed in the *cls2* deletion mutants ($\Delta cls2$ and *cls1::Tn Δ cls2*), *cls2* deletion mutants with empty vector pMSP3535 ($\Delta cls2$ pEmpty and *cls1::Tn Δ cls2* pEmpty), or the complementation strain $\Delta cls2$ *pcls2* and *cls1::Tn Δ cls2* *pcls2* (**Figure 4.3A**). However, Cls2 expression was restored in $\Delta cls2$ *pcls2* and *cls1::Tn Δ cls2* *pcls2* strains in the presence of nisin (**Figure 4.3B**), suggesting that Cls2 protein expression was complemented in $\Delta cls2$ *pcls2* and *cls1::Tn Δ cls2* *pcls2* complementation strains in the presence of nisin.

Figure 4.3 Cls2 protein is not expressed in *cls2* in-frame-deletion mutant and is complemented in pMSP3535::*cls2* in the presence of nisin. Cells were harvested and lysed for western blot analysis using antibody raised either against SecA or Cls2. All *cls* mutants were grown (A) in the absence of nisin or (B) in the presence of nisin, respectively. (C) Representative images of western blot data used to quantify Cls2 protein expression levels. The Cls2 bands on the film are indicated by short arrows and the protein bands below Cls2 are unspecific bands. (D) Quantification, normalizing Cls2 to SecA levels for the same sample, is shown. Data graphed are the mean from three independent biological replicates, error bars represent standard error of the mean (SEM).

Because we observed such strong Cls2 expression upon nisin induction at 200 ng/ml, we subsequently optimized the nisin concentration in order to restore Cls2 expression in the complementation strains to levels similar to WT. Cells grown in media supplemented with increasing concentrations of nisin were again used for western blot analysis using antibodies raised either against SecA or Cls2. We observed a dose-dependent increase in Cls2 expression with increasing nisin concentrations from 5 ng/ml to 100 ng/ml, and we did not observe any detectable Cls2 without nisin (**Figure 4.3C**). To quantify Cls2 expression in different nisin concentrations, the Cls2 band intensity was normalized to SecA band intensity for the same sample. Based on this analysis, we observed that induction with 5 ng/ml nisin in the

complementation strain resulted in Cls2 expression that best matched WT Cls2 levels (**Figure 4.3D**). There was no growth defect of nisin to all *cls* strains (**Appendix Figure 1**). Therefore, we used 5 ng/ml nisin for subsequent complementation experiments when we wanted to approximate WT protein levels. Taken together, using α -Cls2 antibody, Cls2 protein expression is not detectable in $\Delta cls2$ and can be complemented by the complementation plasmid pMSP3535::*cls2*.

4.4.3 Cls2 contributes to daptomycin (DAP) sensitivity

It has been reported that *cls1* related mutations were found in DAP-resistant *E. faecalis* both *in vivo* (Arias et al. 2011) and in *in vitro* evolution experiments (Palmer et al. 2011), and mutation in *cls2* is associated with the development of DAP resistance in *S. aureus in vivo* (Peleg et al. 2012). To test the role of *cls1* and *cls2* in DAP resistance, we tested the minimum bactericidal concentration (MBC) of DAP in *cls1::Tn*, $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ mutants, compared to WT. The MBC results (**Table 4.5**) showed that the MBC of *cls1::Tn* (16 μ g/ml) was similar to WT (16 μ g/ml), whereas the MBCs of $\Delta cls2$ (4 μ g/ml) and *cls1::Tn* $\Delta cls2$ (2 μ g/ml) were decreased compared to WT, suggesting that $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ were more sensitive to DAP. To address whether the sensitivity to DAP resulted from deletion of *cls2*, we performed MBC tests of all *cls* mutants and complementation strains grown in the presence of 5 ng/ml nisin to approximate WT levels and 200 ng/ml nisin for over-expression. In the presence of nisin, all strains became slightly more resistant to DAP, and the MBCs of each strain were increased as the nisin concentrations increased, suggesting that the cell stress response after nisin treatment results in resistance to DAP in *E. faecalis*. In addition, exposure to 200 ng/ml nisin rendered WT *E. faecalis* resistant to DAP with a >4-fold increase in MBC. At each nisin concentration examined, *cls2* complementation in $\Delta cls2$ p*cls2* and *cls1::Tn* $\Delta cls2$ p*cls2* restored DAP susceptibility to WT levels, suggesting that Cls2 contributes to DAP susceptibility in *E. faecalis*.

Table 4.5 MBC results of *cls* mutant at different concentrations of nisin against DAP

MBC of DAP (μg/ml)	Nisin=0 ng/ml	Nisin=5 ng/ml	Nisin=200 ng/ml
WT	16	32	256
<i>cls1::Tn</i>	16	32	256
$\Delta cls2$	4	4	64
<i>cls1::Tn</i> $\Delta cls2$	2	2	64
WT pEmpty	16	32	256
<i>cls1::Tn</i> pEmpty	16	32	256
$\Delta cls2$ pEmpty	4	4	64
<i>cls1::Tn</i> $\Delta cls2$ pEmpty	2	4	64
<i>cls1::Tn</i> <i>pcls1</i>	16	32	256
$\Delta cls2$ <i>pcls2</i>	4	64	256
<i>cls1::Tn</i> $\Delta cls2$ <i>pcls1</i>	2	4	64
<i>cls1::Tn</i> $\Delta cls2$ <i>pcls2</i>	2	64	256

4.4.4 Localization of sortase is not CL synthasedependent in *E. faecalis*

In **Chapter 3**, we observed that SrtA was delocalized in the *pgsA_{ind}* mutant at low ATc concentrations (**Figure 3.1**), suggesting that PG levels are involved in normal SrtA focal localization. As another important anionic phospholipid in the cell membrane, CL is synthesized from PG molecules using Cls in most bacteria. Thus, we hypothesized that SrtA would be delocalized in Cls deficient mutants in *E. faecalis*, including *cls1::Tn*, $\Delta cls2$ and *cls1::Tn* $\Delta cls2$. To address this, we transformed the plasmid pAK1::*srtA*-2HA into the *cls* mutants and performed immunofluorescence microscopy (IFM) to visualize SrtA localization using an α -HA antibody.

We observed that SrtA focal localization was not altered in the *cls* deficient mutant in *E. faecalis*, compared to WT (**Figure 4.4A**), with enrichment at the septum and foci at the nascent septum regularly observed. However, the percentage of HA stained cells was significantly decreased in $\Delta cls2$ *psrtA*-2HA and *cls1::Tn* $\Delta cls2$ *psrtA*-2HA, whereas the percentage of HA stained cells in *cls1::Tn* was not altered, compared to WT (**Figure 4.4B**). We therefore hypothesized that the decreased percentage of HA stained cells could result from decreased SrtA-2HA expression in $\Delta cls2$ *psrtA*-2HA and *cls1::Tn* $\Delta cls2$ *psrtA*-2HA. Thus, we performed western blot to quantify SrtA-2HA expression. Cells harvested from mid-exponential phase were used for western blot analysis and probed using antibodies raised either against HA or SecA as a protein loading control. We observed strong SrtA-2HA expression in WT *psrtA*-2HA and *cls1::Tn* *psrtA*-2HA, and reduced SrtA-2HA expression in $\Delta cls2$ *psrtA*-2HA and *cls1::Tn* $\Delta cls2$ *psrtA*-2HA (**Figure 4.4C**). To quantify SrtA-2HA expression in the different strains, the protein band intensity of SrtA-2HA was normalized to the band intensity of SecA for each same sample. Based on the western blot quantification, we observed that SrtA-2HA expression in $\Delta cls2$ *psrtA*-2HA and *cls1::Tn* $\Delta cls2$ *psrtA*-2HA was significantly decreased (**Figure 4.4D**). Taken together, these data suggest that SrtA focal localization in *E. faecalis* is not CL synthase dependent, but rather that SrtA-2HA expression from a plasmid is dependent on *cls2* transcription.

Figure 4.4 SrtA localization pattern is not altered in *cls*-deficient mutant in *E. faecalis*. Different *cls* mutants containing plasmid pAK1::*srtA*-2HA were harvested during mid-exponential phase. SrtA-2HA localization was visualized via IFM using an anti-HA antibody. SrtA-2HA expression was quantified by western blot analysis using antibody against HA and SecA expression served as a loading control. (A) Representative images of SrtA-2HA localization in each strain are shown, scale bar = 2 μ m. (B) Percentage of α -HA stained cells were quantified, in which at least 300 cells of each strain were counted. (C) Representative images of western blot data used to quantify SrtA-2HA protein expression levels. (D) Quantification of normalized HA to SecA levels for the same sample is shown. All data graphed are the mean of three independent biological replicates, error bars represent standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA, the symbol ns indicates p-value >0.05, **** indicates p-value <0.0001.

To determine whether the reduction of SrtA-2HA expression was due to a reduced plasmid copy number in *cls2* mutant backgrounds, or whether SrtA expression was truly decreased, we performed western blot to quantify the native chromosomally-encoded SrtA expression level in *cls* mutants, compared to WT. Cells harvested during mid-exponential phase were used for western blot analysis using antibodies raised either against SecA or SrtA. We observed reduced SrtA band intensity in $\Delta cls2$ and *cls1::Tn* $\Delta cls2$, compared to WT (**Figure 4.5A**). To quantify SrtA expression in different *cls* mutants, SrtA band intensities were normalized to the SecA band intensity for each same sample. Normalized SrtA intensity of *cls1::Tn* was similar to WT, whereas SrtA intensity in $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ was significantly lower (**Figure 4.5B**). Furthermore, we confirmed the decrease in protein production by qPCR, where we observed that *srtA* gene expression was not altered in *cls1::Tn* but decreased in $\Delta cls2$ and *cls1::Tn* $\Delta cls2$, compared to WT (**Figure 4.5C**). Both the western blot and qPCR analyses suggest that SrtA expression is decreased in *cls2*-deficient *E. faecalis*.

Figure 4.5 Native *srtA* expression is decreased in Δ *cls2* and *cls1::Tn* Δ *cls2*. (A) Representative images of western blots used to quantify native SrtA protein expression levels. (B) Western blot quantification of normalized SrtA to SecA for the same sample. (C) Relative fold change of *srtA* expression by qPCR analysis. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol ns indicates p-value >0.05, * indicates p-value <0.05, **** indicates p-value <0.0001.

Next, we questioned whether the increased membrane protein expression in the absence of *cls2* was specific to SrtA. To address this, we examined the expression of another sortase, SrtC, and another membrane protein, HtrA (high temperature requirement A) in *cls* mutants. Using qPCR analysis, we found *srtC* expression was also not altered in *cls1::Tn* but decreased in Δ *cls2* and *cls1::Tn* Δ *cls2*, compared to WT (**Figure 4.6A**). However, the *htrA* expression was not altered in any *cls* mutant (**Figure 4.6B**). These qPCR data suggested that decreased expression of SrtA and SrtC in the absence of Cls2 is sortase-specific in *E. faecalis*. Based on the altered SrtC expression in *cls2* mutant background, we did not further examine SrtC focal localization in these mutants.

Figure 4.6 Native *srtC* expression is decreased in $\Delta cls2$ and *cls1::Tn* $\Delta cls2$, whereas *htrA* expression is not altered in any *cls* mutant. Relative fold change of (A) *srtC* and (B) *htrA* expression by qPCR analysis. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol ns indicates p-value >0.05, **** indicates p-value <0.0001.

To investigate whether HtrA localization is *cls*-dependent in *E. faecalis*, we transformed the plasmid pGCP123::*htrA*-HA into *cls* mutants to visualize HtrA localization via IFM using an α -HA antibody. HtrA foci were observed at the septa in all *cls* mutants, including *cls1::Tn*, $\Delta cls2$ and *cls1::Tn* $\Delta cls2$, similar to WT (**Figure 4.7**). We also note that HtrA localization was not PG-dependent (**Figure 3.4**). Taken together, these data suggested that HtrA focal localization is not anionic phospholipid dependent and that HtrA expression is not *cls*-dependent in *E. faecalis*.

Figure 4.7 HtrA foci were not altered in *cls* mutants. HtrA-HA was visualized via IFM using an anti-HA antibody. HtrA-HA localization of different *cls* mutants with plasmid pGCP123::*htrA*-HA was visualized via IFM using an anti-HA antibody. Representative images are shown, scale bar =2 μ m.

4.4.5 EbpC expression is increased in *cls2*-deficient strains in *E. faecalis*

As a SrtA and SrtC substrate, the endocarditis- and biofilm-associated pili (Ebp) are high molecular weight polymers which consist of three subunits: EbpA, EbpB and EbpC (Nallapareddy et al. 2006). The anti-Ebp-stained pili are observed in a subpopulation in *E. faecalis* (Nallapareddy et al. 2006). SrtC is responsible for Ebp subunit polymerization while SrtA is required for cell wall anchoring of Ebp, and deletion of *srtA* in *E. faecalis* results in increased levels of Ebp in whole cell lysates (Nielsen et al. 2013). Since we observed expression of *srtA* and *srtC* was both decreased in *E. faecalis* *cls2*-deficient strains (**Figure 4.5 and 4.6**), we next investigated the effect of *cls*-deficiency on EbpC expression, as EbpC is the major subunit of Ebp in *E. faecalis*.

To investigate whether the expression and localization of EbpC was affected in *E. faecalis* *cls*-deficient strains, we performed IFM to visualize EbpC localization using an α -EbpC antibody. We observed a similar hemispherical and septum exclusive localization pattern of EbpC in

each *cls* mutant, compared to WT (**Figure 4.8A**). Although *srtA* and *srtC* expression was decreased in *cls2*-deficient strains, the percentage of EbpC stained cells within the overall population was not altered in *cls* mutants, compared to WT (**Figure 4.8B**). To quantify the expression of EbpC on single cells in *cls* mutants, we performed image analysis to quantify the mean fluorescence intensity of each Ebp positive individual cell at early division stage using Projected System of Internal Coordinates from Interpolated Contours (PSICIC). In contrast to our observation for the population, we observed that the overall EbpC intensity on single cells in $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ was increased whereas the EbpC intensity of *cls1::Tn* was not altered, compared to WT (**Figure 4.8C**). These localization data suggested that EbpC piliation was not altered on a population level, whereas EbpC expression on individual cells was increased in *cls2*-deficient strains in *E. faecalis*.

Figure 4.8 EbpC piliation is not *cls*-dependent on a population level, but EbpC production is increased on individual piliated cells of *cls2*-deficient strains in *E. faecalis*. EbpC localization was visualized via IFM using an α-EbpC antibody and analyzed by PSICIC software. (A) Representative images of EbpC localization in different *cls* mutants, scale bar = 0.5 μm. (B) Percentage of α-EbpC stained cells were quantified, in which at least 300 cells of each strain were counted. Data graphed are the mean values from three independent experiments, error bars represent standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA, the symbol ns indicates p-value > 0.05. (C) The mean fluorescent intensity profiles at the perimeter of cells from each strain were quantified by PSICIC. The cell perimeter was quantified from at least 200 early-division stage cells, in which cells without fluorescence were excluded from the analyses. Units are displayed in arbitrary units (AU). The cartoon depicts predominant sites (red) of EbpC hemispherical localization and the coordinates of the perimeter correspond with the x-axis.

We observed decreased SrtA expression in the *cls2*-deficient strains, and SrtA is required for Ebp anchoring to the cell wall. To determine whether EbpC anchoring to the cell wall was affected in *cls*-deficient strains in *E. faecalis*, we performed western blot to determine EbpC presence in different cellular fractions, including whole cell lysates, the cell wall fraction, and

the cell wall-free protoplast fraction using an α - EbpC antibody. To test the ability of EbpC anchoring to cell wall, we included $\Delta srtA$ as positive control in which EbpC-containing polymerized pili accumulate in the cell protoplast fraction. We also performed western blot of SecA for the same sample in different fractions as a protein loading control and as a control for the cytoplasmic fraction. As expected, in the positive control $\Delta srtA$ sample, we observed the pilus high molecular weight ladder (HMWL, which resulted from the polymerized pili with different molecular weights) accumulated in the protoplast fraction instead of cell wall fraction, compared to WT (**Figure 4.9**). Unlike $\Delta srtA$, the HMWL in each *cls*-deficient strain was found in the cell wall (CW) fraction (**Figure 4.9**), suggesting that pili anchorage to the cell wall was not affected by *cls2*-deficiency in *E. faecalis*, even though SrtA expression was decreased.

Figure 4.9 EbpC accumulates in the cell wall fractions in *cls*-deficient strains in *E. faecalis*. After lysis with lysozyme, the whole cell lysate (WCL) fraction was separated into a cell wall (CW) fraction and a protoplast (Proto) fraction by centrifugation. All three fractions were analyzed by western blot using antibody raised either against SecA or EbpC. Immunoblot of the three fractions from each strain are separated by lines for visualization, but a single blot is shown. The $\Delta srtA$ blot was run separately. Top: Immunoblot with α -EbpC antibody; Bottom: Immunoblot with α -SecA antibody.

In the analysis of EbpC accumulation in different cell fractions, we also observed increased levels of EbpC in $\Delta cls2$ and $cls1::Tn\Delta cls2$, compared to WT (**Figure 4.9**). To confirm that the increased EbpC resulted from deletion of $cls2$, we performed western blot of the different cls mutants as well as the complementation strains. The whole cell lysate fractions from each strain were used for western blot analysis using antibodies raised either against SecA or EbpC. To quantify the EbpC expression in different strains, the whole HMWL intensity was normalized to the SecA band intensity for the same sample. We observed a similar protein distribution profile (banding pattern) of HMWL in the different strains, suggesting that the polymerization of EbpC was not affected (**Figure 4.10A**). We also observed a stronger intensity of pilus HMWL in $cls2$ deletion strains (**Figure 4.10A**), and quantification revealed that EbpC expression was not altered in $cls1::Tn$, but was significantly increased in $\Delta cls2$ and $cls1::Tn\Delta cls2$ (**Figure 4.10B**). A similar increase was also found in the $cls2$ deletion strains with empty vectors. Complementation of $cls2$ in $\Delta cls2$ and $cls1::Tn\Delta cls2$ restored EbpC expression to WT levels, whereas complementation of $cls1$ in $cls1::Tn\Delta cls2$ was not able to restore EbpC expression levels, suggesting that the increased Ebp expression resulted from $cls2$ deletion, and $cls1$ expression was not involved in Ebp expression in *E. faecalis*.

(A) Representative images of western blot data used to quantify native EbpC protein expression levels. Immunoblot of different strains are separated by lines for visualization, but a single blot is shown. Top: Immunoblot with α -EbpC antibody; Bottom: Immunoblot with α -SecA antibody (B) Western blot quantification of normalized HMWL of EbpC to SecA for the same sample. Statistical significance was determined by one-way ANOVA, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001.

4.4.6 Cardiolipin synthase contributes to *E. faecalis* fitness during wound infection

Previously, our lab published that multiple peptide resistance factor (Mprf), which can modify PG to lys-PG to mediate resistance to cationic antimicrobial peptides (CAMP), contributes to *E. faecalis* fitness during wound persistence, suggesting that CAMP resistance contributes to persistence during wound infection (Chong et al. 2017). As cardiolipin was found to be responsible for sensitivity to daptomycin and is involved in resistance to antimicrobials in other species (Peleg et al. 2012, Adams et al. 2015), we performed mouse wound infection and hypothesized that the CL-deficient *E. faecalis* *cls1::Tn Δ cls2* would be less competitive in this infection model. Based on preliminary data obtained from one experiment, we found that the *cls1::Tn Δ cls2* mutant was significantly less competitive, compared to WT (**Figure 4.11**). Although the preliminary data suggests that CL synthase contributes to *E. faecalis* fitness during wound infection, more biological replicates must be performed to verify this conclusion.

Figure 4.11 *E. faecalis* fitness during wound infection is decreased in cardiolipin synthase deficient mutant (*cls1::TnΔcls2*). Wounds were infected with a 1:1 ratio of *E. faecalis* OG1RF:OG1X or OG1RF *cls1::TnΔcls2*:OG1X, at 10^6 CFU per inoculum. After 3 days of infection, the recovered bacteria were enumerated on selective media for each strain. Each dot represents 1 mouse, and the solid horizontal lines indicate the median, N = 1, n = 5. Statistical analysis was performed using Mann–Whitney U test, the symbol * indicates p-value <0.05.

4.4.7 Cardiolipin synthase does not contribute to *E. faecalis* survival under high salinity

In *E. coli*, CL content is increased upon exposure to osmotic stress (Romantsov et al. 2007), and in *B. subtilis* a *cls2* deletion mutant grows slower in medium with high salinity (Lopez et al. 2006). Thus, we hypothesized that the *cls*-deficient *E. faecalis* strains would be more sensitive to high salinity and overexpression of *cls* would result in resistance to high salinity. To address this, we performed growth kinetic assays of the *cls* strains in BHI medium supplemented different percentages of sodium chloride (NaCl), in addition to the intrinsic NaCl composition in BHI medium (0.5% NaCl). The data revealed that all strains grew more slowly in medium supplemented with increasing concentrations of NaCl and were inhibited at the highest NaCl concentration (8%) (**Figure 4.12A-D**). However, we observed similar growth curves for all of the different *cls* strains at each NaCl concentration, suggesting that *cls*-deficiency does not affect cell growth under high salinity.

Figure 4.12 Cardiolipin does not contribute to high salinity survival for *E. faecalis*. Normalized cells (10^5 cells/ml) were incubated in BHI medium supplemented with (A) 0% NaCl, (B) 2% NaCl, (C) 4% NaCl and (D) 8% NaCl for overnight (~18 hours). Absorbance readings were measured at 0.5-hour intervals. Data graphed are the mean from three independent biological replicates.

Next, to test the hypothesis that overexpression of *cls* could result in resistance to high salinity, we performed similar growth kinetic assays of *cls* strains grown in the presence of nisin 200 ng/ml to maximally induce *cls* expression (**Figure 4.3**). Similar growth defects were observed in the *cls* overexpression strains (nisin 200 ng/ml) grown in increasing NaCl concentrations (**Figure 4.13 A-D**), suggesting that overexpressed *cls* does not affect cell sensitivity to salinity. Taken together, these data revealed that CL synthase does not contribute to *E. faecalis* sensitivity to high salinity.

Figure 4.13 Overexpressed *cls* does not result in resistance to high salinity. Normalized cells (10^5 cells/ml) were incubated in BHI medium supplemented with Erm 25 μ g/ml, nisin 200 ng/ml and (A) 0% NaCl, (B) 2% NaCl, (C) 4% NaCl and (D) 8% NaCl for overnight (~18 hours). Absorbance readings were measured at every 0.5-hour interval. Data graphed are the mean from three independent biological replicates.

4.4.8 Cardiolipin synthase does not contribute to macrophage survival in *E. faecalis*

CL levels are increased in *S. aureus* after phagocytosis by polymorphonuclear leukocytes (PMN) (Femling et al. 2005, Koprivnjak et al. 2011). Based on this, we predicted that deficiency of CL synthase would alter survival of *E. faecalis* after phagocytosis by another immune cell type (macrophages). To test this, we performed macrophage survival assays to determine the intracellular survival percentage of the *cls*-deficient mutant *E. faecalis* *cls1::TnΔcls2* within macrophages, compared to WT. After 1 hour of infection, gentamycin and vancomycin was added to the growth media to kill the extracellular bacteria. The percentage of intracellular bacteria was quantified over time, compared to time 0. As the incubation time increased, the percentage of surviving WT and *cls1::TnΔcls2* both gradually

decreased (**Figure 4.14**). At each time point, the percentage of intracellular survival for WT and *cls1::TnΔcls2* was not significantly different, suggesting that CL synthase does not contribute to macrophage intracellular survival in *E. faecalis*.

Figure 4.14 Cardiolipin synthase does not contribute to macrophage intracellular survival. Macrophage cells were incubated with *E. faecalis* cells (MOI=10) for 1 hour. The extracellular bacteria were killed by antibiotics. The remaining macrophages with intracellular bacteria (0h) were incubated for different times. At each time point, the macrophages were lysed and the remained intracellular *E. faecalis* cells were enumerated by dilution plating. Statistical significance was determined by two-way ANOVA with Sidak's test for multiple comparisons, N = 3 independent biological replicates. Error bars represent standard error of the mean (SEM), the symbol ns indicates p-value >0.05.

4.5 Discussion and conclusion

In this chapter, to characterize the functions of CL in *E. faecalis*, we identified the CL synthase genes in *E. faecalis* OG1RF and constructed *cls1::Tn*, $\Delta cls2$, *cls1::Tn* $\Delta cls2$ and corresponding complementation strains. We found *cls* genes were not essential in *E. faecalis*, which is consistent with reports in many other species of bacteria (Tan et al. 2012, Czolkoss et al. 2016, Kawai et al. 2004, Ohniwa et al. 2013).

Although two *cls* genes were identified in *E. faecalis*, we observed that the *cls2* encoded enzyme was the major enzyme responsible for the differentially altered phenotypes in this chapter. In *E. coli*, the *clsA* encoded enzyme was the only CL synthase responsible for CL production and no detectable CL was found in $\Delta clsA$ mutant during exponential phase (Shuichi Hiraoka 1991), while other two CL synthase genes (*clsB* and *clsC*) were responsible for CL accumulation during stationary phase (Guo and Tropp 2000, Tan et al. 2012). In a *B. subtilis* $\Delta clsA$ mutant, CL levels was not detectable at exponential phase but was decreased at stationary phase; therefore, two additional putative CL synthase genes encoded by this organism may be more important during stationary phase (Kawai et al. 2004). In *S. aureus*, *cls2* contributes much more to CL synthase than *cls1* in both exponential and stationary phase (Koprivnjak et al. 2011). These data are consistent with our findings that *cls2* contributes more to the differentially altered phenotypes during exponential phase. Thus, we hypothesized that *cls2* encoded enzyme is the major enzyme responsible for CL levels, resulting in CL levels decreased in *cls2*-deficient mutants at mid-exponential phase. More experiments need to be done to validate the hypothesis and further investigate whether *cls1* contributes to CL accumulation during stationary phase in *E. faecalis*.

As a component of the bacterial membrane, CL has been found to be involved in resistance to membrane-targeting antibiotics in other species (Swain et al. 2018, El Khoury et al. 2017, Wassmann et al. 2018). Daptomycin (DAP) is a clinical used antibiotic for many Gram-positive bacteria. Although CL has been implicated in DAP resistance, its net effect on DAP activity is not well-defined. In studies of DAP resistance, a mutation in *cls2* was found in DAP-resistant *S. aureus* and multiple different mutations in *cls1* were found in DAP-resistant *E. faecalis* and *E. faecium* (Peleg et al. 2012, Arias et al. 2011, Tran et al. 2013). The *cls1* related mutations were found in *in vitro* adapted and clinically isolated DAP-resistant *E. faecalis*, and expression of mutated *cls1* alleles, found in the adapted DAP-resistant *E. faecalis*, expressed on a plasmid

confers *trans*-dominant DAP resistance to a DAP susceptible *E. faecalis* strain (Palmer et al. 2011). However, in this chapter, disruption of *cls1* function in *cls1::Tn* did not affect DAP sensitivity. Although enzyme activity analysis revealed that Cls1 purified from DAP-resistant *E. faecalis* had higher enzyme activity than native Cls1 (Davlieva et al. 2013), here we showed that overexpression of neither *cls1* nor *cls2* in *E. faecalis* conferred resistance to DAP. We further showed that $\Delta cls2$ and *cls1::Tn* $\Delta cls2$ mutants became more sensitive to DAP. However, since *cls* overexpression did not confer resistance to DAP, together these findings suggest CL may not be a direct target for DAP, but additional lipid analysis needs to be done to verify this prediction. This conclusion is also supported by membrane lipid analysis of DAP-resistant *S. aureus* (Mishra and Bayer 2013) and DAP-resistant *E. faecalis* (Mishra et al. 2012), in which CL levels were not altered, whereas PG levels were significantly decreased. In another research, the DAP resistance in *E. faecalis* correlates with redistribution of CL microdomains, as supported by visualizing the distribution of CL stained by NAO (Tran et al. 2013). However, NAO is not a CL-specific dye, and can bind PG as well (Oliver et al. 2014). Fluorescently labelled DAP also preferentially binds to PG domains and depletion of PG results in resistance to DAP in *B. subtilis* (Hachmann et al. 2009). Genetic reconstruction studies of DAP-resistant *B. subtilis* show that replacement of mutant *pgsA* alleles that arose in DAP-resistant strains with WT *pgsA* is able to restore DAP sensitivity to WT levels, suggesting that changes in *pgsA* were primarily responsible for DAP resistance (Hachmann et al. 2011). As CL is synthesized from the precursor PG by *cls* genes, and our DAP sensitivity data in this chapter suggests that CL may not a direct target. Thus, we hypothesize that PG is the direct target of DAP, and the previously reported *cls* gain-of-function alleles in DAP-resistant *E. faecalis* merely contributed to reduction of PG content. However, more research needs to be done to investigate the role of CL and PG in DAP resistance.

In **Chapter 3** we found that SrtA localization was PG-dependent (**Figure 3.1**), but in this chapter we found SrtA localization was not CL-dependent. We observed SrtA expression was decreased in *cls2*-deficient strains and in *pgsA*_{ind} at low ATc concentrations (**Figure 3.3**), suggesting that SrtA localization does not consistently correlate with SrtA expression levels. In *Streptococcus pneumoniae*, the septal localization of SecA and HtrA was diminished in a CL-deficient mutant (Tsui et al. 2011), whereas HtrA focal localization in *E. faecalis* was not affected by absence (decreased) of CL. Our data suggest that the localization of HtrA in *E. faecalis* relies on CL-independent mechanism(s).

One aim in this chapter was to study the role of CL in cell wall-associated virulence factor assembly, which relies on a conserved secretion and sorting pathway. SrtC is responsible for EbpC polymerization and SrtA is responsible for Ebp anchoring to the cell wall (Nielsen et al. 2013). In *E. faecalis* $\Delta srtA$, the percentage of Ebp positive cells in a population increased, but this is a post-transcriptional effect since *ebpC* transcription is not altered in this mutant (Afonina 2017). However, in this chapter, we found the expression of *srtA* was decreased in *cls2*-deficient *E. faecalis* but percentage of piliated cells in the population was unchanged and EbpC protein levels on piliated cells was significant increased. We speculate that this phenotype may be transcriptional because we observed significant upregulation of *ebpA* (the first gene of a three gene operon containing *ebpA*, *ebpB*, and *ebpC*) in RNA sequencing analysis of *cls1::Tn Δ cls2* compared to WT (data not shown). However, qPCR validation needs to be done to confirm the increased transcription of *ebpC*. The polymerization of EbpC was not altered in *cls2*-deficient *E. faecalis*, even though *srtC* expression was decreased, suggesting that decreased *srtC* expression may not affect SrtC function in Ebp polymerization. Although SrtC is transcribed in the *ebpA-srtC* operon of *E. faecalis* OG1RF, the promoter of *srtC* is independent of *ebpA* promoter (Nallapareddy et al. 2006). SrtC expression is not affected, even *ebpABC* transcript is undetectable in a $\Delta ebpR$ mutant (Bourgogne et al. 2007). Taken together we propose a model wherein in the *cls2*-deficient *E. faecalis*, EbpC accumulates in the cell wall, which is somehow sensed by the cell, resulting in a feedback response that results in downregulation of *srtA* and *srtC* transcription (**Figure 4.15**).

A) Wild-type

B) *cls2*-deficient mutant

Figure 4.15 Models for Ebp assembly in *E. faecalis* wild-type and *cls2*-deficient mutant. Pili anchoring to cell wall relies on a conserved sortase assembled pathway in *E. faecalis*. (A) Schematic diagram of pili assembly in *E. faecalis* wild-type cells. Ebp consists of three subunits: EbpA, EbpB and EbpC. After polymerization of SrtC, Ebp is anchored to cell wall by SrtA. (B) *cls2* deficiency results in increased Ebp production in the cell wall, which is somehow sensed by the cell, resulting in a feedback response that results in downregulation of *srtA* and *srtC* transcription.

Many bacteria remodel their membrane composition in response to high osmolality , wherein the CL proportion was increased when bacteria were grown in high salinity condition (Lopez et al. 1998, Lopez et al. 2000, Romantsov et al. 2007, Machado et al. 2004). A *B. subtilis* *cls2* deletion mutant grows slower in medium with high salinity (Lopez et al. 2006). By contrast, we observed similar growth curves for *E. faecalis* *cls*-deficient strains under high salinity and overexpression of *cls* in *E. faecalis* did not result in resistance to high salinity, suggesting that CL does not contribute to *E. faecalis* survival under high salinity. This suggested that *E. faecalis* uses another stress response pathway to high salinity conditions. In the analysis of *E. faecalis* transcriptional response to NaCl-induced stress, type II fatty acid synthesis related genes and *epa* genes, which encode enterococcal polysaccharide antigen (Epa), were upregulated under high NaCl stress (Solheim et al. 2014). However, only *epa* mutants were more sensitive to high concentrations of NaCl, demonstrating an osmoprotective role for *epa* in *E. faecalis*, and suggesting a cell envelope regulatory response of *E. faecalis* to high salinity stress.

In conclusion, we report the following discoveries:

- 1) Cardiolipin synthase genes (*cls1* and *cls2*) are not essential in *E. faecalis*, and the *cls2* encoded enzyme may be the major CL synthase responsible for CL production in *E. faecalis* during exponential phase under laboratory conditions.
- 2) *cls2*-deficiency results in increased EbpC production and decreased *srtA* and *srtC* expression, however the piliation and localization of EbpC is not altered, nor is the localization SrtA altered.
- 3) Cls2 contributes to DAP sensitivity and *E. faecalis* fitness during wound infection but does not contribute to *E. faecalis* survival under high salinity or within macrophages.

4.6 References

- [1]. Adams, H. M., X. Li, C. Mascio, L. Chesnel & K. L. Palmer (2015) Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and Enterococcus species. *Antimicrobial agents and chemotherapy*, 59, 4139-4147.
- [2]. Afonina, I. 2017. Characterizing the relationship between Sortase A-dependent substrates and the consequences of SrtA deletion in *Enterococcus faecalis*. 180. Nanyang Technological University.
- [3]. Arias, C. A., D. Panesso, D. M. McGrath, X. Qin, M. F. Mojica, C. Miller, L. Diaz, T. T. Tran, S. Rincon, E. M. Barbu, J. Reyes, J. H. Roh, E. Lobos, E. Sodergren, R. Pasqualini, W. Arap, J. P. Quinn, Y. Shamoo, B. E. Murray & G. M. Weinstock (2011) Genetic basis for in vivo daptomycin resistance in enterococci. *N Engl J Med*, 365, 892-900.
- [4]. Beisson, J., M. Betermier, M. H. Bre, J. Cohen, S. Duharcourt, L. Duret, C. Kung, S. Malinsky, E. Meyer, J. R. Preer, Jr. & L. Sperling (2010) Immunocytochemistry of Paramecium cytoskeletal structures. *Cold Spring Harb Protoc*, 2010, pdb.prot5365.
- [5]. Bernal, P., J. Munoz-Rojas, A. Hurtado, J. L. Ramos & A. Segura (2007) A *Pseudomonas putida* cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. *Environ Microbiol*, 9, 1135-45.
- [6]. Bourgoigne, A., K. V. Singh, K. A. Fox, K. J. Pflughoeft, B. E. Murray & D. A. Garsin (2007) EbpR Is Important for Biofilm Formation by Activating Expression of the Endocarditis and Biofilm-Associated Pilus Operon (ebpABC) of *Enterococcus faecalis* OG1RF. *Journal of Bacteriology*, 189, 6490-6493.
- [7]. Bryan, E. M., T. Bae, M. Kleerebezem & G. M. Dunny (2000) Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid*, 44, 183-90.
- [8]. Chong, K. K. L., W. H. Tay, B. Janela, A. M. H. Yong, T. H. Liew, L. Madden, D. Keogh, T. M. S. Barkham, F. Ginhoux, D. L. Becker & K. A. Kline (2017) *Enterococcus faecalis* Modulates Immune Activation and Slows Healing During Wound Infection. *The Journal of infectious diseases*, 216, 1644-1654.
- [9]. Cronan, J. E., Jr. (1968) Phospholipid alterations during growth of *Escherichia coli*. *J Bacteriol*, 95, 2054-61.
- [10]. Czolkoss, S., C. Fritz, G. Holzl & M. Aktas (2016) Two Distinct Cardiolipin Synthases Operate in *Agrobacterium tumefaciens*. *PLoS One*, 11, e0160373.
- [11]. Dale, J. L., K. B. Beckman, J. L. E. Willett, J. L. Nilson, N. P. Palani, J. A. Baller, A. Hauge, D. M. Gohl, R. Erickson, D. A. Manias, M. J. Sadowsky & G. M. Dunny (2018) Comprehensive Functional Analysis of the *Enterococcus faecalis* Core Genome Using an Ordered, Sequence-Defined Collection of Insertional Mutations in Strain OG1RF. *mSystems*, 3, e00062-18.

- [12]. Davlieva, M., W. Zhang, C. A. Arias & Y. Shamoo (2013) Biochemical characterization of cardiolipin synthase mutations associated with daptomycin resistance in enterococci. *Antimicrobial agents and chemotherapy*, 57, 289-296.
- [13]. Ducret, A., E. M. Quardokus & Y. V. Brun (2016) MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat Microbiol*, 1, 16077.
- [14]. Dunny, G. M., B. L. Brown & D. B. Clewell (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci U S A*, 75, 3479-83.
- [15]. El Khoury, M., J. Swain, G. Sautrey, L. Zimmermann, P. Van Der Smitten, J.-L. Décout & M.-P. Mingeot-Leclercq (2017) Targeting Bacterial Cardiolipin Enriched Microdomains: An Antimicrobial Strategy Used by Amphiphilic Aminoglycoside Antibiotics. *Scientific reports*, 7, 10697-10697.
- [16]. Femling, J. K., W. M. Nauseef & J. P. Weiss (2005) Synergy between extracellular group IIA phospholipase A2 and phagocyte NADPH oxidase in digestion of phospholipids of *Staphylococcus aureus* ingested by human neutrophils. *J Immunol*, 175, 4653-61.
- [17]. Guo, D. & B. E. Tropp (2000) A second *Escherichia coli* protein with CL synthase activity. *Biochim Biophys Acta*, 1483, 263-74.
- [18]. Hachmann, A.-B., E. R. Angert & J. D. Helmann (2009) Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. *Antimicrobial agents and chemotherapy*, 53, 1598-1609.
- [19]. Hachmann, A.-B., E. Sevim, A. Gaballa, D. L. Popham, H. Antelmann & J. D. Helmann (2011) Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. *Antimicrobial agents and chemotherapy*, 55, 4326-4337.
- [20]. Hernandez-Villa, L., M. Manrique-Moreno, C. Leidy, M. Jemiola-Rzeminska, C. Ortiz & K. Strzalka (2018) Biophysical evaluation of cardiolipin content as a regulator of the membrane lytic effect of antimicrobial peptides. *Biophys Chem*, 238, 8-15.
- [21]. Humphries, R. M., T. Kelesidis, R. Tewhey, W. E. Rose, N. Schork, V. Nizet & G. Sakoulas (2012) Genotypic and phenotypic evaluation of the evolution of high-level daptomycin nonsusceptibility in vancomycin-resistant *Enterococcus faecium*. *Antimicrobial agents and chemotherapy*, 56, 6051-6053.
- [22]. Jyothikumar, V., K. Klanbut, J. Tiong, J. S. Roxburgh, I. S. Hunter, T. K. Smith & P. R. Herron (2012) Cardiolipin synthase is required for *Streptomyces coelicolor* morphogenesis. *Mol Microbiol*, 84, 181-97.
- [23]. Kandaswamy, K., T. H. Liew, C. Y. Wang, E. Huston-Warren, U. Meyer-Hoffert, K. Hultenby, J. M. Schröder, M. G. Caparon, S. Normark, B. Henriques-Normark, S. J. Hultgren & K. A. Kline (2013) Focal targeting by human β -defensin 2 disrupts

- localized virulence factor assembly sites in *Enterococcus faecalis*. *Proc Natl Acad Sci U S A*, 110, 20230-5.
- [24]. Kawai, F., M. Shoda, R. Harashima, Y. Sadaie, H. Hara & K. Matsumoto (2004) Cardiolipin domains in *Bacillus subtilis* marburg membranes. *J Bacteriol*, 186, 1475-83.
- [25]. Kelesidis, T., R. Tewhey & R. M. Humphries (2013) Evolution of high-level daptomycin resistance in *Enterococcus faecium* during daptomycin therapy is associated with limited mutations in the bacterial genome. *The Journal of antimicrobial chemotherapy*, 68, 1926-1928.
- [26]. Kline, K. A., A. L. Kau, S. L. Chen, A. Lim, J. S. Pinkner, J. Rosch, S. R. Nallapareddy, B. E. Murray, B. Henriques-Normark, W. Beatty, M. G. Caparon & S. J. Hultgren (2009) Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in *Enterococcus faecalis*. *J Bacteriol*, 191, 3237-47.
- [27]. Koprivnjak, T., D. Zhang, C. M. Ernst, A. Peschel, W. M. Nauseef & J. P. Weiss (2011) Characterization of *Staphylococcus aureus* cardiolipin synthases 1 and 2 and their contribution to accumulation of cardiolipin in stationary phase and within phagocytes. *J Bacteriol*, 193, 4134-42.
- [28]. Kornspan, J. D., S. Rottem & R. Nir-Paz (2014) Cardiolipin synthetase is involved in antagonistic interaction (reverse CAMP phenomenon) of *Mycoplasma* species with *Staphylococcus aureus* beta-hemolysis. *J Clin Microbiol*, 52, 1622-8.
- [29]. Kristich, C. J., V. T. Nguyen, T. Le, A. M. Barnes, S. Grindle & G. M. Dunny (2008) Development and use of an efficient system for random mariner transposon mutagenesis to identify novel genetic determinants of biofilm formation in the core *Enterococcus faecalis* genome. *Appl Environ Microbiol*, 74, 3377-86.
- [30]. Lopez, C. S., A. F. Alice, H. Heras, E. A. Rivas & C. Sanchez-Rivas (2006) Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity. *Microbiology*, 152, 605-16.
- [31]. Lopez, C. S., H. Heras, H. Garda, S. Ruzal, C. Sanchez-Rivas & E. Rivas (2000) Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress. *Int J Food Microbiol*, 55, 137-42.
- [32]. Lopez, C. S., H. Heras, S. M. Ruzal, C. Sanchez-Rivas & E. A. Rivas (1998) Variations of the envelope composition of *Bacillus subtilis* during growth in hyperosmotic medium. *Curr Microbiol*, 36, 55-61.
- [33]. Machado, M. C., C. S. Lopez, H. Heras & E. A. Rivas (2004) Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane. *Arch Biochem Biophys*, 422, 61-70.
- [34]. Mileykovskaya, E. & W. Dowhan (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. *Biochim Biophys Acta*, 1788, 2084-91.

- [35]. Mishra, N. N. & A. S. Bayer (2013) Correlation of cell membrane lipid profiles with daptomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*, 57, 1082-1085.
- [36]. Mishra, N. N., A. S. Bayer, T. T. Tran, Y. Shamoo, E. Mileykovskaya, W. Dowhan, Z. Guan & C. A. Arias (2012) Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content. *PLoS one*, 7, e43958-e43958.
- [37]. Musatov, A. & E. Sedlak (2017) Role of cardiolipin in stability of integral membrane proteins. *Biochimie*, 142, 102-111.
- [38]. Nallapareddy, S. R., K. V. Singh, J. Sillanpaa, D. A. Garsin, M. Hook, S. L. Erlandsen & B. E. Murray (2006) Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest*, 116, 2799-807.
- [39]. Nielsen, H. V., A. L. Flores-Mireles, A. L. Kau, K. A. Kline, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *Journal of Bacteriology*, 195, 4484-4495.
- [40]. Nielsen, H. V., P. S. Guiton, K. A. Kline, G. C. Port, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2012) The metal ion-dependent adhesion site motif of the *Enterococcus faecalis* EbpA pilin mediates pilus function in catheter-associated urinary tract infection. *MBio*, 3, e00177-12.
- [41]. Ohniwa, R. L., K. Kitabayashi & K. Morikawa (2013) Alternative cardiolipin synthase Cls1 compensates for stalled Cls2 function in *Staphylococcus aureus* under conditions of acute acid stress. *FEMS Microbiol Lett*, 338, 141-6.
- [42]. Oliver, P. M., J. A. Crooks, M. Leidl, E. J. Yoon, A. Saghatelian & D. B. Weibel (2014) Localization of anionic phospholipids in *Escherichia coli* cells. *J Bacteriol*, 196, 3386-98.
- [43]. Palmer, K. L., A. Daniel, C. Hardy, J. Silverman & M. S. Gilmore (2011) Genetic basis for daptomycin resistance in enterococci. *Antimicrob Agents Chemother*, 55, 3345-56.
- [44]. Peleg, A. Y., S. Miyakis, D. V. Ward, A. M. Earl, A. Rubio, D. R. Cameron, S. Pillai, R. C. Moellering, Jr. & G. M. Eliopoulos (2012) Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus*. *PLoS One*, 7, e28316.
- [45]. Poger, D., S. Pöyry & A. E. Mark (2018) Could Cardiolipin Protect Membranes against the Action of Certain Antimicrobial Peptides? Aurein 1.2, a Case Study. *ACS omega*, 3, 16453-16464.
- [46]. Randle, C. L., P. W. Albro & J. C. Dittmer (1969) The phosphoglyceride composition of Gram-negative bacteria and the changes in composition during growth. *Biochim Biophys Acta*, 187, 214-20.

- [47]. Romantsov, T., Z. Guan & J. M. Wood (2009) Cardiolipin and the osmotic stress responses of bacteria. *Biochim Biophys Acta*, 1788, 2092-100.
- [48]. Romantsov, T., S. Helbig, D. E. Culham, C. Gill, L. Stalker & J. M. Wood (2007) Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*. *Mol Microbiol*, 64, 1455-65.
- [49]. Scheinpflug, K., O. Krylova, H. Nikolenko, C. Thurm & M. Dathe (2015) Evidence for a novel mechanism of antimicrobial action of a cyclic R-,W-rich hexapeptide. *PLoS One*, 10, e0125056.
- [50]. Short, S. A. & D. C. White (1971) Metabolism of phosphatidylglycerol, lysylphosphatidylglycerol, and cardiolipin of *Staphylococcus aureus*. *J Bacteriol*, 108, 219-26.
- [51]. Shuichi Hiraoka, K. N., Nobuyuki Uetake, Akinori Ohta, Isao Shibuya (1991) Amplification and Substantial Purification of Cardiolipin Synthase of *Escherichia coli*. *The Journal of Biochemistry*, 110, 443-449.
- [52]. Solheim, M., S. L. La Rosa, T. Mathisen, L. G. Snipen, I. F. Nes & D. A. Brede (2014) Transcriptomic and functional analysis of NaCl-induced stress in *Enterococcus faecalis*. *PLoS One*, 9, e94571.
- [53]. Swain, J., M. El Khoury, J. Kempf, F. Briée, P. Van Der Smissen, J.-L. Décout & M.-P. Mingeot-Leclercq (2018) Effect of cardiolipin on the antimicrobial activity of a new amphiphilic aminoglycoside derivative on *Pseudomonas aeruginosa*. *PloS one*, 13, e0201752-e0201752.
- [54]. Tan, B. K., M. Bogdanov, J. Zhao, W. Dowhan, C. R. Raetz & Z. Guan (2012) Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. *Proc Natl Acad Sci U S A*, 109, 16504-9.
- [55]. Tran, T. T., D. Panesso, H. Gao, J. H. Roh, J. M. Munita, J. Reyes, L. Diaz, E. A. Lobos, Y. Shamoo, N. N. Mishra, A. S. Bayer, B. E. Murray, G. M. Weinstock & C. A. Arias (2013) Whole-genome analysis of a daptomycin-susceptible *enterococcus faecium* strain and its daptomycin-resistant variant arising during therapy. *Antimicrobial agents and chemotherapy*, 57, 261-268.
- [56]. Tran, T. T., D. Panesso, N. N. Mishra, E. Mileykovskaya, Z. Guan, J. M. Munita, J. Reyes, L. Diaz, G. M. Weinstock, B. E. Murray, Y. Shamoo, W. Dowhan, A. S. Bayer & C. A. Arias (2013) Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. *MBio*, 4.
- [57]. Tsai, M., R. L. Ohniwa, Y. Kato, S. L. Takeshita, T. Ohta, S. Saito, H. Hayashi & K. Morikawa (2011) *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiol*, 11, 13.
- [58]. Tsui, H. C., S. K. Keen, L. T. Sham, K. J. Wayne & M. E. Winkler (2011) Dynamic distribution of the SecA and SecY translocase subunits and septal localization of the

HtrA surface chaperone/protease during *Streptococcus pneumoniae* D39 cell division. *MBio*, 2.

- [59]. Wassmann, C. S., L. C. Lund, M. Thorsing, S. P. Lauritzen, H. J. Kolmos, B. H. Kallipolitis & J. K. Klitgaard (2018) Molecular mechanisms of thioridazine resistance in *Staphylococcus aureus*. *PloS one*, 13, e0201767-e0201767.
- [60]. Werner, J. N., E. Y. Chen, J. M. Guberman, A. R. Zippilli, J. J. Irgon & Z. Gitai (2009) Quantitative genome-scale analysis of protein localization in an asymmetric bacterium. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 7858-7863.
- [61]. Wood, J. M. (2018) Perspective: challenges and opportunities for the study of cardiolipin, a key player in bacterial cell structure and function. *Curr Genet*, 64, 795-798.
- [62]. Yong, M. H. 2019. HtrA and CroRS two-component signal transduction system monitor sortase-assembled pilus biogenesis in *Enterococcus faecalis*. Nanyang Technological University
- [63]. Zhang, T., J. K. Muraih, N. Tishbi, J. Herskowitz, R. L. Victor, J. Silverman, S. Uwumarenogie, S. D. Taylor, M. Palmer & E. Mintzer (2014) Cardiolipin prevents membrane translocation and permeabilization by daptomycin. *J Biol Chem*, 289, 11584-91.

Chapter 5 Conclusion and future perspectives

5.1 Conclusion

As the third most common cause of nosocomial infections, these enterococci-associated infections are increasingly resistant to multiple antimicrobials, limiting therapeutic options. In this study, we investigate the role of anionic phospholipids in *E. faecalis* physiology, including virulence factor assembly and antimicrobial resistance, which may contribute to the development of new bacterial targets in enterococci.

In **Chapter 2**, we constructed a *pgsA* inducible mutant, *pgsA_{ind}*, to deplete PG levels in *E. faecalis*. Using the *pgsA_{ind}* mutant and corresponding complementation strain *pgsA_{ind} ppgsA-2HA*, we demonstrated that *pgsA* is essential and septum localized in *E. faecalis*, which is similar to many other bacteria. When the concentrations of the inducer, anhydrotetracycline (ATc), were low, *pgsA_{ind}* contained two different populations, in which one population displayed WT-like morphology and the other displayed WT-altered morphology. We proposed that the population displaying WT-like morphology is a subpopulation with suppressor mutations, because we obtained suppressor mutants which displayed WT-like morphology but grew in the absence of ATc. At high ATc concentrations, the total PG levels were not altered, even though the *pgsA* transcription was decreased, compared to WT. However, at low ATc concentrations, the presence of a subpopulation of *pgsA_{ind}* suppressor mutants may result in increased PG levels, giving rise to an apparent increase in PG levels in the whole population.

In **Chapter 3**, using the *pgsA_{ind}* mutant, we found *pgsA_{ind}* at low ATc concentrations became more sensitive to cell membrane targeting antimicrobials but more resistant to cell wall targeting antimicrobials, suggesting that the cell membrane is disrupted whereas cell wall synthesis is induced upon PG depletion in *pgsA_{ind}* at low ATc concentrations. The focal localization pattern of the sortase enzymes SrtA (SrtA) and Sortase C (SrtC) was altered in *pgsA_{ind}* at low ATc concentrations, and protein expression of SrtA was decreased as well. However, localization of another membrane protein, high temperature requirement A (HtrA), was not PG-dependent in *E. faecalis*. Upon induction of cell wall synthesis in *pgsA_{ind}* at low ATc concentrations, the cell wall was less cross-linked and cell wall surface deposition was altered as well, suggesting a compensatory cell wall reaction to PG depletion in *E. faecalis*. In the study of cell responses to PG depletion in *pgsA_{ind}* at low ATc concentrations, we obtained

unexpected results, in which deletion of the upregulated genes in *pgsA*_{ind} at low ATc concentrations resulted in resistance to ATc limitation. These unexpected data may result from the presence of suppressor mutations in *pgsA*_{ind} at low ATc concentrations.

In **Chapter 4**, we identified two putative CL synthase genes, *cls1* and *cls2* in *E. faecalis*, but the phenotypes we observed suggested that the *cls2* encoded enzyme is the major CL synthase in *E. faecalis* during exponential phase. The *cls2* was responsible for daptomycin (DAP) sensitivity and *E. faecalis* fitness during wound infection. The *cls2*-deficiency resulted in increased expression of the virulence factor endocarditis- and biofilm-associated pili (Ebp) on a single cell level, but decreased expression of the sortases (SrtA and SrtC) involved in focal virulence factor assembly. However, piliation of the overall population and localization of Ebp subunit, EbpC, was not altered, nor was the localization SrtA altered. Unlike other bacteria, CL synthase does not contribute to *E. faecalis* survival under high salinity stress or macrophage intracellular stress.

In summary, we demonstrated that both of PG and CL contribute to virulence factor assembly and antimicrobial resistance in *E. faecalis*, in some cases, in unexpectedly different ways. Firstly, we constructed a regulatable mutant of an essential gene, *pgsA*, in *E. faecalis* using a tetR system, which is the first demonstration of such an application in *E. faecalis*. Secondly, synthesizing the studies of two anionic lipids PG and CL, our findings suggested that sortase localization is PG-dependent only whereas, sortase production is both PG and CL dependent, which are also new findings in *E. faecalis*. Subsequent antimicrobial resistance analysis also revealed different roles of PG and CL in antimicrobial sensitivity, which will provide meaningful evidence for advancing strategies for treating enterococcal infections.

5.2 Future perspectives

In **Chapter 2 and 3**, we have shown that a subpopulation of suppressor mutants may exist in *pgsA*_{ind} at low ATc concentrations, resulting in unexpected PG levels and gene transcription profile. To quantify the *pgsA* gene expression in subpopulations or at an individual cell level, fluorescence *in situ* hybridization (FISH) for RNA should be performed to quantify *pgsA* transcription within subpopulation cells. We hypothesize that the *pgsA* expression in morphology-altered cells (subpopulation) is lower than the WT-like cells (subpopulation). In parallel, since we also observed that *pgsA* expression at ATc 0.25 ng/ml was higher than at other ATc concentrations, qPCR analysis should be performed to quantify *pgsA* expression in the Tet repressor mutants and the *E. faecalis* *P*_{srtA-tetO}-*pgsA* (*pgsA*_{ind} without *ptetR*), to confirm the hypothesis that the overall increased *pgsA* expression results from Tet repressor mutations. Also, the significantly differently regulated genes in *pgsA*_{ind} at low ATc concentrations should be validated by the same approach. In addition, we should determine whether the synthesis of PG derivatives including lys-PG and CL are affected by the depletion of PG, thus qPCR analysis of *mprF1*, *mprF2*, *cls1* and *cls2* and corresponding lipid analysis should be performed.

Since we observed decreased SrtA production and delocalization of SrtA in *pgsA*_{ind} at low ATc concentrations, future experiments should include immunoblot analysis for Ebp in different cellular fractions including whole cell lysates, cell wall fractions, and protoplast fractions. We hypothesize that we would observe Ebp in the protoplast fraction in *pgsA*_{ind} at low ATc concentrations, because SrtA is responsible cell wall anchoring of Ebp (Nielsen et al. 2013). The expression and piliation of Ebp may also be affected by PG depletion since Ebp expression and piliation are increased in a *srtA* deletion mutant in *E. faecalis* (Afonina 2017). Since the focal localization of SrtC was also altered in *pgsA*_{ind} at low ATc concentrations, we should similarly perform immunoblotting to quantify whether SrtC levels are altered as well. And because we observed cell wall synthesis was altered in *pgsA*_{ind} at low ATc concentrations, we should perform time lapses using the using peptidoglycan targeting fluorescent d-amino acids (FDAA) dyes to further visualize cell wall synthesis patterns upon PG depletion (Hsu et al. 2017).

To further the studies reported in **Chapter 4**, lipid analysis should be performed to investigate the contribution of each CL synthase to CL production in *E. faecalis* during exponential phase as well as during stationary phase. To confirm their abilities in CL production, we could also

express *cls1* or *cls2* under their native promoters in a plasmid in an *E. coli* CL-deficient mutant to check whether they can produce CL and a heterologous manner. Since our preliminary data has showed that CL contributes to *E. faecalis* fitness during wound infections, more biological replicates should be repeated to confirm the observation.

Additionally, minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) experiments for *cls*-deficiency mutants should be performed using different cell wall targeting antimicrobials to investigate whether similar cell wall compensation can be observed since CL and PG are both anionic phospholipids in bacterial cell membrane. Other cell responses to *cls*-deficiency can be further examined based on the RNA sequencing analysis; for example, the two-component system (TCS) *walK/walR* would be interesting to follow up on. Because *walK/walR* is essential in *E. faecalis*, we can construct a nisin inducible CRISPRi knockdown constructs of *walK* in the *cls*-deficiency mutants and test for altered cell envelope functions.

In addition, we showed that *cls2*-deficiency in *E. faecalis* results in increased EbpC accumulation in the cell wall and hypothesized that Ebp accumulation is somehow sensed by the cell, resulting in a feedback response that results in downregulation of *srtA* and *srtC* transcription. To investigate the role of depleted SrtA in virulence factor assembly, a *srtA* inducible mutant can be constructed using the “Tet-off” system. This *srtA* inducible mutant can be used to investigate the relationship between SrtA expression and subcellular localization and its impact on SrtA function.

In summary, these experiments will be performed in the future to better understanding the role of anionic lipids in *E. faecalis* physiology.

5.3 Reference

- [1]. Afonina, I. 2017. Characterizing the relationship between Sortase A-dependent substrates and the consequences of SrtA deletion in *Enterococcus faecalis*. 180. Nanyang Technological University.
- [2]. Hsu, Y. P., J. Rittichier, E. Kuru, J. Yablonowski, E. Pasciak, S. Tekkam, E. Hall, B. Murphy, T. K. Lee, E. C. Garner, K. C. Huang, Y. V. Brun & M. S. VanNieuwenhze (2017) Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem Sci*, 8, 6313-6321.
- [3]. Nielsen, H. V., A. L. Flores-Mireles, A. L. Kau, K. A. Kline, J. S. Pinkner, F. Neiers, S. Normark, B. Henriques-Normark, M. G. Caparon & S. J. Hultgren (2013) Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *Journal of Bacteriology*, 195, 4484-4495.

Appendix

The appendix figures and tables are referenced in each chapter.

Appendix table 1 Protein sequencing of the genes used in this study

Gene	Protein sequencing
<i>pgsA</i>	MNLPNKLTVLRIFMIPFIIIVSVPMDWGTISFGDTTLAVTQLVGAIIFAVASFTDW LDGKIARAQGLVTNFGKFADPLADKMLVMTAFIVLVGQGKVPWIVAIIVCRE LAVTGLRLLLVEHGEVMAAAWPGKVKTATQMVAIILLFINNIPFSALHPLDQI MLYACLIFTIYSGVDYFAKNKDVFKGSM
<i>cls1</i>	MILSVLTVIYFINAIIAGITILLKPRDVAAIWAWLLVLIALPVFGFFLYLFFGRGLT DKKKFYLLQQSDLRELENFQNFQEESELYSQKMPTEEQQQFTDFFSSLNRMPLT KKNDVEIFTDGTTEKFNALMADIKKAQHSIHIEYYAFVTDHIGTKILNLLEEKAAE GVEVRLLYDAFGSKGTKVHHLNELKKNGGFVQTFITSQKALLKFRLNYHDHRK IVVIDGKVGYYGGFNVADQYAGTTKKFGYWRDTHLRIQGPATSLQMRFLMD WNVSSPEKNRVA YQLDYFFKLEALVPEANTS IQMIASGPNSDREQIKLAFIKLIT SAKKRVWIQTPYLPDDSVLAALKVAAASGVDVKIMIPDKPDHPFIYRATQYY GRLLMKENIEILYNGGFLHAKTMIMDDEVCTVGSANQDIRSYKLNFEANAVLY DKKIIDQLEAIFLEDKCKCTTMTPEVVRDMSKWLIFKQKISRLFSPIIL
<i>cls2</i>	MKIFVWILLFLLVINVIAALITVFRKPRSISSVLAWMMTLIFLPGIGFIIYLCGRGI DGQEVFKLSDDDEKQMVQRIKEKVDVDNKKAGRDKRYDLLYDAKVLNRYFRN MDASPLAKRNSLQLFTDGQEKFQALFEDIRAAKETVHVEYYAFFNDTIGNQFLD VLIEKLHEGVEVYLIYDPWGSFGANKKFFARYVDAGGKVAPFITSRDMIRKTRL NYHLHRKIVVIDGKIGWTGGFNVGDQYLVNTEKFGYWRDTHIRLVGTAVFSLQ EIFIMDWNASVKYPEERMITYHEKYFKLPEDHEVEHLSLQVVS DGPDS EEEILKS GFVRMIFSAEKS VWIQTPYLPDDSMINALLVAVRSGVDVRIMIPCMPDHPFIYR ATQYYANYLHKGRIKIYIYDSGFIHAKTMVIDDELAMVGTTNQDIRSYSLNFEV STFIYNPDIAWMLAQVFEEDIEKSVLLTDEIHKKQGYWLRFKQNF S RLLSPVL

Appendix table 2 Expression of differentially regulated genes in *pgsA*_{ind} at ATc 0.25 ng/ml, compared to ATc 16 ng/ml

Gene locus	Log ₂ FC	Symbol	Name	Annotation
OG1RF_10051	-2.66	<i>acdA</i>	zinc ABC transporter substrate-binding protein	ABC transporters
OG1RF_10099	2.70	<i>arcA</i>	arginine deiminase	Arginine biosynthesis
OG1RF_10100	2.43	<i>arcB</i>	ornithine carbamoyltransferase	Arginine biosynthesis
OG1RF_10101	2.72	<i>arcC</i>	carbamate kinase 1	Arginine biosynthesis
OG1RF_10102	3.27	<i>ntcA</i>	Crp/Fnr family transcriptional regulator	Arginine biosynthesis
OG1RF_10103	3.04	-	C4-dicarboxylate ABC transporter	Arginine biosynthesis
OG1RF_10107	-2.27	-	glycosyl hydrolase	Carbohydrate metabolism
OG1RF_10160	1.79	<i>rpsQ</i>	30S ribosomal protein S17	Genetic Information Processing
OG1RF_10171	1.97	<i>secY</i>	preprotein translocase subunit SecY	Genetic Information Processing
OG1RF_10172	1.68	<i>adk</i>	adenylate kinase	Nucleotide metabolism
OG1RF_10225	1.69	<i>fabI</i>	enoyl-ACP reductase	Lipid metabolism
OG1RF_10281	1.90	-	serine protease; secreted antigen	Glycan biosynthesis and metabolism
OG1RF_10328	3.52	-	peptidase M23; peptidoglycan binding protein	Glycan biosynthesis and metabolism
OG1RF_10369	2.38	<i>nhaC2</i>	Na ⁺ /H ⁺ antiporter NhaC	signaling and cellular processes
OG1RF_10395	-4.39	-	hypothetical protein	hypothetical protein
OG1RF_10417	2.28	<i>pbp2A</i>	penicillin-binding protein 2A	Glycan biosynthesis and metabolism
OG1RF_10499	-2.10	-	hypothetical protein	hypothetical protein
OG1RF_10516	2.53	-	acyltransferase	
OG1RF_10535	3.87	-	hypothetical protein	hypothetical protein
OG1RF_10629	1.88	-	phosphomevalonate kinase	Metabolism of terpenoids and polyketides
OG1RF_10651	1.85	-	hypothetical protein	hypothetical protein
OG1RF_10654	1.63	<i>yxkK</i>	two-component sensor histidine kinase	two-component system
OG1RF_10671	2.35	-	peptidase M23	

OG1RF_10673	-1.91	-	hypothetical protein	hypothetical protein
OG1RF_10720	1.93	-	hypothetical protein	hypothetical protein
OG1RF_10752	2.09	-	PTS lactose transporter subunit IIC	Carbohydrate metabolism
OG1RF_10753	3.16	<i>bglA2</i>	glycosyl hydrolase	Carbohydrate metabolism
OG1RF_10760	1.90	-	membrane protein	Signal transduction
OG1RF_10834	2.07	<i>coaE2</i>	hypothetical protein	hypothetical protein
OG1RF_10850	-2.02	<i>galE2</i>	UDP-glucose 4-epimerase GalE	Carbohydrate metabolism
OG1RF_10864	-2.19	-	hypothetical protein	hypothetical protein
OG1RF_10889	1.88	<i>lepB</i>	signal peptidase I	Genetic Information Processing
OG1RF_10910	3.79	-	CBS domain-containing protein	
OG1RF_10984	2.37	-	LytR family transcriptional regulator	transcriptional regulator
OG1RF_11027	1.75	-	hypothetical protein	hypothetical protein
OG1RF_11041	-2.20	<i>ylxR</i>	DNA-binding protein	unknown
OG1RF_11055	-2.71	-	phage major tail protein, TP901-1 family	phage associated
OG1RF_11059	-4.22	-	phage tail family protein	phage associated
OG1RF_11061	-2.43	-	hypothetical protein	phage associated
OG1RF_11062	-2.90	-	holin	phage associated
OG1RF_11073	3.14	-	LysR family transcriptional regulator	transcriptional regulator
OG1RF_11074	4.29	<i>mgtA</i>	magnesium-translocating P-type ATPase	unknown
OG1RF_11087	6.32	-	cro/CI family transcriptional regulator	transcriptional regulator
OG1RF_11163	1.75	-	hypothetical protein	hypothetical protein
OG1RF_11192	2.03	<i>mutS</i>	endonuclease MutS2;;DNA mismatch repair protein MutS	Genetic Information Processing
OG1RF_11240	2.05	-	E1-E2 family cation-transporting ATPase	unknown
OG1RF_11253	2.16	<i>tig2</i>	peptidyl-prolyl cis-trans isomerase	drug resistance, (CAMP) resistance
OG1RF_11302	2.06	<i>amiD3</i>	N-acetylmuramoyl-L-alanine amidase	
OG1RF_11377	3.92	-	conjugal transfer protein TraX	hypothetical protein

OG1RF_11398	2.51	<i>hlyIII</i>	hemolysin III	signaling and cellular processes
OG1RF_11407	2.10	<i>rpsP</i>	30S ribosomal protein S16	Genetic Information Processing
OG1RF_11422	4.38	<i>cah</i>	carbonate dehydratase	Carbohydrate metabolism
OG1RF_11521	3.82	-	alkaline phosphatase;LTA synthase family protein	Lipid metabolism
OG1RF_11522	2.48	-	multidrug MFS transporter	drug resistance
OG1RF_11535	-2.54	-	hypothetical protein	hypothetical protein
OG1RF_11539	2.51	-	hypothetical protein	hypothetical protein
OG1RF_11591	-2.91	<i>glpO</i>	alpha-glycerophosphate oxidase	Lipid metabolism
OG1RF_11656	2.04	-	peptide ABC transporter permease	ABC transporter
OG1RF_11657	1.67	-	ABC transporter ATP-binding protein	ABC transporter
OG1RF_11709	2.67	-	MOP superfamily multidrug/oligosaccharidyl-lipid/polysaccharide flippase transporter	
OG1RF_11710	3.43	-	polymerase	
OG1RF_11724	2.33	<i>epaO</i>	glycosyl transferase family 2	Glycosyltransferases
OG1RF_11727	2.76	<i>epaL</i>	ABC transporter permease	ABC transporters
OG1RF_11728	3.13	<i>epaK</i>	glycosyltransferases	
OG1RF_11730	1.88	<i>epaI</i>	glycosyl transferase family 2; group 2 glycosyl transferase	Glycosyltransferases
OG1RF_11735	3.02	<i>epaD</i>	alpha-L-Rha alpha-1,3-L-rhamnosyltransferase	Glycosyltransferases
OG1RF_11736	2.70	<i>epaC</i>	glycosyl transferase family 2	Glycosyltransferases
OG1RF_11737	3.12	<i>epaB</i>	glycosyl transferase family 2	Glycosyltransferases
OG1RF_11738	2.74	<i>mraY2/epaA</i>	undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase	Glycosyltransferases
OG1RF_11743	2.48	-	TetR family transcriptional regulator	transcriptional regulator
OG1RF_11779	-2.56	-	hypothetical protein	hypothetical protein
OG1RF_11814	-2.12	-	hypothetical protein	hypothetical protein
OG1RF_11870	3.08	<i>uppP</i>	undecaprenyl-diphosphatase	Glycan biosynthesis and metabolism
OG1RF_11914	2.80	<i>cdsA</i>	phosphatidate cytidyltransferase	Lipid metabolism

OG1RF_12030	1.90	<i>femA</i>	methicillin resistance factor FemA	Glycan biosynthesis and metabolism
OG1RF_12061	2.80	-	hypothetical protein	hypothetical protein
OG1RF_12071	3.62	-	transcriptional regulator	transcriptional regulator
OG1RF_12096	2.29	-	membrane protein	membrane protein
OG1RF_12107	2.49	-	hypothetical protein	hypothetical protein
OG1RF_12109	3.68	<i>dltD</i>	D-alanyl-lipoteichoic acid biosynthesis protein DltD	drug resistance, (CAMP) resistance, TCS
OG1RF_12110	3.50	<i>dltC</i>	D-alanine--poly(phosphoribitol) ligase subunit 2	drug resistance, (CAMP) resistance, TCS
OG1RF_12111	3.53	<i>dltB</i>	D-alanyl-lipoteichoic acid biosynthesis protein DltB	drug resistance, (CAMP) resistance
OG1RF_12112	3.31	<i>dltA</i>	D-alanine--poly(phosphoribitol) ligase	drug resistance, (CAMP) resistance, TCS
OG1RF_12132	2.71	<i>yniG</i>	multidrug MFS transporter,EmrB/QacA family drug resistance transporter	drug resistance
OG1RF_12158	2.45	<i>penA</i>	penicillin-binding protein 2B	Glycan biosynthesis and metabolism
OG1RF_12180	2.00	<i>accC</i>	acetyl-CoA carboxylase biotin carboxylase subunit	Carbohydrate metabolism
OG1RF_12210	1.78	<i>ktrA</i>	potassium transporter Trk;NAD+ binding protein	signaling and cellular processes
OG1RF_12247	-2.75	<i>ulaA2</i>	PTS ascorbate transporter subunit IIC	Carbohydrate metabolism
OG1RF_12255	-1.97	<i>phoB</i>	alkaline phosphatase	Metabolism of cofactors and vitamins
OG1RF_12267	3.99	-	permease	ABC transporters
OG1RF_12269	3.09	-	RND transporter	signaling and cellular processes
OG1RF_12320	2.37	-	RpiR family transcriptional regulator	transcriptional regulator
OG1RF_12331	3.16	-	lipase	Glycan biosynthesis and metabolism
OG1RF_12400	-3.31	<i>sorB2</i>	PTS sorbose transporter subunit IIB	Carbohydrate metabolism
OG1RF_12401	-2.77	<i>sorD</i>	PTS mannose transporter subunit IID	Carbohydrate metabolism
OG1RF_12449	2.15	-	hypothetical protein	hypothetical protein
OG1RF_12460	-1.93	<i>lrgB</i>	antiholin	Signal transduction
OG1RF_12500	2.10	-	LytR family transcriptional regulator;cell-envelope associated acid phosphatase	transcriptional regulator
OG1RF_12535	2.59	<i>croR</i>	DNA-binding response regulator	Two-component system

OG1RF_12536	2.60	<i>croS</i>	two-component sensor histidine kinase	Two-component system
OG1RF_12567	-2.55	<i>citC</i>	citrate (pro-3S)-lyase ligase

Appendix Figure 1 Growth of *E. faecalis* is not affected by high nisin concentrations. Normalized cells (10^5 cells/ml) were incubated in BHI medium supplemented with (A) 0% nisin, (B) 500 ng/ml for overnight (~18 hours). Absorbance readings were measured at 0.5-hour intervals. Data graphed are the mean from three independent biological replicates.